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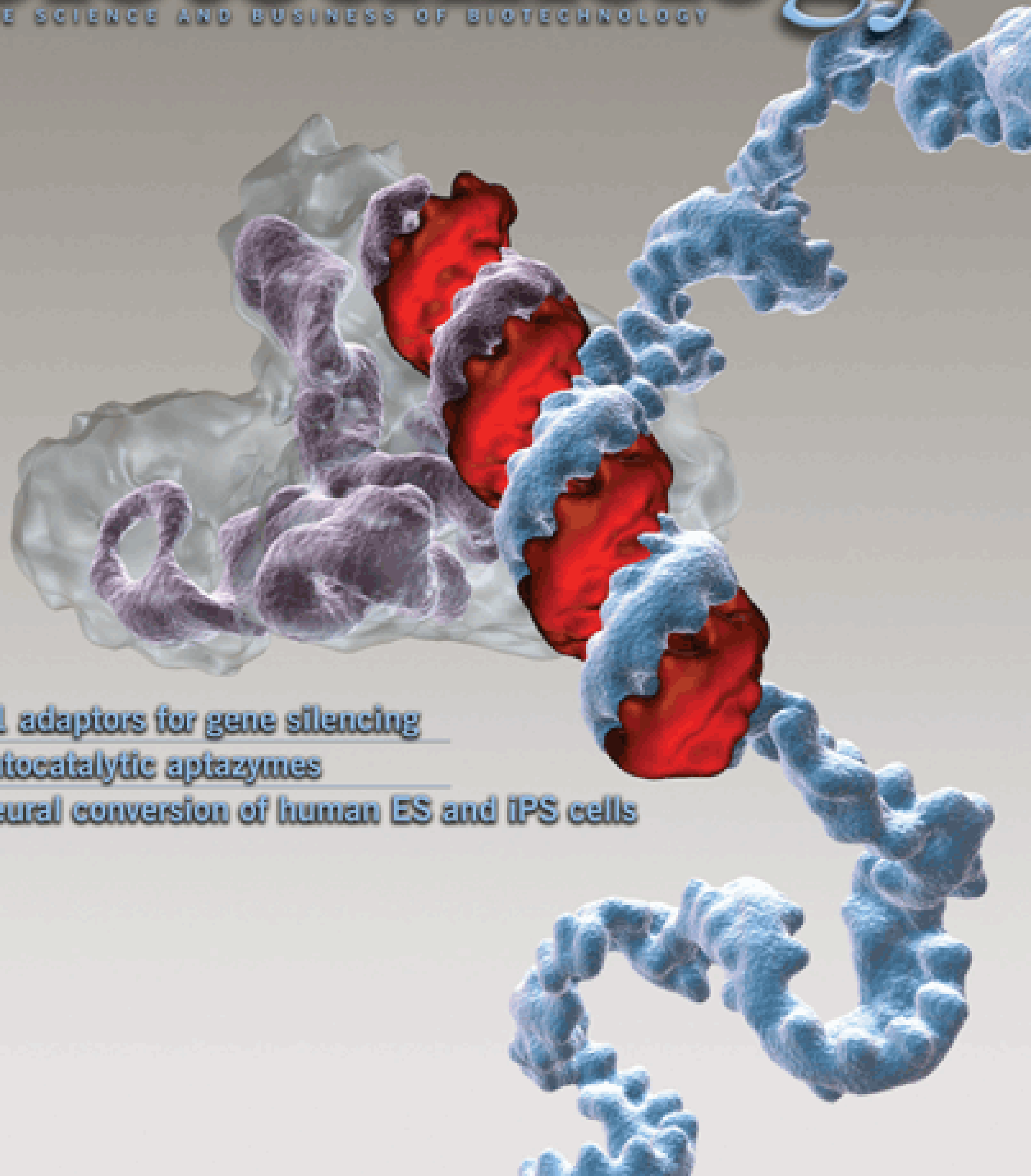

U1 adaptors for gene silencing

Autocatalytic aptazymes

Neural conversion of human ES and iPS cells

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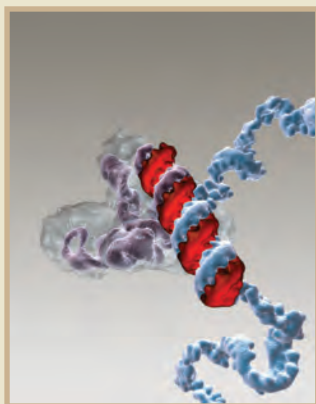

Artist's impression of a U1 adaptor oligonucleotide (orange) recruiting the U1 snRNP (snRNA in purple) to the 3' end of a target pre-mRNA (blue). Goraczniak *et al.* show that the resulting inhibition of transcript polyadenylation silences expression of the target gene (p 257). Credit: Ken Eward © Biografx, with data provided by Holger Stark.

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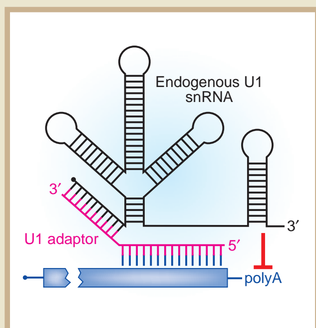

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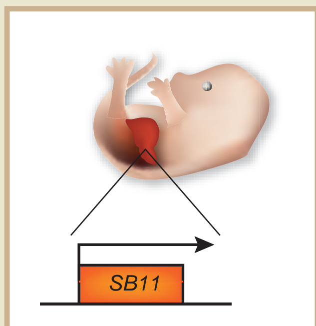

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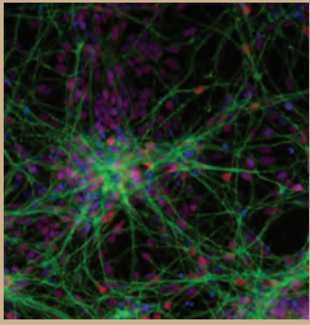

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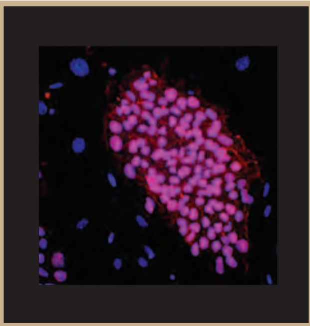

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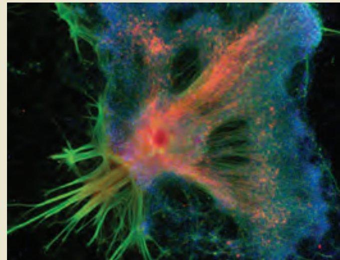
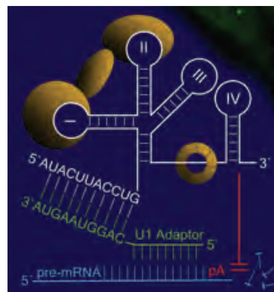
Neural conversion of pluripotent stem cells



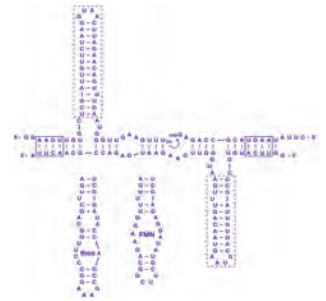
Although human embryonic stem (hES) cells can be readily differentiated into neural cells *in vitro*, existing protocols suffer from limitations such as low yield, coculture with stromal cells, the use of embryoid bodies and lengthy induction times. These problems have been solved by Studer and colleagues with a new method based on Noggin and SB431542, two inhibitors of SMAD signaling. Starting with dissociated hES cells cultured on Matrigel and treated with the ROCK inhibitor Y-27632 (*Nat. Biotechnol.* **25**, 681–686, 2007), the authors demonstrate rapid neural induction at a very high efficiency of >80%. By changing the initial cell density, the ratio of central nervous system cells to peripheral nervous system cells can be modulated. The utility of the protocol is confirmed by showing that neural cells derived from both hES cells and human induced pluripotent stem cells can be further differentiated into midbrain dopaminergic neurons and spinal motor neurons. [**Letters, p. 275**] *KA*

mRNA maturation blockers



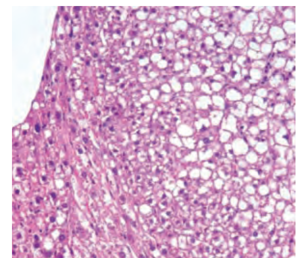
Gunderson and colleagues introduce an oligonucleotide-based alternative to RNA interference and antisense-mediated gene silencing. When their ~25-nucleotide U1 adaptors (green) tether the nuclear U1 small nuclear ribonucleoprotein to a region near the polyadenylation signal of a target pre-mRNA of interest (blue), gene-specific inhibition of polyadenylation (pA) destabilizes the pre-mRNA. The same laboratory previously described U1 interference, which involves engineering the terminal residues of the 164-nucleotide U1 small nuclear RNA to bind directly to the target pre-mRNA. The oligonucleotide version of this principle now opens the way for applying the range of delivery and chemical modification strategies pioneered for siRNAs and antisense oligonucleotides to this gene-silencing approach. Accordingly, various combinations of locked nucleic acid, phosphorothioate and 2'-*O*-methyl modifications enhance the efficacy of U1 adaptors against targets in cultured cells without apparent effects on the splicing of nontarget pre-mRNAs. As their action involves a mechanism distinct from those used by siRNAs and antisense oligonucleotides, U1 adaptors may find particular use as adjuncts to siRNAs when RNAi-mediated silencing is not sufficiently effective or when side effects associated with high siRNA doses need to be avoided. In one instance in the paper, U1 adaptors enhanced siRNA-mediated inhibition about tenfold. [**Articles, p. 257**] *PH*

Aptazyme ligand detection



In a notable feat of *in vitro* evolution, the Joyce laboratory recently developed RNA enzymes capable of efficient exponential cross-replication (*Science*, published online 8 January 2009, doi:10.1126/science.1167856). The system consists of a pair of RNA ligases, each of which catalyzes the synthesis of the other by ligation of two RNA substrates. Now, the same group has adapted this concept to ligand detection by inserting allosteric aptamer domains into cross-replicating ligases in such a way that catalysis occurs only in the ligand-bound conformation. These autocatalytic aptazymes amplify exponentially at a rate that reflects ligand concentration, creating an approach that is analogous to qPCR but that can recognize a broader range of targets, including small molecules and proteins. Enzyme amplification is quantified on gels or with a luciferase assay that measures the released pyrophosphate reaction product. The authors demonstrate the method using one aptamer that binds theophylline and another that binds flavin mononucleotide. Although aptamer binding affinities, which limit the sensitivity of detection, are considerably weaker in the context of the enzymes than in isolation, additional optimization should be possible. The authors also show multiplexing (discriminating two ligands in a sample using two enzyme pairs) as well as dual epitope recognition (requiring two ligands in a sample for amplification of a single enzyme pair). With further development, autocatalytic aptazymes may find application in clinical and environmental assays. [**Letters, p. 288**] *KA*

Jumping into oncogenes



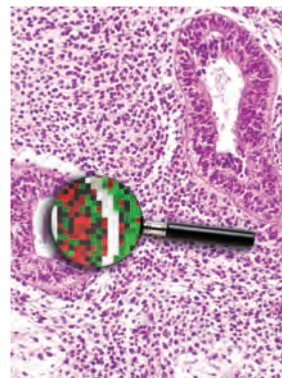
Keng *et al.* describe a conditional transposon-based insertional mutagenesis approach to generate tissue-specific cancer models. They restrict somatic mutagenesis to hepatocytes by placing expression of a Cre recombinase under the control of a liver-specific enhancer/promoter sequence and use high-throughput sequencing to identify tens of thousands of Sleeping Beauty transposon insertion sites in cells from 68 hyperplastic liver nodules, mostly from mice predisposed to oncogenesis by a dominant-negative *Trp53* allele. From a list of ~8,000 nonredundant insertion sites, the authors distill their data to identify 19 common insertion sites, many of which are subsequently reflected in analyses of human hepatocellular

Written by Kathy Aschheim, Michael Francisco, Peter Hare & Lisa Melton

carcinoma samples. Some have been previously implicated in hepatocellular carcinoma, whereas others are potential new targets. Preliminary validation experiments implicate the *Egfr* and *Ube2h* genes and their human homologs as oncogenes in hepatocellular carcinoma. The approach should be applicable to any tumor type for which tissue-specific Cre expression is possible and promises to augment other approaches to cataloging the full complement of cancer genes. [**Articles, p. 264**] *PH*

Survivin teratomas

Implant a sufficient number of human embryonic stem (hES) cells into an immunodeficient mouse and they will produce a benign tumor known as a teratoma. This is a routine assay of pluripotency in many hES cell laboratories, but the tumorigenic process it induces is very unusual because the teratoma-initiating cells can be normal diploid cells with no signs of transformation. Given that teratoma formation is a safety concern in any therapeutic strategy involving hES cells, Benvenisty and colleagues have carried out an in-depth study of this phenomenon. A comparison of tumors arising from mouse and human diploid ES cells shows that the human tumors are less aggressive than the mouse tumors and, unlike the latter, contain no detectable malignant, embryonal carcinoma–like cells. Global transcriptome analysis to find genes implicated in teratoma development identifies 21 genes that are highly expressed in hES cells and teratomas but not in hES cells differentiated *in vitro* into embryoid bodies. Of these 21 genes, the strongest candidate is the oncofetal gene survivin. The authors also show that inhibition of survivin in hES and teratoma cells increases apoptosis, both in culture and in established teratomas *in vivo*. Greater understanding of hES cell teratomas should aid efforts to ensure the safety of hES cell–derived therapies. [**Letters, p. 281**] *KA*



Patent Roundup

Alnylam Pharmaceuticals' patent for the use of RNA interference technology has been revoked by the European Patent Office. The patent, which belongs to the Kreutzer-Limmer series, was deemed too broad; the Cambridge, MA-based company intends to appeal the ruling. [**News, p. 213**] *LM*

China has overhauled its patent laws in a bid to support domestic innovation and attract biopharma companies to the country. [**News, p. 214**] *LM*

Does the US Federal Circuit's decision in *In re: Bilski* further restrain patenting on biotech and pharmaceutical inventions? Simmons discusses the legal challenges facing companies in determining patent eligibility as a result of this important change in patent law. [**Patent Article, p. 245**] *MF*

Recent patent applications in RNA interference. [**New patents, p. 249**] *MF*

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- Using imputation in genome-wide association studies
- Snapshots of the cell-surface glycoproteome

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EDITORIAL

nature biotechnology

A balancing act

Contrary to Genentech's claims, turning over all *in vitro* diagnostics to the US Food and Drug Administration (FDA) is the wrong approach to achieve better clinical validation of tests.

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It's not often that a drug maker approaches the FDA requesting more stringent oversight. So when Genentech filed a citizen's petition late last year asking the US regulator to expand its jurisdiction to encompass all *in vitro* diagnostic tests, the diagnostics industry took notice. In recent weeks, the company has argued that across-the-board FDA oversight of diagnostics used to guide therapeutic decisions is needed because many so-called home-brew tests currently marketed under the Centers for Medicare and Medicaid Services' Clinical Laboratory Improvement Amendments (CLIA) lack both "analytical and clinical validity." What's more, Genentech wants The FDA to immediately pull many of these tests from the market until adequate "scientific evidence of their validity" can be provided.

Several motivations lie behind Genentech's move. The first, mentioned frequently in the petition, is "the potential risks to patient safety" associated with the current regulatory situation. In basing its position on patient safety, the company is unsubtly poking an FDA hot button. However, as the American Clinical Laboratory Association (ACLA)—an umbrella group representing many home-brew providers—argued last month in its response to Genentech, no substantive evidence of harm resulting from the use of any of the thousands of home-brew tests approved under CLIA has yet come to light.

A second motivation—and one only obliquely mentioned in the petition—is the potential threat to Genentech's business. The company's strongest objection is to the proliferation of what it sees as diagnostic tests that make unsubstantiated claims intended to guide specific drug or biologic therapeutic decision making. Among the tests that Genentech would like to see examined closely by the FDA are home brews used for assessing patient suitability for Herceptin treatment, uses that erode Genentech's royalties from sales of 'official' companion diagnostic kits. The company also cites a range of home brews that physicians can use to exclude certain patient groups from using Genentech drugs, such as Rituxan, Avastin and Tarceva. These tests clearly have an impact on the company's revenue from drug sales and bottom line, although this is not mentioned in the petition.

The fact that more stringent regulations are aligned with Genentech's business interests does not invalidate its concerns for patient safety or its desire to ensure that diagnostic methods conform to an appropriate standard of scientific validity and clinical utility. The company's petition argues that, as more diagnostics are designed for high-volume, complex diseases, clinical utility is more difficult to ascertain and validate. In this respect, it has a point. At present, and probably for many years to come, the association of human molecular variation with disease and drug response will be exploratory and rudimentary. Far from being able to say what genetic variation means, researchers are still merely cataloging somatic and germline variation in the genome. In parallel, next-generation sequencing and whole genome association studies are broadening the seam of information from which diagnosticians can draw. So, yes, clinical utility is, and will be, difficult to pin down. But Genentech's call for FDA intervention is almost certainly not an appropriate response, and for several reasons.

First, the FDA currently has insufficient staffing and financial resources to carry out its existing responsibilities. Without a significant investment in staff and training, it certainly could not review the thousand or so diverse home-brew approaches currently on the market, let alone the burgeoning number of new tests stemming from ramped-up sequencing projects. One need only look at the rapid evolution of knowledge relating to *KRAS* mutations in predicting patient responses to epidermal growth factor receptor inhibitors to appreciate how fast the diagnostic field is moving.

Second, the FDA's approach is too stultifying to be appropriate for regulating a field that is so unstandardized and in which the technology and knowledge is evolving so rapidly. The FDA does regulate diagnostics kits through its 510K or premarketing approval pathways. But there are significant costs tied to that regulation, the two most important of which are the delay in reaching the market and the chilling impact on innovation in a sector that already has low margins and poor investment.

Third, the FDA would be regulating in direct opposition to market forces. One of the principal drivers for home-brew diagnostics is their ability to deliver cost savings to the US healthcare system. If a diagnostic system can help avoid expensive-to-treat adverse drug effects or can help avoid wasteful use of noneffective drugs, then there will be a strong incentive for payors to seek out those tests. Indeed, the size of the incentive for the payors to undertake the test is exactly the same as that for Genentech to get the tests removed from the market. Genentech might need to look as closely at its revenue model as payors are looking at theirs.

The final reason that FDA regulation is a bad idea, at least for now, relates to raising the level of awareness about the significance of molecular genetic data. Genentech has argued in its petition, and rightly so, that the clinical claims made by some of the home-brew manufacturers have not been independently verified. However, it also seems to believe that independent verification by the FDA is the only way of informing the patient, physician and payor communities. In reality, channeling all tests through the FDA would serve only to make physicians and payors look for a tick in the 'FDA-approved' box. It would divert them from acknowledging the uncertainties attached to these tests and from regarding the underlying techniques or conclusions in the right context.

If there is a key message from Genentech's intervention, it is that skepticism and a spirit of enquiry with respect to these tests should be maintained by the research community, physicians and payors: leaving verification to the FDA would stop that intellectual pursuit dead. And it may even set the field of personalized medicine back by years.

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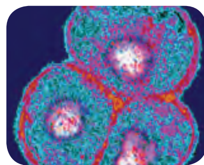

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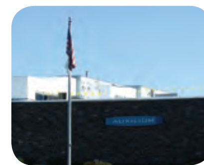
NICE-style cost effectiveness spreads across the globe p215



Pfizer looks to 'niche busters' to fill revenue gap p218

Comparative effectiveness casts first shadows across US industry

Stuffed into the US stimulus package is a provision that biotech companies fear will allow the federal government to restrict patients' access to approved drugs. The bill, which President Obama signed into law on 17 February, allots $1.1 billion for research aimed at comparing the effectiveness of treatments already on the market, but the language of the bill offers little instruction on how the research should be conducted or how its conclusions should be used. Companies and patient advocacy groups worry that treatments will be weighed against each other based on cost, and that the government will deny public health coverage of expensive drugs—generally the newest and most innovative treatments.

Patient access is a key concern. "Nobody thinks it's a bad idea to distribute information to doctors and patients," says Robert Moffit, a director at the Heritage Foundation in Washington, DC. "The issue is whether there will be restrictions on access to treatments and services," he says. "It seems very clear to me that that's exactly what sponsors of the House bill want to do."

Particularly troubling is a report from the House implying that cost may become an important consideration. Treatments "found to be less effective and in some cases, more expensive, will no longer be prescribed," says the report, which accompanied the House's original version of the stimulus bill.

But when members of the House and Senate later released a compromised bill, a joint explanatory statement gave policy experts a different impression. The report says that the conferees of the bill "do not intend" for the funding "to be used to mandate coverage, reimbursement, or other policies for any public or private payer." But how this language will be interpreted is unclear. "It's not how we would have written it, but it's an improvement," says David DiMartino, a spokesperson for DC-based advocacy group Partnership to Improve Patient Care, of which the US Biotechnology Industry Organization (BIO) is a member. "We're pleased that the language takes steps to ensure the research is centered on the needs of patients and providers."

The stimulus package divides $1.1 billion between three federal agencies. The National Institutes of Health (NIH) and the Department of Health and Human Services (HHS) are each to receive $400 million; the Agency for Healthcare Research and Quality (AHRQ), which already conducts comparative effectiveness research, is to receive $300 million. The provision offers little instruction on how to spend the money and gives the Secretary of the HHS wide discretion in setting priorities.

The bill also mandates the creation of a 15-member 'coordinating council', including representatives from the Food and Drug Administration (FDA) and the Centers for Medicare and Medicaid Services. The council will be charged with driving the initiative and advising Congress and the president.

Policy analysts say the bill's language could allow a centralized technology-rationing agency similar to the UK's National Institute for Health and Clinical Excellence (NICE) (see pages 215–217) or Germany's Institute for Quality and Efficiency in Health Care. These agencies use cost effectiveness to determine the worth of treatments and make coverage recommendations. NICE has determined, for example, that if a treatment is found to cost more than $45,000 (£30,000) per "quality-adjusted life-year," the treatment is not considered "cost effective," and such treatments are rarely covered.

But some biotech companies' experiences with NICE have made them leery of such a program in the US. "I am very worried that if the US were to track what's going on in the UK, it could have a devastating effect on oncology research," says Walter Moore, vice president of government affairs at Genentech in South San Francisco.

The oncology industry is moving toward personalized medicine and targeted therapies, which can be highly effective, but in small populations. Such therapies "don't fit into a comparative effectiveness regime very well," says Moore. Indeed, NICE has in the past restricted access to Herceptin, a targeted breast cancer therapy developed by Genentech and marketed by Roche of Basel, Switzerland.

The government's stimulus bill will give $1.1 billion to study the comparative effectiveness of different treatments, but there are fears that cost considerations may restrict access to novel drugs.

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npg

IN brief

Genentech grapples with direct offer

AP Photo/Mark Lennihan

Severin Schwan, CEO of Roche, prays their low offer suceeds.

Genentech shareholders have until midnight, EST, on 12 March to decide whether they want to sell their shares directly to Basel-based F. Hoffmann–La Roche at $86.50 apiece following Roche's hostile cash tender offer, launched on 9 February. That's a drop in price from July last year, when Roche proposed to buy Genentech for $89 per share ($43.7 billion overall), giving Roche the 44% of Genentech shares it didn't already own. Closing the hostile bid is contingent on the majority of outstanding stakeholders tendering their shares to Roche, and on Roche securing the money for the deal—it says it will use its own cash reserves, commercial paper, bonds and 'traditional' bank financing to cover the cost. In response to the latest offer Genentech publicly asked shareholders to "take no action at this time." (Genentech in fact wants a much higher offer: filings by Roche with the Securities and Exchange Commission show that in a December meeting, representatives for Genentech indicated a willingness to pursue a transaction at $112 per share.) If the offer is successful in winning Roche 90% or more of Genentech shares, it would then merge the companies. The failure to reach a conclusion on the original offer with Genentech, of S. San Francisco, is behind the hostile attempt, and the lower per-share offer reflects "the fact that a public purchase is a much more complicated transaction than a direct agreement with the board," says Severin Schwan, Roche CEO. But there is no shortage of theories as to why Roche made the move now, most interesting perhaps is the pending phase 3 data for Avastin (bevacizumab) as an adjuvant in colorectal cancer, due in April. A positive result there could build as much as $15 into Genentech's stock, some analysts surmise, making Genentech prohibitively expensive. Others have pointed out that if the negotiations drag out further Roche might have trouble finding financing in what is universally described as a horrible environment for securing bank loans, especially as New York–based Pfizer recently announced it will purchase Madison, New Jersey–based Wyeth for $68 billion, and would seek financing, too. Regardless, the tender offer has the industry buzzing (again) about Genentech's ability to retain its vaunted scientific talent with a future so cloudy. "I'm sure people are a bit unsettled," says the CEO of a southern California startup, who requested anonymity because he successfully recruited two ex-Genentech scientists to join his firm. "But people have been peeling off [from Genentech] from the beginning, for a whole variety of reasons. That's common to biotech anyway.

Brady Huggett

Moore has spoken with US policymakers about an oncology exclusion, but to no avail. "I'm concerned that we as a country are looking for a "one-size-fits-all answer," he says.

There is still hope that the bill will not be used to restrict access based on costs. At a January 27 Senate Finance Committee hearing, Wyoming senator Mike Enzi said he'd like to see the $1.1 billion used to support "research on clinical effectiveness, not cost effectiveness" and that the bill should prohibit a comparative effectiveness infrastructure from "making any clinical guidelines or coverage decisions."

The outcome will set an important precedent, as the comparative effectiveness provision is among the first of several health-related policies and reforms to come this year. "It will set the tone for future bills," says DiMartino, "It would be really difficult to undo."

Questions around healthcare reform are in such a state of flux, says Ted Buckley, director of economic policy at BIO, that it is impossible to predict how it will all turn out. For the biopharma industry, the looming healthcare reform could present a mixed picture. In announcing GlaxoSmithKline's 2008 results, CEO Andrew Witty in February told analysts that the company sees both upsides and downsides to the coming healthcare reform. "On the downside, clearly there is discussion…around price levels and comparative effectiveness," he says. On the upside, President Barack Obama pushed for affordable healthcare coverage during his election campaign. Drug company executives hope to work with the new administration to expand coverage and reach new customers. Comparative effectiveness studies are nothing new, however. US public and private organizations have been conducting them for years, with varying objectives and criteria. Portland-based Oregon Health and Sciences University's Center for Evidence-Based Policy has been conducting literature reviews on drug classes since 2003 and has so far covered 34 topics. The project's subscribers—14 states and Canada—choose the topics of the reports, which are produced at a cost of about $60,000–180,000 each. "Most states use them to determine which [treatments] make their preferred drug lists [for Medicaid]," says Alison Little, medical director of the project, called the Drug Effectiveness Review Project, or DERP. The reports do not consider the costs of treatments, she says.

One of the advantages of such studies is that they have positive impacts on public health by providing information to doctors and patients. A year before New Jersey–based Merck withdrew the anti-inflammatory drug Vioxx (rofecoxib) from the market, DERP released a report highlighting the treatment's heart attack risk. As a result, several states, including Minnesota, Washington, Oregon and Montana, did not put the drug on their preferred drug lists. "The reports can be to industry's benefit when a product is shown to be very effective," adds Jean Slutsky, a director at the AHRQ.

Slutsky's agency, which is to receive $300 million from the stimulus bill, has been publishing comparative effectiveness studies since 2005 after it was authorized to do so by the Medicare Prescription Drug, Improvement, and Modernization Act. Until now, the AHRQ's annual budget has been $15–30 million, though the agency's spokespeople would not comment on how the additional funding from the stimulus bill might be used. In the past, the Medicare Modernization Act and the secretary of the HHS prioritized the disease areas that should be studied. Top priorities tended to be those "health care items" that "impose high costs on Medicare, Medicaid or SCHIP [State Children's Health Insurance Program]," for instance, cancer, diabetes and mental health. Right now, Slutsky says, AHRQ studies and reviews do not factor in costs, but use data from electronic medical records, registries and administrative claims data. She would not speculate on whether the stimulus bill might change that once it is implemented.

The lack of standard definitions complicates matters further. There is "no single correct way" to conduct such research, noted a 2007 white paper from BIO. With so many different kinds of groups conducting their own comparative effectiveness research, studies range in quality and aim to answer different questions. "Here is also where AHRQ or NIH could make the biggest impact: through a less biased assessment of what questions need answering most urgently," says Sebastian Schneeweiss, a pharmacoepidemiologist at Harvard University in Cambridge, Massachusetts.

How $1.1 billion might change the comparative effectiveness research community is unclear. Says Little at Oregon Health and Sciences University: "This is more money than we've ever seen to do this kind of work in this country."

Emily Waltz *New York*

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Geron gets green light for human trial of ES cell–derived product

After an eight-month delay, on 23 January, the US Food and Drug Administration (FDA) approved the first human trials of embryonic stem (hES) cells, a surprise decision that came on the eve of President Barack Obama's expected policy change concerning hES cell research.

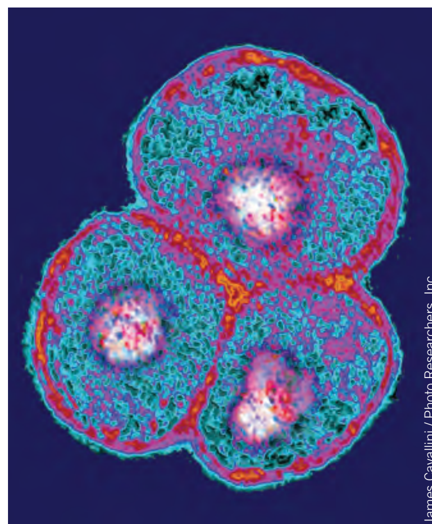
This summer, Geron Corporation of Menlo Park, California, will begin treating ten patients who have suffered a complete thoracic-level spinal cord injury in a phase 1 multicenter trial. The pioneering therapy is Geron's 'GRNOPC1 product', which contains hES cell–derived oligodendrocyte progenitor cells that have demonstrated remyelinating and nerve growth–stimulating properties.

For a company that held its ground during the Bush administration's assault on hES cell research, the FDA's clearance is part triumph, part vindication for sticking with the Sisyphean task of preparing a gargantuan 22,000-plus page Investigational New Drug (IND) application for their product. "There was plenty of crying going on here when we received notification that the FDA had cleared our IND," says Thomas Okarma, Geron's president and CEO.

Although they may not have shed tears of joy, many players in the budding regenerative medicine sector sounded a note of relief, and even optimism. "I've been talking to my colleagues in this field, and the overall feeling is that this is an important milestone because it means that FDA will approve clinical trials using human embryonic stem cells, and that, in fact, there is [regulatory] support for developing therapies based on embryonic stem cells," says Michael West, CEO of BioTime and Embryome Sciences, both in Emeryville, California, who cofounded Geron in 1990.

Other CEOs of stem cell firms are also upbeat: "Clearly, this opens the door not only for Geron, but other companies that develop strong IND packages for stem cell–based therapeutics. And given the compelling evidence that there's a reasonable chance for clinical success, this is a very positive development for the regenerative medicine field," says Richard Garr, president and CEO of Rockville, Maryland–based Neuralstem, which in December filed an IND to use human neural stem cells for treating amyotrophic lateral sclerosis. On the same day, StemCells, Inc. received approval to begin clinical trials of a purified human neural stem cell to treat Pelizaeus-Merzbacher disease—a fatal brain disorder that affects children.

But West, who spurred Geron's support for the research of human stem cell pioneers James



James Cavallini / Photo Researchers, Inc.

Embryonic stem cells. Companies are rapidly gearing up to follow in Geron's footsteps, as the firm receives the first approval to carry out embryonic stem cell work in humans.

Thomson and John Gearhart before leaving the company in 1998 and then ran Worcester, Massachusetts–based stem cell company Advanced Cell Technology (ACT) until 2007, says there's also a sense of unease with Geron's planned trial. "While we all want [the clinical trials] to work, there's a concern among many of us that some of these patients will develop ectopic growths, and that would be a disaster."

Ectopic growths, also known as teratomas, are encapsulated, usually benign tumors that may grow from residual hES cells. They can occur naturally, but the fear, based on some animal studies, is that some proportion of the cells derived from hES cells injected into the body could stray from their intended developmental pathway. Last month, a group of Israeli researchers reported that a boy with ataxia telangiectasia who had received several fetal neural stem cell transplants developed teratomas in his brain and spinal cord four years after treatment (*PLoS Med.* **6**, e1000029). "Concerns about tumorigenicity are bang on," says Melissa Carpenter, a San Diego–based independent consultant on stem cell therapeutics. "Yes, Geron and others have done extensive testing in rodents that show that teratomas don't form from their preparations, but a rat's lifespan is short. What we really don't know is how these cells will behave in a human that might live 10–50 years after receiving treatment."

Although acknowledging that teratoma formation might be a concern, Geron's Okarma

IN brief

Alnylam dealt blow

The European Patent Office (EPO) has revoked a patent covering RNA interference (RNAi) technology from Alnylam Pharmaceuticals. The '945 patent (EP 1214945), which belongs to the Kreutzer-Limmer patent family, protects the use of small interfering RNAs 15–49 nucleotides long. Alnylam's claim was disputed by London-based Silence Therapeutics, Abbott Park, Illinois–based Abbott and San Francisco–based Sirna, owned by Merck. The ruling—made in part because the patent was deemed too broad—is not final and will be appealed. "In an area like this, companies don't expect to get their patents through unobjected to," says patent lawyer Simon Cohen, of Taylor Wessing, a European law firm. "They start off with broad claims and they realize they have to narrow down their scope." Cambridge, Massachusetts–based Alnylam had another of its Kreutzer-Limmer patents revoked by the EPO last December. A spokesman for Silence Therapeutics says the whole Kreutzer–Limmer patent series may eventually fall, creating space for other companies who want to work with RNAi. This is "very much the start of litigation and opposition, rather than the last phase of it," Cohen stresses. In the US, Alnylam received recent FDA approval for phase 1 trials of an RNAi-based treatment for liver cancer. *Asher Mullard*

C-Path sets diagnostics standard

A newly launched diagnostics evaluation service for companies could help standardize tests and ease their transition to market. The United States Diagnostic Standards (USDS), a nonprofit organization set up by the Critical Path Institute (C-Path), will provide independent test evaluations, effectively functioning as a voluntary "Underwriters Labs" for diagnostics companies, says Jeffrey Cossman, chief scientific officer at C-Path, of Rockville, Maryland. Analytic evaluations performed by the new entity will take place at carefully selected neutral sites. Under USDS policy, the clinical samples (e.g., blood, tumor tissue) used as standards in the evaluation of diagnostic assays must be approved by an independent, outside panel of experts. In some instances, well-established clinical samples may serve as standards so that assays from different suppliers can be compared. Although the group has no regulatory authority, diagnostic test manufacturers can use evaluation results to support an application for FDA approval. Alternatively, as one of its many services, the USDS will certify a Laboratory Developed Test (LDT) and ensure its performance. As Cossman explains, "The information [USDS provides] would be useful for [insurance] payers, clinical pathology laboratories, providers, as well as for regulators such as FDA, [and] might help with reimbursement decisions, as well as approval or assurance that an LDT (not evaluated by FDA) performs as labeled." *Jim Roberts*

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IN brief

Vatican cheers GM

A closed door meeting to be held at the Vatican in Rome in May will see leading scientists gathering to discuss a campaign backing agricultural biotech. The study week has been organized by Ingo Potrykus, co-inventor of the fortified Golden Rice technology and president of the Golden Rice Humanitarian Board, on behalf of the Pontifical Academy of Sciences. The Vatican has long been concerned about food security, and advisors from the academy, which holds a membership roster of the most respected names in twentieth-century science, have recognized that plant biotech has the potential to benefit the poor. "I think we are heading in the right direction with this meeting and it will help to dispel some of the myths about GM crops," argues Peter Raven, director of the Missouri Botanical Garden in St. Louis and an academy member. Participants are expected to issue a definitive declaration and work on a roadmap for science-based regulations for genetically modified (GM) crops. "I would hope the moral high ground of the Vatican is relevant at least in Catholic countries," says Potrykus, whose Golden Rice project has been held up by political hurdles. It will be particularly interesting to see reactions in Italy, where a nine-year ban on open field trials recently ended. Some of the 'regions', into which Italy is subdivided, "still jeopardize field studies by failing to identify [planting] locations," says Piero Morandini of the University of Milan. *Anna Meldolesi*

China overhauls patent law

China's top legislature has amended its patent laws in a bid to support domestic innovation and entice foreign biopharma companies to do business in the country. The revised law, passed late last year by the Standing Committee of the National People's Congress, will take effect on 1 October. The intent is to raise the novelty benchmark by requiring that a patent application must be new worldwide. In the past, patents could be granted as long as the technology was novel in China. The revised law will allow inventors to apply for patents in other countries before obtaining them domestically. They must, however, first get an approval from China's patent administration department, which will determine whether the invention should be made a 'national secret'. The development is welcomed by the international patent community, says Michael Vella, head of the Shanghai-based China Intellectual Property Practice. "It is a signal that China's patent law is increasingly brought into line with international standards." The revised law should encourage foreign companies to do business with China, says Vella, by increasing patent enforcement. The new law also allows the granting of a compulsory license in cases of national emergency, and includes a provision requesting that patent applicants disclose the source of materials to affirm that they are lawfully obtained. "China will be the first major economic power that requires this," says Vella. *Jane Qiu*

says those worries are misplaced because of the extensive purification steps that the company takes to produce hES cell–derived oligodendrocyte progenitor cells. "These aren't totally undifferentiated cells, but rather, they are 90% of the way to being a glial cell. Getting the cells to that state is a critical part of the manufacturing process, and it's integral to every product we're developing."

The bigger worry is that any safety issues that arise during Geron's clinical trial could have a devastating impact on the ability of stem cell companies as a group to raise funds. "We do worry about the potential negative impact a safety signal could have in this trial on the investment community, particularly among those investors that don't have a lot of history in the regenerative medicine space," says Joseph Pantginis, senior vice president at Merriman Curhan Ford in San Francisco. "Safety is obviously an issue, but having said that, you just have to look at the 22,000-page IND to see that the company went out of its way to address the potential for adverse events." And on a lighter note, Neuralstem's Garr adds, "The venture capital community hasn't been in this space for years, so I don't worry about scaring anyone off should Geron's trial run into trouble, which I actually don't expect."

Safety concerns aside—and the verdict will be out until phase 1 trials are complete in late 2010 or early 2011—researchers and investors alike worry that Geron's hES cell–derived oligodendrocyte progenitor cells simply won't work. "It's hard to think of an indication more difficult to treat than severe spinal cord injury in a human," says Aileen Anderson of the University of California, Irvine, who has had some success in using stem cells to treat spinal cord injury in rats. One issue is that the rodent spinal cord and primate spinal cord differ markedly both functionally and physiologically, "so extrapolating from rats to humans is not straightforward," she explains.

Of particular concern, says Arnold Kriegstein of the University of California, San Diego, is the fact that patients can experience some improvement in function without treatment, and so unless the positive effects of stem cell treatment are marked, phase 1 results could prove equivocal. "There's a real problem for Geron in that there is no way to track the fate of these cells once they are injected into the patient," he explains, "so in the absence of a big clinical response, which I'm not expecting, we may not get an answer as to whether this approach works or not."

Then there is the matter of perception and hype. On the day Geron announced the trial, the company's phone system crashed under the influx of calls from patients wanting to take part in the clinical trials. "This is a landmark study, potentially game changing, but expectations need to be realistic," says Pantginis. "We can't expect people to get up and walk following this therapy. Even the most optimistic of us don't expect that to happen." Indeed, experts such as Kriegstein, Carpenter and Anderson all agree that an improvement in lower body sensation or bladder control would represent huge benefits to patients.

In the meantime, Geron and others, including Neuralstem, BioTime, ACT and Stem Cells in Palo Alto, California, are pushing ahead with other stem cell–derived products, and Carpenter, for one, believes that everyone in the field owes Geron a debt of gratitude. "Geron has had such a difficult road," she says. "The company has been in the spotlight for years and it's been criticized up and down, but to its credit, it persevered, and as a result, everyone in the field is benefitting. And despite the safety concerns, the bottom line is that this trial is not premature. The safety of Geron's stem cell product has been tested as well as the current animal models allow. The next step is to take these stem cells into humans."

Joe Alper *Louisville, Colorado*

SELECTED research collaborations

Partner 1	Partner 2	$ (millions)
Micromet (Munich)	Bayer Schering Pharma (Leverkusen, Germany)	395
Santaris (Horsholm, Denmark)	Wyeth (New York)	100
Arcadia (Davis, California)	Advanta India (Bangalore)	*

* Not disclosed.

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Report blames NICE for hastening decline of UK biotech

The UK biotech sector is faltering, and the cause is not solely lack of finance. A British government–sponsored report published in January singles out the National Institute for Health and Clinical Excellence (NICE) as one of the main factors holding back biotech companies and their products. With the World Health Organization (WHO) touting NICE's Health Technology Assessment (HTA) approach as the model for other countries around the globe to follow, companies that fail to take into account cost effectiveness issues in trial design do so at their peril.

NICE is the body charged with ensuring the UK's annual £11 billion drug budget is spent (or rather rationed) equitably. By weighing the additional expense of a newly approved drug against its increased effectiveness over standard treatment, the institute provides an HTA that is used by the UK's National Health Service (NHS) to determine which drugs to make available in public hospitals (see **Box 1**). Although the HTA has its roots in the 1970s, it is only since the turn of the century that the focus has widened from clinical effectiveness to encompass cost-effectiveness. And with the rising cost of healthcare, its use is spreading. Following on from the UK's example, France, Sweden, Canada and Germany are among the countries that have recently introduced cost into drug appraisals.

According to Sir David Cooksey, chair of the Bioscience Innovation and Growth Team that prepared the recent 'Review and Refresh of Bioscience 2015' report for the UK government, although the NICE approach has reduced the national drug bill in the short term, it also has had a pernicious effect: "to delay the introduction of new therapies and shorten patent-protected marketing periods." This has resulted, among other things, in "increased risk for the investor," says Cooksey, who thinks that new drugs will no longer be developed in the UK because their development there is unsustainable. Cooksey is calling for an independent enquiry to assess the long-term impact of NICE upon cost, access to and uptake of medicines in the UK.

Indeed, under NICE, fewer drugs are coming to the market in the UK than in other countries. It is worrying for the biotech industry as a whole, then, that NICE-style HTAs have been commended by the World Health Organization as "an important model for technology appraisals internationally."

"Lots of countries are increasingly formally requiring the same sort of evidence as NICE requires. The outlier is the US," says Rod Taylor, associate professor of health services research at the Peninsula Technology Assessment Group at Exeter University, one of seven academic groups that are under contract to carry out assessments for NICE.

Twenty-four countries in Europe have HTA agencies, according to EUnetHTA, a network set up three years ago by the European Commission in Brussels to share best practice and avoid duplication in the expensive and time-consuming business of carrying out assessments. Europe has gone furthest down the HTA pathway, but the movement has also taken hold in Latin America, Asia and Australia. In North America, Canada uses HTAs to decide which drugs will be reimbursed through the public system upon which roughly half the population depends. And in the US, 'Comparative Effectiveness Research' has been introduced as part of the economic stimulus plan (see pages 211–212).

Details
Bayer Schering will pay $6 million for a one-year option on one of Micromet's preclinical BiTE antibodies against an undisclosed oncology target. Micromet could earn another $389 million in additional fees and milestone payments.
The two companies will collaborate to discover, develop and commercialize RNA-based therapeutics using Santaris' Locked Nucleic Acid drug platform. Santaris will receive $7 million in cash up front and a $10 million equity investment from Wyeth. A further $83 million may be payable in milestones for each of ten undisclosed targets selected by Wyeth, which will have exclusive, worldwide rights to develop and commercialize any resulting compounds.
Arcadia has agreed to collaborate with Advanta India to develop nitrogen use–efficient sorghum. Under the deal, Advanta will have exclusive global rights to use Arcadia's technology in sorghum, in return for an upfront payment, milestone fees and share in sales.

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IN brief

Norway's swift bail out

The Norwegian government has unveiled a rescue package for the biotech industry as part of a national financial rescue plan. The stimulus package worth NOK20 ($2.87) billion contains explicit measures worth about $400 million to support the biotech industry and prevent companies from going bankrupt. The government's move came in response to a proposition made by the Oslo Cancer Cluster, an industry and research cluster representing 25 Norwegian groups. Over half of the group's member companies, which together have more than 50 oncology products in the pipeline, were in danger of running out of cash in the next 12–18 months. In other countries where similar requests have been made, the response has been slow (*Nat. Biotechnol.* **27**, 1, 2009). "The Norwegian government understood that they had to react quickly," said Jónas Einarson, chairman of the Oslo Cancer Cluster. Key measures in the package include a tripling of the funds allocated to innovations loans for biotech and information technology, an additional $279 million for the government-owned fund Argentum to invest in private venture capital funds focusing on life sciences, and extra tax breaks for individual small-to-medium enterprises. "Norway has a small but growing industry with a very strong pipeline, mostly in the oncology sector," says Einarson. "The Norwegian government wants to make sure that this fragile industry survives the ongoing financial crises." *Nayanah Siva*

Green fuels thrust

By 2020, all road transport fuel in Europe must include 10% from renewable sources, be it from biofuels, hydrogen or green electricity. The European Parliament's decision, reached last December, is a step down from the original aim of sourcing 10% of transport fuels from biofuels alone. Across the Atlantic, the US Department of Energy announced $200 million in funding from 2009 to 2014 for pilot and demonstration-scale biorefineries to develop cost-effective biofuels such as bio-butanol, 'green gasoline' and advanced biofuels technologies, such as algal biomass. But first-generation biofuels manufacturers have been trading at an all-time low. In January, Pacific Ethanol, of Sacramento, California, suspended operations at one of its sites, and last November, the world's largest corn-based ethanol producer, VeraSun Energy Corporation, of Sioux Falls, South Dakota, filed for bankruptcy citing huge losses and a $1.5 billion debt. The situation for corn ethanol producers could arguably improve as the US gears up to accommodate the 36 billion gallons per year of annual domestic renewable-fuel production stipulated in the Energy Policy Act. "Corn ethanol is not going away anytime soon," says Pavel Molchanov, analyst at Raymond James in St. Petersburg, Florida. "With the current costs and low rates of return, I see no real investment going into the sector apart from VC [venture capital] and public money, so it will take some time to figure out the economics of second-generation technologies." *Victor Bethencourt*

Zuma/Zuma Press/Newscom

Anne Marie Rogers in the UK launched a legal action against her local health authority in 2006 after she was denied Herceptin to treat early-stage breast cancer. NICE guidelines restricted the drug's use to 'exceptional circumstances', but guidance has since been revised to include all HER2-positive breast cancer patients.

But Exeter University's Taylor thinks that the biotech industry needs to face up to the fact that some form of HTA will be applied in all major markets. "The movement is bound to spread: as far as governments are concerned, they are interested in cost effectiveness because they can't pay for everything. The question will be, Does this drug give you more healthcare bangs for your healthcare bucks?"

Negative rulings by NICE are leading companies to strike 'creative pricing' or 'risk-sharing' deals to meet the institute's cost-effectiveness criteria. Examples include Velcade (bortezomib) for treating multiple myeloma, for which manufacturer Johnson & Johnson of New Brunswick, New Jersey, reimburses the NHS for patients who do not respond, and Lucentis (ranibizumab), for which the NHS pays for the first 14 injections and manufacturer Genentech of S. San Francisco pays if more treatment is required.

Although these are portrayed as risk-sharing deals, Keiron Sparrowhawk, partner at the pricing and reimbursement consultancy PriceSpective, believes that they are merely a form of discounting. "The industry is prepared to do it when it doesn't have to lower the list price," he said. This is important because although the UK represents just 6%

Box 1 How does NICE judge cost-effectiveness?

To decide if the UK's NHS should pay for a drug, NICE assesses the treatment's additional cost over that of the current standard therapy, set against the extra health benefits it confers. The tool for comparing the value or health gain of different drugs is the quality-adjusted life year, or QALY, which, at its crudest, measures the increase in life expectancy and quality of life derived from any treatment.

The main difficulty with QALYs is that this measure does not take account the severity of the underlying condition. A second major problem is the question of who decides what is an acceptable cost per QALY. Any drug with a cost per QALY below £20,000 will automatically get the nod; those between £20,000 and £30,000 will need additional evidence; and it is rare for drugs with a cost per QALY of over £30,000 to be approved.

Given an unacceptable price per QALY, there are two ways forward for companies to get NICE's approval: provide more compelling data for benefits or lower the price. In Australia, negotiating price is an explicit part of the HTA process. Similarly, in France the clinical added value, as determined through an HTA, is the key factor in agreeing on a price. *NM*

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of the global market, it is used as the reference point when setting drug prices in around 25% of the world market.

Most companies see such deals as a fallback if they do not reach NICE's cost-effectiveness threshold, but a better approach is to build NICE's evidence requirements into the thinking when designing phase 3 trials. NICE is considering whether it should routinely offer advice on trial design. In France and Sweden, the HTA bodies already provide advice to help companies design trials that will generate data that can be fed into pricing and coverage decisions. "Companies may not like getting into bed with NICE," says Sparrowhawk, "but I don't think you can get to understand what NICE wants without engaging with them in advance."

For those companies dealing with the reality of a NICE rejection, Sparrowhawk argues that the key is to start with the clinical data to frame a deal. "If you are going to offer free treatment after a particular point, you need to have an idea how many nonrespondents there are likely to be. In effect you are making a judgment that ten percent, say, will be free." The risk is that the clinical trial does not play out in the real world: "You could end up with fewer responses, giving away 50% free," says Sparrowhawk.

Thus, the overheads of getting involved in a NICE submission are such that some companies have declined to do so. Roche, for instance, declined to submit a dossier for Avastin (bevacizumab) in breast cancer. "We did our own calculations and knew Avastin wouldn't meet NICE's parameters and would be turned down," says a spokesman. With Avastin, patients are healthier for longer, but as NICE does not weigh social factors such as wellness, or even costs such as hospitalization, Avastin adds to the cost of treatment. (Avastin is currently licensed for treating breast cancer in the UK if patients pay for it.)

This is the classic biotech dilemma—although many treatments improve outcomes, they do so at an additional cost. But two significant concessions have been made recently. The first is that the government has agreed that if patients pay for drugs themselves, they can still have the rest of their treatment free on the NHS.

The second is that NICE will loosen its cost-effectiveness rules for end-stage disease treatments, to allow reimbursement of drugs that offer three months of extra life if no alternative treatment exists. This is expected to open the door to the reimbursement of Sutent (sunitinib), Nexavar (sorafenib), Torisel (temsirolimus) and Avastin in treating kidney cancer when NICE issues its final guidance on these drugs this month.

As the case of NICE demonstrates, the inclusion of cost effectiveness in drug assessments can have a huge impact on the marketing strategies of biotechnology companies, and the current prospect is that it will start to affect investment decisions across the discovery and development cycle. Industry needs to coordinate its views and set an agenda for how it will engage with HTAs. A good starting point would be to lobby for the price of drugs to be put into the overall context of the cost of healthcare.

Nuala Moran *London*

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IN their words

"Roche is not big pharma—Roche is the leading biotech company in the world."

The Swiss drug firm's CFO Eric Hunziker at a recent JPMorgan Healthcare Conference. (*San Francisco Chronicle*, 18 January 2009)

"We must continue to develop an environment in which scientific discourse and at times disagreement is accepted and respected, where politics has no standing in regulatory decisions."

Frank Torti, Acting Commissioner of Food and Drugs (27 January 2009)

"People would say to me 'Are you wearing false eyelashes?'—even my own mother asked."

Clinical trial participant Cindy Ross, of Wethersfield, Connecticut, on the glaucoma drug Latisse's (bimatoprost) unexpected side-effects that make eyelashes grow longer and fuller. *(New York Times*, 13 January 2009)

"It's tougher than getting into Harvard."

Vertex CEO Joshua Boger says his company took in 11,000 applications last year for 300 positions, a sign of how pharma's downsizing is affecting unemployment in the sector. (*Seekingalpha*, 14 January, 2009)

"In some ways it may have been a failure of us all actually to stand behind the science."

Sir Terry Leahy, chief executive of British retail giant Tesco, fesses up to the food industry's failure to recognize scientific consensus that GM food should be considered on a case-by-case basis. (City Food Lecture, London, January 28, 2009)

"I'll make a killing at some point during the year on the small caps."

Sven Borho of OrbiMed, on the bargains to be had among companies who have seen their stocks plummet by 70–90%. (*BioCentury*, 1 January 2009)

Pfizer swallows Wyeth, validates niche buster

In late January, Pfizer made the headlines with its purchase of Wyeth, of Madison, New Jersey, for $68 billion. Though the merger made the bigger splash, another Pfizer deal, unveiled in December, could have equally substantial repercussions for biotech. The partnering agreement for Xiaflex with Auxilium Pharmaceuticals may be axiomatic of the type of future deal that New York–based Pfizer, and the rest of the pharma field, hopes to ward off pending revenue loss.

With much of large pharma facing near-term patent expirations on key products, the pressure to quickly replace revenues remains intense. And pharma won't be very selective about the areas involved, one analyst noted. "They'll take anything, as long as it wiggles and it works," says Andrew Weiss, an analyst at Zurich-based Vontobel. That will include 'niche busters'—drugs that successfully target limited markets—in the hopes they will fill in the revenue gaps formed by the multibillion-dollar drugs going off patent (**Box 1**).

For Pfizer, the deal with Auxilium, of Malvern, Pennsylvania, seems to fit the niche-buster profile, though Xiaflex (clostridial collagenase for injection), a bacterial collagenase, might grow into something more. The deal with New York–based Pfizer, which includes an upfront payment of $75 million as well as regulatory and commercial milestones totaling $410 million, is noteworthy not only because of the niche indications involved but also because of its limited geographical scope. Auxilium retains North American rights to the product, while Pfizer has rights for the European Union plus 19 other European and Eurasian countries.

Auxilium's manufacturing plant in Horsham, Pennsylvania. The biotech company has clinched a partnership deal with Pfizer for a novel, first-in-class, late-stage bacterial collagenase to treat painful diseases resulting from collagen build-up.

"It might be unique from that perspective," says Eric Schmidt, senior biotech analyst at Cowen & Co. in New York. "Maybe it's a sign that the biotechnology industry has greater leverage over the US pharma industry than it used to. That can't be a bad thing."

Xiaflex, based on a combination of several subtypes of collagenase enzyme derived from the bacterium *Clostridium histolyticum*, has completed a phase 3 trial in Dupuytren's contracture, and a biologics license application (BLA) filing is expected imminently. It is also undergoing a phase 2b study in Peyronie's disease, with top-line data expected later this year. Each condition arises from a thickening of collagen fibers. Dupuytren's contracture

Box 1 How niche is niche?

Niche medicines have had a good track record of late, featuring prominently in the tally of 25 new drugs and biologics that the US Food and Drug Administration approved last year. For example, two thrombopoietin receptor agonists gained approval for treating immune thrombocytopenic purpura: Nplate (romiplostim), developed by Amgen, of Thousand Oaks, California; and Promacta (eltrombopag), developed by GlaxoSmithKline and Ligand Pharmaceuticals, of San Diego, California. Cinryze, a plasma-derived human C1 inhibitor, gained approval for hereditary angioedema, which is also rare.

In some cases, ultra-niche indications (those with less than 1,000 patients) can lead in to mainstream areas with more commercial potential, says Andrew Weiss, pointing to the research of Mark Fishman, president of Novartis Institutes for BioMedical Research in Boston. His work on the genetically inherited autoimmune condition Muckle-Wells syndrome (MWS) yielded ACZ885 (canakinumab), an anti-interleukin-1 (IL-1) beta antibody. The product is undergoing regulatory review for MWS, but it also could have potential in rheumatoid arthritis, psoriasis, type 2 diabetes and other conditions. "From that point of view you may see more activity in ultra-niche indications," says Weiss.

Regeneron Pharmaceuticals, of Tarrytown, New York, is following a similar strategy. It gained approval in MWS and related cryopyrin-associated periodic syndromes last year for a fusion protein called Arcalyst (rilonacept), which blocks IL-1 beta by acting as a soluble decoy receptor. It is now testing the product in patients with gout.

Edward Stuart of HS LifeSciences is critical, however, of the increasing focus on specialty medicines, which he says misses the most significant contribution that biotech can make in medicine: bringing high levels of innovation to bear on major medical problems. The focus on specialty medicines, he says, is driven by the short-term concerns of venture capital funds. "They have systematically tried to beat the innovation out of companies and turn them into specialty pharma companies," he says. "There's nothing wrong with that, but they shouldn't dress it up as biotech."

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Table 1 Wyeth's approved biologics and vaccines[a]

Product	Indication	2008 revenue (billions)
Enbrel (etanercept)	Rheumatoid arthritis Plaque psoriasis Psoriatic arthritis Ankylosing spondylitis Juvenile idiopathic arthritis	$3.8
Prevnar (pneumococcal-7 valent conjugate vaccine)	Invasive pneumococcal disease	$2.7
Mylotarg (gemtuzumab ozogamicin)	$CD33^+$ acute myeloid leukemia	Not available
Neumega (oprelvekin)	Prevention of thrombocytopenia	Not available
ReFacto[b] (recombinant Factor VIII)	Hemophilia A	Not available
Xyntha (plasma/albumin-free recombinant Factor VIII)	Hemophilia A	Not available

[a]Does not include animal health products. [b]ReFacto no longer available after 31 May 2009.

affects the hand, and makes straightening and extending the fingers difficult. Peyronie's disease causes an upward curvature of the erect penis and can result in pain and impaired sexual function. In each condition, surgical rupture of the collagenous tissue has been the only treatment option.

Schmidt says the niches targeted in the alliance simply constitute business as usual. "Biotech has made a living out of taking exotic indications few have heard of in the past and turning them into cash machines," he says. And that's what Auxilium (and now Pfizer) expect to do here: "It looks like about 3% to 6% of the Caucasian population in the US and Europe have some form of Dupuytren's contracture," says Auxilium spokesman Will Sargent. And Auxilium estimates that about 475,000 men visit urologists in the US and Europe every year seeking treatment for Peyronie's disease. "Pfizer did its own market research, and it matched what we had done," he says.

Though Auxilium sees the product as an eventual blockbuster, that is up for debate, of course. Jon LeCroy, analyst at Natixis Bleichroeder in New York, remains unimpressed with the deal, saying that Xiaflex won't move "Pfizer's earnings at all."

But the Wyeth merger would, or at least would add sales. Through the buyout, Pfizer gets Wyeth's stable of approved drugs, though many of those are facing patent expiration, too. It also gets Wyeth's share of the behemoth biologic Enbrel (etanercept), which has no generic competition, as well as vaccines (**Table 1**).

Though Pfizer's need to fill a revenue gap is the most acute among its peers—its lucrative Lipitor (atorvastatin) franchise, worth $12.4 billion in 2008, will start to unravel in 2010—plenty of companies are under similar pressure and need to make moves (**Box 2**). For example, the hypertension treatment drug Diovan (valsartan), marketed by Novartis, of Basel, Switzerland, which achieved $5.7 billion in sales in 2008, loses patent exclusivity in 2011. "They have a pipeline they can work on, but they have to make sure that it works out," says Weiss.

Perhaps there is a different way forward for pharma, beyond the buyouts and traditional licensing. Edward Stuart, cofounder of HS LifeSciences, of Zurich, is managing a new evergreen, early-stage investment fund called QureInvest. HS LifeSciences has developed a distinctive approach to developing companies, in which it actively avoids venture capital investment and seeks early-stage deals for its portfolio firms that will minimize drug development time and maximize patent-protected sales. Some pharmaceutical firms are open to the model, Stuart says, though successful partnering always takes long-term commitment and a sense of realism on both sides of the table. Big pharma companies have to deploy their power intelligently, while biotech needs to relinquish its traditional swagger. The whole industry must take a step back and re-examine.

It could happen. "Everybody is looking at it pragmatically at the moment," Stuart says.

Cormac Sheridan *Dublin*

Box 2 More pharma consolidation?

The Pfizer-Wyeth merger has some worried that a wave of consolidation may occur among large pharmaceutical firms, leaving cash-strapped biotech companies with fewer potential partners or buyers. The Pfizer-Wyeth merger already scuppered one deal: Wyeth's reported $1.35 billion bid for the vaccine maker Crucell, of Leiden, The Netherlands. But not all pharma firms are planning to take the mega-merger route. London-based GlaxoSmithKline CEO Andrew Witty and Basel, Switzerland–based Novartis CEO Daniel Vasella both recently ruled out such a move, saying they would continue to seek smaller transactions and diversification. AstraZeneca, of London, has said it doesn't need a merger to help itself.

It's not easy telling pharma's future anyway. Edward Stuart of HS LifeSciences points to the recent appointments of new CEOs at many of the top ten pharma companies—including Severin Schwan at Roche, which is attempting to buy Genentech (see p. 212). The fresh blood could affect the culture and the partnering behavior of the companies concerned, and it's hard to know in what direction they will pilot their ships. "These guys are the next generation," he says. "All of these guys are going to put their approaches to work in their [respective] companies." But the basic overall dynamic of big pharma–biotech partnering is unlikely to change dramatically. "I'm guessing it might be difficult to get the attention of the Wyeth business development people in the next couple of weeks. I don't think it's going to take them out of the game in terms of the deals they were looking at," says David King, who sold one of his ventures, the protein engineering firm BioRexis Pharmaceutical, of King of Prussia, Pennsylvania, to Pfizer in 2002.

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Willem 'Pim' Stemmer

One of the most pioneering protein engineers of his generation is also a serial entrepreneur with a flair for spotting new business opportunities.

The same year that Pim Stemmer, a native of Holland, obtained his PhD from the University of Wisconsin, Madison, he founded his first company, Genetic Designs. It was 1985, and his firm was pioneering three key protein engineering technologies: peptide phage display, codon-based synthesis and antibody expression in *Escherichia coli*. Eight years later, working as a research scientist at Affymax, Stemmer invented and developed 'gene shuffling', a process that has since transformed protein engineering. Rather than mutagenize sets of single residues in existing proteins or design new proteins from scratch, Stemmer's technique mimicked natural DNA recombination, producing modified proteins with enhanced or new activities over time frames suitable for commercial development.

The gene shuffling approach proved exceptionally powerful, and the resulting intellectual property formed the basis for Redwood City, California–based biotech Maxygen in 1997. Paradoxically, however, Stemmer is today barred from using it himself. "The perspective that Maxygen took on the portfolio was to control very tightly close to 100 patents," Stemmer says. When he left Maxygen in 2003 to spin off a new company, Avidia, no license was made available. "What I think is too bad is that Maxygen has never widely out-licensed the technology and it is still widely underutilized, or utilized and called something else," he notes rather wistfully.

There seem to be no hard feelings, however. Russell Howard, Maxygen's CEO, has known Stemmer since 1992, when the two worked together at the Affymax Research Institute. "My first impression of him—excited about science; born optimist; creative and always prepared to think differently to provoke debate," Howard recalls. "He is the ideal person to have in the ferment of discovering technologies for a specific purpose when the precise technology path is not clear but the goal is clear and the path seems feasible, if only remotely."

It is not just Stemmer's approach to scientific conundrums that impresses Howard. "He is an entrepreneur prepared to place his money, reputation and bets on ideas that he has for new ways to create biotechnology products."

Stemmer admits to being excited by the intellectual challenges of both business and research. "I really enjoy science concepts, but the entrepreneurial part is especially exciting because I am still learning at a rapid pace. The concepts are newer to me and I have to prove myself more."

Indeed, Stemmer has just launched Versartis, his sixth biotech company, based on the 'versabody' technology held by his current company Amunix, based in Mountain View, California. "Our business model is to spin off additional product development companies for different disease areas," explains Stemmer. "Amunix creates the technologies and products. The spin-off gets a basket of specific compounds to take into the clinic, but the technology stays with Amunix."

The versabody format is inspired by the toxins produced by poisonous snakes, spiders, scorpions and leeches, which are disulfide dense and non-immunogenic. In a versabody, the hydrophobic amino acids that typically form the protein's hydrophobic core are replaced by a disulfide scaffold, resulting in a disulfide-rich 'microprotein' that is smaller, more resistant to proteases and heat, and not recognized by the immune system. Amunix was the brain child of Stemmer and cofounder Volker Schellenberger.

Amunix has also developed what it terms 'recombinant PEG' or 'rPEG'. The rPEG technology is a long (at least 40 residue) unstructured poly amino acid chain (with glycine, aspartate, alanine, serine, threonine, glutamate and proline comprising ~80% of the chain) that, when genetically fused to a therapeutic protein, extends serum half life in a similar manner to polyethylene glycol. Unlike polyethylene glycol, however, rPEG requires no chemical conjugation or repurification, greatly simplifying the manufacturing process. Versartis' first products will be to create rPEG versions of existing biologics (for example, extending a daily injectable therapeutic to a weekly product).

Stemmer has high hopes for his product-driven business model. "The typical biotech scenario starts out with a couple years of research, and as soon as [you have] a product candidate, [you] go out and hire a development group and then lay off most of the research group. Then you sell the whole company in phase 1 or phase 2a," says Stemmer. "For VCs [venture capitalists], this is fine. For scientists, this is not ideal."

Instead, Stemmer thinks that discovery and development should plug and play quite differently. "Amunix is a stable group of about 30 people who invent new, practical technologies and create new products based on them. A set of products then serves as the basis for a spin-off company that is led by an experienced development team of five or six people who outsource everything else," he explains. "It's designed to be a bite-sized piece that a pharmaceutical company can easily acquire."

"I really enjoy science concepts, but the entrepreneurial part is especially exciting because I am still learning at a rapid pace."

Stemmer's capacity to make difficult seem easy has always set him apart. Stephen Del Cardayre, vice president of research for the South San Francisco–based biofuels company LS9, met Stemmer in 1996. "Aside from being one of the most insightful evolutionary technologists around, he is a fire hose of ideas and is unique in his ability to get them implemented," states Del Cardayre.

The power and breadth of gene shuffling means that Stemmer also worries about its use, or more specifically its misuse: "Arguably, it's the most dangerous thing you can do in biology," he says. What's more, he is worried about the potential for "many blue-sky projects to be misunderstood." Indeed, Stemmer is no stranger to controversy, having been criticized by both environmentalists and agrochemical companies when he came up with a scheme to produce non-transgenic seeds from transgenic crops (*Nat. Biotechnol.* **20**, 215–216, 2002).

For that reason, he is happy for others to wrestle with the ethical and societal problems associated with breeding new life forms. But snake venom? No problem.

Crispin Littlehales, Covelo, California

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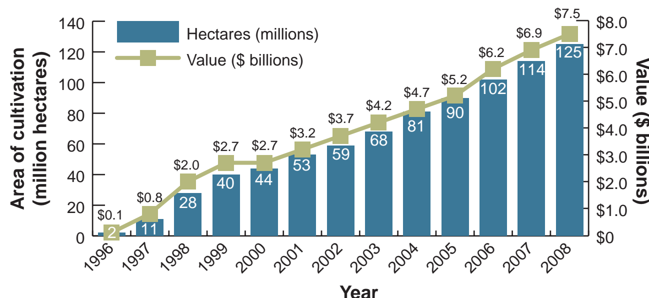
13.3 million farmers cultivate GM crops

Andrew Marshall

Last year 13.3 million farmers in 25 countries planted transgenic crops, over 90% of them in developing nations. It was also the year the second billionth acre of transgenic crop was planted—only 3 years after the first billionth acre was achieved. In Canada and the US, Monsanto (St. Louis) successfully introduced a new biotech crop, glyphosate-resistant sugar beet. Latin America, India and China continued to rapidly adopt GM varieties; 7 of 27 European Union countries cultivated the only transgenic crop approved there (*Bt* maize); France illegally froze its commercial plantings. Stacked traits continue to rise in popularity.

Historical global area of transgenic crops

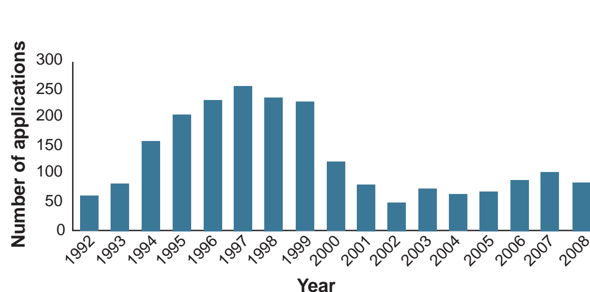
The area planted with transgenic crops rose by ~10% in 2008, with their estimated value climbing by $750 million.



Source: International Service for the Acquisition of Agri-Biotech Applications, Cropnosis

EU transgenic crop field trials

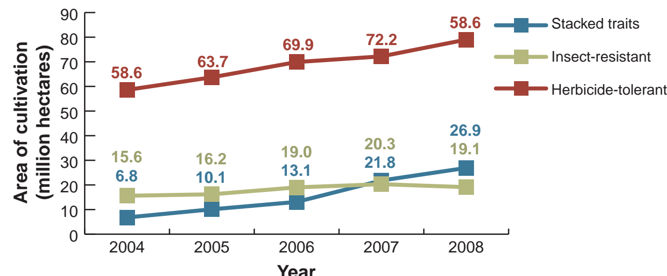
The number of field trials decreased partly as a result of the freeze on planting in France.



Source: European Union, GMO Compass

Global area by transgenic trait

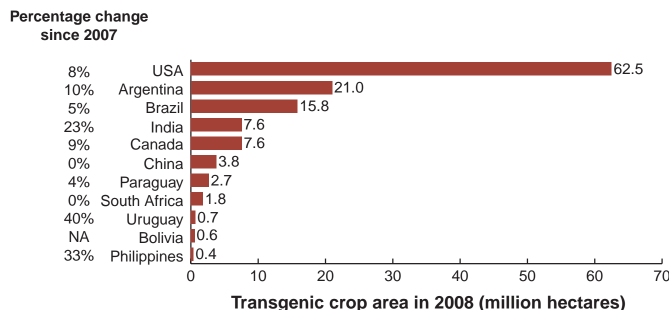
Stacked traits continued to grow in 2008, with 10 countries planting ~27 million hectares.



Source: International Service for the Acquisition of Agri-Biotech Applications

Global area of biotech crops by country

Bolivia became the ninth South American country to plant transgenic crops; India's GM acreage continued to grow, equalling Canada's.



Source: International Service for the Acquisition of Agri-Biotech Applications

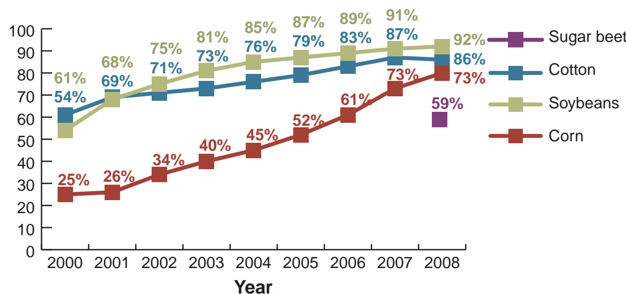
2008 transgenic crop approvals in US and EU

Country	Company	Description	Approval type
US	Pioneer Hi-bred International	98140/Maize resistant to glyphosate and ALS-inhibiting herbicides	Food/feed
US	Syngenta Seeds	MIR162/maize resistant to lepidopteran pests via expression of Bt Vip3Aa	Food/feed
US	Monsanto	MON89034/maize resistant to lepidopteran pests via expression of Bt Cry1A.105 and Cry2Ab2	Environment
US	Pioneer Hi-bred International	DP356043/Soybean resistant to glyphosate and ALS-inhibiting herbicides	Environment
EU	Bayer CropScience	LLCotton25/Cotton resistant to glyphosate herbicide via expression of phosphinothricin acetyl transferase	Food/feed
EU	Aventis CropScience	A2704-12/Soybean resistant to glyphosate herbicide via expression of phosphinothricin acetyl transferase	Food/feed
EU	Monsanto	MON89788/Glyphosate-tolerant soybean	Food/feed

Source: agbios.com

Transgenic crops as a share of total US crops

Herbicide-tolerant sugar beet constituted 59% (258,000 hectares) of the US crop in its first year of adoption.



Source: National Agricultural Statistics Service

Andrew Marshall is Editor, Nature Biotechnology

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Fresh from the biologic pipeline

Randy Osborne reports on the latest product approvals.

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Though still underfunded and low on staff, the US Food and Drug Administration (FDA) managed to approve 21 new molecular entities (NMEs) in 2008, picking up momentum in the second half of the year and breaking a slide in approvals that had continued since the turn of the present century—except for the 'blip' year 2004, when the agency's Center for Drug Evaluation and Research gave its blessing to 36 products. Included in 2008 approvals for NMEs were five new biologics (**Fig. 1**, **Table 1**), a slight uptick from 2007's 16 NMEs and two new biologics. In all, about 80 new drug applications and biologic license applications for medications won clearance in 2008, about the same as 2007, but most involved combo drugs, added dosing forms or new formulations of already approved compounds. The FDA is more focused on safety than ever, as Congress and the nervous public holds regulators and industry to harsh scrutiny.

Sara Radcliffe, vice president of science and regulatory affairs for Washington, DC's Biotechnology Industry Organization, cited "no huge, unwelcome surprises" in approvals. The Food and Drug Administration Amendments Act (FDAAA), signed into law in 2007, is "still shaking out."

New rules

New this year is the Risk Evaluation and Mitigation Strategy (REMS) mechanism, made possible through the FDAAA, which is intended to deal with 'a known or potential' serious hazard of a drug or biologic, taking the surveillance measures one step beyond post-marketing studies. Whether REMS will add steam to approvals, given the other pressures on the FDA, remains an open question.

"The value of REMS will depend on the kinds of processes, tools and methodologies that are available for monitoring drug safety after marketing, and that is something that is very much evolving," Radcliffe says. Ways to carry out the routine, active monitoring and surveillance called for by FDAAA are still evolving too, as officials try to collate information from a wide selection of databases on drugs, patients and how they interact. "A lot of work will have to be done to put in place a system that is what we all want it to be," Radcliffe says. "It's literally a complicated scientific and technical task to get these databases to talk to one another, and then figure out how they can be used to draw conclusions."

Kim Egan, an attorney with DLA Piper in Washington, DC, who often provides consultation for biotech and pharma firms facing potential FDA roadblocks, does not believe the REMS will fling open the window for drug approvals. In fact, she believes just the opposite. "It's having a sort of backward effect," she says. "REMS is simply making Congress more aware and more focused on FDA's safety practices. In a sense, it's attracting unwanted attention and causing [the agency] to be more cautious." The REMS, which includes various provisions whereby safe use is assured and drug reactions are tracked, can be applied to compounds previously approved with the less-stringent risk minimization action plans (RiskMAPs).

Among the higher-profile drugs cleared by the FDA with REMS attached in 2008 are Cimzia (certolizumab pegol), the PEGylated anti–tumor necrosis factor (TNF)-α antibody for adults with Crohn's disease from Brussels-based UCB; Entereg (alvimopan), the μ-opioid-receptor antagonist for postoperative ileus from Adolor, of Exton, Pa., and London-based GlaxoSmithKline (GSK); and Lexiscan (regadenoson), a stress agent for use in radionuclide myocardial perfusion imaging in patients unable to undergo adequate exercise stress, from CV Therapeutics, of Palo Alto, Calif., and Deerfield, Ill.-based Astellas Pharma US.

Egan feels the agency remains "gun shy" after the criticism it received after the high-profile withdrawal in 2004 of the cyclooxygenase-2 (COX-2) inhibitor Vioxx (rofecoxib) for pain from Merck, of Whitehouse Station, N.J., which voluntarily pulled the drug from the market worldwide after data revealed an increased risk of heart attack and stroke in patients dosed for 18 months or more. "It wasn't the start [of the safety crackdown], but it was the event that most normal Americans know about, because so many people know about Vioxx," Egan says. "We thought [the pharmaceutical industry's reputation] was bad when Vioxx was withdrawn, but we feel it's worse now," she adds, as public opinion regarding drug prices and profit margins continues to boil.

The FDA's continued recoil has been strong, and maybe too strong, in her view. A behind-the-scenes trend, she says, has FDA "more and more deciding not to approve something because they don't think it's needed—which is arguably illegal," because the agency's mandate is to determine whether the risk–benefit profile merits marketing clearance, rather than decide whether another drug works similarly on a given indication. "That's happened a lot in the last year," she says. "I think it's going to get worse."

As an example from history that could repeat, Egan cites Arcoxia (etoricoxib), the second-generation COX-2 inhibitor from Merck. At an FDA advisory panel meeting several years ago, Arcoxia's favorable risk-benefit profile "taken in isolation" was acknowledged, Egan notes, though the drug had a potential liver toxicity issue. Arcoxia failed to win approval mainly because New York–based Pfizer's Celebrex (celecoxib) and other nonsteroidal anti-inflammatory drugs already served the ailments that Arcoxia would have targeted, she says, and Arcoxia was considered just one of a group of already available, effective medications rather than as one that should get separate consideration of benefits weighed against risk.

A more recent example is Pfizer's Fablyn (lasofoxifene), a selective estrogen-receptor

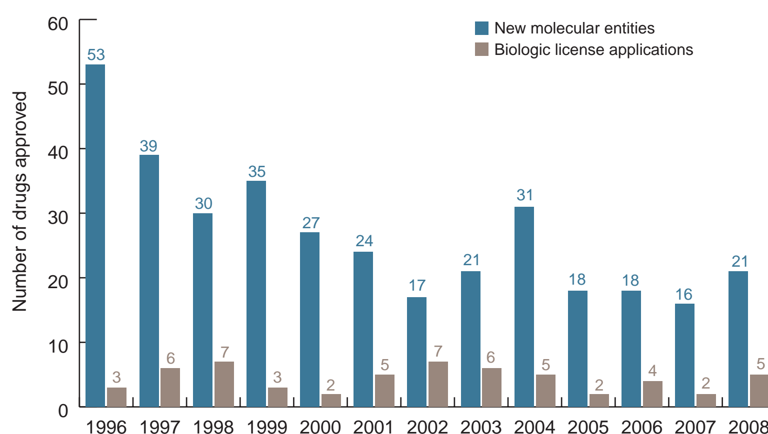

Figure 1 2008 FDA drug approvals.

Table 1 Biologic approvals in 2008[a]

Company/partner	Product	Indication
Amgen	Nplate (romiplostim), a 60-kDa peptide with a thrombopoietin receptor (Mpl)–binding domain	Thrombocytopenia in adults with idiopathic thrombocytopenic purpura
Avant (now Celldex; Needham, Mass.)/GSK	Rotarix (oral live attenuated rotavirus vaccine)	Prevention of rotavirus gastroenteritis
Regeneron	Arcalyst (rilonacept), a single-chain fusion of the extracellular binding domains of IL-1 receptor I and IL-1 receptor accessory protein coupled to Fc portion of a human IgG	Cryopyrin-associated periodic syndromes
UCB	Cimzia (certolizumab pegol), a PEGylated fragment of a humanized antibody to TNF-α	Crohn's disease
ViroPharma	Cinryze (serum-derived complement factor C1-esterase inhibitor)	Prevention of angioedema attacks in patients with HAE
ZymoGenetics (Seattle)/ Bayer (Leverkusen, Germany)	Recothrom (recombinant thrombin)	Inadequately controlled bleeding during surgery

[a]For a list of CDER approvals, see http://www.fda.gov/cder/rdmt/InternetNME08.pdf.

modulator (SERM) designed for postmenopausal osteoporosis (PMO). In September, the FDA's Reproductive Health Drugs Advisory Committee voted 9-3, with one member abstaining, that Fablyn's benefits outweigh its risks in certain populations, but panel members could not agree which groups should get the drug. At the same time, much talk centered on how Fablyn stacked up against Evista (raloxifene), Indianapolis-based Eli Lilly's SERM, the only drug in the class approved for PMO. Fablyn, which Pfizer was developing in partnership with Ligand Pharmaceuticals, of San Diego, had run into problems with the FDA previously. Once branded Oporia, the drug was declared not approved for PMO in 2005, and not approvable for vaginal atrophy the following year, because of concerns regarding the risk of endometrial cancer. Fablyn was thought by some to be a candidate for approval under the REMS umbrella, but the FDA in mid-January

Box 1 The class of 2008

This year's class of approved biologics includes some first-in-class drugs and some drugs for unmet needs, as well as the usual collection of expanded indications of already-approved compounds (**Table 1**). Of the five new drugs, two will be undergoing REMS.

Among the more novel approved drugs is the first using 'trap' technology, Regeneron's Arcalyst (rilanocept), an interleukin (IL)-1 trap—so-called because it combines two receptor domains for IL-1, creating a high-affinity binder, which is fused to an Fc portion of the human immunoglobulin G (IgG) antibody molecule for stability. Rilanocept was approved in February for a niche set of inflammatory diseases, cryopyrin-associated periodic syndromes (CAPS) affecting about 300 people in the US. Most, but not all, CAPS cases are caused by mutations in the NLRP-3 (previously known as CIAS1) gene. The typically used anti-inflammatory drugs do not work against CAPS. Regeneron is testing rilanocept in gout and has a vascular endothelial growth factor trap in phase 3 clinical trials for various cancers and for age-related macular degeneration, so the coming years might see more trap approvals.

A second fusion protein among the new approvals is Amgen's Nplate (romiplostim), a fusion of two thrombopoietin receptor-binding domains and a human IgG1 Fc domain, indicated for chronic thrombocytopenic purpura. Cleared in September, this thrombopoietin mimetic is the only approved drug that raises platelet counts. However, the drug is not without serious side effects, which delayed the approval for several months as the company worked out a REMS program with the FDA. Some Nplate patients end up with fibrous deposits in the bone marrow. When they stop therapy, patients can undergo a 'backlash' effect that sends platelet counts falling even lower than levels measured before treatment.

One milestone approval—and a triumph for its developers—was the October clearance by the FDA of Cinryze, the complement factor 1 (C1)-esterase inhibitor for prophylactic treatment of hereditary angioedema (HAE) developed by Lev Pharmaceuticals of New York. It's the first drug ever for the rare genetic disorder. The compound had been delayed for eight months after regulators asked for more information about manufacturing, and the holdup cast a minor pall over Lev's planned $443 million merger with ViroPharma, which went ahead. Lev had hoped to win approval of Cinryze in prevention and treatment of acute HAE; prophylaxis was an add-on to the original biologics license application. The FDA wanted more data before blessing the drug in acute cases, but in early February it granted Cinryze a priority review in that indication.

Another first: the approval of Recothrom, the only recombinant, plasma-free thrombin cleared for use as a topical hemostat—and the first product to be developed and commercialized by ZymoGenetics, of Seattle. Formerly known as rThrombin, Recothrom won the FDA's nod for use during surgery to control minor bleeding when standard surgical techniques for stopping blood loss are ineffective. The product is made in Chinese hamster ovary cells engineered to produce human thrombin. ZymoGenetics pocketed a $40 million milestone payment from partner Bayer HealthCare Pharmaceuticals, of Wayne, N.J., which owns rights to Recothrom in all markets outside the US.

Cimzia, UCB's PEGylated anti-TNF-α fragment, joins an elite group of antibody fragment drugs, of which only three have been approved in the US. Approved in April for Crohn's disease, Cimzia faces stiff competition in the marketplace from a cadre of TNF-α inhibitors and antibodies, among them Remicade (infliximab, Johnson & Johnson), Humira (adalimumab, Abbott, Abbott Falls, Ill.) and Enbrel (etanercept, Amgen). Working against Cimzia may be some fairly serious side effects, including the potential development of lymphomas and other malignancies; it alone is subject to a black box advisory. The FDA allowed that clinical trials turned up no tumors, but pointed out that the studies were somewhat small and didn't last very long.

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issued a 'complete response' letter, expressing concern over increased mortality among patients taking Fablyn in trials. (A complete response tells a company its new drug application will not be approved in its present form.) In Europe, Committee for Medicinal Products for Human Use recommended the product's approval last December.

A further signal of the hyper-cautious era that might hinder approvals in the future was the FDA decision in December to attach warnings about suicidal thoughts to the labels of marketed antiepileptic drugs, such as Pfizer's Lyrica (pregabalin) and Neurontin (gabapentin); GSK's Lamictal (lamotrigine); and Topamax (topiramate), from the Ortho-McNeil unit of New Brunswick, N.J.-based Johnson & Johnson. The edict followed a July meeting of outside experts to consider the FDA's meta-analyses of 199 placebo-controlled trials of 11 drugs in all, which suggested that patients taking those medications have about twice the risk of suicide versus those taking placebo. In January the FDA had issued an alert about the medications.

One disturbing aspect about the decisions concerning such label warnings, according to Egan, is the emphasis on postmarketing data, which are "unreliable for really assessing risk," even according to opinions previously aired by the FDA itself. At most, such findings should be "tools to identify things to take a real look at," she argues. "Under the new regime, they're being used as final evidence." Egan notes that another drug to get a suicide warning on its label is Paxil (paroxetine), GSK's selective serotonin reuptake inhibitor for depression and panic disorder, "and there's not a single completed suicide in that entire database."

No real trends

Among the standout success stories in 2008 was Treanda (bendamustine hydrochloride), the cell-death promoter for cancer from Frazer, Pa.-based Cephalon. The only NME cleared for two cancer indications in 2008, Treanda won approval for chronic lymphocytic leukemia (CLL) in March and for non-Hodgkin's lymphoma (NHL) in late October. Hopes are high for the compound, though its exact mechanism of action is unknown. Oppenheimer analyst Bret Holley wrote in mid-January that the sales ramp for the product in NHL is likely to beat expectations. Holley pegs the revenue potential in NHL at about twice that of CLL's, adding about $400 million to peak sales that could reach $628 million.

Eisai of Tokyo also scored two NME approvals, but for different compounds, both at the end of 2008. Banzel (rufinamide) won clearance in November as an adjunctive treatment for seizures in adults and children with Lennox-Gastaut syndrome, and given the nod in December was Lusedra (fospropofol disodium), an intravenous sedative-hypnotic agent for monitored anesthesia in adults.

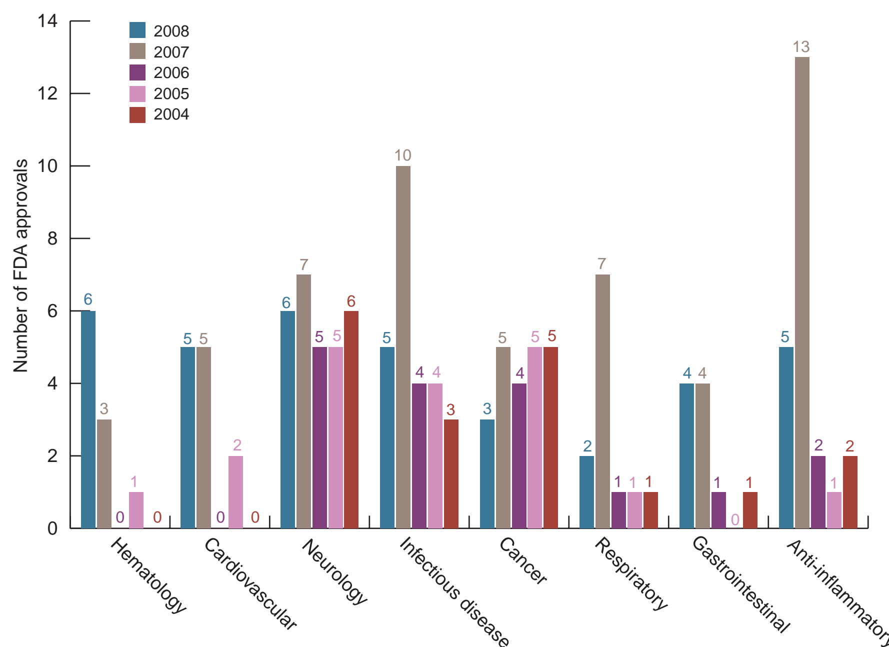

Figure 2 FDA approvals since 2004 according to therapeutic indication. Tallied numbers may be in more than one indication (for example, anti-inflammatory and gastrointestinal). Source: Biocentury, BCIQ.

But biotech firms held their own, coming up with five new biologics that passed muster at the FDA's Center for Drug Evaluation and Research (CDER). These included UCB'S Cimzia (certolizumab pegol); Thousand Oaks, Calif.-based Amgen's platelet booster Nplate (romiplostim); and Tarrytown, New York-based Regeneron Pharmaceuticals Arcalyst (rilonacept), an interleukin (IL)-1 blocker for the treatment of two rare autoinflammatory conditions (**Box 1**). In December, Framingham, Mass.-based Genzyme also received an approval for its CXC-motif chemokine receptor 4 (CXCR4) antagonist Mozobil (pleraxifor) for hematopoietic stem cell mobilization.

The Center for Biologics Evaluation and Research (CBER) also approved seven new biologic license applications last year, with the approval process taking an average of 15.3 months, compared with 18.3 months at CDER. Unlike CDER approvals (almost one-third of which missed their deadlines), CBER met its user fee goals in 2008. Most of the CBER approvals were for combination vaccines, but the center did oversee the approvals of Seattle-based Zymogenetics's Recomthron (recombinant thrombin) for controlling bleeding during surgery and Exton, Pa.-headquartered ViroPharma/LevCinryze (a inhibitor).

Overall, the area of hematology had an unusually productive year, capped with Mozobil's approval and the registration for market of GSK's small molecule Promacta (eltrombopag) and Amgen's biologic Nplate—two thrombopoietin agonists for idiopathic thrombocytopenic purpura (**Fig. 2**). Similar numbers of approvals of medications for gastrointestinal, cardiovascular, infectious, neurological and inflammatory disease were seen last year, although the number of approvals in oncology was down from previous years, which may perhaps reflect a raised bar at the FDA for cancer products (*Nat. Biotechnol.* **26**, 967–969, 2008).

The FDA remained hobbled by lack of personnel, and this showed in missed deadlines. Egan points out that the FDA had funding to hire 1,300 new people but still had about 200 vacancies near the end of the year because it could not find qualified applicants. "It's not like there's a graduate program that feeds into that kind of job," she says. Only 11 drugs approved in 2008 gained a six-month priority review by CDER, and most were NMEs or new biologics from biotech firms. Other compounds and biologics ended up with reviews of 10 months or longer.

R.T. 'Terry' Hisey, vice chairman and US life sciences leader of the consulting firm Deloitte, finds "nothing overly remarkable" in 2008's approvals, and he holds out hope that REMS—and the new administration—will speed them in the year ahead. "What gets approved in a

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given year is a function of so many things," Hisey says. "We're heading for a period of time when the FDA is going to have more resources at their disposal to execute against their mission," thanks to new US president Barack Obama. "I'm actually quite optimistic." REMS, in particular, could be a "significant enabler," he opines, because it involves genotyping patients and figuring out how to handle risks in those who respond.

Hisey has direct experience, having consulted with Biogen Idec, of Cambridge, Mass., in developing the RiskMAP that led to market reintroduction in the summer of 2006 of the troubled multiple sclerosis drug Tysabri (natalizumab), which was compromised by cases of a rare but dangerous brain infection. (The drug is partnered with Dublin-based Elan.) Presumably more rigorous than RiskMAPs, REMS are "conceptually pointed at the right thing—understanding the safety profile, and how you make certain the right people get the right medicines," Hisey says, adding that negotiations with the FDA "went pretty smoothly." He adds that the newer, REMS approach lets the industry "take advantage of a broad set of tools it has at its disposal," such as electronic medical records and those kept by public health agencies. He says he is not against "overt monitoring, when that's indicated as a result of clinical information."

Thanks to the credit crunch and worsening economic picture, many firms will not reach the point where they need to worry about how quickly the FDA will act on a submission. They will run out of cash first. "We're going to see a more acquisitive year in 2009," he says, as biotech companies scramble for cash and pipeline-dry pharma firms snatch up bargains. Such deals, and the shapes they take, will make or break the chances of many new therapies ever reaching the market. Toward helping craft collaborations, Deloitte in June made an acquisition of its own, taking over Recombinant Capital, a San Francisco-based advisory firm that has kept life-sciences industry data for more than 20 years.

Though it will not have an immediate and obvious impact on the number of FDA approvals, the ongoing evolution of commercial models brought about by pressure on capital will keep the pharma pipelines stocked well enough until the crisis is over, Hisey believes. Regulators will have enough to keep them busy. "You'll see a greater use of contract research organizations," he says. "One version of that is less dependency on your own research and more partnering with academic centers and people who are receiving US National Institutes of Health grants."

Richard Hendriks, project analyst with the research and advisory firm Nerac of Tolland, Conn., notes the paucity of NME approvals in cancer as compared with such areas as gastroenterology, but stops short of drawing conclusions. "I can't say there's a trend, because some areas get a lot approvals, others don't, and it varies from year," he says. More biologics are winning approvals for niche indications. He cites Toronto-based Cangene's Accretropin (somatropin), the recombinant human growth hormone for pediatric patients with growth failure caused by inadequate levels or Turner syndrome. Cangene developed the product with Apotex of Weston, Ont., its majority shareholder.

Nervousness at the FDA

Hendriks predicts that approvals will be stymied further by the FDA's safety jitters this year, even with the REMS rule in place. "I don't think it's that simple, though," he says, pointing to a problem larger than regulators. Would-be blockbuster drugs are disappearing, and developers will find themselves focused on smaller disease areas, attempting to personalize their medicines more and more—as a way of making money and, possibly, dodging the side effects that regulators worry so much about. "We're coming to the realization that there are no completely safe drugs," he says, adding that drug companies "obviously can't hide the data, which they have tried to do in the past."

REMS could lead to deeper data drilling and to findings that do not hasten approvals but slow them down, as previously ignored early signs of trouble are pinpointed. Nerac, Hendriks says, found through its own research that some side effects that caused drugs to be taken off the market were clearly recorded in early tests. "It's a complicated story, with lots of money involved," he says dryly. Hendriks is careful to accuse no one of wrongdoing, but notes that his staff's research into tests before clinical work with Avandia (rosiglitazone), the diabetes drug from GSK, found "three key references [that] indicated it had some quite significant effects on the cardiovascular system." That there would be trouble ahead "should have been pretty obvious," he says. For GSK, the scenario has grown even worse lately, as published reports have cited internal emails that suggest the firm's own scientists were concerned about heart-attack risks, though the company had denounced an independent study showing the link.

Hendriks, though, does not blame the drug developers entirely. "Clinical trials are a very inaccurate way" of assessing the final outcomes of drugs, especially with regard to safety, he says, because studies are hard pressed to determine all effects everywhere in the body. "Once the preclinical stuff is done, they really focus on the specific area that the drug is supposed to treat," he adds. "If the drug treats the gastrointestinal tract, they focus there, and not so much on effects in the heart." Therapies tailored through genotyping and a targeted, personalized approached "will overcome that, maybe, to some extent. I don't know what else the drug companies can be doing."

DLA Piper's Egan has a suggestion—though it may not be heeded by most firms, wary of rankling the FDA. If post-marketing "monitoring" is defined as something more scientific and less anecdotal, she notes, fewer drugs will be withdrawn—which will lead to a calmer approach and, ultimately, more approvals. "Hopefully, participants in the industry will be emboldened to say, 'We need a better set of data to look at than phone calls coming in from patients,'" Egan says. "It's the same issue for food safety. It's not acceptable for world commerce to stop selling tomatoes because somebody got sick." Meanwhile, though, Egan believes the FDA will keep, and possibly deepen, its conservative stance. "I bet they're not going to come out very bold in the next year," she says. "They'll use every option they have to delay approvals."

Randall Osborne, Mill Valley, Calif.

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When times get tough

Simcha Jong

With the major economies around the world in recession, what strategic actions should you be taking?

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Turmoil in the financial markets is making the business environment for life sciences companies challenging. The situation is becoming particularly difficult for those involved in running small firms and startups. Although many of the factors in a financial downturn are out of your hands, history suggests several simple steps to steel your business against the long economic winter that lies ahead.

Short-term liquidity: finding new sources of money

Of all the issues troubling small biotech firms today, liquidity has become the most important, for two reasons. First, the initial public offering (IPO) as a near-term exit option for investors has been taken off the table. According to data from Jefferies and Company of New York, capital raised by publicly traded biotech companies was down 62% over the first 9 months of 2008 as compared with the same period in 2007. Across all industries, 2008 was the worst year for IPOs of venture-backed companies in at least 31 years. Most observers agree that the IPO window will not reopen anytime soon.

Second, available venture capital is dwindling fast as a growing number of investors are unable to make good on commitments to existing venture capital funds. Moreover, funding that venture capitalists *do* have at their disposal is mostly frozen into existing investments, and exiting from these investments has become ever more difficult.

This means a liquidity problem for many biotech firms. According to data compiled by the Biotechnology Industry Organization, 25% of the 370 public US biotech companies have less than six months' worth of cash. Among privately owned biotech firms, this figure is likely to be much higher. This makes securing access to cash and credit lines an immediate priority. How should you go about it, and what are your options?

If you need short-term liquidity, you should first seek support from existing private investors—an option that, surprisingly, companies today often overlook. Existing investors already have a stake in a firm's future. In addition, bringing in new investors during periods of financial duress usually comes at a comparatively high price to existing investors in terms of the dilution of their equity. Therefore, existing investors have an interest in providing bridge loans or other types of short-term cash infusion to give managers time to get their act together and reposition their firms.

Second, you may be able to monetize some of your firm's assets. Several specialized financial firms, such as Paul Capital Healthcare of San Francisco, offer financing to early-stage biotech firms against future royalty payments. If you are fortunate enough to be managing a firm with products reaching the clinic, then pursue investors such as Symphony Capital of New York and Cowen Healthcare Royalty Partners of Stamford, Connecticut, as well. These firms specialize in offering financing against existing or future revenues associated with specific clinical development programs. Finally, firms such as Oxford Finance in Alexandria, Virginia, that specialize in offering loans to companies in the life sciences industry might help you access credit lines specifically tied to equipment purchases.

Third, consider a reverse merger. These have become an increasingly popular option for biotech firms seeking to shore up their liquidity. Biotechs with promising pipelines often team up with publicly traded firms with plenty of cash reserves but weak pipelines (see **Box 1**). However, as the number of reverse mergers increased over 2008, the number of public targets for such mergers has decreased, so finding an appropriate partner might be tough.

Fourth, you should exploit the growing number of funding opportunities outside the commercial sector. Apart from enhancing your cash position and credibility in the marketplace, funding from the government or charities generally comes with the added benefit of not diluting equity. As with recently ailing banks, the best hope for you to stay afloat may well be turning to the government for help. Public funding agencies have continued to expand their role in biotech during the current downturn, particularly in fields such as stem cell research, regenerative medicine and cancer research. Data from BioWorld show that public funding agencies committed more than $275 million in support to biotech firms from March to October last year.

You should also approach nonprofit foundations, particularly if your company works in a therapeutic niche area not served by the major pharmaceutical companies. In addition to the larger foundations such as the Wellcome Trust in London and the Bill and Melinda Gates Foundation in Seattle that have shown a growing interest in supporting research and development in biotech, foundations dedicated to specific disease areas have become receptive to funding promising compounds in these areas. According to data from Thomson CenterWatch, funding by patient advocacy groups has increased 13-fold over the level in 2000. For example, the Cystic Fibrosis Foundation of Bethesda, Maryland, has awarded biotech companies more than $300 million over the past 10 years[1].

Recession-proofing your firm: repositioning for long-term success

Once you've gotten some much-needed cash back into your organization, it's time to strategically reorient your business. Convene a meeting among your top managers, and spend time reassessing the firm's long-term objectives and planning. You should reach a consensus about where your company should be in the life

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sciences industry value chain, and then work out a detailed action plan that will lead it in that direction.

The reduced funding available these days coupled with the collapse of several exit options means that you must be more focused than ever in setting long-term objectives. Small biotech firms are notoriously unfocused, with their futures hedged against multiple business opportunities. Many follow two-track strategies, focusing both on the further development of their drug discovery platforms and on various downstream clinical development projects. Before the meeting, prepare a comprehensive list of your firm's current programs and activities. Go over each of the different project and activity items on the list during the meeting and prioritize by assessing for each item how, given the firm's limited resources, it contributes to long-term objectives.

Coming out of such a meeting, the strategic focus of the firm is most likely also going to be different from what it was beforehand. The new strategy will need to neutralize the effects of the changed funding environment by avoiding the financial markets and focusing on growth plans that rely on alternative sources of funding.

For example, firms with innovative drug discovery platforms are currently in favor with pharmaceutical firms. Although the R&D productivity of pharma firms remains abysmal, they continue to have significant cash reserves and excellent credit ratings. As a result, spending by pharma on discovery-stage deals has skyrocketed and can represent an important business opportunity for you. Data from Signals Magazine show that 6 out of 13 $1-billion-plus biopharma deals in 2007 were pure 'discovery deals' (deals that did not involve compounds in clinical trials). Another four were 'discovery oriented' (involving still-to-be-discovered compounds, as well as compounds in clinical trials). The consolidation wave that is set to hit the biotech industry is in many ways a race to create the product offerings that will drive improvements in the R&D performance of pharma firms over the coming years.

Therefore, your firm will be among the winners if you manage to focus on consolidating existing expertise, intellectual property and skills in integrated 'technology suites' for the R&D organizations of pharmaceutical firms. Maintaining a wait-and-see approach toward the consolidation wave will leave you with a dwindling set of options. You should thus try to catch this wave sooner rather than later, and the development of a partnering strategy should be central in any action plan. You should identify potential partners that hold complementary technologies and lay the basis for an exploration of 'horizontal mergers' that will broaden both your drug discovery and development platforms.

This isn't possible for everyone, of course. In particular, if your firm is organized around a virtual business model or focused on a pharmaceutical niche market, then you might have a problem. So far, these are the companies among the first to move into administration. Nevertheless, there are modes of action for even these firms to avoid bankruptcy and salvage shareholder value (see **Box 2**).

Box 1 Cash + pipeline = new company

Publicly traded firms that encounter unexpected hurdles in the clinical trials process sometimes end up with a large pile of cash without projects to spend it on, and they therefore make desirable merger targets. The recent reverse merger announced in September 2008 between publicly traded Novacea, of South San Francisco, and Transcept Pharmaceuticals, of Point Richmond, California, is a good example. Novacea had to abandon phase 3 trials for its core clinical asset, the prostate cancer drug Asentar, and was looking for new investment projects for its $84 million in cash reserves. The management of cash-strapped Transcept convinced Novacea's management that gaining final-stage approval and launching Transcept's insomnia drug Intermezzo would put these reserves to good use.

In a similar deal, privately held and cash-strapped ARCA Biopharma, of Broomfield, Colorado, announced in September its intention to merge with publicly traded Nuvelo of San Carlos, California. Nuvelo had $76 million in cash at the end of June but no meaningful pipeline following the failure of alfimeprase in clinical trials. ARCA Biopharma, in contrast, had a heart failure drug, bucindolol, for which it had filed a new drug application that is under consideration by the FDA.

To make a reverse merger work, it is particularly important to gain support from shareholders in the cash-rich 'shell' company. Shareholders' concerns about the valuation of the cash reserves that go into the new company form a key obstacle in such mergers. Failure to preemptively address these concerns early on will mean significant problems for the merger down the road. This is how the reverse merger, announced in November 2008, between cash-strapped Archemix, of Cambridge, Massachusetts, and publicly traded NitroMed, of Lexington, Massachusetts, ran into trouble. Nitromed had sold its most important clinical asset for a one-off $24.5 million payment in cash and, unlike Archemix, had no promising compounds in the pipeline to spend this cash on. However, NitroMed's shareholder, Chicago-based Deerfield Management, disputes the value that Archemix is able to contribute to the new combination and is trying to block the merger. It instead wants to liquidate NitroMed's assets and distribute the firm's cash reserves directly to shareholders.

Your key stakeholders

Your investors are probably going to pressure you to slash costs, regardless of any strategic realignments you initiate. There are a range of pitfalls associated with cutting costs. To get your business through an economic crisis, you're likely to need to convince key stakeholders, such as your employees, partners and customers, to make sacrifices. However, indiscriminate cost cutting potentially alienates these stakeholders. To avoid harming the firm's long-term strategic position, adhere to several simple principles.

Have a plan. Make any necessary cutbacks sooner rather than later. However, one of the worst mistakes possible is asking key stakeholders to make sacrifices without presenting a clear plan for how those sacrifices will help secure the firm's future. Seemingly arbitrary measures, such as across-the-board budget cuts or requests to trim the budgets of different departments, may create an image of management as incompetent and lost. Be specific.

Transparency. It is particularly important to keep suppliers, partners and customers on board during uncertain times. The economy is making it hard on everyone right now, and survival is largely tied to your success in mobilizing stakeholders behind the new direction. It is therefore advisable to intensify communication with partners by providing regular updates and by being open about the progress you're making in your realignment process. In addition, efforts to strengthen ties with key stakeholders during difficult times often translate into competitive advantages once economic conditions improve.

Negotiate. The economic turmoil is going to decrease demand for key services and products, which means the negotiating position of buyers will strengthen. You will have a number of

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opportunities to renegotiate payment terms. For example, the economic slump has put significant downward pressures on prices of office leases, creating an opportunity to renegotiate tenancy agreements. This could save you considerable money.

Use equity rather than cash. One strategy to preserve cash while also offering key stakeholders an interest in the success of the company is renegotiating terms of contracts so that payments are made in equity rather than cash. Some firms put into effect voluntary salary reduction programs, in which employees may opt to receive a part of their remuneration in equity instead of cash. Hemispherx BioPharma, of Philadelphia, announced in December that it will be using restricted stock to pay up to half the salary of its senior staff members. Consider this option for your own firm.

Layoffs. Management often uses layoffs to signal that it is in control and doing 'something' about the dire situation a firm is in. Although such announcements often have a positive short-term effect on investor confidence, research shows that forced redundancies often have a negative long-term impact on a firm's performance[2]. Apart from the loss of important skills, expertise and experience, redundancies often lead to a breakdown in trust between management and the employees who are left behind. As a result, you could face problems retaining key employees after forced redundancies. Try for voluntary redundancies, salary reduction programs and initiatives through which employees are hired as external consultants—in many cases these are preferable.

If forced redundancies are unavoidable, you should keep several guidelines in mind. First, it is imperative to cut staff once and to make sure such cuts do not drag on. There are few things more damaging to morale than a work environment in which there is a constant threat of further dismissals. Second, communicate clearly how layoffs (and decisions to retain certain employees) link into a long-term strategic plan. This will strengthen workers' confidence in your ability to lead the firm forward. Moreover, it empowers employees who remain during a period of uncertainty and turmoil. Third, it is important to remember that how you interact with those laid off has a direct impact on how survivors view their future. Therefore, magnanimous layoff packages and procedures often repay themselves through the goodwill they create among the remaining employees.

The silver lining

Despite the funding squeeze and the likely slowdown of the broader economy, the long-term prospects for the biotech industry as a whole remain good. Consumers who are the end users of products of biotech firms are not (yet) cutting back on healthcare spending. In fact, markets for healthcare products are, for obvious reasons, among the most recession-proof of markets. Moreover, demand by the major pharma firms for the innovative technologies developed by biotech firms remains strong. Finally, the wave of consolidation hitting the industry means that firms able to weather the current economic slowdown will face less competition in the future. If you recognize the challenges that lie ahead and adjust your financial and strategic plans accordingly, you're likely to be rewarded.

Box 2 Survival tips for niche firms

If you are a manager of a more development-oriented biotech firm without cash and income to fund clinical R&D, you face a particularly hostile business environment. A key priority at this moment should be to make an assessment of what other companies your clinical assets may be valuable to and explore two strategic options.

First try to turn existing partners into buyers. In many cases, they have a stake in the continuity of your operations and an interest in increased control over your assets. As a result, existing partners are often willing to pay market premiums for your business.

This has happened several times in recent months. GeneLabs Technologies in Redwood City, California, in October convinced partner GlaxoSmithKline, of London, to buy it at a price that included a 565% market premium. Memory Pharmaceuticals in Montvale, New Jersey, in November convinced its partner F. Hoffmann–La Roche, of Basel, Switzerland, to buy it at a 319% market premium.

If you have a richer pipeline but not the cash to get any of your products to the market, you should pursue long-term partnership deals with more mature biotech or pharma companies that have significant cash reserves and complementary clinical assets. Under such deals, of which the original Genentech-Roche partnership is the best-known example, an investment is made by the larger firm that gives the biotech firm time and resources to move key compounds through the pipeline. In exchange, the biotech gives up an equity stake and a chunk of the commercialization rights related to its most promising compounds (usually outside its home market).

An example is the November deal between cash-strapped Infinity Pharmaceuticals, of Cambridge, Massachusetts, and privately held Purdue, of Stamford, Connecticut, and its Basel-based affiliate Mundipharma. In the deal, Purdue/Mundipharma will cover Infinity's R&D expenses until the end of 2013 in exchange for an equity stake in Infinity and commercialization rights on most of Infinity's pipeline outside the United States. Infinity maintains commercialization rights for the US market.

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A survey of South-North health biotech collaboration

To the Editor:

In recent years, biotech companies in North America and Europe have increasingly looked to developing countries to find new partners and develop new collaborations. Even though the growth rates of emerging economies like China and India, as well as several sub-Saharan African countries, have been hampered by the current global recession, over the past five years their economies have grown faster than economies anywhere else in the world[1]. This growth has been reflected by growing indigenous private sectors in health biotech that are also taking active steps to strengthen their innovation capabilities[2–4], thereby allowing collaboration to become a two-way street.

In health biotech, substantial benefits are accrued from collaboration between firms in high-income (developed) and low- and middle-income (developing) countries, or what we define as 'the North' and 'the South'. (Note: even though some developed countries, such as Australia, are South of the equator, we still refer to them as from the North. Likewise we refer to some developing countries North of the equator, such as China, as from the South.) Collaboration can minimize costs and share risks because expenditures for R&D and clinical trials are typically lower in the South than in the North[5]. Collaboration between firms in the North and South can also facilitate access to strategic knowledge and resources. This flow of resources is not solely North to South, with developed countries being the providers of knowledge; developing countries have been increasing their expertise in this field and possess other resources, such as indigenous materials, important for health biotech development[6,7]. Furthermore, South-North collaboration can open firms' access to each other's markets. For developing countries, it can be key to gain access to the rich markets in the North, but market opportunities are also flourishing in the South. For example, the economic growth and growing middle-class populations of such countries as China and India are creating an increased demand for resources from abroad. In 2025, the urban middle-classes of China are expected to reach 612 million, increasing their spending fivefold to more than $2.3 trillion a year[8]. In addition, many developing countries have masses of poor people who, because of their large numbers, represent great market opportunities for affordable health products[9].

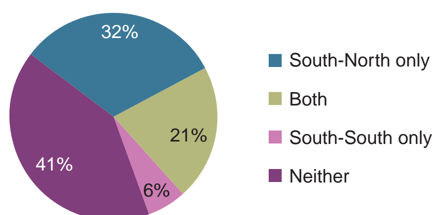

Figure 1 Extent of North-South and South-South firm collaboration, as revealed by percentages of developing countries' firms in South-North versus South-South collaboration.

Very little information is available about the global spread of health biotech alliances and the extent to which the linkages cross the North and the South boundaries. Here, we present data that fill this gap using results from a survey on South-North entrepreneurial collaboration in this field. We surveyed firms working in the health biotech sector in six developing countries—Brazil, China, Cuba, Egypt, India and South Africa—about their international collaborations (see **Supplementary Methods** online for a fuller description of the methodology). These countries were selected on the basis of their position as southern leaders in the field, as identified through previous research on health biotech in developing countries[10,11]. We followed a broad definition of 'collaboration', considering it to be any work jointly undertaken by firms and organizations in developed and developing countries that contributes to the production of knowledge, products or services in health biotech.

We sent the survey to 467 firms and received responses from 288 firms, which constitutes a 62% response rate. We feel this is a solid response rate, given that participation was voluntary, and the fluidity and secrecy of the sector can make it challenging to collect responses. In the analysis, we mapped the extent of South-North entrepreneurial collaboration at the aggregate level reported by firms and organizations in our six focal countries, and compared it with their South-South collaboration levels. We further compared the extent of international collaboration initiatives in each of these main countries and explored where the main linkages lie.

Extent of collaboration

Our survey reveals that South-North entrepreneurial collaboration is considerable (**Fig. 1**). Over half of these firms (53%) reported collaborations with partners in developed countries (32% of firms in South-North collaboration only, and 21% of firms in both South-North and South-South collaborations). Most of the firms reported several collaborations with northern countries amounting to a total of 433 reported South-North collaboration initiatives. This reflects an average of 2.8 collaborations for each firm that is actively engaging in South-North collaborations and is an indication that health biotech firms in developing countries seem to be closely involved in networks with those of developed countries. In comparison, their collaboration with other developing countries is more modest, with about a quarter (27%) of firms reporting at least one South-South collaboration. Almost all the firms in South-South collaboration are also active in South-North collaboration. A total of 41% of the firms in the developing countries we surveyed reported that they had no international collaborations. Collaboration in health biotech is therefore roughly twice as likely to be along the South-North axis as the South-South one.

One example of such a South-North collaboration is that between the major biomedical institution in Brazil, the Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, Brazil), and the large US biotech firm, Genzyme (Framingham, MA, USA). In July 2007, the two came together to further drug discovery in neglected diseases—Fiocruz uses its bioinformatics expertise to identify novel drug targets in *Trypanosoma cruzi*, the causal agent of Chagas

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disease, which are then tested by Genzyme against its high-throughput screening libraries. In this project, the partners are leveraging complementary capabilities in their collaboration and facilitating knowledge flow through initiatives like Genzyme's hosting of a scientist from Fiocruz at its laboratories. Through its Humanitarian Assistance for Neglected Diseases program, Genzyme has an explicit focus on contributing to the public health interests of the countries in which it has strong market interest. And as part of the Brazilian Ministry of Health, Fiocruz is committed to basic applied health research on regional diseases of relevance to Latin America[12].

The countries we studied differed with respect to their extent of international collaboration (**Fig. 2**). Whereas the rates of South-North entrepreneurial collaboration for Brazil, Cuba, India and South Africa ranged from 60–75% of firms, only 33% of Chinese firms and 14% of Egyptian firms reported partnerships with the North. In the latter two countries, collaboration with developed countries does not seem to be a common practice for health biotech firms. This cannot be explained solely by the fact that these countries have large populations and therefore do not need to collaborate outside their borders. Another country with a large population, India, is among the countries that collaborate most with developed countries. India may collaborate more with developed countries because of the greater predominance of English as a second language and the stronger historical links with the UK. Different policy emphasis could also explain the wide difference between countries in their levels of collaborations. Further research is needed to understand why China and Egypt collaborate less with northern countries in the health biotech sector than other countries.

We also observed that comparatively small countries, such as Cuba, are actively collaborating with the North and engaging in a relatively high number of such collaborations (10.5 per firm). We can see that Cuba is also active in South-South collaboration and is the only country we surveyed that has an equal percentage of firms involved in North and South collaboration. Cuba's small size is likely to encourage a more outward orientation. It is also notable how relatively rarely South-South collaboration is done by Chinese and Indian firms. The health biotech firms in China have generally limited international collaboration, whereas Indian firms emphasize much more collaboration with developed than with developing countries, as we saw above. We also observed

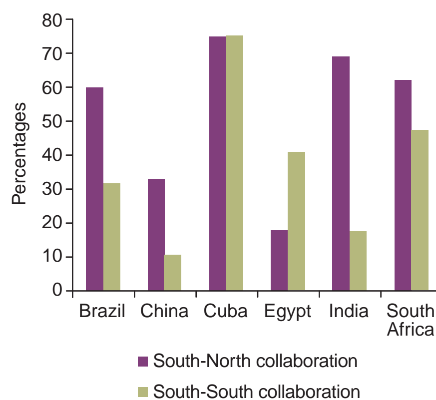

Figure 2 Percentage of firms in international health biotech collaboration, comparing South-North with North-South collaboration in the countries we studied.

that Egypt is the only country we surveyed that has more South-South collaboration than South-North collaboration. It may be because it actively collaborates with other Arabic-speaking countries in North Africa and the Middle East.

Geography of South-North collaborations

To better examine where South-North collaborations in health biotech lie, we created a map of the main linkages (**Fig. 3**) using the software Ucinet 6. This map reveals that firms in the United States are the most common partners (35% of all partnerships) of firms in developing countries. In the map, we observe that China-US and India-US collaborations are particularly frequent. This most likely reflects the dominant position of the United States in the biotech field, both in number of firms and revenues[13]. Even though the United States is not the main northern collaborator of Cuban biotech, entrepreneurial collaborations with the United States still exist, despite the trade embargo against them.

European countries are also common partners of developing countries' health biotech firms, with collaborations with firms in Germany, the UK and France, comprising 8%, 7% and 6% of partnerships, respectively. It is notable that Brazil seems to partner with a relatively high number of European countries, more so than any other surveyed country. Collaboration with Canada is also relatively common, with 6% of the developing countries' collaborations occurring with Canadian partners.

To normalize the data according to the size of the respective biotech sectors in each country, we adjusted our analysis using data from the Organization for Economic Cooperation and Development (OECD; Paris)[13] on the number of firms in each region (see **Supplementary Methods** online). Even when adjusting for this, the United States stands out as developing countries' main collaborator, with 0.0747 South-North collaborations per US biotech firm. This compares with 0.0571, 0.0550 and 0.0263 southern collaborations per firm from Canada, Europe and Australia, respectively.

Regional biases in collaborations were also evident; for example, our data show that among the six developing countries we surveyed, China has the highest number of collaborations with firms in Japan, South Korea and Australia, indicating East Asia and Pacific networks. This is also the case with South-South collaboration where firms originate within Latin America; those within southern Africa also partner heavily within their regional networks. Furthermore, we observe the influence of past historical ties on South-North partnering patterns. Developing countries collaborate relatively frequently with their former colonial powers, which may be partly because they share languages and/or have similar institutional environments. For example, Brazilian firms collaborate as frequently with Portugal as with the UK (even though the former country is ranked by OECD to be relatively weak in health biotech, whereas the latter country is ranked as a strong country in the field)[13]. Cuban firms' collaborations with Spanish organizations are also notable in our survey, as are the relatively high rates of collaboration of Indian and South African firms with the UK.

Conclusions

By examining the extent and geography of South-North collaboration of six leading developing countries, our survey provides an indication of how closely developing countries' firms are tied to northern health biotech networks. The findings of the study may be useful for firms in the North and the South considering global expansion, for research groups considering entrepreneurial alliances and in informing innovation, development and foreign affairs policies in both developed and developing countries. The data presented on the level and characteristics of firm collaboration can also be used as a reference by future studies on this topic, thereby affording the possibility to evaluate changes over time and the successes of initiatives for promoting South-North collaboration.

As with any survey, several caveats apply. For logistical reasons, we had to restrain our data collection to a few (six) select countries

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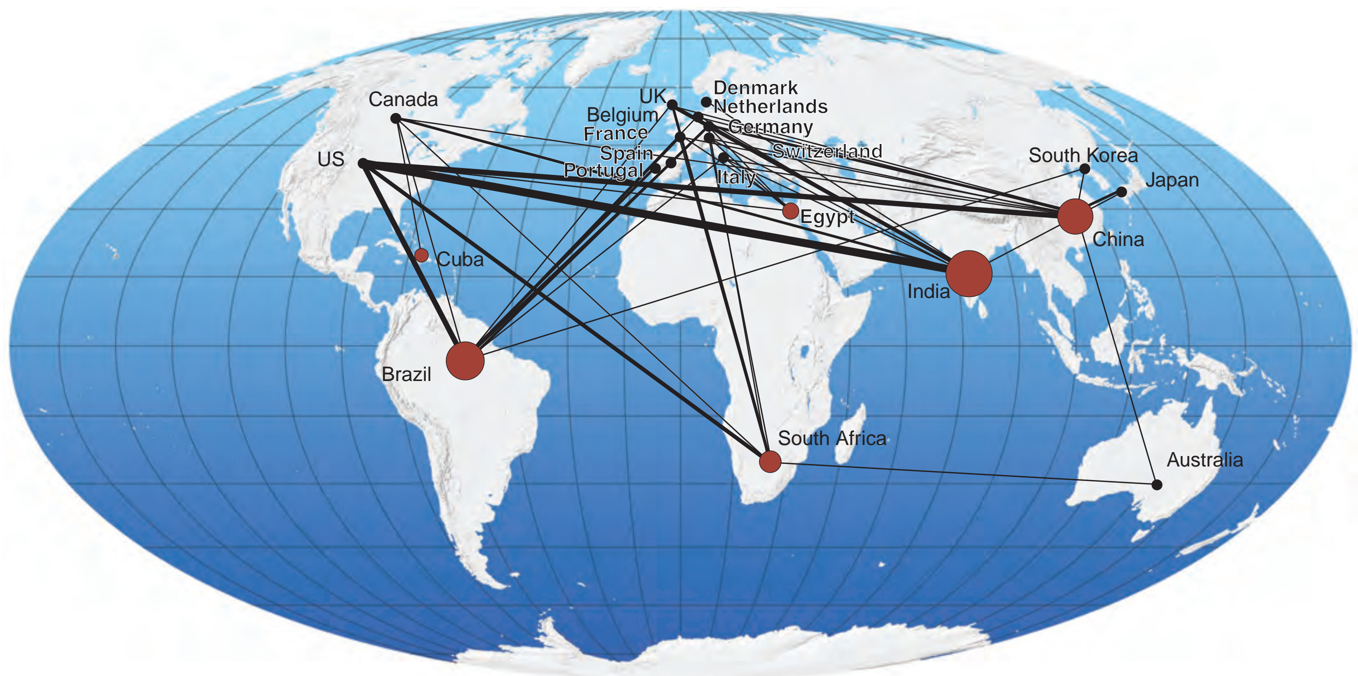

Figure 3 North-South firm collaboration in health biotech. The size of node represents the total number of South-North collaborations for the country, whereas the width of the lines represents the number of collaborations between two linked countries. To ease the representation of partnerships, only linkages of three or more collaborations were included on this world map.

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that were likely to represent the bulk of developing countries' firms most active in health biotech and therefore most likely to be collaborating with northern firms. We have also not been able to receive information from every firm active in health biotech in the six countries surveyed, and the firms we contacted may not have reported to us the extent of characteristics of all their South-North collaboration. As we obtained a reasonable response rate, we nonetheless believe that the results accurately represent the extent and geography of South-North firm collaboration in the health biotech field.

Our main conclusions are that South-North collaborations are common practice in health biotech. The results show that over half of the firms in Brazil, China, Cuba, Egypt, India and South Africa are actively collaborating with countries from the North. In comparison, only around a quarter of these firms are actively collaborating with other developing countries. Developing countries' firms therefore seem to be tied more closely in South-North health biotech networks than South-South networks.

Second, the extent of collaboration varies widely among countries from the South. Some of the developing countries we focused on seem to rely heavily on collaboration with northern countries (e.g., over two-thirds of Indian firms actively collaborate with developed countries), whereas others do not (e.g., in China only around one-third of firms collaborate with developed countries). Further research is needed to identify whether Chinese firms don't need international collaboration or if a lack of English knowledge or other hindrance tends to limit the international collaboration of Chinese firms.

Third, South-North collaborations are focused heavily on the United States for all the developing countries surveyed, except Cuba. This likely reflects the dominance of the United States in the global biotech scene in both capitalization and expertise, two factors that are likely to facilitate the flow of knowledge and catalyze collaboration.

And finally, it is clear that collaboration is influenced by historical ties between countries. The effects of colonial legacies are evident in the regional patterning of partnerships, and some developing countries have stronger relationships with their former colonial power than we would have expected, considering the latter's respective strength in the health biotech sector. This is somewhat surprising as colonial ties were formally severed in these countries before the birth of the biotech sectors during the 1980s and 1990s. Even so, shared bureaucratic structures as well as official languages are likely to continue to promote tighter South-North linkages between such countries.

As a large proportion of health biotech firms in the leading developing countries are collaborating with firms in North America and Europe, they are positioning themselves to access different types of strategic knowledge (e.g., technical, marketing, regulatory) that can be gained only through tight firm linkages with a northern partner. Developing countries with little or no South-North collaboration should thoroughly assess whether they are losing out on such opportunities by not emphasizing South-North collaboration more fully, and, if so, come up with ways to strengthen their international collaborations.

Collaboration with developing countries can also be directly beneficial to northern countries. When developed countries experience economic recession, they should pay attention to global opportunities to help them survive hard times, as South-North collaboration can give firms in developed countries increased opportunities to reach developing countries' markets. This helps mitigate the losses they might experience in difficult economic conditions in their own countries. But they can also go further and collaborate with developing countries' firms in the research and development stages of their health biotech activities, thereby lowering the cost of getting products on the market. It is important that firms, researchers and governments all over the world realize that opportunities in the health biotech field are not confined to a handful of northern coun-

tries and that a global approach is required to advance the development of this field.

Note: Supplementary information is available on the Nature Biotechnology website.

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BIO and the next generation

To the Editor:
As president and CEO of the Biotechnology Industry Organization (BIO; Washington, DC, USA), I fully support the premise of the editorial in your December issue[1]: educating, cultivating and encouraging young people to lead the next generation of biotech innovation are critical to the continued growth and success of biotech. That is why BIO developed the Biotechnology Institute (Arlington, VA, USA) in 1998 to help promote early and expanded science, technology, engineering and math education and training initiatives.

BIO continues to promote increased investment in education. Currently, we are working with Battelle, a leading science and technology enterprise, and the Biotechnology Institute to survey all 50 states to identify how states are promoting science education. We will release the results of this survey during the BIO International Convention being held in Atlanta on May 18–21, 2009.

More directly, the career fair we hold during our international convention tactically assists companies in recruiting young talent while our "Growing the Biotech Workforce Track" of educational sessions provide attendees with detailed information, recruitment and training tools.

And we are reaching out to young people where they congregate—online. We are leveraging the power of Facebook, Twitter, YouTube, Delicious, LinkedIn and other social networking sites to develop communities to educate, proselytize and promote biotech. We have launched several blogs and podcasts covering a range of issues, including food and fuel, innovation, climate change and general biotech advances that spotlight the scope and power of biotech. And through our new, broad-based public education and communications effort, we are establishing new online communities, such as http://iam-biotech.org/, to showcase the contributions being made by the biotech community to address the world's most pressing issues.

We agree it is imperative to support entrepreneurship as well as education. That is why BIO runs events like the BIO National Venture Conference. This event features seed-stage and A-round companies that go through an aggressive screening process. Our efforts to find appropriate companies for this event include working with key technology transfer offices and outreach to business plan competitions.

In addition, BIO conducts considerable education courses for new entrepreneurs. BIO has, for several years, conducted CEO presentation workshops. These workshops are dedicated to new entrepreneurs, providing guidance and real-time practice in how to make presentations to investors, which is one of the key hurdles for scientists seeking funding.

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Bonsai biotech

To the Editor:

Your editorial on bailing out biotech[1] makes some good points, but misses a key one. The UK biotech industry is not a tale of unalloyed woe. Some companies' stock prices have paced the market or beaten it, some have even risen in the past six months. Both US and UK biotech stock indices have risen at a time of substantial stock market falls[2]. In general, the successful companies are those that have not had venture capital (VC) backing and have based their business on revenue rather than capital financing. Those that have been successful and were once VC-based have shed that legacy and have built up cash reserves through doing real trading. In the UK at least, business angel investment in startups seems to be buoyant, providing that those companies' business plans do not call for substantial further investment that would destroy angel value through punitive investment terms. In other words, the parts of the industry that are not dependent on institutional investors are suffering but surviving. It is only the VC-backed UK biotech industry that, in Sir Christopher Evans' words, is looking into the abyss of "an indefinite downward spiral until the very real prospect of short-term extinction."

The reasons for this are complex and need not be rehashed here. I have elaborated on them at length elsewhere[3]. Suffice to say that the VC management teams' business model in the UK, and largely elsewhere in Europe, differs greatly from that of the classic 'venture' investor in the United States, depending on management fees and rapid, continual fundraising for its profits rather than carried interest in companies well invested, well managed and well exited. This dependence on fundraising means that investee companies are used to support fundraising in a way that is often not compatible with their long-term survival, let alone growth. The result is a UK industry, which was on a par with the United States on a *per capita* basis in the early 1980s, that has shrunk steadily to a bonsai imitation of a real industrial sector in 2009. The credit crunch and associated banking collapse is just one more deadly chop at the roots of a sector already pruned to near extinction.

To give £1 billion to the industry that created those problems seems foolish, especially to establish a fund for mergers and acquisitions (M&A) when work published in your journal[4] has shown that mergers, in the hands of UK investors, are at best neutral to company survival, instead being principally a vehicle for shareholding manipulation. It would, of course, be attractive to investors: 2% management fee on two £500 million funds is £20 million fee income per annum. But it will do nothing for the biotech industry. Co-investment with overseas investors would dilute the European investment model, but with the associated risk of infecting US investors with a fees-driven, profit-blind, business model.

A much better use of such funding would be to revitalize the science base with capital for genuinely visionary programs. The biotech revolution arose out of the science of the 1960s, when funding was famously unconstricted by demands for obvious gain or guaranteed output in terms of papers, patents and spin-outs. Plasmid biology and phage host range genetics, which created the tools of recombinant DNA technology on which Amgen (Thousand Oaks, CA, USA), Genzyme (Framingham, MA, USA), Genentech (S. San Francisco, CA, USA), Chiron (Emeryville, CA, USA), Cetus (Emeryville, CA, USA) and Biogen (Cambridge, MA, USA) were founded, were odd backwaters with no obvious use and researched by tiny, maverick groups that would get no funding today. More than bailing out a failed investment model, we need to reinvigorate brilliant, curiosity-driven, blue-skies research. Good business will follow. It would keep scientists in jobs, too.

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Proliferation-resistant biotechnology: an approach to improve biological security

Ali Nouri & Christopher F Chyba

Is there a way to design DNA synthesis technology with safeguards that prevent its cooption for nefarious purposes?

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To prevent the application of pathogenic genes and genomes to the production of biological weapons, some commercial DNA providers now screen orders so that potentially dangerous sequences are not synthesized. However, new and innovative approaches and declining development costs could enable the diffusion of advanced synthesizers from a few centralized locations to an increasing number of facilities and perhaps even individual laboratories, rendering the current risk-management framework obsolete. To prepare for this possibility, we propose the development of 'proliferation-resistant biotechnology'—safeguards intrinsic to emerging technologies that will ensure that nefarious applications are hindered while benefits are preserved. As biotechnologies become increasingly automated, such safeguard strategies can become effective tools for managing risks in the life sciences.

Emerging technologies

Biotechnological advances underlie a scale and pace of biological research never before seen. Plunging DNA sequencing costs have made a $1,000 human genome a realistic goal. *De novo* DNA synthesis technologies now automate the assembly of long DNA molecules from sequence data alone. Just recently, the J. Craig Venter Institute (Rockville, MD) announced the chemical synthesis of a minimal bacterial chromosome—over half a million nucleotides[1]. Further cost reductions for this technology may transform molecular biology, resulting in the replacement of conventional gene cloning techniques by automated DNA synthesis. But the technology is dual use: although beneficial for biological research, it can also be applied toward the production of biological weapons by states, non-state groups and even individuals. For instance, many viral genomes have been sequenced; these are typically small and well within the limits of commercial DNA synthesis. High-profile scientific publications have already demonstrated the application of *de novo* DNA synthesis to the creation of the poliovirus[2], as well as the otherwise extinct Spanish influenza virus[3]—the agent that is estimated to have killed around 50 million people in the pandemic that began in 1918.

As DNA synthesis technology increases in power, proliferation-resistant approaches should be incorporated to prevent its cooption for nefarious purposes.

Although DNA synthesizers can be readily purchased, using these to build large genes and viral genomes is technically challenging and time-consuming and requires considerable material. At the same time, these DNA molecules can be obtained almost effortlessly, within days or weeks, from commercial entities that employ more advanced technologies. These DNA providers are aware of the risks,

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and, to protect against illicit acquisition of sequences that could subsequently be transcribed and translated into infectious agents, some have begun to regulate themselves. In the United States, these procedures include screening all incoming DNA orders so that genomes and genes of federally regulated pathogens and toxins, respectively, are not synthesized (except for researchers permitted to work with these regulated agents)[4–6]. These voluntary measures are likely to be formalized into mandatory ones; the major US biosecurity panel, the National Science Advisory Board for Biosecurity (Washington, DC), has called for governmental oversight of commercial DNA synthesis[7].

These regulatory schemes may help to prevent the misuse of commercially provided DNA molecules, but they will only be effective so long as the underlying technologies remain centralized at a relatively small number of facilities. Meanwhile, increasing demand has made large-DNA synthesis a competitive area, resulting in the development of multiple platforms[8–10], all of which have potential for automation. One possible outcome of this could be the diffusion of more advanced synthesizers to large numbers of users, making the current risk-management framework increasingly irrelevant. To prepare for this possibility, alternative nonproliferation proposals need to be explored.

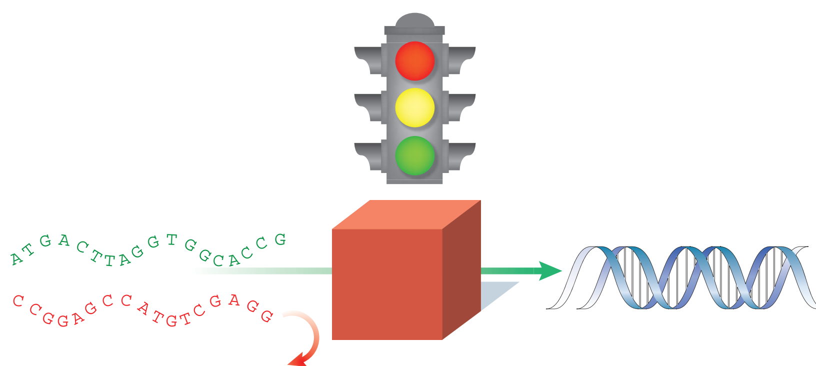

Figure 1 Proliferation-resistant biotech. Advanced biotechnologies, such as DNA synthesis, are becoming increasingly automated and black boxed, providing even novice researchers with powerful tools. This automation, however, also provides an opportunity to incorporate intrinsic safeguards that block illicit sequences (red) from being synthesized, while allowing legitimate ones (green).

Conventional nonproliferation strategies

An important consideration for nonproliferation strategies in any dual-use area is that the efforts not unnecessarily hamper the technology's benefits. This is especially true for biotech, which has critical implications for improving human health and agriculture. What makes this an even greater challenge is that the biological research process—whether legitimate or nefarious—lacks obvious bottlenecks that might be amenable to safeguards.

This is profoundly different from the situation with nuclear energy, where nonproliferation strategies take advantage of the fact that paths toward nuclear weapons pass through the severe bottlenecks of either highly enriching uranium, or producing and reprocessing plutonium. These bottlenecks serve as the basis for the extensive monitoring that underlies the nuclear nonproliferation regime. As a recent illustration, consider the monitoring conducted by the International Atomic Energy Agency (IAEA) at the Chinese gas-centrifuge uranium enrichment plant at Shaanxi. The IAEA installed into this facility equipment that monitors the uranium flow rate and enrichment levels, to "provide continuous unattended monitoring of enrichment levels and quantity of the product"[11]. Any efforts to divert uranium, or enrich it beyond the level appropriate for peaceful use, would thereby be automatically detected.

This difference between most industrial-level nuclear processes and laboratory biotech guarantees that many analogies between nuclear and biological nonproliferation strategies fail badly and that very different approaches are needed for the two cases[12]. Meeting the challenge of biotech requires a web of measures, most of which little resemble the approaches deployed to prevent nuclear proliferation[13]. Nevertheless, an important strand in this web of prevention may prove to be nonproliferation measures—but only if appropriately conceived.

Rather than depending on any industrial-scale bottleneck processes analogous to those needed to produce weapons-useable nuclear material, biotech predominantly relies on skilled, knowledgeable individuals who employ readily available tools and even renewable reagents (such as bacterial-derived restriction enzymes or competent bacteria for transforming DNA). The lack of obvious intervention points has left the life sciences with overly broad nonproliferation proposals of a restrictive (for example, curbing access to technologies and know-how) or intrusive nature (for example, physical inspections of laboratories that conduct biological research)[14].

There are legitimate concerns, however, that restrictive or intrusive nonproliferation proposals will hurt scientific progress, as well as hindering robust responses to any disease outbreak, whether natural or intentional. So instead, proposals of a softer nature have gained traction. For example, through the Biological Weapons Convention, countries have come to rely on self-reporting of research activities, increased awareness-raising and the adoption of codes of conduct as primary mechanisms by which dual-use biotech is addressed. Although these strategies are essential for establishing and strengthening norms against misuse, by themselves they cannot prevent any aspiring illicit actor. Proliferation-resistant technologies could begin to fill this gap.

Proliferation resistance

Proliferation-resistance strategies arose as a way to help manage dual-use nuclear technologies. Such intrinsic safeguards are intended to hinder the diversion of technologies for weapons-grade nuclear material production while allowing the peaceful applications of the technology. Currently, life science tools are undergoing rapid transformation, from manual technologies to ones that are increasingly automated. This automation provides an opportunity to incorporate safeguards into the technologies themselves, so that only their nefarious applications are hindered. In the case of DNA synthesis, for instance, safeguards could include the implementation of DNA screening software into synthesizers so that a subset of sequences, such as toxins and pathogen genomes, cannot be illicitly synthesized (**Fig. 1**). To determine the set of sequences that would be disallowed, a pre-existing regulatory framework that applies to the possession of pathogens and toxins of concern could be extended to their DNA sequences. In the United States, for instance, possessing these agents of concern requires licensing by the Centers for Disease Control and Prevention or the Department of Agriculture. Sequencing discrimination would be more challenging for genetic material that is very similar, but not identical, to agents of concern, such as those belonging to particular vaccine strains. Any

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future regulatory framework that attempts to extend licensing procedures from pathogens and toxins to their sequences would have to address this, perhaps by restricting sequences that cross a certain threshold and become too similar to an agent of concern.

For researchers who would be registered to perform experiments with the genetic material of agents of concern, a software update (such as a downloadable patch) could be obtained to bypass certain restrictions. Just as US federal law prohibits the transfer of these agents of concern to unauthorized users, software updates and patches could follow a similar regulatory framework, while also incorporating requirements specific to individual machines. To account for regulatory changes, such as those needed to address novel pathogens or toxins, software could be updated on a regular basis.

Detection of illicit activity by users might be accomplished if synthesizers were designed to operate only when online, in a transparent manner whereby any software manipulation would be revealed to the online community. This approach does have some drawbacks, such as increasing access and, thus, vulnerability of synthesizers to the online community. These concerns could be alleviated in part via stronger schemes that might include the incorporation of computer chips into synthesizers to block the production of certain sequences. Moreover, because biotech advances tend to outpace the government regulatory process, data chips or even synthesizers could be regularly modified or replaced with improved versions that were also updated to comply with any changes to the regulated list of pathogens and toxins.

This system of DNA synthesis screening at the machine level would not replace the university and agency-level human experiment review that occurs in some countries but would rather complement it as another strand in an overall web of prevention.

These approaches might also be applied more broadly to other emerging biotechnologies, such as some in the fledgling field of proteomics. The *de novo* synthesis of amino acids from chemical precursors, for instance, enables construction of proteins of around 300 amino acid residues in size—putting a number of human protein toxins well within reach. The technology lowers required expertise in molecular biology and biochemistry techniques, enabling a relative novice to construct proteins of any sequence with any desired amino acid modifications, which can further influence protein activity. Similar to *de novo* DNA synthesis safeguards, screening software could be employed in future automated protein synthesizers to prohibit the construction of particular toxic gene products.

A concept analogous to these strategies might be found in the V-chip, a feature that can block the display of television programming of a particular rating. The V-chip, however, is intended only to exert parental control over television viewing and can easily be reprogrammed and even disabled. In the case of dual-use biotechnologies, such security measures would require more stringent criteria along the lines discussed above.

The way forward

Proliferation-resistant safeguards could be designed during the initial development of new technologies. A way to achieve this is to create incentives through special funding for innovators. Since the 2001 mail anthrax attacks, the US federal government has spent over $40 billion just on civilian biodefense projects[15]. A large portion of this is dedicated to developing countermeasures (such as vaccines and drugs) and surveillance and detection tools, but to our knowledge, virtually no funding is allocated for developing biotechnologies that are intrinsically more secure. Designing and deploying these would help to prevent misuse of the technology, thus relieving some of the need to develop measures aimed at neutralizing laboratory-generated pathogens.

Deploying proliferation-resistant biotechnologies first requires that rules for possessing organisms and toxins of concern be extended to their genetic sequences. A greater challenge, however, will be to extend these rules internationally. Many countries lack a regulatory framework for dealing with such agents, let alone their genetic material. And for those that do have a framework in place, perceived biological threats vary greatly, leaving many challenges to the creation of a harmonized global framework. Given the international dimension of life science research, however, any comprehensive biological security strategy should be international in scope and should include improved rules for the possession and sharing of biological agents—and their genetic material—both within and among nations. Finally, by themselves, the technical safeguards discussed here will not alleviate all the risks that arise from the illicit genetic engineering of pathogens and toxins. Rather, these safeguards should be regarded as one component that, together with other measures, constitute a web of prevention[13] to reduce the likelihood of production and deployment of biological weapons.

Although certain advanced biotechnologies still occupy a niche market, declining costs will make them increasingly dominant. Conventional molecular biology techniques that are used to construct and manipulate DNA molecules, for instance, are likely to eventually be replaced by the faster, cheaper and almost effortless *de novo* synthesis. As these new automated technologies begin to replace older, manual ones, there is an opportunity to introduce proliferation-resistant safeguards into the newer generation of biotechnologies. Gradually, improved automated technologies that are also safeguard-friendly will replace the older, less efficient and difficult-to-safeguard tools. This means that, if managed properly, the revolution in synthetic biology need not increase the risk of misuse but could rather improve biological security.

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Biotech under Barack

Jeffrey L Fox

The Obama administration looks to be a welcome shot in the arm for the scientific endeavor, but the current economic crisis is likely to keep several issues of key interest to biotech firmly on the back burner.

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Barack Obama came into office with campaign promises of keen interest to the biotech industry, including commitments to overhaul the US healthcare system, to lift restrictions on federal funding for human embryonic stem (hES) cell research and to increase focus and funding for science (**Box 1**). But the economic crisis that weighs down the US economy, as well as skirmishes with the US Senate over key Cabinet and top-level appointees, has kept the new administration from dealing with much else during its first weeks in office. The anticipated changes in the healthcare system, if realized, are widely expected to lower the pricing of biological therapeutics by ushering in biogenerics, also known as follow-on biologics, sooner rather than later. Healthcare reforms also might introduce a system for evaluating therapeutic regimens on a cost and comparative-efficacy basis.

In addition to politically thorny issues embedded in healthcare reform, other more familiar matters regarding how well the US Food and Drug Administration (FDA) functions and whether it has strong leadership and adequate resources are attracting attention from those tracking biotech policy as this new regime settles in. Many other issues, however, remain unresolved and there is a general sense that continuity will reign until some bigger and more-pressing issues are under a semblance of control. Nonetheless, some Obama comments, including his strong endorsement of the value of science during the inaugural address, are lifting spirits among researchers inside and outside the biomedical sciences, including many within the biotech industry.

Positive signals

Even with little in terms of explicit biotech policies being spelled out since the election, diverse policy watchers who follow biotech developments express optimism about the Obama administration, its Cabinet and other changes in leadership, including in Congress. Thus, for example, Obama's anticipated change in policy that promises to provide strong and broadened federal support for research on hES cells is boosting morale among researchers across many sectors.

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MATTHEW CAVANAUGH/epa/Corbis

Obama puts his signature on his first bill, the State Children's Health Insurance Program (SCHIP), a "down payment," he says, on his commitment to cover every American.

In practical terms, Obama can reverse Bush's restrictive stem-cell policy simply by means of an executive order. However, that approach risks leaving the issue open to further reversals by his successors. Another possibility is that Obama might join forces with Congress to push through legislation ensuring more-permanent federal support for hES cell research. If the votes are there, congressional staffers say, this issue might help build momentum to address other more challenging political issues, including healthcare reform itself.

In February, Representative Diana DeGette, (D-CO) introduced legislation to authorize hES cell research and to provide for the development of guidelines for such research by the National Institutes of Health (NIH; **Table 1**).

"Until they're ready for a public announcement, the Obama [administration] is very disciplined, and everyone is being careful" before formally changing the policy, according to Robert Cook-Deegan, director of the Center for Genome Ethics, Law & Policy at the Duke Institute for Genome Sciences and Policy (Durham, NC, USA). "Obama has said he'd like legislation, which is more permanent, but neither he nor DeGette is taking the possibility of an executive order off the table."

Jim Greenwood, president of the Biotechnology Industry Organization (BIO, Washington DC, USA) "applauds" Obama's intentions to change hES cell research policies at the federal level. Of course, Greenwood

Box 1 Excerpts from Obama campaign priorities

- Double federal funding for basic research over ten years
- Support investments in biomedical research, fund biomedical research and make it more efficient by improving coordination within government and across government, private and nonprofit partnerships
- Support increased hES cell research on a wider array of stem cell lines
- Build capacity to mitigate the consequences of bioterror attacks
- Create new drugs, vaccines and diagnostic tests, and manufacture them more quickly and efficiently
- Promote international efforts to develop new diagnostics, vaccines and medicines that will be available and affordable in all parts of the world
- Make the R&D tax credit permanent
- Foster a business and regulatory landscape in which entrepreneurs and small businesses can thrive and startups launch
- Protect American IP, give the PTO resources to improve patent quality and open up the patent process to citizen review

adds, getting this particular policy "right" is not enough to ensure commercial development. That kind of success will depend on many federal policies, including those affecting FDA, drug pricing, patents, taxes and the Small Business Administration. Broadening federal support for hES cell research, he says, is "just the beginning of a long process leading to lifesaving drugs."

Nonetheless, with a simple gesture that now could also be backed by legislation, Obama is poised to remove a nagging obstacle blocking researchers keen on moving forward in this biomedical subspecialty, which many see as highly promising. On a more symbolic level, this change also will help to repair a badly damaged breach between biologists and the White House, which also appeals widely to those working in other areas of science and engineering. "No doubt the [Obama] administration will be different in a good way, with a real commitment to medical research and science policy in terms of stem-cell research and for funding for the National Institutes of Health," says Michael Werner, who heads the Werner Group, a biotech consulting group (Washington, DC, USA). "And industry will see this as appropriate and necessary as the US tries to keep its competitive edge."

Perhaps Obama's arrival in the White House has already emboldened some federal regulators who deal with matters involving stem cells. Thus, before any explicit change in federal policy, FDA officials cleared Geron (Menlo Park, CA, USA) to begin a phase-1 clinical trial of GRNOPC1, an hES cell-based therapy, in patients with acute spinal cord injuries—the first such trial of its kind in the United States. FDA notified Geron that its investigational new drug application was being approved only a few days after Obama took office—timing that some observers suggest is not entirely accidental. "Geron's stem cell approval certainly suggests that some doors that have been closed for various reasons may be loosening now," says one insider following biomedical policy developments.

Policy shifts play out more broadly

"It is heartening to see the Obama administration embrace science as an important input of government and science policy as a driver of the American economy," says Thomas Murray, president of the Hastings Center (Garrison, NY, USA), which focuses on bioethics. "Instead of muzzling or ignoring science, it will sit at the table, along with the appointment of a set of remarkable science advisors."

"Obama is clearly a science buff, and is really, honestly, into knowing the facts, having them laid out, and then making the best choices that can be mustered," says a policy watcher who was close to the transition team but is outside the federal government. "It is a whole different approach compared to the 'How can we spin this information?' approach of the [Bush administration]. Back to 'honest-to-goodness' curiosity, which is, yes, incredibly refreshing."

Thus, there is solid enthusiasm for some of Obama's early choices for key Cabinet posts, including Nobelist Steven Chu, physicist from Lawrence Berkeley Laboratory (Berkeley, CA, USA), for secretary of the Department of Energy (DOE), and former Iowa governor Tom Vilsack for secretary of the US Department of Agriculture (USDA) as well as for high-level science advisors, such as Harold Varmus, also a Nobelist, president of the Memorial Sloan-Kettering Cancer Center (New York) and a former director of the NIH, and Eric Lander, who is founding director of the Broad Institute of the Massachusetts Institute of Technology and Harvard (Cambridge, MA, USA).

"We're jazzed," says an insider at DOE, referring to Energy Secretary Chu. "He's a great pick, and it's a huge boost to think of someone who can speak science to anybody—even to the OMB," he adds, alluding to the federal Office of Management and Budget, whose top officials in recent years have habitually blocked federal research or policy initiatives.

"With this president, a lot of policies are going to change, and a number of them are likely to be exciting for us," says Willy De Greef, secretary general of EuropaBio (Brussels). He points to USDA Secretary Vilsack as but one example of Obama appointments that look positive for biotech. The new USDA secretary "understands what biotech crops can do and has a deep interest in putting agriculture in play, including for energy independence and biofuels," De Greef says. Although no details are available, he adds, Vilsack's attitudes toward and familiarity with biotech-related agriculture issues "are very good for our sector."

The appointment of Vilsack is "nothing but positive for biotechnology," says Val Giddings, a Washington-based industry consultant and former USDA official. "There's not been an ag [USDA] secretary who comes in so familiar with biotech issues and who doesn't have to be briefed for the first time, but is favorably disposed to biotech for farmers. Plus, he respects data and evidence." As for Energy Secretary Chu, Giddings says, "He can't help but advance the [DOE] biotech portfolio. There will be greater openness, and it's nothing but positive."

Other positive changes anticipated

Before taking office, Obama's outreach to the scientific community was not limited to gestures about hES cell research. Shortly before the election, for example, he vowed to federal scientists—specifically, to scientists at the Environmental Protection Agency (EPA), who are represented by the American Federation of Government Employees—that "the principle of scientific integrity will be an absolute, and I will never sanction any attempt to subvert the work of scientists."

Members of the 111th Congress are also pledging support for policies that promote scientific integrity, aiming them at anyone working within the federal system or receiving federal support. In February, for example, the House of Representatives made the omnibus fiscal stimulatory legislative package a vehicle for instituting such policies. Thus, the House bill includes language that makes it an "abuse of authority" for anyone in federal agencies to take action "that compromises the validity or

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accuracy of federally funded research or analysis," disseminates "false or misleading scientific, medical or technical information," or restricts or prevents "any person performing federally funded research or analysis from publishing in peer-reviewed journals or other scientific publications or making oral presentations at professional society meetings or other meetings of their peers." Furthermore, an amendment to the bill protects government scientists who report efforts to "alter or suppress federal research." Incorporating these provisions into the final version of the stimulus package would broaden and extend Obama's campaign promise to all federal researchers.

Meanwhile, much in the rest of the stimulus package is less about symbolism and more about supplementary outlays, tax cuts and other financial resources to aid a beleaguered economy. There is plenty in the way of new resources being promised to support federal research programs across a broad range of disciplines.

Notwithstanding the political vulnerabilities of the specific proposals, the stimulus package includes a slew of concrete provisions that could benefit biotech on several levels. For example, it earmarks an extra $8.5 billion for the NIH to support extramural grants and contracts (**Fig. 1**).

Other noteworthy provisions in the bill affect other agencies and programs within the Department of Health and Human Services (HHS): $400 million for the Agency for Healthcare Research and Quality to analyze the comparative effectiveness of medical treatments, with another $400 million funneled to NIH for comparative effectiveness research.

Outside HHS, DOE is slated for an infusion of $1.6 billion for science programs, with $800 million earmarked for research on biomass, whereas the National Science Foundation is to receive $3.0 billion. Furthermore, the National Institute of Standards and Technology within the Department of Commerce is being provided with $360 million for building research facilities and $220 million for scientific and technical research and services.

Shortages of private capital

These "pledges of increased federal money and getting the government back to supporting science are good for the biotech industry," says Arthur Klausner of Pappas Ventures (Durham, NC, USA), a venture capital (VC) investment firm. "More money for NIH and easing the embryonic stem cell research ban are all in the right direction."

Klausner is not alone among venture capitalists who count on the federal stimulus package to boost investments in federal research programs. The Washington-based National Venture Capital Association (NVCA), which represents 460 VC firms, recently urged congressional leaders to "invest in basic, long-term, discovery-oriented research and development," according to NVCA president Mark Heesen. Noting that federal R&D budgets in recent years were "virtually flat and occasionally declining," he and NVAC also called for the stimulus package to include "additional funding for key science agencies [to] enable the development of innovative technologies…."

Beyond the circle of federal R&D programs, however, the economic climate for research-intensive companies continues to be dismal. The virtual disappearance of capital in the private sector is proving especially burdensome

Table 1 Legislation to watch

Issue	Legislation	Sponsors
Small business	*Small Business Act* extends through 2010 provision of the tax code (Section 179) providing tax relief for small businesses purchasing new equipment	Landreiu (D-LA), Snowe (R-ME), Kerry (D-MA)
	Strengthening our Economy through Small Business Innovation Act (*S.177*) amends and extends the Small Business Innovation Research (SBIR) and Small Business Technology Transfer (STTR) programs, increases allocations for these programs, adds energy, water, transportation and domestic security to topics	Feingold (D-WI)
Conflict of interest	*Physician Payments Sunshine Act (S.301)* requires disclosure of all payments of $100 or more to physicians; imposes fines, up to $1 million for "knowing failure to report," individual infractions between $1,000 and $10,000 per infraction with up to $150,000/year/company	Grassley (R-IA), Kohl (D-WI), Klobuchar (D-MN)
Access to health care	*Americare Health Care Act (HR 193)* provides health insurance for all Americans; modeled after Medicare (only for those who want it)	Stark (D-CA)
Patents	Pilot program in certain district courts (HR 628, S.299) encourages enhancement of expertise in patent cases among district judges	Issa (R-CA), Schiff (D-CA), Specter (R-PA)
Drugs	*Medicare Prescription Drug Savings and Choice Act (S.330)* delivers meaningful benefit and lower prescription drug prices under Medicare	Durbin (D-IL)
	Pharmaceutical Market Access Act (S.80) amends the Food, Drug and Cosmetic Act with respect to importation of drugs	Vitter (R-LA)
	Preserve Access to Affordable Generics Act (S.369) prohibits brand-name drug companies from compensating generic drug producers to delay entry into the market	Kohl (D-WI)
FDA	*Food and Drug Administration Globalization Act (S 80 HR 759)* gives FDA authority to recall unsafe drugs and devices and institutes fees to cover cost of inspections	Dingell (D-MI), Vitter (R-LA)
	Food Safety Modernization Act (HR 875) establishes a Food Safety Administration within the FDA	DeLauro (D-CT)
Nanotechnology	*National Nanotechnology Initiative Amendments Act (HR 554)* strengthens and provides transparency to the research effort to understand environmental, health and safety risks of nanotechnology	Gordon (D-TN)
Biofuels	*New Manhattan Project for Energy Independence (HR 513)* supports the development and production of a biofuel that does not exceed 105% of the cost for the energy equivalent of unleaded gasoline	Forbes (R-VA)
Finance	Permanent extension of research credit (S.37)	McCain (R-AZ)
Cloning	*Human Cloning Prohibition Act* prohibits cloning of individuals without otherwise restricting research	Fortenberry (R-NE)
Stem cell research	*Ethical Stem Cell Research Tax Credit Act (S.99)* provides federal income tax credit for "ethical" stem cell research	Vitter (R-LA)
	Stem cell research (HR 872, 873) provides for embryonic stem cell research and for the NIH to provide guidelines for such research	DeGette (D-CO)
	Sanctity of Human Life Act (HR 227) provides that human life begins at fertilization	P. Broun (R-GA)

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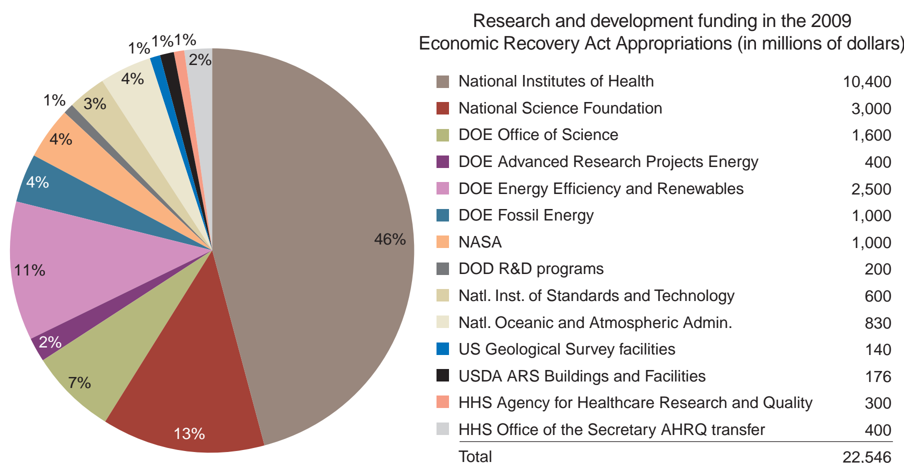

Figure 1 Funding for research and development in 2009 Economic Recovery Act Appropriations. Source: American Association for the Advancement of Science Programs in Science and Policy, Washington, DC, USA (accessed Feb 13, 2008).

on smaller companies, for which the path to initial public offerings is virtually "sealed shut," says BIO's Greenwood. Before and while the economic stimulus package was being negotiated, he was conferring with members of Congress on what he described as several "very dense tax policy matters," including R&D tax credits and zeroing capital gains liabilities to offset investments in biotech companies. Such financial breaks could bring near-term benefits to the industry.

The tax relief being sought is not a "bailout," Greenwood says. Instead, it is something of a stopgap that would put tax credits into the hands of biotech companies to invest in R&D, buying time and keeping research teams working together until the credit markets thaw. Such measures make particular sense for the research-intense biotech sector, for which the timeline from idea to commercial product typically takes from 10 to 15 years before revenues from product sales begin to bring a return on investment, he says.

"The sickness of the capital markets is the main problem now facing biotech companies," says Mark Leuchtenberger of Targanta Therapeutics (Cambridge, MA, USA). "It's not that the 'window' for financing closed; the house fell down. This is like the 'nuclear winter' for biotech companies." Pointing to a recent merger-acquisition involving Pfizer and Wyeth, he predicts "further convulsions" throughout the pharma and biotech industries, including similar mergers involving many of the smaller biotech companies. Thus, he endorses efforts to change tax credit rules to benefit small companies even in the absence of their having current tax liabilities. "Allowing them to monetize is another area where government could do something meaningful for struggling biotech companies," he says.

"There were no IPOs [initial public offerings] in the last quarter, and there were a few acquisitions, but not much money from them because of the ridiculously low valuations," says Jenny Mather of Macrogenics (San Francisco). "Funding is way down, and it's a hardship for many companies. Some people think we're headed to a recovery, but until we're there, it's pretty grim and there are a lot of layoffs. There isn't much money going into the industry, and it won't regain its health until that happens."

One offbeat move to overcome current domestic capital shortages and attendant worries about declining price structures for biological therapeutics might be to look to Asia and particularly China for partners and markets, says Jie Liu D'Elia of BioBridge China in Seattle. As companies anticipate US healthcare reform, they will be seeking to "lower costs of prescription drugs," she says. "Companies will dig deeper and look for ways to cut costs by outsourcing to China, [where] a PhD level scientist costs at least 30–40% less than their counterpart in the US."

Plus, she adds, the Chinese market could become "a growth driver" as healthcare reform puts "negative pressure on the growth rate of the drug market in the US," which is expected to grow by 4.4% annually, compared with the Chinese drug market's projected annual growth of up to 17%. "I expect to see more leading biotech companies in the US and EU reach out to China either directly or via partnerships, instead of staying focused on the US domestic market," she says. "The economic climate and a combination of other forces, including the likelihood of healthcare reform, could prod companies to seek other outlets for their therapeutics as Obama takes office."

Uncertainty in healthcare reform

Early during the transition, Obama named former senator Tom Daschle to become secretary of HHS and also overseer of healthcare reform within the White House. Daschle, the lead author in 2008 of a book laying out a plan for reforming the US healthcare system, *Critical: What We Can Do About the Health-Care Crisis*, seemed poised to run with healthcare reform as soon as a majority in the Senate, a body he once led, confirmed his appointment. However, in early February, with a political uproar over his tardy payments of income taxes growing ever louder, Daschle withdrew from consideration, leaving the Obama administration to seek a new candidate to head healthcare reform efforts and also HHS and its many agencies, including FDA, NIH and the Centers for Medicare and Medicaid Services.

No obvious candidate appeared immediately, although as Nature Biotechnology was going to press, Kansas governor Kathleen Sebelius seems the most likely choice. (**Box 2**). Nonetheless, the administration is maintaining keen interest in pursuing healthcare reform, even if the economic crisis and Daschle's abrupt departure add further uncertainty as to when those reform proposals will be put forward. Shortly after Daschle withdrew, key Senate Democrats Ted Kennedy (D-MA) and Max Baucus (D-MT) reaffirmed plans to develop healthcare reform legislation.

Meanwhile, Congress also passed and Obama signed legislation reauthorizing the State Children's Health Insurance Program (SCHIP), promising to expand its enrollment to include 11 million children by 2013. Former President Bush had twice vetoed similar legislation, which authorizes federal payments that are matched with state funds providing health coverage. The rapid turnaround in February reflects some of the momentum Obama and Congress hope to bring to healthcare reform. "As I see it," Obama said, "providing coverage for 11 million children is a down payment on my commitment to cover every single American." Thus, although the timeline is even more uncertain than it was before the Daschle debacle, the commitment to reforming healthcare is not.

Expect follow-on biologics

In terms of healthcare reform, regardless of timing, "the President and Congress want something bold and universal, and it could cost trillions of dollars," says Greenwood. Because they also will need to seek "savings to pay for" that reform, he expects the Obama administration to review drug-pricing issues, even though

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therapeutics account for only a "small fraction" of healthcare costs, with "biologics a small subset of that." If healthcare reform leads the new administration to "squeeze down" prices, Greenwood is concerned about the risks of "starving the drug discovery" process.

"Healthcare reform will cost [biotech] in some ways, and I think it will be a front-end burden," says Klausner of Pappas Ventures. But the overall impact is difficult to predict if reforms lead both to "lower prices" but also to "more insured people so a greater volume of prescriptions." However, he adds, "If biotech becomes less attractive because the value of the enterprise is less, then less venture-capital investment goes into it, and that will hurt new company formation. How much is hard to say, but it won't be immediate."

"The weight and cost of regulation is inherently making new product development a very risky and expensive business," says EuropaBio's De Greef. "This one issue is the same for the US and the EU in the development of new drugs." A major source of those costs is clinical testing that is "not risk, but hazard based…[making] trials with unknown molecules and generics subject to the same administrative burden, and that's wasteful," he adds. Moreover, high costs and defensive testing help to "deny access to new drugs." One mitigating step, not "a silver bullet," would be to develop agreements permitting "more mutual acceptance of data," along with a "lot of other small improvements to cut costs."

Greenwood is continuing to visit key members of Congress, reminding them that even very costly biologics can provide healthcare savings, particularly when they alleviate chronic conditions. Thus, he calls it "criminal to enact public policies that limit explorations of that potential," namely development of new therapeutics, that bring both "humanitarian benefits and cost savings." Although biogenerics may be inevitable, Greenwood says that biotech companies should be entitled to "14 years of data exclusivity to recover their investments in drug discovery."

Some insiders following this debate wonder why some factions within the industry continue to resist follow-on biologics. Those who resist the onslaught of follow-on biologics are at risk of "showing hysteria about the replacement of 20-year-old proteins" and "have forgotten how to be optimistic" about innovative products, says one observer. With more than 600 biologics under development but fewer than 10 of them being approved per year by FDA, why spend so much time worrying about follow-on biologics? Bigger challenges for those developing therapeutic products lie elsewhere.

James Bianco, CEO of Cell Therapeutics (Seattle), calls it "bizarre" that there is still no US law allowing generic biologics on the market. "It works for small molecules, and it will work for biologics," he says. "This is one area that could be the first piece of healthcare reform legislation, especially with Waxman," he adds, referring to Representative Henry Waxman (D-CA), who recently wrested the chairmanship of the House Energy and Commerce Committee from Representative John Dingell (D-MI). Not only did Waxman play a key role in shepherding the Hatch-Waxman Act of 1984, the gateway for conventional generic drugs, he introduced a similarly minded follow-on biologics bill early in 2007, *Access to Life-Saving Medicine Act*, that is likely to be reintroduced soon.

Although Waxman is a "strong advocate" for such legislation, ironically his new responsibilities as chair of the committee may prove a "distraction" for him, according to Gregory Conko of the Competitive Enterprise Institute (Washington, DC, USA). Nonetheless, there appears to be "enough political will in the House and Senate to move follow-on legislation forward," he says. "I'd be surprised if the legislation is not passed soon, even in 2009 and surely within the first two years."

Several observers expect Senator Orrin Hatch, (R-UT), who joined forces with Waxman in 1984, to do the same with biologic follow-ons. Last year, Hatch, fellow Republican Senator Mike Enzi (R-WY), as well as Democrats Kennedy and then-Senator Hillary Clinton (D-NY), who is now Secretary of State, co-sponsored the "Biologics Price Competition and Innovation Act." Although not an official co-sponsor of that legislation, Obama was on record while in the Senate for supporting such legislative efforts.

That earlier Senate bill allowed for 12 years of data exclusivity for the brand company before a biogenerics product could be approved among other provisions. Some contend that Waxman will not go along with such prolonged exclusivity. So far, however, no one in the House or Senate has introduced a bill covering biologic follow-ons, although Anna Eshoo (D-CA) will reintroduce her bill (HR 5629, 110th Congress, Eshoo Barton Pathway for Biosimilar Biological Products), according to Erin Katzelnick-Wise, legal assistant to Eschoo.

Intellectual property and the FTC?

"Healthcare reform and patent reform dovetail," says Targanta's Leuchtenberger. "Even the staunchest defenders of biotechnology realize that it's subject to the same caveats as other sectors. But the bargain we've struck allows innovation to be rewarded."

In terms of patents and intellectual property (IP) issues, Arti Rai from Duke University Law School served as Obama's point person in this arena (**Box 2**). Some observers see her as promoting reforms that could prove detrimental to both biotech and traditional pharmaceutical companies, making it more difficult and costly for them to obtain and then enforce patents.

There is also concern that proposed rule changes from the US Patent and Trademark Office that make it more difficult to obtain or enforce patents could prove harmful for companies. Furthermore, uncertainties from federal court rulings raise the concern that all claims of some patents could be declared unenforceable in cases where even the slightest information submitted to PTO is construed as misleading or inadequate. Meanwhile, if a patent reform bill that was sponsored last term by Democratic Senator Patrick Leahy (D-VT) and Orrin Hatch is reintroduced, Greenwood of BIO vows to work toward blocking it again.

For the moment, however, IP legislative reform is "a second order issue, and very hard to predict" in terms of timing before this Congress and the Obama administration, according to Duke's Cook-Deegan. During the last term, there was "a big push" for reform in Congress, with "software companies working in the information technology (IT) sector pushing hard" and others, including biotech and pharma, "suspicious of changes." Since then, various court decisions eased some of the reform pressure coming from the IT sector, he says. "There was a battle, and inertia won."

Assuming follow-on biologics become part of the biotech landscape, they could further encourage the Federal Trade Commission (FTC) to enter biotech territory, according to Greenwood and others. Thus, because FTC keeps an eye on generic and proprietary drug companies through its mandate to guard against anticompetitive practices, this oversight would likely extend to follow-on biologics. Late in 2008, FTC convened a roundtable on "Follow-on Biologic Drugs: Framework for Competition and Continued Innovation."

With another more muscular sign of interest in such matters, FTC filed a complaint in federal district court early in February, challenging agreements in which they say Solvay Pharmaceuticals paid generic drug makers Watson Pharmaceuticals and Par Pharmaceutical Companies to delay generic competition to Solvay's branded testosterone-replacement drug AndroGel. Although that suit involves conventional drugs, FTC appears not only interested in the economics of follow-on biologics but also ready and perhaps eager to enforce legislation that would promote the introduction of such products.

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Box 2 People to watch

While the Senate and Obama wrangle over top-level appointments, the thinking of Obama's closest advisors provides some clues as to the path the administration will be taking, which seems likely to have a strong emphasis on reform in general and healthcare reform in particular.

Physician and bioethicist Ezekiel Emanuel. If you think you've heard that name before, it's because you have. Zeke Emanuel is the older brother of Obama Chief of Staff Rahm (and some say the smartest of the three Emanuel brothers, the third, Ari, being a Hollywood mover and shaker). A star in his own right, Zeke is a physician and bioethicist who held numerous prestigious positions within the medical community, most recently, the chair of NIH's Department of Bioethics, before being reassigned to chief counsel at the White House's Office of Management and Budget in January. Author of the 2008 book on healthcare, *Healthcare, Guaranteed: A Simple, Secure Solution for America*, his appointment may have more to do with healthcare reform than with bioethics *per se*. But he has expertise in both areas, and will, one would imagine, have unfettered access to the highest levels of government.

Health economist David Cutler. Trained as an economist at Harvard and MIT, Cutler has worked both in academia, on the faculty of Harvard since 1991, and in the public sector, as a member of President Clinton's Council of Economic Advisors. Cutler was also an advisor on Hillary Clinton's failed healthcare reform package of the 1990s. Author of the book, *Your Money or Your Life: Strong Medicine for America's HealthCare System*, Cutler takes a somewhat different approach to healthcare reform, focusing on value gained rather than dollars spent. Cutler served on Obama's transition team and announced in January a move to Washington, DC.

Healthcare reformer Jeanne Lambrew. A health policy expert who literally wrote the book on healthcare reform with Tom Daschle (*Critical, What Can We Do About the Health-Care Crisis*?), Lambrew was appointed deputy director of the White House office on health reform, second in command under the chief, who was to be Daschle, before his tax problems came to light. In addition to writing together, the two were fellows at the liberal think tank, Center for American Progress. Through her writing, Lambrew has advocated a federal healthcare board, modeled after the Federal Reserve, expanding the Federal Employees Health Benefits to include all those in need of insurance, and a wellness trust, to provide preventive medicine.

Health policy expert and governor Kathleen Sebelius. Democratic governor of Kansas since 2003 and an early supporter of Obama, she was on the Obama short list for vice president and hence thoroughly vetted. Considered among the most powerful women in healthcare by *Modern Healthcare* in 2001, Sebelius served as insurance commissioner in Kansas for eight years, during which she advocated for consumer rights. Sebelius was called by salon.com "a passionate advocate of political moderation, as oxymoronic as that sounds." The daughter of a one-term governor of Kansas, John Gilligan, she is a very popular governor in a mostly Republican state.

Patent lawyer Arti Rai. Classmate of Barack Obama's at Harvard Law School, Rai served on the transition team as a member of the agency review team for science, technology, space and the humanities. An expert in patent law and the pharmaceutical industry, she could well be the administration's point person on patent reform, as part of the overall healthcare reform effort.

Laura DeFrancesco

Concerns over comparative effective analyses

The economic stimulus package being considered in February included $700 million for the Agency for Healthcare Research and Quality in HHS to analyze the comparative effectiveness of medical treatments, with $400 million of that outlay reserved for NIH to conduct comparative effectiveness research [**p. 211**]. Some analysts, notably Scott Gottlieb of the American Enterprise Institute (Washington, DC, USA), consider such activities a threat to development of innovative therapeutics.

Gottlieb, a former official at FDA, argues that this approach of insisting on evaluating comparative effectiveness is modeled on the UK National Institute for Health and Clinical Excellence (NICE; London) whose hidden agenda, he says, is to "protect the British healthcare budget" by withholding lifesaving but high-cost new drugs from individuals. Such practices also "distort future investment decisions," he notes. "The last time policy makers waged a concerted effort to control the price of, and the access to, the most innovative but expensive new drugs as part of broader healthcare reform in the mid-1990s, the percentage of venture capital going into biotech fell by almost half in a single year."

Gottlieb also thinks it's important for the FDA to lower the threshold for diagnostic tests. rather than step-up regulations, as Genentech's (S. San Francisco, CA, USA) recent citizen's petition asked (p. 211). Gottlieb believes a relaxation of oversight would enable doctors to target, or personalize, medicines to those most likely to benefit from them.

Others in biotech are also wary of the NICE model. "There is talk of a system like [NICE], which calculates cost-benefits and decides what to reimburse. We don't want to go there, and don't think Americans want to do that either," says Mather of Macrogenics.

Elsewhere, Murray of the Hastings Center thinks there is a middle ground to be found. "We don't have to think that the British have it exactly right to take their core message that we can't infinitely expand healthcare," he says. "Paying greater attention to quality care is not bad news for all biotechnology companies, and it may result in a shift in strategies to think more about cost effectiveness. And any sensible policy will give innovations a very important place. It is a challenge for companies…to strike a fruitful balance among quality, access and innovation. And I know people in the industry who will be eager to take on this challenge."

Muscular FDA may split in two

"How FDA functions is of huge importance, and we want this agency that regulates us

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muscularly funded and staffed," says Greenwood. "It is critical that our [product] applications are reviewed fairly, transparently and efficiently while meeting the gold standard of being safe and efficacious. It is critical that Obama appoint a commissioner who is confirmed by the Senate [and has] the confidence to manage FDA adroitly." For several periods during the Bush administration, FDA was led by acting commissioners—a practice that Greenwood considers "tragic" for the agency.

"Our key objective for the new administration is that it make sure there is a good and capable commissioner at FDA," says Alan Goldhammer, who is deputy vice president of regulatory affairs at the Pharmaceutical Research and Manufacturers of America (PhRMA, Washington DC, USA). "We will also work to ensure the agency has adequate resources to meet its public health mission, particularly reviewing new drugs in a timely manner."

"FDA deals with a huge number of products requiring review, which requires resources, but they have not kept up," says Leuchtenberger of Targanta. "Without a leader, they fall behind in recruiting. I think they could do much better." FDA is a "key agency for all of us, not just the biotech industry, and it being less politicized is a good thing," adds Murray of the Hastings Center. "But it desperately needs strong leadership and resources to boost morale."

Greenwood points out that FDA regulates products accounting for as much as 25% of the overall US economy, yet another reason for making sure that it runs smoothly and its leadership is in step with the White House. He would also like to see the "black box" of FDA demystified. "Even with [recent reform legislation], companies don't get enough meetings, and we're always scratching our heads," he says. "There is a lot of frustration when applications are denied, and we're always open to making FDA more efficient."

During the past year, FDA has been on a hiring and building binge as it prepares to move more operations into brand new office and lab space at its suburban campus, situated on the grounds of a former munitions depot. Moreover, the first-ever FDA chief scientist, Frank Torti, who became acting commissioner in January, began actively making the case last year for strong, in-house scientific expertise to support agency missions. Toward that end, FDA recently created a new position in the Office of Chief Scientist dedicated to coordinating activities involving genomics and related fields of science. The Obama administration is thought likely to continue these expanded efforts.

"On the food side, I expect biotechnology to be a fairly unimportant issue for the next couple of years," says Conko of the Competitive Enterprise Institute. Instead, he and others recognize that conventional safety issues, with the salmonella-laced peanut butter problem the most recent example, will be predominant. One exception directly involving biotech could be a move to reinstate a premarket notification rule for genetically engineered plants, a move that was blocked by Bush but could be brought back by the Obama administration. "There is no reason to think the [Obama] administration would go toward more deregulation, much to my chagrin," he says.

Meanwhile, Conko anticipates a "concerted effort to evaluate how FDA looks at general food safety" amid renewed talk of splitting it into two agencies. "The food people at FDA are really underfunded, and CFSAN [Center for Food Safety and Applied Nutrition] is seen as the ugly stepsister to the medical products side," he says. Experts have debated the possibility of separating those two FDA responsibilities into different entities for more than two decades, and there are plenty of obstacles standing in the way, he adds. "But I expect to see some congressional hearings and internal FDA investigations within the next two years."

Indeed, Representative Rosa DeLauro (D-CT) in February introduced a bill, HR 875, seeking to establish a separate "Food Safety Administration." Here again, despite such signs of renewed interest in separating food from drug and possibly splitting FDA into two agencies, Washington insiders provide plentiful reminders of how complicated and potentially contentious it will be to legislate that move. Even with bipartisan agreement, many congressional committees have partial jurisdiction over FDA programs, ensuring that such a restructuring effort will be a bureaucratic nightmare.

Bioethics stature likely to change

With hES cell research as part of the impetus, Werner, Murray and others expect the Obama administration to reestablish a broad-based bioethics commission. However it is recast, Werner says, it probably "won't lean on the biotech industry quite so much" as did President Bush's Council on Bioethics, particularly while Leon Kass served as its director. Biotech issues surely will come forward in a bioethics context, Werner says, but "not from the perspective that the industry is bad and immoral. With the intellectual curiosity of this administration, we'll see nuances and a thoughtful outlook on bioethics."

The Bush bioethics "apparatus is very unlikely to continue, just as Bush let Clinton's [National Bioethics Advisory Commission] expire," says Murray of the Hastings Center. "But what will take its place, I don't know." For now, delays in reformulating and rechartering such a body make sense politically and practically, he adds. "Bioethics issues of tremendous importance will be coming up, but most of them can wait a little while. I want my President to revivify the economy today."

Rick Weiss of the Washington-based Center for American Progress expects, like Werner and Murray, that the Obama administration will put together a bioethics commission with a broad mandate, one that will likely include issues such as "scientific integrity" and the "business of doing science," Weiss says. "I've not heard anyone suggest that they will pull business out of science, and public-private partnerships remain in vogue. But there are concerns in terms of transparency and conflicts of interest."

Congress will be part of this debate as well. For instance, Senator Chuck Grassley (R-IA) introduced a bill, S.301, in January calling for "transparency in the relationship between physicians and manufacturers of drugs, devices, biologicals, or medical supplies." The bill is formulated as an amendment to the Social Security Act, meaning it exerts jurisdiction over payments under the Medicare, Medicaid and SCHIP programs. In the House, Waxman is likely to take on similar issues, including conflict of interest and transparency, as it applies to biomedical researchers at NIH and elsewhere, as well as for physicians, according to Cook-Deegan.

Much as there is little definite yet known about the structure a bioethics forum will take in the Obama administration, not much can be said about who will be part of it. However, insiders point to R. Alta Charo of the University of Wisconsin, Jonathan Moreno at the University of Pennsylvania and the Center for American Progress, and Ezekiel Emmanuel, now at the OMB and formerly at NIH, as among the front-runners for such duties (**Box 2**). Emmanuel also is mentioned as a candidate for HHS secretary.

Biofuels, biodefense and biocrop bickering

Beyond the metaphorical cold snap affecting the economic climate, Obama sees warming trends in the global climate as a different kind of high-priority threat, a point he reiterated soon after the November elections. What's more, the new president comes from Illinois in the Corn Belt, where corn-based ethanol is a major source of revenue for farmers throughout the region. Greenwood views this situation as an opportunity for biotech to play a "crucial role" in reducing greenhouse gases through biofuels, particularly by supplying technology for processing cellulose more efficiently.

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With oil prices falling, biofuels programs may be on "autopilot" for the near-term future, according to Conko. Even so, and in agreement with Greenwood, he anticipates recent research initiatives, particularly those focusing on non-food sources for biofuels, to go forward under a broad agenda being managed by DOE Secretary Chu.

"We're enormously impressed with Obama's pick to run DOE; it speaks volumes," says Jonathan Wolfson, CEO of Solazyme (S. San Francisco, CA, USA), which is working on renewable energy and alternative chemicals development. He also speaks favorably of the EPA administrator-designate Lisa Jackson. "There were very dedicated 'lifers' at those agencies who care about the science," he says. "With new choices for leadership, we hope they will be free to go where the technology leads and make decisions based on hard science."

With oil prices hovering near "$40 per barrel and financial markets dormant, there are substantive challenges" facing his and similar companies working on alternative energy technologies, Wolfson admits. "But you have to recognize that climate problems and energy security have people concerned, and really need to be addressed. Our big hope is that [federal policy] will set out incentives in a technology-neutral way that is ends-driven—not dictating the type of fuel, but only that it reduces carbon. We think there will be policies to enable adoption of technologies from this space. Even if biofuels are now taking a beating, there are some advanced technologies that can provide real environmental benefits."

What happens with biofuel development ties in with developments and policies affecting agriculture and, here again, Obama's selection of Tom Vilsack for USDA secretary is drawing praise from biotech analysts. "Agbiotech is regarded as important, but let's have no illusions," says Washington-based consultant Giddings. "The economy and Middle East are first-tier issues, and Vilsack won't get Obama's attention for quite a while. And, even if they [administration officials] could be specific about agbiotech, they wouldn't because they will set it on the shelf and get to it once they deal with other stuff."

In terms of regulatory policies affecting genetically modified crops, little is expected to change anytime soon during the Obama presidency, except perhaps for a greater emphasis on transparency. "It is likely that the Obama administration will be more open than Bush's to a wide range of stakeholders," says Gregory Jaffe, who directs the Biotechnology Project at the Washington-based Center for Science in the Public Interest. More generally, the new administration is more likely to seek additional regulatory authority or even to ask Congress to amend laws in cases where rule-making becomes too much of a stretch for those already on the books. However, he adds, with so many other pressing food-safety issues to face having to do with microbially or chemically contaminated products, "I don't think biotech foods will be high on Obama's agenda."

"Expect more scrutiny of new varieties and more disclosures and transparency about biotechnology in food and agriculture," agrees Mark Mansour, an attorney with Bryan Cave (Washington, DC, USA). He, too, does not anticipate "much change" from recent policies in the near term, except for "some concessions to watchdog groups. But this will take a while, and will be expressed in due course."

One area where agricultural policy might change course is internationally, particularly with Secretary of State Clinton revitalizing international outreach programs, according to Mansour. This could take shape as an "aggressive engagement of USDA and USAID [Agency for International Development] with developing countries in Africa and other parts of the world, using agriculture as a means of engagement," he says. Unlike the Bush administration, for which such programs were, at best, "an adjunct to security, this [Obama] administration could see agricultural biotechnology as a constructive tool." Of course, "there will be obstacles to overcome, but a lot of opposition to biotechnology could melt with a prolonged recession."

"We're spending about $22 billion per year for the region [Africa], and candidate Obama called for doubling resources, and to put agricultural resources among the top ten," says Robert Paarlberg of Wellesley College (Wellesley, MA, USA), and author of *Starved for Science: How Biotechnology is Being Kept out of Africa*. "Science-based assistance does seem to have a voice." However, biotech will not soon make inroads into African agriculture because so many countries there remain dominated by Europe through custom and because Europe provides them much more assistance than does the United States, he adds. Thus, although USAID "has tried to throw its weight around, that doesn't work in Africa."

"The EU approach has helped keep African countries from adopting GM [genetically modified] crops," agrees De Greef of EuropaBio. "We hope if the EU and US become less adversarial, it could remove pressure from Africa, which feels forced to choose between US or EU regulations."

In terms of global agbiotech disputes, there are "tricky dossiers" to be faced, De Greef says. Even though the US won a round against the EU in a long-standing World Trade Organization (Geneva) case about genetically modified organism imports, "no official appeal" from the EU has been filed yet, he says. "If EU does not appeal or comply, the US, Argentina and Canada can take unilateral measures, but the US probably will prefer to negotiate, which seems more Obama's style. I'd like to see agreements rather than litigation, and a real victory would be to have science-based regulations."

Biodefense is another "critical" area for biotech companies, but "we haven't heard comments yet from Obama," Greenwood says. What's more, despite repeated efforts by the Bush administration to develop programs to meet the US Department of Defense and Department of Homeland Security needs, "companies can't get government to say what it's looking for. We chalk that up to relative newness and the general difficulties in dealing with government programs," he says.

Development of the biodefense slice of the biotech industry has been a "tortured" process through its early stages, says Gerald Epstein, a senior fellow at the Center for Strategic and International Studies (Washington, DC, USA). One major challenge from the outset was "how to get industry interested in government," he says. "How biodefense needs get filled is still a challenge. Some small biotechs were attracted but not pharma. Even after BioShield put money in a pot, it hasn't worked as intended." Meanwhile, increased funding through the omnibus stimulus package for the Biomedical Advanced Research and Development Authority (BARDA; Washington, DC, USA) within HHS, another segment of the sprawling federal biodefense initiative, could make BARDA a genuine "new player," even if it is still dwarfed by multibillion dollar NIH funding.

Epstein expects the Obama administration to approach biodefense policy and practices with "continuity." Current programs "won't change much, although BARDA might grow," he says. "For now, the administration continues to see the threat from weapons of mass destruction as true." But, figuring out how to organize such efforts is a looming challenge, he points out. "A lot of White House offices have something to do with these issues, and they will have to accommodate one another with this inherently broad subject. Right now biodefense looks balkanized, but maybe it will come together in the Office of Science and Technology Policy. For now, though, it's not a mainstream discussion."

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Bilski blundering biotech

William J Simmons

Is the Federal Circuit's decision in *In re: Bilski* yet a further restraint on patenting biotech and pharmaceutical inventions?

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Given recent changes in the law, the state of the global economy and the recent election of a new president and executive government in the United States, there is a palpable apprehension regarding pharmaceutical and biotech intellectual property (IP), specifically in patent procurement and enforcement. Even though there are uncertainties in navigating such changes, biotech companies and universities must nonetheless pursue their fundamental goal of developing innovative new products. Navigating change is further complicated by the reality that almost every biological innovation, particularly those having significant potential to mature into drug candidates, fails at some juncture of development.

The uncertainties now confronting biotech and pharmaceutical companies and universities are accompanied by economic factors arguably unique at this point in history. Indeed, Roger Newton, Esperion Therapeutics CEO and co-inventor of the blockbuster drug Lipitor (atorvastatin) recently predicted that nearly one-third of the small US biotech companies will go out of business within a year[1]. Industry experts say that it is imperative that the industry address several realities that require pharma and biotech companies and universities and their licensees to "fundamentally reinvent [their] business models." These include factors such as key patent expirations, the advancement of personalized medicine and globalization[2]. For example, between 2007 and 2012, more than three dozen drugs will lose patent protection, which will result in a loss of annual sales to generic competition of an estimated $67 billion.

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Given this uncertainty in the IP portfolios of companies and universities, it is more important than ever for them to exploit their existing pipelines as well as to develop new strategies for streamlining R&D to preemptively compensate for the expiration and erosion of IP rights. As biotech entities attempt to grow by advancing R&D, it is paramount that they both develop and adhere to sound strategies for protecting their inventions. But statistics suggest increasing problems in the process of procuring and enforcing biotech and pharma patents. After a spike in patenting in the 1990s, there was a slowing of patent application filing at most patent offices throughout the world in the early 2000s (ref. 3). The number of international biotech patent applications filed dropped from over 10,000 applications in 2002 to 7,200 in 2005, a 7.5% drop compared to an increase of 20.2% on average between 1995 and 2000 (ref. 3). The trend is reversed for total international patent applications filed, which continued to increase by an annual average of 4.7% from 2000. In addition, between the mid-1990s and the early 2000s in many countries, there is an observed decrease in the relative weight of biotech subject matter in all international patent application filings. "On average, biotech patents represented 5.8% of countries' patent portfolios from 2003 to 2005, compared to 9.4% in the mid-1990s." (ref. 3). These trends may become more pronounced as the practical impact of the World Health Organization proposals to modify IP practices and policies is felt by member states.

The *Bilski* decision

Recent sweeping decisions in non-biotech patent cases such as *KSR International Co. v. Teleflex Inc.*[4], wherein the court held that a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results; *Quanta v. LG Electronics*[5], wherein the court held the authorized sale of an article that substantially embodies a patent exhausts the patent holder's rights and prevents the patent holder from invoking patent law to control post-sale use of the article; and *In re: Seagate Technology, LLC*[6], wherein the court decision makes it much more difficult for a patentee to prove a claim for willful infringement, have raised barriers to obtaining and protecting patent rights for biotech inventions. In addition to unsettling recent court precedent, Congress continues to rewrite the Patent Act and the US Patent and Trademark Office (USPTO) continues to seek implementation of proposed patent rule changes that might arguably severely affect the procurement and enforcement of biotech patents. To consider the current state of patent law in the United States as unprecedented is an understatement.

A recent change in the law, articulated in *In re: Bilski*, applies to pharma and biotech subject matter and may have a dramatic impact on the procurement and enforcement of patent rights in the US. In this en banc decision, the Federal Circuit reconsidered and significantly modified the standard for determining whether a process is statutory subject matter and therefore eligible for patenting under the Patent Act, 35 USC §101 (ref. 7). While the facts at issue involved the patentability of a method of hedging risks in commodity trading, the holding was said to be "the governing test" to be applied in determinations of process patentability, including processes encompassing biotech and pharma subject matter. Indeed, contemplating without clarifying the ramifications of the decision on biotech and pharma claims, the Court pointed out that a chemical reaction was an example of a "self-evident" physical transformation of matter.

Does the claim recite biotech or pharma methods or processes?
NO → Cease *Bilski* analysis
YES ↓
Does the claim recite a fundamental principle of nature (a mathematical algorithm) or a purely mental process?
NO → Further evaluate for novelty non-obviousness, written description, enablement, etc.
YES ↓
Does the claim apply the fundamental principle or mental process and not preempt its uses?
NO → Unpatentable
YES ↓
Is the claimed method tied to a machine or apparatus?
YES → Is the machine use limited to data gathering?
YES → Unpatentable
NO ↓
Does the machine impose a meaningful limitation on the claim's scope?
NO → Unpatentable
YES ↓
Is the machine activity merely a post-solution activity?
YES → Unpatentable
NO ↓
Patent Eligible
NO ↓
Does any chemical or physical transformation occur in the recited method?
NO → Unpatentable
YES ↓
Is the transformation limited to data gathering?
YES → Unpatentable
NO ↓
Does the transformation impose a meaningful limitation on the claim's scope?
NO → Unpatentable
YES ↓
Is the transformation merely a post-solution activity?
YES → Unpatentable
NO → Patent Eligible

Figure 1 A flow chart for putting claims to the *Bilski* test.

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The *Bilski* test is therefore critical to understand and apply because it requires a tie to a particular machine or apparatus or transformation of a particular article into a different state or thing.

In deciding *Bilski*, the court ultimately affirmed the decision of the USPTO Board, finding that the method claims at issue in Bilski and Warsaw's patent application were not directed to patentable subject matter and that Bilski and Warsaw were not entitled to a patent for the claims. The court applied a newly announced test, which the court referred to as the "machine-or-transformation" test, for determining patent eligibility under the governing statute. The court indicated that the machine-or-transformation should be used to determine whether any process claims are patentable in the United States. In its most reduced form, the test aims to determine if a claimed process is tied to a particular machine or apparatus, or transforms a particular article into a different state or thing. If either prong of the test is met, the subject matter claimed is deemed eligible for continued analysis for patentability (e.g., novelty, nonobviousness, etc.).

The Bilski patent application did not include claims directed to biotech processes. However, the court pointed out that the articulated machine-or-transformation test governs regardless of the nature of the technology. Bilski claims related to methods for hedging risk in commodities trading, the claims at issue in the case. The USPTO rejected the claims, arguing that the process claims were not patentable because the method described by the claims did not involve the "technological arts." The USPTO also argued that the method claims were not patentable because the claims were not limited by a specific apparatus (e.g., a digital computer).

In an administrative appeal, the USPTO Board of Appeals held that the office personnel were incorrect in relying on the "technological arts" test. Interestingly, the board also held that the office was incorrect in requiring a specific machine in the claim language. Even though the board admitted that the office applied the wrong tests, the board's ultimate conclusion was that Bilski's hedging risk claims were unpatentable because the claims were to nonpatentable subject matter. The rationale for the board's conclusion was that Bilski's claims were to an abstract idea, and that abstract ideas were

ineligible for patent protection. Bilski appealed the board's decision to a higher court, the Federal Circuit.

Without request by Bilski, the Federal Circuit ordered an en banc review of the issues, meaning that the case was heard and decided before all judges of the court, a procedure sometimes used if a case is of unusual significance. In resolving the issues, the court analyzed the language of the relevant statute and noted that four categories of patent-eligible subject matter are recited explicitly, including processes, machines, manufactures and compositions of matter. The court pointed out that the simple dictionary definition of "process" is not the meaning accorded to the process described in the governing law because the Supreme Court has held that the meaning of "process" as used in 35 USC §101 is narrower than its ordinary meaning, excluding, for example, a process that embodies a "fundamental principle," such as a law of nature, natural phenomenon or abstract idea. The court held that a process claim that includes a fundamental principle is patentable only if the claim recites a particular application of the fundamental principle (**Fig. 1**). The court indicated that under a proper application of the machine-or-transformation test, "[a] claimed process is surely patent-eligible under §101 if: (i) it is tied to a particular machine or apparatus, or (ii) it transforms a particular article into a different state or thing."

The *Bilski* court included limited guidance on the machine-or-transformation test in determining whether claims include patent-eligible subject matter. The court indicated that one could demonstrate that patentable subject matter is in the language of the claim by demonstrating that the process is tied to a particular machine. Alternatively, an applicant can demonstrate patentable subject matter by demonstrating that the process includes transforming an article. But the court warned that mere mention of a machine or transformation was not enough—the use of a "specific machine or transformation of an article must impose meaningful limits on the claim's scope to impart patent-eligibility."(**Fig. 1**) The court also indicated "the involvement of the machine or transformation in the claimed process must not merely be insignificant extra-solution activity." Regarding the transformation aspect of the test, the court held that a claimed process must transform an article into a different state or thing to be patent eligible and that the transformation must be central to the purpose of the claimed process. The court warned that "the raw materials of many information-age processes…are electronic signals and electronically manipulated data" and may involve the "manipulation of even more abstract constructs such as legal obligations, organizational relationships, and business risks."

In deciding *Bilski*, the court warned the public, however, that other courts may need to modify the machine-or-transformation test because "future developments in technology and the sciences may present difficult challenges to the test," drawing into question the general applicability and the longevity of the holding.

Possible ramifications

On January 28, 2009, Bilski filed a petition for certiorari with the US Supreme Court. Bilski challenged the requirement that a patent-eligible process must be tied to a particular machine or apparatus, or transform a particular article into a different state or thing. Bilski argued that the lower court's test was incongruent with the Supreme Court's prior determination to not limit the broad statutory grant of patent eligibility for any new and useful process. Bilski also requested that the court determine whether the Federal Circuit's machine-or-transformation test for patent eligibility contradicts the broadly articulated intent of Congress that patents protect "method[s] of doing or conducting business"[8]. In the request for Supreme Court review, Bilski argues that the machine-or-transformation test is inconsistent with Supreme Court precedent and the intent of Congress and is contrary to the court's rejection of the test in two prior precedential cases. Bilski's petition also asserts that method patents must include emerging technologies (that is, biotech).

The potentially devastating extension of *Bilski* from business methods to biotech is illustrated by two recent district court cases using the test to invalidate pharmaceutical claims. In *King Pharmaceuticals v. EON Labs.*[9], King Pharmaceuticals sued Eon Labs, and Eon argued that King claimed non-patentable subject matter under the *Bilski* rule. The subject matter involved a method of increasing the oral bioavailability of Skelaxin (metaxalone) to a patient receiving Skelaxin therapy, comprising administering to the patient a therapeutically effective amount of Skelaxin in a pharmaceutical composition with food. The court held that because increased oral bioavailability was an inherent property of the prior art, informing a patient of that inherent property does not constitute patentable subject matter (that is, informing a person of the phenomenon does not transform the Skelaxin into a different state or thing). Thus, the test articulated in *Bilski* was, according to the court, not satisfied and the claims were directed to subject matter that was not patentable.

A second case is *Prometheus Labs v. Mayo Collaborative*. In the case, a lower court determined that the patent at issue was invalid under §101 for claiming unpatentable subject matter (that is, natural phenomena). The patent at issue contains claims that describe the process of testing levels of certain metabolites in the blood of patients taking thiopurine drugs, which permits doctors to monitor a patient's metabolite level and thereby adjust medication levels to reach certain therapeutic goals. The court characterized the correlations between thiopurine drug metabolite levels and toxicity as natural phenomena. The court based its decision, at least in part, on the steps in the process directed to "administering" the medication and "determining" metabolite levels, indicating that determining the correlation was not patentable subject matter. Several briefs have been filed in *Prometheus*, including those submitted by Novartis Pharmaceuticals, the Biotechnology Industry Organization and the American Intellectual Property Law Association. The case is now pending before the Court of Appeals for the Federal Circuit. On January 9, 2009, Prometheus Labs filed its arguments for appeal, stating in terms found in the court's holding in *Bilski*, "the whole point of these processes is to transform the patient's body…the patient's body is transformed by administration of a synthetic thiopurine drug…[and] a sample of bodily fluid or tissue is transformed [using] sophisticated laboratory machines…and the resulting data is transformed into a warning… ." Prometheus Labs asserts that the lower court's finding would "threaten to invalidate the entire field of medical treatment and diagnostic patents on which the innovative and lifesaving biotech industry is built."

Another important case before the court is *Ariad Pharmaceuticals Inc. v. Eli Lilly & Co.*[10] The biotech process claim language at issue in this case describes a method for modifying effects of external influences on a eukaryotic cell, which external influences induce NF-κB-mediated intracellular signaling, the method comprising altering NF-κB activity in the cells such that NF-κB-mediated effects of external influences are modified, wherein NF-κB activity in the cell is reduced, and wherein reducing NF-κB activity comprises reducing binding of NF-κB to NF-κB recognition sites on genes which are transcriptionally regulated by NF-κB. Recently, Ariad and Eli Lilly argued before the Federal Circuit, wherein Lilly asserted that NF-κB inhibition to reduce the expression of genes was not patent eligible subject matter because it was no more than a "fundamental principle of nature. … They may have

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discovered something about that, but it's always been there and that's always the way it's been." Ariad rebutted this assertion, arguing that "the reduction of NF-κB … is very much a transformation," sufficient to meet the standard set for under the "machine-or-transformation" test the Federal Circuit recently announced as law in *In re: Bilski*. Ariad argued that the subject matter of the claims at issue is "a method for transforming the state of living cells, which are compositions of matter, which is a traditional transformative process. … It does not appropriate anything that exists in nature." He elaborated that the transformation was "changing the way a cell responds to its environment." In response, Lilly asserted, "it's not a particular transformation of a particular article" because "restricting the use to reducing NF-κB to reduce gene expression" fails to limit the invention so as to preempt all uses of a principle of nature.[11]

Going forward

Without doubt, the decision in *Bilski* significantly affects patent eligibility in the biotech arts. With respect to strategies for obtaining patents under the new law, *Bilski* indicates that claims must be considered as a whole, not analyzed as individual steps. In preparing a patent application, one should consider explicit reference to articles undergoing a transformative process. Such transformations can be explicitly included in the written application or by way of reference, for example, by incorporating the contents of a paper describing the transformation or by way of reference to what is already known in the state of the art. *Bilski* suggests that tying a claim to a machine (e.g., a computer) is not in and of itself sufficient to make allegedly nonstatutory subject matter patent eligible. The case also indicates that insignificant data gathering (that is, accumulating scientific data without specifying how it affords meaning to claim language) and post-solution or other extra-solution activity is insufficient to meet the threshold requirements for patentability. Rather, the implementation of a machine or the transformation of an article must impress meaningful limits on the claim's scope before the subject matter is patent eligible. The mere recitation of physical steps in a claim is insufficient to render the process patentable if the claim is not tied to a particular machine and does not result in a transformation. Thus, it may be advantageous to add claims that integrate steps and machines in the language of the claims.

Additional strategies to minimize risks associated with noneligible subject matter issues in patent procurement include preparing a description of the invention that adequately emphasizes various technical aspects of the biotech invention. There is an advantage in implementing this strategy in both the written description of the invention and the drawings depicting the invention as both serve to describe the invention. It is best to include various embodiments of the invention and to assess, before attempting to obtain a patent, secondary positions in the event that a *Bilski* issue arises. Additional strategies include providing descriptions, in biotech and pharma applications, of generic computer or machine processes, as well as very specific examples of machine implementation in the claimed processes. In doing so, it might be advantageous to have multiple recitations of the machine-implemented process, so that it can be argued that the machine or apparatus plays a fundamental role in the overall claimed process. Be aware that extra-solution activity is not enough to confer patent eligibility. If possible, prepare examples and information that defines transformed data as physical phenomena (that is, the visual depiction is not simply the manifestation of a single algorithm). The *Bilski* court observed that "transformation of that raw data into a particular visual depiction of a physical object on a display" may constitute a sufficient transformation to confer patent eligibility.

Regarding the protection of vital claims in patents that have already issued but arguably may not meet the test set forth in *Bilski*, it is possible to request a reissue under 35 USC §251. In this process, a patent is re-considered by the USPTO even though it was previously determined that the claims are patent eligible based on the patentee's assertion that there is an error in the claims or specification. To be eligible for this process, the "error" (that is, failure to comply with the test set forth in *Bilski*) must have been made without any deceptive intent. During this process, once claims are amended to comport with the machine-or-transformation test and subsequently allowed, the patentee must surrender the patent at issue to obtain a new patent. However, if this strategy is undertaken, it is important to remember that compliance with other provisions in the patent statute (such as novelty, nonobviousness and enablement) must also be determined and that there is no guarantee that the reissue process will proceed expediently.

Regarding addressing *Bilski* issues during litigation of an issued patent, in prelitigation negotiations or settlement talks, it is of value to fully understand the potential for compliance with the machine-or-transformation test of every process claim central to the dispute because each potentially susceptible claim may provide a new ground for an adversary to launch an invalidity attack. It is also important to consider whether or not a jury would be more or less sympathetic to and understanding of the science of biotech, as a *Bilski* issue is a legal issue, not an issue of fact for a jury to decide.

Conclusions

It is clear that as a result of the fundamental change in patent law and enforcement of the test articulated in *Bilski*, further limiting patent subject matter eligibility, the preexisting legal challenges facing biotech companies will only increase. This is particularly true for those companies seeking procurement or enforcement of IP involving medical diagnostics, therapeutic methods and personalized medicine. As companies and universities await potential modification of the *Bilski* test by the Supreme Court, they must act to ensure the future of their biotech R&D.

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8. 35 USC §273.
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Recent patent applications in RNA interference

Patent number	Description	Assignee	Inventor	Priority application date	Publication date
US 20090013433	A transgenic plant comprising a brassinosteroid receptor BRI1 kinase inhibitor 1 (BKI1)-type polypeptide or modulator of *bki1*-type gene expression; useful for displaying modulated brassinosteroid response and plant phenotype. The modulator in the transgenic plant is an RNA interference or antisense molecule that inhibits translation of an mRNA that encodes a BKI1-type polypeptide.	Salk Institute for Biological Sciences (La Jolla, CA, USA)	Chory J, Wang X	1/10/2007	1/8/2009
WO 2009002440	A nucleic acid molecule that downregulates expression of an epidermal growth factor receptor (EGFR) gene via RNA interference; useful for preparing a composition for treating or preventing EGFR-expressing cancer.	Liu Y, Xie FY, Yang X	Liu Y, Xie FY, Yang X	6/22/2007	12/31/2008
WO 2008155918	A method of promoting or inhibiting activity of hypoxia-inducible factor-1α (HIF-1α), involving increasing or reducing the interaction of intracellular inhibitor of factor inhibiting HIF-1 (iFIH) protein and factor inhibiting HIF-1 (FIH-1) protein, or increasing or reducing the interaction of intracellular membrane type 1–matrix metalloproteinase–cytoplasmic tail binding protein (MT1-MMP-CP) and FIH-1 protein. The interaction of iFIH-1 and FIH-1 protein is reduced by decreasing the expression of iFIH-1 protein by RNA interference.	University of Tokyo (Tokyo)	Sakamoto T, Seiki M	6/20/2007	12/24/2008
US 20080311081, WO 2008156702, WO 2008156661	An invasive bacterium comprising small interfering RNA that interferes with the mRNA of human papilloma virus (HPV) oncogenes; useful for treating or preventing a viral disease or disorder—e.g., HPV infection.	Cequent Pharmaceuticals (Cambridge, MA, USA); Fruehauf J, Laroux FS, Sauer NJ, Vaze MB, Beth Israel Deaconess Medical Center (Boston)	Fruehauf J, Laroux FS, Sauer NJ, Vaze MB, Li C	6/15/2007	12/18/2008, 12/24/2008, 12/24/2008
WO 2008152131	A new RNA interference agent comprising sense strand; useful for treating a human subject afflicted with cystic fibrosis or Liddle syndrome, and for treating and/or preventing hypertension and/or renal insufficiency in a human subject.	Novartis (Basel, Switzerland)	Danahay HL, Geick A, Hickman E, Tan P, van Heeke G, Vornlocher H	6/15/2007	12/18/2008
JP 2008301812	A kit comprising a vector containing a promoter derived from phage used for producing double-stranded RNA having an RNA interference effect in lactic acid bacteria. The lactic acid bacteria are useful in compositions for killing intestinal parasites and treating enteric diseases—e.g., *Caenorhabditis* and *Ascaris*.	Okayama University (Japan)	Otsuki T, Shishido M	5/10/2007	12/18/2008
WO 2008148304, CN 101314775	An HIV-targeted RNA interference target sequence; useful for treating HIV infection.	Xiamen University (Xiamen, China); Yang Sheng Tang Co. (Haikou, China)	Cheng T, Miao J, Xia N, Zhang J, Zhang T, Zhang Y	5/31/2007	12/11/2008, 12/3/2008
CN 101246169	A diagnostic reagent for oral squamous cell carcinoma comprising a reagent capable of detecting the expression level of RACK1 protein and its mRNA content; useful for preparing a kit or biochip for oral mucosa carcinoma. The drug comprises an RNA interference molecule of RACK1 protein.	University of Sichuan (China)	Ceng X, Chen Q, Huang C, Wang Z	5/23/2007	8/20/2008
JP 2008167739	A novel modified double-stranded RNA having RNA interference activity, comprising sense strand RNA having a complementary base sequence to a target gene and an antisense strand RNA for suppressing expression of the target gene.	National Institute of Advanced Industrial Science and Technology (Tokyo)	Bakalova R, Kubo T, Oba H, Zhelev Z	6/14/2006	7/24/2008

Source: Thomson Scientific Search Service. The status of each application is slightly different from country to country. For further details, contact Thomson Scientific, 1800 Diagonal Road, Suite 250, Alexandria, Virginia 22314, USA. Tel: 1 (800) 337-9368 (http://www.thomson.com/scientific).

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A splicing component adapted to gene silencing

Xavier Roca & Adrian R Krainer

U1 adaptors offer an alternative to siRNA for targeted gene knockdown.

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Methods for gene silencing, such as RNA interference[1] and antisense[2], have transformed the study of molecular, cellular and organismal biology and opened up new therapeutic opportunities. In this issue, Gunderson and colleagues[3], present an alternative technology for reducing gene expression based on ~25-nucleotide sequences called U1 adaptors. This approach could be applied to genes that are refractory to silencing by current methods or could be used in combination with other methods to achieve greater silencing potency.

The U1 small nuclear ribonucleoprotein particle (snRNP) comprises the 164-nucleotide U1 small nuclear RNA (snRNA) and 10 associated polypeptides. A component of the cellular splicing apparatus, it is best known for its role in recognizing the 5′ splice sites of introns through hybridization between these sequences and the 5′ end of U1 snRNA. The U1 snRNP has also been shown to inhibit polyadenylation of some pre-mRNAs by binding to a 5′-splice-site-like sequence in the 3′ untranslated region (3′ UTR), leading to degradation of the pre-mRNA[4,5].

This well-established mechanism of repression was previously exploited by the Gunderson laboratory for targeted silencing of endogenous genes in an approach dubbed U1 interference[6]. In this method, the 5′ end of U1 snRNA is mutated to enable it to bind the terminal exon of a target pre-mRNA (**Fig. 1a**). Although transfection of a plasmid expressing the mutated U1 snRNA efficiently knocks down the target transcript, U1 snRNAs with altered 5′ ends can bind nontarget transcripts and have unintended effects on splicing and polyadenylation, potentially limiting the utility of the approach. Moreover, the size of U1 snRNAs necessitates their expression from a plasmid or viral vector. In the present study, Gunderson and colleagues[3] provide an elegant solution of these difficulties by using ~25-nucleotide sequences to recruit endogenous U1 snRNPs to the terminal exon or 3′ UTR of the target pre-mRNA (**Fig. 1b**). U1 adaptors comprise a 5′ sequence that binds the target and a 3′ sequence that binds the 5′ end of the U1 snRNA. Bifunctional oligonucleotides, as well as oligonucleotide-peptide conjugates, have been used previously to recruit splicing factors or their activation domains to specific sites on pre-mRNAs to change their splicing patterns[7,8].

The authors show that U1 adaptors effectively silence two reporter and two endogenous transcripts with IC_{50}s in the nanomolar range. They test different nucleotide chemistries[1], including 2′-*O*-methyl, phosphorothioate and locked nucleic acid modifications, at different positions in U1 adaptors to increase binding affinity and oligonucleotide stability and to avoid degradation of the target transcript by endogenous RNase H. Remarkably, the best U1 adaptor proved effective at subnanomolar concentrations, similar to the best short interfering RNAs (siRNAs). Further refinement will be required to derive general rules for optimal U1 adaptor design and target choice.

As intended, U1 adaptors offer substantial advantages over the mutated U1 snRNAs developed previously[6]. First, the use of short, chemically synthesized oligonucleotides rather than longer plasmid-expressed sequences allows one to draw on the considerable accumulated experience with delivery of siRNAs and antisense oligos in cultured cells and *in vivo*[1]. An ever-increasing array of chemical modifications is being developed to enhance the efficacy of therapeutic oligonucleotides by increasing their stability, reducing off-target effects and abrogating side-effects such as immunostimulation. Several approaches to targeted oligonucleotide delivery are also available[1].

Second, U1 adaptors rely on the endogenous U1 snRNP, which is extremely abundant in all cells and is present in excess over other components of the splicing machinery, thereby avoiding the potential off-target effects of mutated U1 snRNAs[6]. Diverting a small fraction of U1

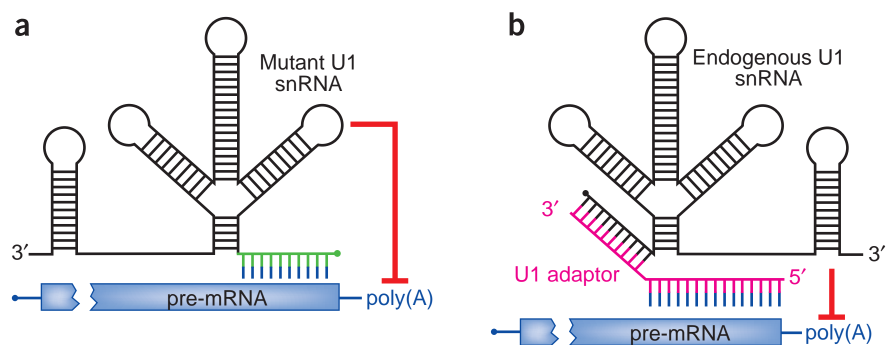

Figure 1 Two gene-silencing methods based on tethering the pre-mRNA of the target gene to the U1 snRNP. In both methods, recruitment of the U1 snRNP to the terminal exon of the pre-mRNA inhibits addition of a poly(A) tail, leading to degradation of the transcript. (**a**) A U1 snRNA with a mutated 5′ end (green) complementary to the pre-mRNA is delivered to a cell. The engineered U1 snRNA is assembled into a U1 snRNP complex and binds to the target within the pre-mRNA. (**b**) Endogenous U1 snRNPs are recruited by U1 adaptors (pink). U1 adaptors are 25-nucleotide sequences containing a 5′ target domain complementary to the 3′ terminal exon of the pre-mRNA and a 3′ U1 domain complementary to the 5′ end of the U1 snRNA. ORF; open reading frame. The dot denotes the 5′ cap.

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snRNPs to a specific pre-mRNA is not expected to affect processing of other transcripts. The authors show that alternative splicing of four human genes likely to be sensitive to the levels of splicing factors is not discernibly affected by U1 adaptors. Moreover, a genome-wide microarray analysis for one U1 adaptor targeting an endogenous gene shows that its off-target effects are indistinguishable from those associated with an siRNA targeting the same gene. In light of our own recent data demonstrating that sequestration of U1 snRNP by specific, transiently expressed RNA decoys changes the splicing of certain reporter pre-mRNAs[9], thorough transcriptome-wide analysis of the effects of U1 adaptors would definitively identify or rule out unspecific effects on splicing.

As Gunderson and colleagues[3] show, U1 adaptors act synergistically with siRNAs to enhance gene knockdown. In one experiment, a U1 adaptor enhanced siRNA-mediated inhibition approximately tenfold. This combinatorial effect highlights the potential of U1 adaptors to boost gene silencing, allowing different effectors to be administered at lower doses, minimizing toxicity and off-target effects, and reducing costs. Specifically, the possible toxicity caused by diverting the RNAi machinery to exogenous siRNAs[1] might be relieved by combining RNAi-dependent and -independent silencing methods. Finally, there are genes for which loss-of-function data have not been obtained because they are refractory to current silencing methods or because they are essential and expressed at limiting levels. U1 adaptors could fill the gap for the former set of genes, such as heterogeneous nuclear ribonucleoprotein L (*HNRNPL*), which appears unresponsive to RNAi in certain cell lines[10].

U1 adaptor–mediated knockdown is a useful addition to the growing list of gene-silencing methods. These methods have been used effectively to study the function of individual genes and for genetic screens. In addition, many therapeutic oligonucleotides are in early-stage clinical trials[1,2]. Expanding the available techniques for targeted gene silencing should improve the odds of achieving successful outcomes in the clinic.

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Jump-starting cancer gene discovery

Anthony Uren & Anton Berns

Targeting transposon mutagenesis to a specific tissue facilitates screening for tumor-associated genes and tracking of tumor lineages.

Tumor induction by insertional mutagenesis has proven very useful in cancer research because of the ease with which cancer-causing mutations can be identified. But the approaches used in these studies—primarily slow-transforming retroviruses and transposon gene traps—are able to induce tumors in only a limited range of tissues. As described in this issue, Keng *et al.*[1] have overcome this drawback with a method that activates the Sleeping Beauty transposon in any tissue of interest. Taking the liver as an example, they have created a mouse model of hepatocellular carcinoma (HCC) and identified new and known genes associated with this cancer.

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The Sleeping Beauty transposon was first adapted for use as an oncogenic mutagen several years ago by combining a transposon gene trap designed to activate and disrupt nearby genes with a transposase transgene expressed in all tissues[2,3]. Whenever random transposition in or near host genes alters their activity and confers a selective growth or survival advantage to a cell, clonal expansion of that cell ensues. Subsequent insertions in the expanding clone can endow it with additional growth potential, and this may eventually give rise to a tumor in which the genes that drive tumorigenesis are marked by transposon insertions. Many of the recurrent mutations found in these tumors are also found in human cancers[2,3]. Nonetheless, despite ubiquitous transposase expression in these mice, the range of tumors generated was limited primarily to T-cell lymphomas[2], except in a p19^{ARF} knockout background, which predisposed the mice to develop sarcomas[3]. Similarly, the tropism of slow-transforming retroviruses has limited their use to the study of hematopoietic and mammary tumors[4].

Keng *et al.*[1] combined a Cre-inducible transposase allele with a transgene expressing Cre recombinase under the control of an albumin-gene promoter to specifically target transposon mutagenesis to the liver (**Fig. 1**). The resulting multifocal tumors arise after a long latency period, although their appearance can be accelerated by the presence of a dominant p53 mutation. The tumors mimic their human counterparts in several ways: they express the HCC marker α-fetoprotein, show a higher incidence in males than in females and frequently metastasize to the lung.

By cloning insertions from 68 preneoplastic nodules, mostly in the p53 mutant background, the authors identified 19 common insertion sites (CISs)—regions of the genome that are mutated more than would be expected by chance. The most prominent of these CISs contains truncating mutations within the *EGFR* gene. Although in humans mutations in *EGFR* are most commonly associated with lung tumors, the gene is also frequently overexpressed in HCC. Keng *et al.*[1] validate the oncogenic capacity of the truncated form by introducing it into the livers of adult mice and documenting the induction of preneoplastic foci. Other CIS genes they identified have also been previously implicated in human HCC. For instance, *HIF1A* is frequently overexpressed in human HCC, and the hepatocyte growth factor receptor gene *MET* was previously shown to induce liver tumors in mice[5] and to be amplified in human hepatocellular carcinoma[6]. Comparison of the list of mouse CIS genes with human genes implicated in HCC by comparative genomic hybridization analysis reveals a number of new loci altered in both datasets. One of the CIS genes, *UBE2H*, whose expression is amplified in certain tumors, confers enhanced proliferation when transfected into a hepatocyte cell line.

Several of the mice in this study had nodules in the lung, a frequent site of metastases in human HCC[7]. These nodules were metastases was confirmed to be metastases in one of the mice by showing that they shared multiple CIS insertions with a liver nodule taken from the same mouse. It will be interesting to see whether

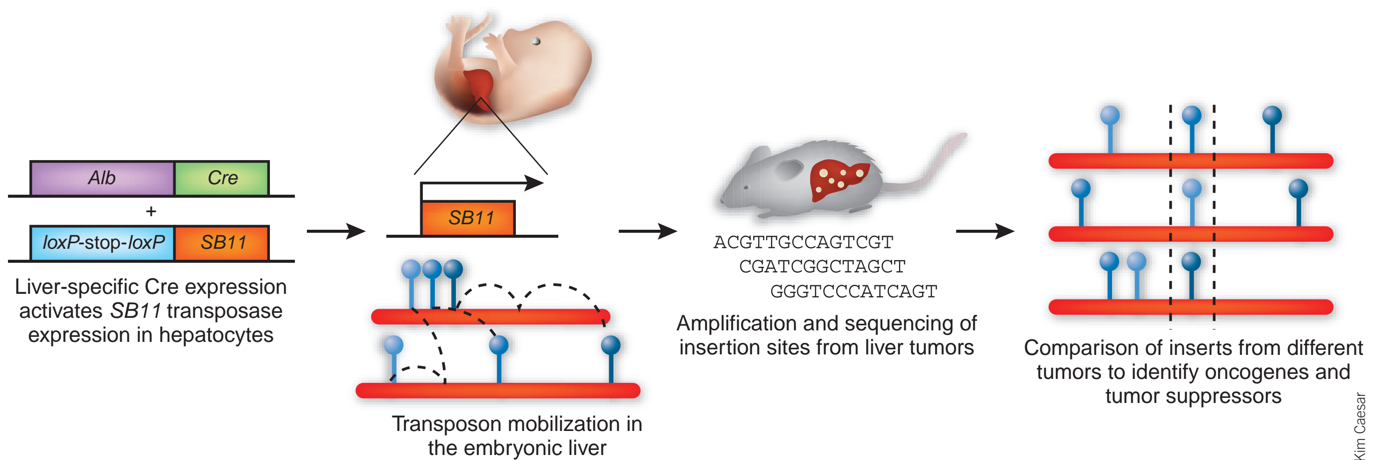

Figure 1 A conditional Sleeping Beauty transposase (*SB11*) allele is crossed to a Cre transgene under control of the albumin-gene (*Alb*) promoter to specifically induce expression in the liver. Liver-specific mobilization of transposons (blue) creates mutations that give rise to liver tumors. Isolating the insertion sites and mapping them onto the genome identifies regions disrupted repeatedly in different tumors, implicating these as oncogenes and tumor suppressors.

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future analysis of larger cohorts of mice with lung metastases can identify mutations that facilitate this process.

The relatedness of different liver nodules in the same mouse is less clear. Whereas the vast majority of insertions within the screen were unique, dozens of identical insertions were identified within the *EGFR* locus, with one of the TA dinucleotides in the 24th intron having 13 insertions in the nodules studied. The presence of recurrent identical *EGFR* insertions in different mice suggests that this concentration of insertions at a single base pair is a product of intense positive selection, coupled with preferential insertion bias of the transposase due to the local chromatin structure or sequence context. However, there were also examples of tumor nodules within the same animal that carried identical insertions, and these could also be explained by a common origin, either as a consequence of intrahepatic metastasis or perhaps because both nodules derive from the same population of mutated premalignant liver cells.

As expression of the albumin gene is detectable in the hepatic endoderm as early as embryonic day 9.5, transposition during early liver development may result in some insertions being present in large sections of the adult organ, particularly if these mutations confer a selective advantage. Nonmalignant clonal expansion has previously been observed in the hematopoietic compartment of mice infected with murine leukemia virus[8], and clonal expansion (field cancerization) is also a known feature in oral squamous-cell carcinomas, which often arise from a field of premalignant cells with normal morphology[9]. If insertions found in *EGFR* are also observed in nonmalignant tissue, this may even suggest a role for these mutations in inducing clonal expansion of hepatocytes, with subsequent mutations giving rise to multiple related tumors. Whether or not this holds true for tumors in this model, insertional mutagens seem uniquely qualified for the study of preneoplastic alterations and the clonal relatedness of apparently independent tumors. The system provides the opportunity to trace the lineage of tumors and define the order in which lesions have occurred.

Given the track record of insertional mutagenesis screens over the past three decades, we are excited at the prospect of using any method of tissue-specific Cre expression to target a range of cell populations. It will be possible not only to screen new tumor types but also to begin detailed examination of the role that the cell of origin plays in tumorigenesis. These screens also provide an invaluable cross-validation platform for the cancer genome sequencing efforts that are underway worldwide[10].

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Combinatorial stem cell mobilization

Mikhail G Kolonin & Paul J Simmons

Different subsets of bone marrow stem cells can be mobilized by varying drug treatments.

The development of strategies to induce the release of stem cells and derivative progenitor cells from the bone marrow into the blood, a phenomenon termed mobilization, has focused almost exclusively on hematopoietic stem cells (HSCs)[1,2]. Thus far, there have been no reports of reproducible, efficient methods for mobilizing other stem or progenitor cells in the bone marrow, including endothelial progenitor cells (EPCs) and stromal mesenchymal progenitors, more commonly referred to as mesenchymal stem cells (MSCs). In a recent issue of *Cell Stem Cell*, Pitchford *et al.*[3] outline new ways of selectively recruiting different subsets of these cell populations into the circulation, an approach that may have considerable therapeutic benefits.

The agent used most frequently to elicit HPC mobilization is the myeloid cytokine

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granulocyte colony-stimulating factor (G-CSF)[2], and such is the efficacy of mobilized peripheral blood for collection of hematopoietic stem and progenitor cells that it has now essentially replaced bone marrow as a source for hematopoietic reconstitution in cancer therapy[2]. Mobilization of EPCs, which subsequently contribute to neovascularization of ischemic tissues, has been achieved with vascular endothelial growth factor (VEGF)[4]. Pitchford *et al.*[3] studied combinations of G-CSF, VEGF and, in addition, the CXCR4 inhibitor AMD3000, also known as Mozobil (plerixafor), which enhances mobilization of HSCs by blocking binding of CXCR4 to stromal cell–derived factor (SDF)-1α. Mice were treated with combinations of these three drugs and the resulting effects on the proliferation and activation of stem and progenitor cells in the bone marrow, as well as their release into peripheral circulation, were measured (**Fig. 1**).

Pretreatment of mice with G-CSF before AMD3000 administration dramatically increased the egress of HSCs and neutrophils, but not EPCs, from the bone marrow. In marked contrast, pretreatment with VEGF before administration of AMD3000 reduced the release of HPCs and leukocytes into the circulation but increased mobilization of EPCs compared with the CXCR4 inhibitor alone. The authors also examined mobilization of MSCs (termed stromal progenitor cells in this study) after treatment with each of the regimens. Mobilization of MSCs was not detected with G-CSF alone or in combination with AMD3000. The only condition that resulted in significant mobilization of MSCs was pretreatment with VEGF before administration of AMD3000.

With their unique combination of multipotent differentiation potential and immunosuppressive properties, MSCs are appropriately considered a promising cell type for regenerative medicine. MSCs normally circulate at low to undetectable frequencies, and there is an ongoing debate as to whether these levels increase in pathological conditions[5]. The capacity to mobilize MSCs in healthy mice, as reported by Pitchford *et al.*[3], is consequently an observation of potentially very great significance given the profound impact that efficient HPC mobilizing agents have had in the field of hematological transplantation.

Nevertheless, one must be cautious in interpreting data on MSCs as this field is fraught with examples of poorly characterized cells analyzed *in vitro* using bioassays that do not predict *in vivo* potency[6]. It is noteworthy that the baseline frequency of fibroblast colony-forming cells (CFU-F) reported by Pitchford *et al.*[3] in the bone marrow is considerably higher than that reported in previous studies. So in the future it will be important to confirm the mesenchymal identity of these cells immediately upon isolation and to distinguish them from diverse populations of plastic-adherent cells, such as monocytes.

Good experiments raise as many new questions as they yield answers. A key question arising from this study concerns the mechanism(s) responsible for the release of EPCs and MSCs elicited by combined VEGF and AMD3000 administration. The release of HPCs into the peripheral blood initiated by G-CSF involves activation of proteases locally within the bone marrow, perturbation of adhesion-molecule function and disruption of SDF-1α/CXCR4 signaling[7], but it is not clear whether similar mechanisms underlie EPC and MSC mobilization. The absence of VEGF receptors on MSCs suggests that, in the case of this class of progenitor, the effects of VEGF must be indirect.

An additional important question concerns the tissue of origin of the circulating progenitors in the model of Pitchford *et al.*[3]. Although bone marrow is often considered the major source of circulating progenitors, accumulating evidence suggests that cells with MSC properties exist in many organs. For example, experiments in rodent models have recently demonstrated that white adipose tissue overgrown in obesity is a source of progenitors that could also undergo mobilization and engage in reparative tissue remodeling.

In conclusion, this exciting study suggests that it may be possible to tailor the mobilization of individual populations to specific pathological conditions. However, it should be emphasized that the introduction of new mobilization regimes into the clinic must proceed with caution. In pathologies such as cancer—ironically the setting in which stem cell reconstitution is needed most—mobilization of progenitors could be a double-edged sword. Indeed, recruitment of vascular progenitors by tumors is observed in patients[8] and drives the progression of cancer through increased tumor blood vessel formation in animal models[9]. Hematopoietic and mesenchymal/stromal progenitors are also recruited by tumors and contribute to cancer progression[9]. As noted above, white adipose tissue is a particularly rich source of progenitors, raising a possibility that MSC and/or EPC mobilization from this tissue could partially account for the association between obesity and cancer progression, which has recently emerged[10]. Thus, any proposed new clinical regimens for stromal progenitor mobilization would require rigorous safety assessments.

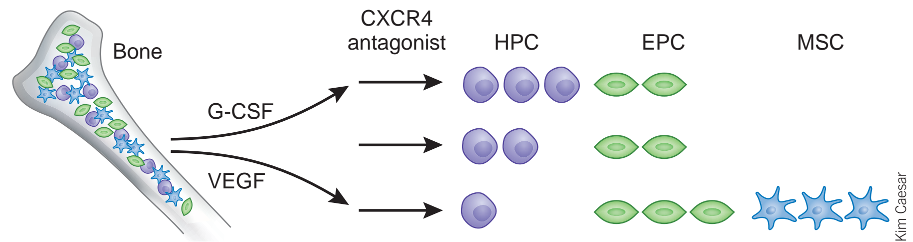

Figure 1 Regimens for differential mobilization of populations of stem and progenitor cells from the mouse bone marrow. Pitchford *et al.*[3] treated mice with a CXCR4 antagonist alone or preceded by G-CSF or VEGF. HSC: hematopoietic stem cell; EPC: endothelial progenitor cell; MSC: mesenchymal stromal progenitor cell. Number of cells drawn corresponds to the relative egress of each progenitor population into peripheral blood induced by treatment.

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Sorghum sequenced

Sorghum bicolor is an important staple for humans and livestock in northern Africa and is increasingly cultivated as a biofuel crop in arid parts of North America and Asia, where farmers like the advantages associated with its hardiness and C_4 photosynthetic pathway. Nonetheless, improvements in sorghum yield have lagged behind those of other agronomically important grains. Assembly of the ~760-Mb genetic blueprint of sorghum, using a modified shotgun approach that takes into account the highly repetitive nature of many large eukaryotic genomes, is especially noteworthy as it is the first genome of a tropical grass. The high conservation of grass gene order (synteny) should facilitate improvement of its close relatives, the bioethanol crops sugarcane and *Miscanthus*. Until now, the only cereal genome has been that of rice, a temperate species with C_3 photosynthesis. Knowing the genetic complement of a C_4 plant could thus accelerate realization of the long-sought goal of improving the photosynthetic efficiency of C_3 species. Although the sorghum genome is ~75% larger than the rice genome, the numbers of genes and sizes of gene families are similar in the two species, with a remarkable >98% concordance in intron position and phase. Most of the extra DNA in the sorghum genome is heterochromatin and largely comprises long terminal repeat retrotransposons. (*Nature* **457**, 551–556, 2009) *PH*

Profiling the common cold

Ninety-nine strains of human rhinovirus, the etiological agent of the common cold, have thus far been identified. To better our understanding of the differences among these strains at the genome level, Palmenberg *et al.* report the sequencing and analysis of all known human rhinovirus genomes. The investigators then use this sequence information to build a phylogenetic tree for the rhinovirus genus, incorporating the handful of previously sequenced rhinovirus genomes and including a recently identified virus species as well as ten clinical isolates from rhinovirus-infected people. Using a protocol optimized to sequence the noncoding ends of the viral genome, and taking into account the potential three-dimensional structure of the virus's single-stranded RNA genome, the researchers identify a noncoding region of the genome that varies even among isolates of the same rhinoviral strain and that is analogous to regions in other viral genomes that determine pathogenic potential. The group's analyses also reveal that surprisingly extensive genetic recombination has occurred throughout rhinovirus evolutionary history. This genetic variation may underlie the recent poor performance in clinical trials of antirhinoviral therapies and suggests that the pursuit of clade-specific treatments may prove fruitful. (*Science* published online, doi:10.1126/science.1165557, 12 February 2009) *CM*

Silencing host and pathogen

Although most antiviral therapies have until recently targeted viral proteins, emphasis is shifting to target host proteins associated with viral replication. Wu *et al.* now combine the two approaches by designing a small interfering (si)RNA-based strategy that targets the expression of both a herpes simplex virus type 2 (HSV-2) protein and a host protein in mice. To achieve this, the investigators apply topically, to the epithelium of the vagina, two siRNAs: one that knocks down nectin-1, a cellular receptor for the viral envelope glycoprotein D; the other that inhibits UL29, a viral DNA binding protein. The siRNAs are conjugated to cholesterol to enhance the uptake by the epithelial cells and protected from cervicovaginal RNases by 3′ phosphorothioate modifications. The onset of the protection is extremely fast, and even treatment 3 and 6 hours after exposure to the virus protects 80% of the mice. The protective effect is long-lasting, with mice resistant for up to a week from an otherwise lethal viral challenge. The early onset effects are mainly due to the direct targeting of the viral mRNA, whereas prolonged protection requires the efficient downregulation of the cellular receptor. The approach may prove useful not only for HSV-2 treatment but also for other sexually transmitted viruses, such as HIV. (*Cell Host Microbe* **5**, 84–94, 2009) *ME*

Reversing brain drain

Brain-derived neurotrophic factor (BDNF) is found throughout the entorhinal cortex and hippocampus where memory and learning are established. The loss of BDNF from those regions in Alzheimer's disease led Tuszynski and colleagues to question whether providing BDNF could reverse or ameliorate symptoms of the disease. Their findings in several animal models of neurodegeneration suggest that it can. In a transgenic mouse model, J20, that expresses human amyloid precursor protein, injecting a lentivirus constitutively expressing BDNF into the entorhinal cortices resulted in improvements in tests of spatial memory compared with control lentivirus– or sham-injected mice. Additionally, more normal expression of 55% of genes whose expression is altered by amyloid plaques was restored, as were synaptic markers in the cortex and hippocampus. Similar improvements in test performance as well as gene expression were obtained with cognitively impaired aging rats. BDNF also prevented cell death both *in vitro* (primary entorhinal neurons exposed to toxic Ab1-2 protein) and *in vivo* (injury-induced neuron loss in rats). Finally, in perhaps the best animal model of neurodegeneration, aging monkeys, BDNF injections improved visual-spatial discrimination a month after treatment. Although delivery into human patients would be challenging, the authors suggest that their results warrant consideration in the clinic. (*Nat. Med.* advance online publication, doi:10.1038/nm.1912, 8 February 2009) *LD*

Nanoscale MRI microscopy

Magnetic resonance imaging (MRI) is widely used in medicine and physiology. Its utility for microscopy and structural biology, however, has been limited by its comparatively low resolution. Degen *et al.* now present a new MRI technique that improves the maximal spatial resolution to <10 nm. Their approach is based on sensitive force measurements between a 200-nm-diameter magnetic tip and the sample. The force is generated by triggering nuclear magnetic resonance in the sample by a radio frequency–modulated magnetic field and is proportional to the density of ^1H in the observation volume. Spatial resolution is achieved by moving the probe tip in a three-dimensional pattern at a distances of 24–62 nm from the surface of the sample. A three-dimensional picture is generated by computer reconstruction of the hydrogen atom densities. Degen *et al.* imaged dried tobacco mosaic virus particles at 0.3 K. In the future, this technique could be used to obtain high-resolution images of any biological cryosample using common MRI contrast techniques. (*Proc. Natl. Acad. Sci. USA* **106**, 1313–1317, 2009) *ME*

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Maximizing power in association studies

Eran Halperin & Dietrich A Stephan

Only a subset of genetic variants can be examined in genome-wide surveys for genetic risk factors. How can a fixed set of markers account for the entire genome by acting as proxies for neighboring associations?

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The etiology of many complex diseases is attributed to a combination of genetic and environmental risk factors. Knowledge of these influences yields insight into disease mechan isms and can thus ultimately enable better preventive, diagnostic and therapeutic strategies. The most common genetic variants in the human genome are single nucleotide polymorphisms (SNPs)—point mutations with multiple possible alleles at a locus across the population. Genome-wide association (GWA) studies examine the set of cases and controls at many polymorphic sites and often identify one or several physical location(s) in the genome where genetic variation contributes to disease susceptibility[1].

Although conceptually straightforward, the statistical and computational aspects of GWA studies are considerable. They encompass the design of a well-powered study, controlling for confounding risk factors (e.g., population structure or exposure to environmental risks), accurate genotyping, correcting for multiple hypothesis testing and defining interactions between different SNPs. Such statistical measures are necessary whether we use current high-density SNP genotyping approaches or complete whole-genome sequencing in the future. We discuss here the foundation that allows us to capture information about regions of the genome that are currently not genotyped using standard high-throughput technologies. Understanding these computational approaches is key to maximizing identification of disease-associated DNA variants.

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Indirect association and linkage disequilibrium

Recent technological advances allow us to rapidly genotype $>10^6$ SNPs in an individual, accounting for 10% of the estimated number of common SNPs (>1% minor allele frequency) across the population[2]. As a result, true associations might be missed if the causal SNP is not genotyped or if the causal variant is an unknown variant. Computational methods have been developed to account for some of the unobserved variants[3–7]. The rationale for these methods is based on the observation that SNPs in close proximity to one another in the genome tend be correlated, or in linkage disequilibrium.

There are a few metrics that measure the linkage disequilibrium between a pair of SNPs. The linkage disequilibrium parameter D measures the linkage disequilibrium between a pair of SNPs s_1 and s_2. D is defined as $D = P_{12} - p_1p_2$, where P_{12} is the frequency of chromosomes with the minor allele present in both SNPs, and p_i is the frequency of the minor allele frequency at SNP s_i. Intuitively, D measures the deviation of the joint distribution from the case where the SNPs are inherited independently. It is largely determined by the recombination rate between the two SNPs. If ρ is the probability of a recombination in a single meiosis in the region spanned by these SNPs, the linkage-disequilibrium parameter should change to $D_n = (1-\rho)D_{n-1}$ in subsequent generations. A more commonly used metric is $D' = D/D_{max}$, where D_{max} is the maximal possible value of D for the given allele frequencies p_1 and p_2. As this metric does not directly depend on the allele frequencies, we can compare 'apples to apples' when contrasting linkage disequilibrium between different pairs of SNPs.

In association studies, linkage disequilibrium between SNPs can be used to replace a direct association test with an indirect one (tagSNP). As current technology does not allow us to genotype all known SNPs, we pick a set of tagSNPs such that the ungenotyped SNPs (or hidden SNPs) are in linkage disequilibrium with the tag SNPs. Thus, if the causal SNP is a hidden SNP, we expect to find a correlation between the phenotype and the tag SNPs due to correlation between the two SNPs. To do so, we first have to decide on a criterion for when one SNP 'captures' another. Although D' is a possible candidate, the relation between D' and the power to detect association is not clear. Alternatively, one can simply measure the correlation coefficient r between the two SNPs. The correlation coefficient is a measure, which ranges from –1 to 1, of how well two variables predict each other; formally, it is defined as

$$r = \frac{D}{\sqrt{p_1(1-p_1)\,p_2(1-p_2)}}$$

Often, the square of the correlation coefficient is used; whereas $r^2 = 1$ indicates that the two SNPs are perfectly correlated, $r^2 = 0$ implies that the two SNPs segregate independently throughout the population. The correlation coefficient is often chosen as the criterion for selecting tagSNPs, as there is a direct relation between r^2 and the power to detect association. If the true causal SNP is s_1, then the power to detect association at s_2 by genotyping N individuals is approximately the power attained by genotyping r^2N individuals at s_1 (ref. 8).

Based on this observation, an ideal set of tagSNPs will be a minimal set of SNPs with a high correlation coefficient between every hidden SNP and its corresponding tagSNP. The definition of 'high' may be somewhat subjective, and it generally depends on the resources available (that is, the total number of SNPs that will be genotyped). As the power to detect association in SNPs depends on their allele frequency, it is advised to use a more stringent threshold for such SNPs. In practice, however,

Table 1 Haplotypes improve the prediction of hidden SNPs

SNP1	SNP2	SNP3	Frequency
A	A	C	23%
A	A	T	1%
G	A	C	40%
G	G	C	2%
G	G	T	34%

SNPs 1 and 2 alone have poor power to predict the genotype at SNP 3, even when the phased haplotype is known. But together, using a multimarker tagSNP approach, SNPs 1 and 2 predict SNP 3 with 97% accuracy.

association studies are normally designed with a fixed threshold in mind for all SNPs; a common choice is a threshold of $r^2 > 0.8$.

Unfortunately, defining the best set of tagSNPs is computationally intractable in its full general form. In practice, an iterative greedy algorithm works well[3]. This algorithm analyzes a reference data set, such as the data provided by the International HapMap Project, in which 270 individuals from four different populations were genotyped at 3.1 million SNPs across the genome[9]. The algorithm finds a set of tagSNPs that 'covers' all other SNPs, where SNP s_1 covers SNP s_2 if the r^2 between them is larger than a threshold specified by the user. The algorithm works in iterations; initially, all the genotypes of the SNPs in the reference data set (in this case 3.1 million) are considered 'uncovered'. An iteration involves finding a tagSNP that covers the maximum number of uncovered SNPs. The tagSNP, as well as the SNPs that it covers, is considered covered from that point further. The algorithm ends when all possible SNPs are covered. This method is effective and widely used to define linkage-disequilibrium structure and tagSNPs in the genome. The scaffold can then be superimposed on the available high-density genotyping platforms, and the subset of hidden SNPs that the platform captures can be identified.

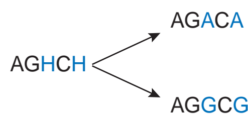

Figure 1 TagSNPs and haplotype information can enhance the ability to identify disease-related loci using linkage disequilibrium. On the left side is a genotype of an individual, where A,G,C,T correspond to homozygous genotypes and 'H' denotes a heterozygous genotype. On the right side are the two haplotypes of the same individual. Another possible pair of haplotypes that explain the genotype is AGACG, AGGCA. Phasing methods use the population information to infer which of the possible haplotypes is correct.

Multimarker methods and haplotypes

We have discussed the possibility of having one tagSNP that covers a hidden SNP. Often, multiple tagSNPs serve as a better proxy for a hidden SNP than any single tagSNP. In **Table 1**, SNPs 1 and 2 cannot serve as a proxy to SNP 3, but together, they correlate almost perfectly to SNP 3 (that is, when SNPs 1 and 2 carry the A allele, then SNP 3 most likely carries the C allele). We can thus predict SNP 3 with a 3% error rate by considering only tagSNPs 1 and 2—a considerably better outcome than when using SNP 1 or 2 alone. Note, however, that we are using the haplotype information and not genotypes. Unlike genotypes, which represent the allelic information on both chromosomes, haplotypes represent the information on only one of the chromosomes (**Fig. 1**).

Current whole-genome platforms can geno type a fixed set of SNPs that cannot be customized per experiment. To take advantage of haplotypes within these constraints, de Bakker *et al.* suggested that for every hidden SNP *s*, one can exhaustively search the HapMap data set for a proxy haplotype for which the square of the correlation coefficient with SNP *s* is higher than a given threshold[4]. Deriving a haplotype proxy is not a computationally trivial task, as the number of potential haplotypes is enormous. In principle, every set of SNPs (not necessarily consecutive) may potentially span a haplotype proxy. Exhaustively searching across all possible sets of SNPs is infeasible; however, to allow for a manageable running time, the algorithm considers only short haplotypes (2–3 SNPs) and only SNPs in close proximity to the hidden SNP. As SNPs that are physically far from the hidden SNP are unlikely to correlate well with it owing to increased probability for recombination between the sites, these restrictions do not cause substantial loss of information. Once the proxy is found, the haplotype can be tested for association with the disease by performing a standard χ^2 test. de Bakker *et al.* have shown that the use of haplotypes is beneficial and consequently increases the power to detect an association[4]. Intuitively, this is because the number of haplotypes in any given region is smaller than the number of genotypes (**Table 2**), resulting in a larger sample size that is used to estimate any given haplotype. More importantly, the haplotypes represent the ancestral genetic structure that is shaped by evolutionary forces such as recombination rates and mutations, and these are implicitly taken into account when haplotypes are analyzed, as opposed to genotypes.

The above discussion deals with the case where the set of genotyped SNPs is not necessarily fixed. However, in practice, high-throughput genotyping platforms are designed so that there is no flexibility in the tag SNP selection.

Table 2 Genotype prediction power

SNP1	SNP2	SNP3	Frequency
A	A	C	5.3%
A	A	H	0.5%
H	A	C	18.4%
H	H	C	0.9%
H	H	H	15.7%
H	A	H	0.8%
H	H	T	0.7%
G	A	C	16%
G	H	C	1.6%
G	H	H	27.2%
G	G	H	1.3%
G	G	T	11.6%

When using genotype information, the same SNPs 1 and 2 have less power to predict the genotype at SNP 3, as the third SNP remains ambiguous even when the full genotype information is given at SNPs 1 and 2. 'H' denotes a heterozygote for that SNP.

Recently, different approaches have been proposed to choose a set of haplotype-based statistical tests that will be performed on the data given a fixed set of tagSNPs. One generalization of the haplotype-based test assigns a weight w_i to each haplotype h_i, and the resulting proxy for a nearby SNP is given by $\sum_i w_i h_i$ (ref. 5). An optimal choice of the weights guarantees improved power compared to the single-SNP or single-haplotype tests as these tests correspond to specific choices of the weights. It turns out that such an optimal set of weights corresponds to the probabilistic 'imputation' of a hidden SNP using the observed SNPs; in other words, we can use the haplotype structure of a reference population such as the HapMap[9] to learn the conditional distribution of a hidden SNP based on the haplotype distribution in the tagSNPs. Currently there is a major effort to improve the methods for imputation of hidden SNPs, as these methods promise to improve the power of association studies and to reach SNPs that have not been genotyped in the study. We will discuss these methods and their applications in genome-wide association studies in a future paper.

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nature biotechnology

Gene silencing by synthetic U1 Adaptors

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We describe a gene silencing method that employs a mechanism of action distinct from those of antisense and RNA interference. U1 Adaptors are bifunctional oligonucleotides with a 'target domain' complementary to a site in the target gene's terminal exon and a 'U1 domain' that binds to the U1 small nuclear RNA component of the U1 small nuclear ribonucleoprotein (U1 snRNP) splicing factor. Tethering of U1 snRNP to the target pre-mRNA inhibits poly(A)-tail addition, causing degradation of that RNA species in the nucleus. U1 Adaptors can inhibit both endogenous and reporter genes in a sequence-specific manner. Comparison of U1 Adaptors with small interfering RNA (siRNA) using a genome-wide microarray analysis indicates that U1 Adaptors have limited off-target effects and no detectable adverse effects on splicing. Further, targeting the same gene either with multiple U1 Adaptors or with a U1 Adaptor and siRNA strongly enhances gene silencing.

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RNA interference (RNAi) to silence specific vertebrate genes has rapidly become a standard method for analyzing gene function and has garnered much attention as a promising molecular therapy[1,2]. RNAi silences gene expression by degrading the target mRNA in the cytoplasm and typically uses synthetic siRNA duplexes[3] or engineered plasmid or viral vectors that express precursor RNAs, such as short hairpin RNAs. We previously reported a gene silencing technology called U1 small nuclear (sn)RNA interference (U1i). In this method, a plasmid vector is used to express a U1 snRNA in which the natural U1 targeting domain is replaced by a ten-nucleotide (nt) sequence complementary to the target's terminal exon[4,5]. The U1i snRNA assembles into a U1 snRNP complex that hybridizes to the target's pre-mRNA and inhibits poly(A) tail addition, an obligatory RNA processing step for nearly all eukaryotic mRNA[5,6]. Without polyadenylation, the pre-mRNA fails to mature and is degraded in the nucleus, thereby reducing cytoplasmic mRNA levels of the target gene. The mammalian U1 snRNP comprises ten proteins bound to the 164-nt U1 snRNA (**Fig. 1**) and functions early in splicing through hybridization between the U1 snRNA and the 5′ splice site sequence[7]. Separate from its role in splicing, the U1 snRNP can also potently inhibit gene expression by binding the pre-mRNA near the poly(A) signal. First shown in papillomaviruses[8] and more recently in certain mammalian genes[9], this property of the U1 snRNP forms the basis of the U1i silencing method. The inhibitory mechanism involves the U1-70K subunit of the U1 snRNP binding to and inhibiting poly(A) polymerase[10].

Although U1i effectively reduces mRNA levels, it has not been widely adopted as a gene silencing technology because of the inconvenience of preparing custom U1i targeting plasmids and concerns over specificity. A 10-nt domain engineered into the 5′ end of the U1 snRNA binds the target mRNA. Lengthening this 10-nt domain paradoxically weakens silencing[11]. Furthermore, the U1i snRNA must be expressed from a plasmid or viral vector and attempts to make it amenable to chemical synthesis by shortening it have failed.

Here we circumvent these problems by developing a class of synthetic oligonucleotides, U1 Adaptors, to recruit endogenous U1 snRNP to the target site (**Fig. 1**). A U1 Adaptor has two domains: a 'target domain' designed to base pair to the target gene's pre-mRNA in the 3′ terminal exon, and a 'U1 domain' that tethers the U1 snRNP to the target pre-mRNA. Bringing the U1 snRNP in contact with the target pre-mRNA inhibits proper 3′-end formation and leads to RNA degradation. Using optimized U1-Adaptor design and chemical modifications to improve binding affinity, we have achieved very high potency and subnanomolar IC_{50} (the concentration needed to inhibit gene expression by 50%). Notably, inhibition is increased by targeting the same gene either with multiple U1 Adaptors or by co-transfection of U1 Adaptors and siRNAs. U1 Adaptors add another technique to the gene-silencing tool kit and can be used either alone or in combination with RNAi.

RESULTS

U1 Adaptor oligonucleotides reduce gene expression

We used the dual-luciferase reporter system to facilitate optimization of the U1 Adaptor system. This involved targeting mRNA encoding *Renilla* (*Renilla reniformis*) luciferase for inhibition by U1 Adaptors and using a co-transfected firefly luciferase reporter as an internal normalization control. The first target studied was *MARK1* (NM_018650), which contains a single natural U1 snRNP binding site (U1 site) in its 3′ untranslated region (3′UTR) that downregulates *MARK1* expression in the wild-type (wt) gene[9]. The reporter pRL-MARK1wt was made from a standard pRL-SV40 *Renilla*-luciferase expression plasmid by replacing the SV40-derived 3′UTR and poly(A) signal sequences with the human *MARK1* 3′UTR and poly(A) signal region, including the 146 nt 3′ of the poly(A) site. The pRL-MARK1mt reporter is identical to pRL-MARK1wt except for a 4-nt

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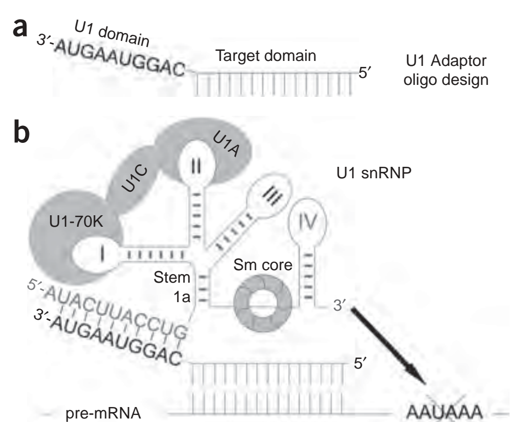

Figure 1 The U1 Adaptor concept. (**a**) The U1 Adaptor is a synthetic oligonucleotide with a target domain that hybridizes with the target gene's pre-mRNA in the 3′ terminal exon and a U1 domain that hybridizes with U1 snRNA, a subunit of U1 snRNP. (**b**) In the U1 Adaptor–mediated inhibitory complex, the U1 Adaptor tethers the U1 snRNP to the terminal exon of the target gene's pre-mRNA, which inhibits poly(A)-tail addition at the poly(A) site. Without a poly(A) tail, the pre-mRNA fails to mature and is degraded. Human U1 snRNP comprises three U1 snRNP–specific proteins (U1A, U1C and U1-70K), the 164-nt U1 snRNA (black line) with four stem loops (I, II, III and IV) and the Sm core comprising seven Sm proteins (ring) bound to the Sm site (adapted from ref. 37). Base pairing between nt 2–11 of the U1 snRNA and the U1 domain recruits U1 snRNP to the pre-mRNA, promoting inhibition of the nuclear poly(A) polymerase by the serine-arginine rich domains of U1-70K[10]. For simplicity, the pseudouridines at U1 snRNA positions 5 and 6 are shown as uridines.

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change in the natural U1 site. Each MARK1 reporter was transfected into HeLa cells along with a control firefly-luciferase reporter. A 17-fold increase in expression of *Renilla* luciferase from the pRL-MARK1mt plasmid relative to the pRL-MARK1wt plasmid indicated that the natural U1 site causes a 17-fold inhibition of expression of the wt reporter (**Fig. 2a**). As the wt *MARK1* 3′UTR can be inhibited by a U1 snRNP-mediated mechanism, this sequence context seemed a good first test for the U1 Adaptor method. A 25-nt U1 Adaptor called U1 Adaptor 6 (UA6) was designed with a 10-nt U1 domain complementary to the 5′ end of the U1 snRNA and a 15-nt target domain complementary to the *MARK1* sequence immediately 3′ to the mutated U1 binding site in pRL-MARK1mt. UA6 comprises 15 locked nucleic acid (LNA) nucleotides and DNA nucleotides at the remaining 10 positions. Co-transfection of HeLa cells by means of the UA6 Adaptor with the pRL-MARK1mt plasmid and the control firefly-luciferase reporter resulted in a 90% inhibition of *Renilla*-luciferase expression at 62 nM concentration outside the cells with an IC_{50} of 6.6 nM (**Fig. 2b**). An RNase protection assay involving an mRNA probe specific for *Renilla* luciferase[12] demonstrated reduced levels of both total and cytoplasmic mRNA encoding *Renilla* luciferase. This indicates that inhibition occurs at the RNA level, with no apparent nuclear accumulation of mRNA encoding *Renilla* luciferase (**Supplementary Fig. 1** online). To demonstrate that inhibition by UA6 requires complementarity with U1 snRNA, we synthesized and tested a mismatch control U1 Adaptor, UA7a, which has a 4-nt mutation in the U1 domain. A mismatch of 4 out of 10 nt in this domain reduces complementarity with U1 snRNA so that it no longer binds the U1 snRNP. We previously used an electrophoretic mobility shift assay (EMSA) involving purified U1 snRNP to demonstrate that pre-mRNAs containing this 4-nt mutation are unable to bind U1 snRNP, unlike a matching pre-mRNA with a wt U1 domain sequence[10,11]. A similar EMSA demonstrated that the UA6 Adaptor can tether the U1 snRNP complex to the target RNA (**Supplementary Fig. 2** online). Co-transfection of the mutant UA7a Adaptor with pRL-MARK1mt plasmid resulted in no inhibition (**Fig. 2b**), demonstrating the importance of the U1 domain.

The chemical composition and design of the U1 Adaptors is crucial for optimizing their activity. All first-generation U1 Adaptors were combinations of LNA and DNA. LNA nucleotides contain a carbon linkage between the 2′-oxygen and the 4′-carbon of the ribose sugar ring, thereby locking the nucleotide in an endo-sugar pucker position and increasing duplex stability and resistance to nuclease degradation[13]. LNA nucleotides were included in the U1 Adaptor to increase binding affinity of the short functional domains present in the 25-nt oligonucleotide. Placement of LNA nucleotides in this pattern also avoids activation of an RNase H–dependent antisense silencing mechanism. Ribose with 2′-modifications (such as 2′-*O*-methyl (2′OMe)), LNA or 2′-fluoro modifications block RNase H activity. RNase H activation requires at least four contiguous DNA residues and does not reach full potency until seven or eight DNA nucleotides are present[14,15]. The fact that all of the active U1 Adaptors in this report have fewer than four continuous DNA nucleotides argues against a role for RNase H in mediating U1 Adaptor activity. It will be interesting to explore U1 Adaptor configurations that support both RNase H activity and U1-snRNP binding in the same molecule, as this may increase potency by exploiting different mechanisms of action.

It is possible that *MARK1* sequences flanking the UA6 binding site contribute to the observed suppression. To eliminate this possibility, we tested the 15-nt UA6 binding site outside of the context of the *MARK1* 3′UTR by constructing a reporter, pRL-UA6, which has one UA6 binding site inserted into the 3′UTR and the poly(A) signal sequence from SV40 (**Fig. 2c**). Co-transfection of pRL-UA6 with increasing amounts of the UA6 Adaptor suppressed expression of *Renilla* luciferase with an IC_{50} value of 7.4 nM, which is close to the IC_{50} of 6.6 nM seen for the UA6 Adaptor that targets the pRL-MARK1mt reporter. As in **Figure 2b**, the mutated UA7a Adaptor did not inhibit pRL-UA6 expression (data not shown). Thus, the 15-nt UA6 binding site is necessary and sufficient to quantitatively direct inhibition by the UA6 Adaptor oligonucleotide. We and others previously demonstrated that multiple U1-snRNP binding sites in the terminal exon show additive levels of inhibition[4–6]. We made a new version of the pRL-UA6 reporter that had two tandem UA6 binding sites, called pRL-$(UA6)_2$. The pRL-$(UA6)_2$ reporter with the UA6 Adaptor showed better knockdown (IC_{50} of 2.2 nM) than the pRL-UA6 reporter (IC_{50} of 7.4 nM), demonstrating that the U1 Adaptor method shows additive suppression if multiple binding sites exist on the same target (**Fig. 2c**). In contrast, multiple siRNAs against the same mRNA do not result in additive inhibition, and instead show suppression at the level expected for the single most-potent siRNA in the pool[1,3,16].

Optimization of U1 Adaptor design

The UA6 Adaptor comprises a 25-nt LNA-DNA sequence having 10 nt complementary to the U1 snRNA and the remainder complementary to the target. The hybridization domains in this U1 Adaptor are short, yet function well because of the high LNA content of this oligonucleotide (15/25 bases are LNA). However, a high LNA content increases the potential of a sequence to self-dimerize and form hairpins, both of which are favored by stable hybridization of LNA strands. This complicates the design of U1 Adaptors targeted at other sites. We

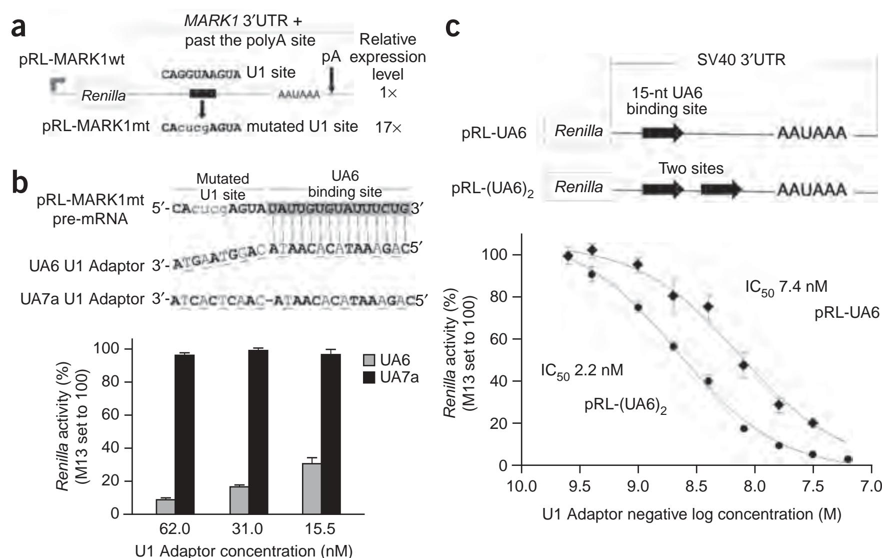

Figure 2 U1 Adaptor–mediated inhibition of reporter plasmid expression. (**a**) pRL-MARK1wt is a standard reporter plasmid expressing *Renilla* luciferase, with the original SV40-derived 3′UTR and poly(A) signal sequences replaced with the 3′UTR and poly(A) signal sequences from the human *MARK1* gene. The *MARK1* gene includes a naturally occurring U1 snRNP binding site (U1 site). pRL-MARK1mt is identical to pRL-MARK1wt, except that the U1 site is inactivated by mutation. HeLa cells were transfected with plasmids and luciferase activity was measured; values for *Renilla* luciferase were normalized to those obtained from a co-transfected plasmid encoding firefly luciferase. The pRL-MARK1wt construct expresses 17-fold less *Renilla* luciferase than the pRL-MARK1mt construct, indicating that the U1 site inhibits *Renilla* luciferase expression 17-fold. (**b**) Co-transfection of the UA6 Adaptor specifically inhibits pRL-MARK1mt. Design of the UA6 Adaptor, which targets a site in the 3′UTR of pRL-MARK1mt, is shown. LNA nucleotides are bold uppercase and DNA nucleotides are underlined uppercase. For examination of the dose-dependent inhibitory activity of the UA6 Adaptor, activity of the M13 oligonucleotide is set to 100%. UA7a is a mutated negative-control U1 Adaptor bearing a 4-nt mutation in the U1 domain. Transfections and normalization of *Renilla* luciferase activities to firefly luciferase activities were carried out as in **a**. (**c**) Co-transfection with the UA6 Adaptor specifically inhibits pRL-UA6 (diamond), which has a single binding site for the UA6 Adaptor. Enhanced inhibitory activity is observed with pRL-$(UA6)_2$ (solid circle), which has two tandem binding sites spaced 8 nt apart. All results with s.d. are from three independent transfections.

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All of the U1 Adaptor sequences studied thus far had the target domain at their 5′ ends and the U1 domain at their 3′ ends. When we tested the effects of switching domain order, we found that U1 Adaptors with the U1 domain at the 5′ end were less effective than the original design (**Fig. 3b**). Curiously, increasing the length of the U1 domain to 13 nt did not improve potency of the U1 Adaptor as much when using this configuration. A 2′OMe-LNA combination should have higher binding affinity than a uniform 2′OMe RNA or a LNA-DNA sequence when hybridizing to an RNA target. We therefore tested use of a mixed 2′OMe RNA and LNA sequence for the U1 domain, using the optimal 13-nt length. A variant of UA17-13 (the most potent U1 Adaptor identified in **Fig. 3b** with an IC_{50} of 1.5 nM) with five LNA nucleotides improved potency threefold and had an IC_{50} of only 0.5 nM (UA17-13b, **Fig. 3c**). These design improvements have therefore increased potency of the original UA6 Adaptor more than tenfold.

These insights into design optimization of the U1 domain should apply to all U1 Adaptors. Additional optimization involved examining similar design variation in the target domain. However, as the optimal length, number, and position or configuration of modified nucleotides may vary for different target sequences, each new target gene and its target sequence may require optimization of the target domain. Versions of the UA6 Adaptor sequence exclusively comprising 2′OMe RNA were inactive (**Supplementary Fig. 3** online), whereas a target domain comprised exclusively of LNA slightly increased activity (**Supplementary Fig. 5** online). As sequences that are fully modified by LNA cannot activate RNase H, these results eliminate the possibility that an RNase H–mediated antisense mechanism might contribute to the observed gene suppression. Assuming that higher binding affinity is helpful, we increased the length of the exclusively 2′OMe RNA target domain incrementally from 15 nt to 25 nt and 35 nt, and observed a loss of activity in both instances (data not shown). Although longer target domains might work at other sites, it would thus seem advisable to use a short, high-affinity sequence, which is most easily achieved using the LNA modification. This may relate in part to the secondary structure of the target sequence. Similar findings have been reported for antisense oligonucleotides: short, high-affinity compounds are generally more potent than long, low-affinity compounds[17,18]. Notably, U1 Adaptors with a phosphorothioate backbone were especially potent (**Supplementary Fig. 6** online), a potentially valuable feature for *in vivo* applications where resistance to nucleases is an important consideration. The ability of U1 Adaptors to inhibit target RNAs with less-than-perfect complementarity was assessed by comparing activities of variants of UA17-13b with 1-, 2- and 3-nt changes in the target domain with those of a wt reporter and a mutated reporter with a compensatory 3-nt base change in the target RNA. The results demonstrate a graded response: a 3-nt mismatch had no activity and a 1-nt mismatch had around half the activity of the

examined ways to decrease the relative LNA content by comparing different chemistries and domain lengths using the UA6 Adaptor as a model system. Although an all–2′OMe RNA version of the UA6 Adaptor showed no inhibitory activity (**Supplementary Fig. 3** online), the 10-nt U1 domain could be replaced with 2′OMe RNA with only a slight loss of activity (UA17-10, **Fig. 3a**). Continuing to use the 2′OMe RNA chemistry, we synthesized a series of U1 Adaptors with lengths of the U1 domain varying from 7–19 nt (**Fig. 3a**). As the length of the U1 domain decreased below 10 nt, activity was gradually lost. As the length of the U1 domain increased, activity increased and peaked at a length of 13 nt. Activity decreased with further increases in length. The UA17-13 Adaptor, which has a 13-nt 2′OMe U1 domain, was threefold more potent than the original UA6 Adaptor, with its 10-nt LNA-DNA sequence. Although it is not clear why U1 domains longer than 13 nt show less activity, these longer sequences may disrupt the folding structure of the U1 snRNA and thus decrease association with the U1-70K protein, the U1 snRNP subunit that inhibits poly(A) site activity[10]. Similar results were observed with a U1 Adaptor specific for a different target sequence, demonstrating that a peak in activity for 13-nt U1 domains is not peculiar to UA6 (data not shown). In designing the UA17 series, we assumed that the inhibitory activities of UA17 Adaptors could be increased by increasing their relative affinities for U1 snRNP, as subsequently shown using an EMSA competition assay (**Supplementary Fig. 4** online).

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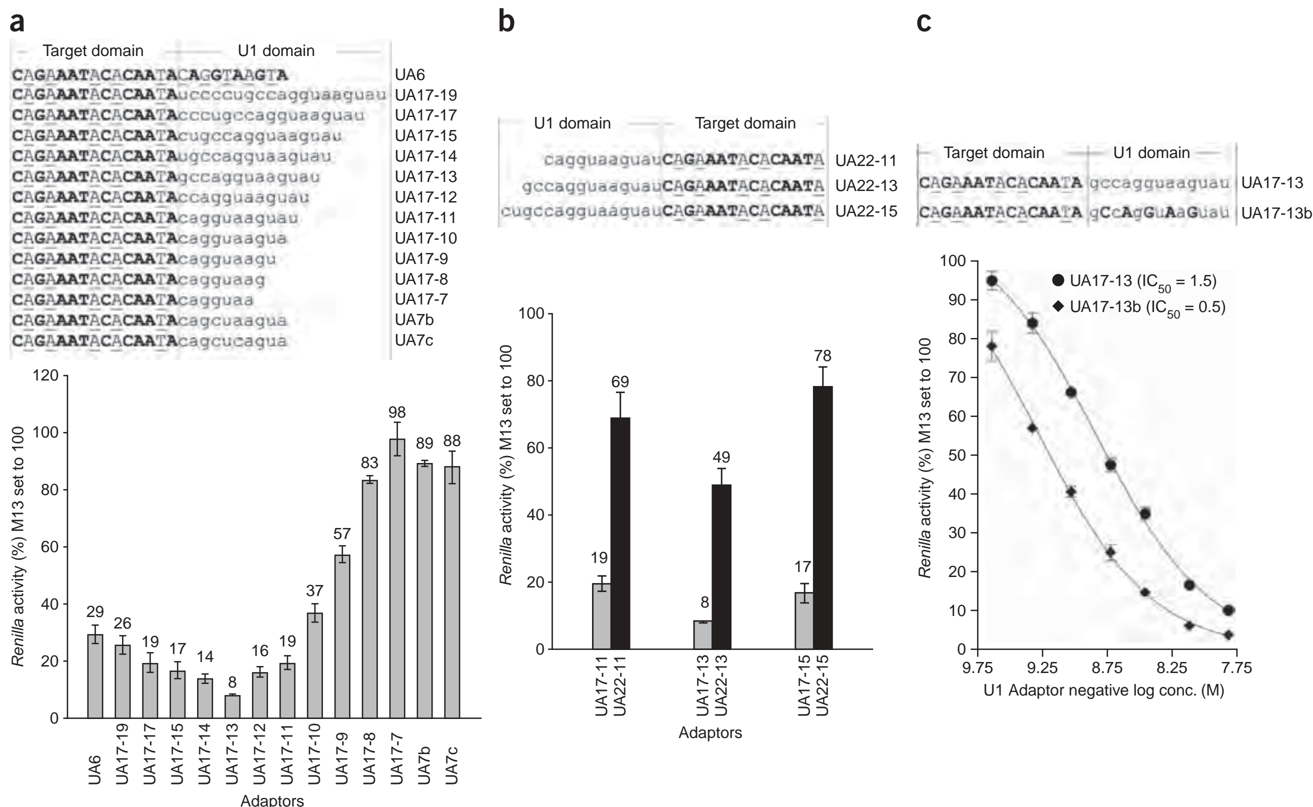

Figure 3 Effect on activity of changes in U1 domain length, location and composition. (**a**) Effect of increasing the length of the U1 domain. (Unless indicated, transfections and analysis are as in **Fig. 2**). The UA17 Adaptor series has the same target domain as the UA6 Adaptor and has a U1 domain comprised exclusively of 2′OMe RNA. Lengths of U1 domains vary from 7 nt (UA17-7 Adaptor) to 19 nt (UA17-19 Adaptor). LNA nucleotides are bold uppercase, DNA nucleotides are underlined uppercase and 2′OMe RNA nucleotides are lowercase. As in **Figure 2c**, HeLa cells were co-transfected with 15 nM of each U1 Adaptor and pRL-UA6 and inhibitory activities were calculated. The UA7b and UA7c Adaptors are negative controls bearing a single (UA7b) or double (UA7c) mutation in the U1 domain, respectively. (**b**) Effect of placing the U1 domain at the 5′ end of the U1 Adaptor. The UA22 Adaptor series is identical to UA17, except that the relative positions of the U1 and target domains in UA22 are reversed so that the U1 domain is positioned at the 5′ end. Transfections and analysis are as in **a**. The graph summarizes the results of testing the UA22 series (black bars) side-by-side with the corresponding UA17 series (gray bars). (**c**) Effect of incorporating LNA nucleotides into the U1 domain. The inhibitory activity of UA17-13, the most active U1 Adaptor from **a**, was compared with a matching UA17-13b Adaptor, which has 5 LNA nucleotides in the U1 domain. All results with s.d. are from three independent transfections.

wt U1 Adaptor (**Supplementary Fig. 7** online). Thus, in terms of base-mismatch discrimination, U1 Adaptors behave similarly to high-affinity antisense oligonucleotides. Although antisense oligonucleotides can show single-base discrimination when using low-affinity (low melting temperature) modifications, like methylphosphonation, this level of specificity is usually not achieved when incorporating high-affinity (high melting temperature) modifications such as LNAs[18,19]. U1 Adaptors differ from antisense oligonucleotides, however, in that they should only suppress expression when tethering the U1 snRNP to the 3′ terminal exon. Terminal exon restriction is a well-established property of U1 snRNP–mediated inhibition of poly(A) sites[4–6]. We confirmed that U1 Adaptors have this same terminal exon restriction by inserting binding sites for U1 Adaptors in a variety of positions within a splicing reporter containing three exons and two introns (**Supplementary Fig. 8**). Thus, any unintended cross-hybridization of U1 Adaptors to upstream exons and introns is unlikely to affect expression of that gene.

Inhibiting endogenous RAF1 with U1 Adaptors

To assess the ability of U1 Adaptors to suppress expression of endogenous genes, we designed the UA25 Adaptor to target human *RAF1* (NM_002880)—an oncogene with potential therapeutic value[20]. siRNAs are part of the RNA-induced silencing complex (RISC), which includes RNA helicases thought to assist in silencing by unwinding target sequences hidden within secondary structures. In contrast, as the U1 snRNP lacks intrinsic RNA helicase activity and the U1 Adaptor–U1 snRNP complex is presumably unlikely to recruit helicases, target-site accessibility will be important for optimal performance. As antisense oligonucleotides have the same requirement for target-site accessibility, it seems likely that good antisense sites might also be good U1 Adaptor sites. Our first *RAF1* target site studied was therefore designed at a site in the terminal *RAF1* exon known to mediate potent antisense-mediated silecing[21]. The UA25 Adaptor employs an 11-nt U1 domain without LNA nucleotides (**Fig. 4a**) because longer U1 domains (12 and 13 nt) with LNA residues were predicted to have a strong potential to form self-dimers and hairpin structures at this site (data not shown).

HeLa cells were transfected with the UA25 Adaptor and cell extracts were analyzed by western blot analysis for *RAF1* expression (**Fig. 4b**). RAF1 protein levels were specifically reduced by the UA25 Adaptor in a dose-dependent manner. The control Adaptor UA25-mt, which has a 2-nt mutation in the U1 domain, was inactive. Silencing of *RAF1* leads to cleavage of poly(A) ribopolymerase (PARP) as part of induction of apoptosis[22]. Reprobing the western blot in **Figure 4b**

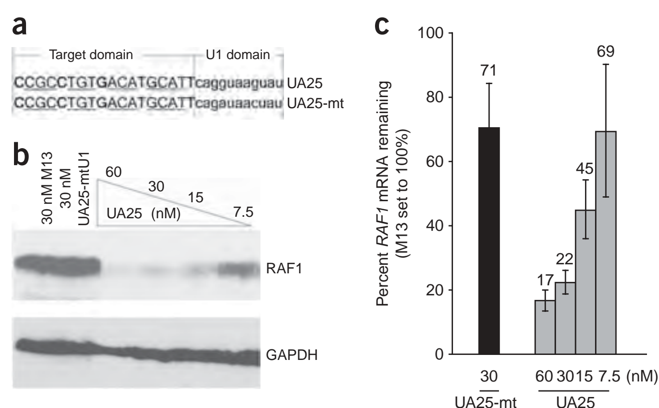

Figure 4 Inhibition of endogenous *RAF1*. (**a**) Design of the UA25 Adaptor, which targets the human *RAF1* gene. UA25-mt is a control Adaptor identical to UA25, except for a 2-nt mutation in the U1 domain. Symbols are as in **Figure 3**. (**b**) Western blot analysis with an anti-*RAF1* antibody demonstrates that the UA25 Adaptor specifically silences *RAF1* protein in a dose-dependent manner when used to transfect HeLa cells. The same blot was stripped and reprobed with anti-GAPDH antibody to control for equal loading. The same set of transfected cells was split into two, with one part being analyzed by western blot analysis and the other by qPCR. (**c**) qPCR analysis demonstrates that *RAF1* silencing by the UA25 Adaptor occurs at the mRNA level. qPCR was performed and levels of *RAF1* mRNA were normalized to the internal standard *GAPDH* mRNA. All results with s.d. are from three independent transfections.

with an anti-PARP antibody demonstrated that suppressing *RAF1* using the UA25 Adaptor induces PARP cleavage (**Supplementary Fig. 9** online). Quantitative real-time PCR (qPCR) demonstrated that the observed reduction in RAF1 protein levels correlated with similar reductions at the mRNA level, with an IC_{50} of 8 nM (**Fig. 4c**). In comparison, out of 34 antisense oligonucleotides analyzed in another study, the best sequence (ISIS5132) had an IC_{50} of 50 nM[21].

Three more U1 Adaptors targeting *RAF1* were designed to target sites in the terminal exon of *RAF1* that fit general antisense design criteria[23] including choosing unstructured areas of the mRNA. All three inhibited *RAF1* expression and were ~50% as active as UA25 (**Supplementary Fig. 10** online). As functional data become available for more U1 Adaptors, it may be possible to develop algorithms that predict effective target sites. To support the generality of the U1 Adaptor method, we targeted a second human gene, *PCSK9* (**Fig. 5**). Two U1 Adaptors that target *PCSK9* each silenced the gene with an IC_{50} in the 4–5 nM range. Importantly, simultaneous targeting of *PCSK9* with both dedicated U1 Adaptors enhanced inhibition, similar to that observed for *Renilla* luciferase (**Fig. 2c**). Although, by definition, U1 Adaptors have two domains, none of our experiments have demonstrated that the domains must be linked. To examine this, we tested so-called half-adaptors that have either an isolated U1 domain or an isolated target domain. Transfection of half-adaptors either alone or together failed to inhibit the target gene, demonstrating that the target and U1 domains must be linked for inhibition to occur (**Supplementary Fig. 11** online). The requirement for an intact bifunctional oligonucleotide to trigger suppression further argues against involvement of an antisense-based mechanism.

Combining U1 Adaptors with siRNAs enhances silencing

As U1 Adaptors and siRNAs use distinct mechanisms of action that occur in different compartments of the cell (nucleus and cytoplasm, respectively), they should have additive effects when combined. Additive inhibition was reported for combinations of antisense oligonucleotides and siRNAs[24]. To test this, the *Renilla* luciferase reporter in the plasmid pRL-UA6 was targeted with both an siRNA (RL-siRNA) and the UA17-13b Adaptor (**Fig. 6a**). Co-transfection of RL-siRNA with UA17-13b improved inhibition relative to the use of either the siRNA or U1 Adaptor alone. Negative control oligonucleotides (control siRNA and the mutated UA7a Adaptor) did not reduce luciferase expression (data not shown). The specificity of this additive inhibition is shown using the pRL-UA6rev reporter, which has the 15-nt UA6 binding site in the reverse orientation. As expected, the RL-siRNA decreased expression of pRL-UA6rev, although the UA17-13b Adaptor had no effect on pRL-UA6rev expression either when used alone or in combination with RL-siRNA. Lack of inhibition when the target site is in the inverted orientation, as with the UA6 Adaptor on pRL-UA6rev, argues against repression at the transcriptional level or being mediated by binding of the UA6 Adaptor to its target site in the double-stranded DNA plasmid. Finally, analysis of additional U1 Adaptors unrelated to UA6 demonstrated that they also function additively with siRNA (**Supplementary Fig. 12** online).

To determine whether combining siRNAs and U1 Adaptors can similarly enhance silencing of an endogenous gene, we targeted *RAF1* by transfecting UA25 and a Dicer-substrate siRNA (DsiRNA)[25,26] specific for *RAF1*, either alone or together. Measurement of *RAF1* mRNA by qPCR demonstrated that combined use of the U1 Adaptor and siRNA enhanced silencing (**Fig. 6b**). Western blot analysis confirmed that RAF1 protein levels were similarly reduced (data not shown). Further, we also observed a similar degree of additive inhibition when an siRNA and U1 Adaptors were used to silence *PCSK9* (**Supplementary Fig. 13** online). We thus conclude that additive suppression is a general property when U1 Adaptors and siRNAs are combined to target the same gene.

The potential for global off-target effects of the U1 Adaptors targeting *PCSK9* was assessed by microarray profiling, comparing them head-to-head with an siRNA directed against *PCSK9*. The results indicate the two methods of gene knockdown have a very high degree of overlap (Pearson correlation of 0.93; **Supplementary Fig. 14** online). This suggests that U1 Adaptors targeting *PCSK9* do not

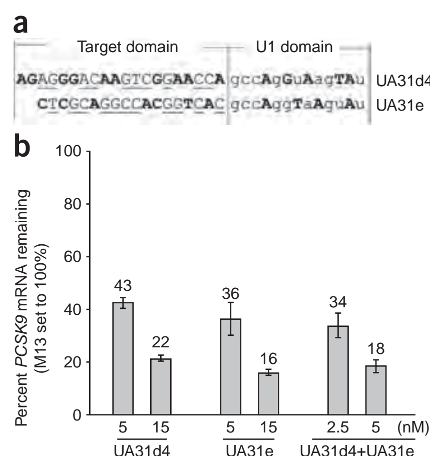

Figure 5 Inhibition of the endogenous *PCSK9* and enhanced inhibition with multiple Adaptors. (**a**) Sequences of two U1 Adaptors that target *PCSK9*. Symbols are as in **Figure 3**. (**b**) HeLa cells were transfected with U1 Adaptors targeting *PCSK9* alone or together. After 24 h, total RNA was harvested and analyzed by qPCR to measure silencing of *PCSK9*. All results with s.d. are from three independent transfections.

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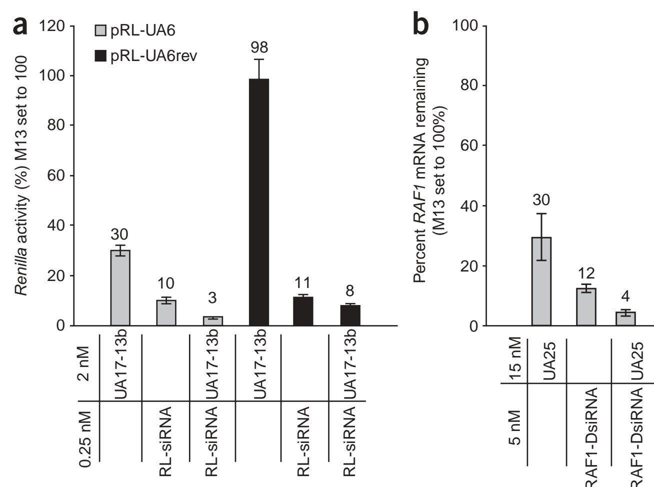

Figure 6 Co-transfection of U1 Adaptors and an siRNA enhances silencing. (**a**) Co-transfection of the UA17-13b Adaptor (**Fig. 3c**) and an siRNA targeting *Renilla* luciferase (RL-siRNA) with the reporter construct pRL-UA6 into HeLa cells gives better silencing than transfection of either the U1 Adaptor or the siRNA alone. pRL-UA6rev is a control plasmid where the UA6 Adaptor binding site is in the reverse orientation and so should not be inhibited by the UA17-13b Adaptor if inhibition occurs at the mRNA level. (**b**) Co-transfection of the UA25 Adaptor targeting *RAF1* with a Dicer-substrate siRNA (DsiRNA) targeting the same endogenous gene enhances silencing when compared with transfection of either the U1 Adaptor or the siRNA alone. Western blotting to detect RAF1 confirmed that enhanced inhibition is also seen at the protein level (data not shown). All results with s.d. are from three independent transfections.

have any new off-target effect profile when compared with siRNA targeting *PCSK9*. The U1 snRNP complex is involved in splicing to produce mature mRNA. It is possible that binding of some U1 snRNP complexes with U1 Adaptors might adversely affect splicing within the cell. We examined the relative splicing patterns of four endogenous genes known to undergo alternative splicing and observed that U1 Adaptors targeting *PCSK9* had no discernable effect on the ratio of alternatively spliced products for these four genes, at least within HeLa cells (**Supplementary Fig. 15** online). U1 Adaptors are therefore unlikely to have a global effect on splicing, a conclusion further supported by the data involving splicing reporter constructs (**Supplementary Fig. 8** online). The determination of U1 Adaptor specificity will ultimately require a significant effort with multiple U1 Adaptors and multiple gene targets using global expression profiling techniques. It is likely that additional improvements to U1 Adaptor potency and design parameters will reduce the potential for off-target effects, as seen with optimization of siRNAs and antisense oligonucleotides.

DISCUSSION

We describe an oligonucleotide-based gene silencing method that reduces gene expression by tethering the U1 snRNP splicing factor to pre-mRNA of a target of interest. Successful inhibition was demonstrated at both the mRNA and protein levels and was studied for both a reporter gene and two endogenous human genes. We observed potent inhibition, with IC_{50}s as low as 0.5 nM. As knowledge of design rules and target site selection improves, we predict that potency in the subnanomolar range will be achieved routinely. Using the limited set of U1 Adaptors studied so far, we achieved an ~50% success rate in obtaining U1 Adaptors with ≤5 nM IC_{50} potency by applying antisense oligonucleotide selection criteria to our target genes.

Although their practical use remains speculative, several considerations support the prospect of using U1 Adaptors *in vivo* for therapeutic indications. First, *in vivo* administration of U1 Adaptors could use the same delivery technologies already pioneered for use with siRNA and antisense methods[27–30]. Second, a range of modified nucleotides, including phosphorothioate modifications, have already been incorporated into U1 Adaptors with the goal of increasing nuclease stability. Further, as no enzymatic activity is required for their function, U1 Adaptors may be compatible with a wider range of modifications than siRNAs or antisense oligonucleotides, which require direct interaction with cellular enzymes, such as Argonaute 2, Dicer and RNase H[27,31,32]. Third, the additive activity of several U1 Adaptors used together or in combination with siRNAs may permit use of lower doses of each individual oligonucleotide, reducing the potential for toxic side effects and lowering costs of administration. Importantly, the most active U1 Adaptors described here comprised exclusively 2′OMe-modified and LNA residues. This chemical composition does not contain motifs known to trigger the innate immune system. A list of sequences for all the U1 Adaptors and siRNAs is provided in the **Supplementary Figures 16** and **17** online. **Supplementary Figure 18** online summarizes the similarities and differences between the U1 Adaptor, antisense and RNAi methods.

Besides U1 snRNP, there are other RNA processing factors that inhibit poly(A)-site activity and hence gene expression[33,34]. It is possible that novel U1 Adaptor–like oligonucleotides could be designed to similarly recruit these other factors, either individually or in combination. However, several features unique to the U1 snRNP prompted our focus on it for this study. First, U1 snRNP is highly abundant, with about 1 million copies present in a typical mammalian nucleus (~0.5 μM U1 snRNP complexes in a HeLa cell, with an even higher concentration in the nucleus), and is in about tenfold stoichiometric excess over the spliceosome[7]. Thus, it is plausible that sequestering a small fraction of all U1 snRNP complexes by interaction with low nM amounts of U1 Adaptors will have little effect on the overall splicing machinery and will not deplete the pool of all available U1 snRNP complexes. Second, the functional *in vivo* concentration of U1 snRNP, defined by the degree of inhibition observed when inserting a binding site for U1 snRNP near a reporter gene's poly(A) signal, is much higher when compared to these other RNA processing factors[5,35]. Third, unlike other RNA processing factors, it is rather straightforward to increase the affinity of U1 snRNP for the U1 Adaptor, as evidenced by the data in **Figure 3**. Nevertheless, it is possible that new types of Adaptor designs can be identified that inhibit gene expression by interaction with other RNA processing factors.

METHODS

Method for transfection and luciferase assays. Cell culture and transfections were done as previously described[12]. For luciferase assays, the cells were harvested after 24–48 h and luciferase measured using the Promega dual-luciferase kit (Promega) measured on a Turner BioSystems Luminometer (Turner BioSystems). For inhibition of endogenous genes, cells were harvested after 24–48 h and either lysed in SDS buffer for western blot analysis or total RNA was extracted using the RNeasy kit (Qiagen). Nuclear and cytoplasmic RNA preparations were performed as described[12]. The siRNA-targeting *Renilla* luciferase was purchased from ABI/Ambion. All of the U1 Adaptors and the siRNAs targeting PCSK9 and RAF1 were manufactured by Integrated DNA Technologies (IDT).

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Enhanced chemiluminescence western blot analysis. This was done as previously described[10] using a 1:10,000 dilution of an anti-GAPDH antibody (Chemicon division of Millipore), a 1:1,000 dilution of an anti-RAF1 antibody (R1912 from BD Biosciences), or a 1:1,000 dilution of an anti-PARP antibody (Ab-2 from Oncogene). The secondary anti-mouse and anti-rabbit antibodies were used at a 1:5,000 dilution (Amersham Biosciences). The membrane used was Immobilon-P (Millipore) and was treated as per manufacturer's instructions.

General method for quantitative real-time PCR (qPCR). RNA from transfected cells was isolated using the RNeasy kit. Complementary DNA (cDNA) was synthesized using 1 μg of RNA, random hexamers and MMLV reverse transcriptase as suggested by the manufacturer (Promega). 50 ng of cDNA was analyzed using qPCR run on a Rotorgene 3000 (Corbett Research) and the QuantiTech SYBR Green PCR kit (Qiagen). Results from test genes were normalized using GAPDH as an internal control. Primer sequences are provided in the **Supplementary Data** online. The comparative cycle threshold (Ct) method was used[36] to analyze the data where the relative values of the amount of target cDNA equal $2^{-\Delta\Delta Ct}$, where ΔCt = difference between the threshold cycles of the target (*RAF1*) and an endogenous reference (GAPDH), and $-\Delta\Delta Ct$ = difference between ΔCt of the target sample and a control (cells treated with M13 oligo).

Accession number. GEO: GSE14434.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS
S.I.G. and R.G. conceived the project and are co-inventors of the U1 Adaptor technology. R.G. performed the bulk of the wet bench experimental work, with some assistance from S.I.G. All authors contributed to experimental design, data interpretation and preparation of the manuscript. M.A.B. provided most of the U1 Adaptors employed and assisted with antisense and RNAi aspects of the project.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/

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A conditional transposon-based insertional mutagenesis screen for genes associated with mouse hepatocellular carcinoma

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We describe a system that permits conditional mobilization of a Sleeping Beauty (*SB*) transposase allele by Cre recombinase to induce cancer specifically in a tissue of interest. To demonstrate its potential for developing tissue-specific models of cancer in mice, we limit *SB* transposition to the liver by placing Cre expression under the control of an albumin enhancer/promoter sequence and screen for hepatocellular carcinoma (HCC)–associated genes. From 8,060 nonredundant insertions cloned from 68 tumor nodules and comparative analysis with data from human HCC samples, we identify 19 loci strongly implicated in causing HCC. These encode genes, such as *EGFR* and *MET*, previously associated with HCC and others, such as *UBE2H*, that are potential new targets for treating this neoplasm. Our system, which could be modified to drive transposon-based insertional mutagenesis wherever tissue-specific Cre expression is possible, promises to enhance understanding of cancer genomes and identify new targets for therapeutic development.

Transposon-tagged mutagenesis has proven invaluable for functional genomics in organisms such as *Drosophila melanogaster*[1,2], but similar progress in mammalian systems has been retarded by the long delay in identifying transposons, such as Sleeping Beauty (*SB*), which are active in mouse cells[3]. Although the low frequency of *SB* transposition in the mouse germ line[4–6] had suggested that the frequency of *SB* mobilization in somatic tissues was too low to induce cancer, *SB* transposons have been mobilized in somatic cells at frequencies high enough to induce cancer in wild-type mice[7] and accelerate the formation of tumors in *p19*^{Arf}-deficient mice[8]. Nonetheless, it was not possible to model specific tumor types when expressing *SB* from a ubiquitous promoter. Whereas *SB* transposition in *p19*^{Arf}-deficient mice accelerated the formation of tumors normally observed in these mice[8], in wild-type mice, it resulted in the formation of aggressive hematopoietic tumors that killed the animals by 4 months of age[7]. Mice did not live long enough to develop other types of tumors such as solid tumors.

To address this need, we sought to develop a conditional *SB* transposition system to screen for genes associated with different types of cancer. For this, we first integrated the *SB* transposase allele *SB11* carrying a *loxP*-flanked ('floxed')-stop (lsl) cassette into the mouse *Rosa26* locus, which encodes a ubiquitously expressed nonessential gene[9]. Genes inserted into the *Rosa26* locus are expressed in most tissues and not subject to epigenetic silencing normally observed with transgenes[9]. Expression of the transposase knock-in (*Rosa26*-lsl-*SB11*), which is normally blocked owing to the presence of the floxed-stop cassette, can be reactivated in any target tissue using a tissue-specific Cre recombinase to drive the transposition of the T2/onc mutagenic transposon[7,8]. The T2/onc vector contains sequences that can both cause misexpression of an oncogene and inactivate a tumor suppressor gene (**Supplementary Fig. 1a** online).

HCC is the third leading cause of cancer-related deaths globally[10], with potential curative treatment available for $<$30% of patients at the time of diagnosis[11]. HCC is prevalent worldwide, with differences in rates of its incidence reflecting regional diversity mostly related to the geographic distribution of viral hepatitis[11]. A greater prevalence in males, relative to females, has been explained by preliminary molecular data[12]. Mutations in the *TP53* gene are commonly found in HCC[13,14]. The presence of many unexplained recurrent chromosomal abnormalities and the identification of mRNA expression–based subsets of HCC suggest the presence of unidentified genetic drivers of this disease[15].

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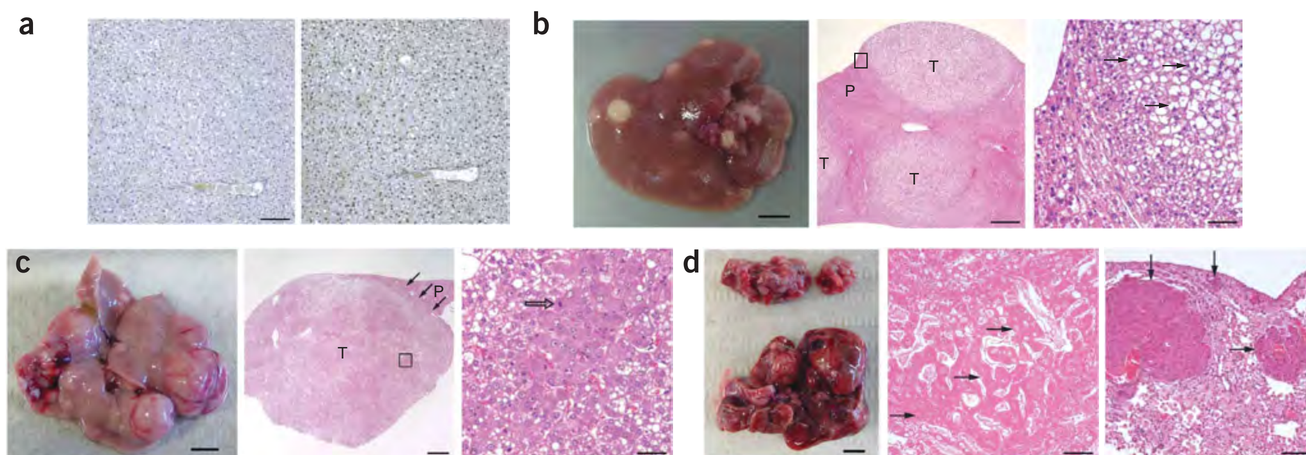

Figure 1 Accelerated tumorigenesis in *p53*-deficient livers compared with nonpredisposed livers of male mice. (**a**) Albumin-Cre (*Alb*-Cre) expression efficiently deletes the floxed-stop (lsl) cassette within the *Rosa26*-lsl-*SB11* transgene, permitting *SB* expression and subsequent somatic transposition. Left panel, immunohistochemistry of (*Alb*-Cre; *Rosa26*-lsl-*SB11*) double-transgenic liver section treated without the primary anti-*SB* transposase (SB) antibody (negative control). Right panel, immunohistochemistry of serial liver section treated with the primary anti-SB antibody. Sections were lightly stained with hematoxylin after immunohistochemistry. Scale bar, 100 μm. (**b**) Liver from quadruple transgenic male mouse (159 d old), displaying many preneoplastic nodules (scale bar in left panel, 0.5 cm). Middle (low magnification) and right (high magnification of boxed area from middle panel) panels show the tumor histology of several adenomas using hematoxylin-eosin (HE) staining. These adenomas often compressed surrounding parenchyma. Cells within the preneoplastic foci and adenomas were frequently vacuolated, containing distinct lipid vacuoles or clear cytoplasm (arrows). Nuclei were in the same size or smaller than those in the normal hepatic parenchyma, and occasionally contained mitotic figures indicative of cell division. Adenomas were frequently bordered by hepatocytes with markedly enlarged nuclei that were occasionally karyomegalic. T, tumor nodule; P, parenchymal liver cells; scale bars for middle and right panels, 500 μm and 100 μm, respectively. (**c**) Liver from triple transgenic male mouse (330 d old) showing advanced tumor development. Many large irregular nodules are well-vascularized (scale bar in left panel, 0.5 cm). Middle panel shows the HE histological section of one large neoplastic nodule typical of hepatocellular adenoma consisting of variably vacuolated hepatocytes filled with lipid. Three arrows indicate the border between the adenoma and nonneoplastic hepatic parenchyma (P), which is slightly compressed. Right panel shows high magnification of boxed area in the middle panel. Note the enlarged nuclei of hepatocytes with moderate variation in nuclear size, prominent nucleoli, and mitotic figure (open arrow). T, tumor nodule; P, parenchymal liver cells; scale bars in middle and right panels, 1,000 μm and 50 μm, respectively. (**d**) Liver with HCC (bottom, left panel) and lung metastases (top, left panel) from triple transgenic male (440 d old; scale bar, 0.5 cm). HE staining of the liver (middle panel) and lung (right panel) show advanced HCC in the liver and its metastasis into the lung. A partial HCC section reveals irregular trabeculae of neoplastic, diffusely necrotic hepatocytes (black arrows) that are multifocally vacuolated. Trabeculae are separated by dilated sinusoids containing variable amounts of fibrin. The lung contains multiple variably sized metastatic nodules of HCC (black arrows) that markedly compress the pulmonary parenchyma. Pulmonary alveoli are filled with many foamy macrophages. Scale bars in middle and right panels, 100 μm.

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We used a hepatocyte-specific albumin-Cre (*Alb*-Cre) transgene to activate transposase expression in mice, specifically in the liver[16]. As mutations in *TP53* are the most frequently described mutations in human HCC, we included a conditional dominant negative *Trp53* transgene[17] in a second construct, named *p53*-lsl-*R270H* (**Supplementary Fig. 1a**). We bred triple transgenic (*Rosa26*-lsl-*SB11*; T2/onc; *Alb*-Cre) and quadruple transgenic (*Rosa26*-lsl-*SB11*; T2/onc; *Alb*-Cre; *p53*-lsl-*R270H*) mice and monitored the onset of liver tumorigenesis in both sets of lines (**Supplementary Fig. 1b**). When combined with high-throughput sequencing, our conditional forward genetics screen identified 19 genes potentially associated with oncogenesis in the liver and prioritized epidermal growth factor receptor (*Egfr*) and ubiquitin-conjugating enzyme E2H (*Ube2h*) for experimental validation. *Egfr* was the most frequently mutated gene in our screen and was especially prevalent as a truncated form lacking the C-terminal half of the gene. Moreover, initial human comparative studies suggest a nonsignificant trend to higher tumor recurrence and poorer survival rates associated with higher expression levels of *UBE2H*. This information enhances insight into the genetic mechanisms associated with HCC and may facilitate development of more effective therapies.

RESULTS

Hepatocyte-specific transposition and tumorigenesis

To demonstrate that the conditional transposase knock-in is activated exclusively in the liver, we used an anti-*SB* transposase (SB) antibody for immunohistochemical analyses of mice carrying both *Alb*-Cre and *Rosa26*-lsl-*SB11* transgenes (**Fig. 1a**). To confirm that transposition is occurring in the livers of experimental transgenic animals, we used excision PCR[8] to demonstrate amplicon excision (**Supplementary Fig. 2a** online) when experimental and control animals from both sexes were initially euthanized ~100 d after birth. No visible lesions were seen in any organs at this stage (data not shown).

Preneoplastic liver nodules were first detected in male triple (nonpredisposed genetic background) and quadruple (predisposed to HCC by expression of a dominant-negative *Trp53* allele) transgenic animals ~160 d after birth. Nodules in the quadruple transgenic animals were larger and more numerous than those from triple transgenic animals (**Supplementary Fig. 2b**). Double and triple transgenic mice carrying all possible combinations of the four transgenes were also generated to provide control cohorts. Throughout our studies, we saw no evidence of tumorigenesis in control male littermates euthanized at a similar age (data not shown).

Of six quadruple transgenic male experimental animals euthanized between 101 and 223 d after birth, four (67%) had livers with macroscopic preneoplastic nodules (**Fig. 1b**); a total of 67 nodules were isolated (**Supplementary Table 1** online). In contrast, we found evidence of neoplasms in only three (43%) of the seven triple transgenic male animals euthanized between 105 and 289 d after birth and isolated 36 preneoplastic nodules from these animals (**Supplementary Table 1**). Excision PCR assays confirmed

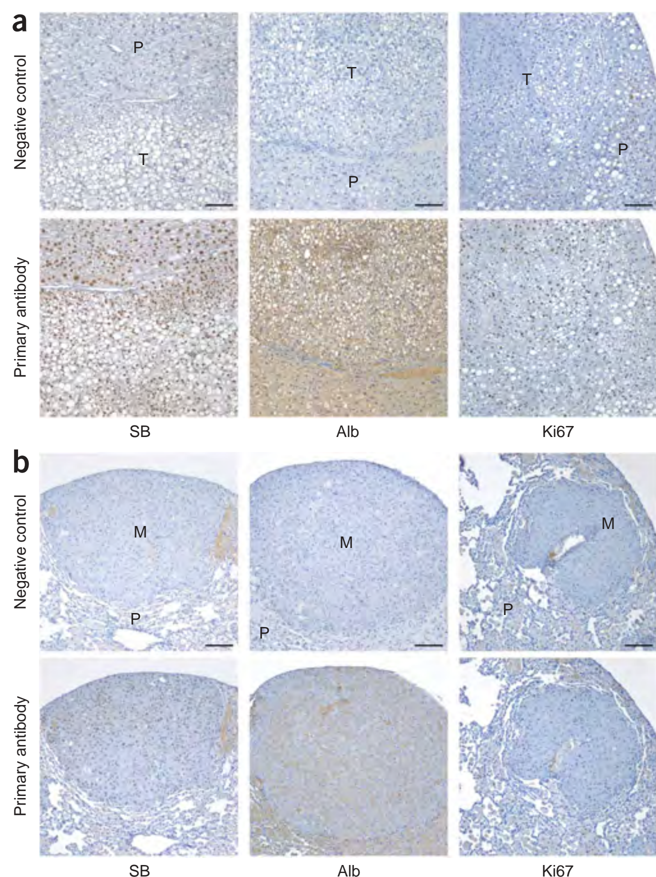

Figure 2 Immunohistochemical analyses of liver adenomas. (a) Paraffin-embedded liver tissue sections from triple or quadruple transgenic male animals stained positive with antibodies against *SB* transposase (SB), albumin (Alb) and the proliferative marker Ki67. Representative immunohistochemical liver sections from a 160-d-old quadruple transgenic male mouse are shown. Top panels, liver sections not treated with the primary antibody (negative controls); bottom panels, serial liver sections treated with primary antibody against the indicated protein; T, tumor nodule; P, parenchymal cells; scale bars, 100 μm. (b) Immunohistochemical analyses of the HCC-derived lung metastasis. Paraffin-embedded lung tissue sections were stained with antibodies against SB, Alb and Ki67. Top panels, lung sections not treated with the primary antibody (negative controls); bottom panels, serial lung sections treated with the indicated primary antibody; P, parenchymal lung cells; M, metastasis from HCC; scale bars, 100 μm.

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transposition in the livers of experimental animals that did not produce tumors (**Supplementary Fig. 2a**).

Detailed histopathological analyses revealed that the livers of triple and quadruple transgenic mice euthanized ~150 d after birth frequently contained preneoplastic foci of cellular alteration that represents the earliest visible stage of neoplastic formation, with a few adenomas (**Fig. 1b**). The liver of a 330-d-old triple transgenic male mouse had multiple large well-vascularized tumors with microscopic features of hepatic adenoma (**Fig. 1c**). Two older triple transgenic male mice (440 and 460 d old) and a 432-d-old quadruple transgenic male mouse displayed lung metastases as well as livers with histopathological features of HCC (**Fig. 1d** and **Supplementary Table 1**). Immunohistochemical analysis of preneoplastic nodules from all triple and quadruple transgenic livers stained positive for SB, albumin and for the cellular proliferative marker, Ki67 (**Fig. 2a**). This indicates that these nodules result from transposition events originated from hepatocytes and have increased rates of proliferation. The lung metastases also stained positive for SB, albumin and Ki67 using immunohistochemistry, indicating that they were derived from the HCC (**Fig. 2b**).

Although RT-PCR revealed that the majority of preneoplastic nodules expressed alpha-fetoprotein (*Afp*), a biomarker for human HCC (**Fig. 3**), only a small subset of nodules expressed enough Afp to enable detection by immunohistochemistry (data not shown). RT-PCR also demonstrated the expression of secreted phosphoprotein 1 (*Spp1*)—a gene associated with HCC metastasis[18]—in all preneoplastic nodules (**Fig. 3d**). Semi-quantitative RT-PCR demonstrated upregulation of *Ssp1* and *Afp* expression as liver tumorigenesis progressed from adenoma to HCC (**Supplementary Fig. 2c**).

Immunohistochemical analyses for β-catenin levels demonstrated increasing levels of expression as tumorigenesis progressed from preneoplastic nodules to hepatic adenoma to HCC (**Supplementary Fig. 3** online). Mutations in the gene encoding β-catenin and its elevated expression are also observed in human HCC[19]. Notably, triple ($n = 4$) and quadruple ($n = 4$) transgenic female experimental animals (euthanized 178–342 d after birth and 178–344 d after birth, respectively) did not have any visible liver lesions (**Supplementary Table 1**). However, two female triple transgenic animals (512 and 575 d old) and one quadruple transgenic animal (432 d old) presented livers with small preneoplastic nodules (**Supplementary Table 1**). The low frequency and prolonged latency of liver nodules in female experimental animals mirrors the strong gender bias in the incidence of HCC tumor seen in humans. Moreover, immunohistochemistry revealed increased expression of Afp and the proliferative marker Ki67 in nontumorigenic liver sections from female mice (**Supplementary Fig. 4a** online). Therefore, our conditional *SB* liver tumor model is useful in elucidating genetic mechanisms for HCC tumorigenesis, including lesions ranging from early hepatic adenomas to fully developed HCC, including metastatic HCC.

Sequencing identifies common insertion sites in tumors

Supplementary Figure 1c online provides a flow chart for *SB* somatic cell mutagenesis and barcode-assisted integration site amplification. Briefly, T2/onc integration sites from 68 preneoplastic nodules (3 from triple and 65 from quadruple transgenic animals) were cloned and sequenced using barcoded primers and linker-mediated PCR. Subsequent pyrosequencing[20] enables tens of thousands of T2/onc integration sites from a mixture of tumors to be characterized in a single sequencing run (**Supplementary Methods** online). Pyrosequencing of linker-mediated PCR products from these tumors generated over 140,000 individual sequences. Sequences containing <16 nucleotides of genomic sequence were eliminated, leaving ~106,000 sequences. From these, 85,652 sequences were uniquely mapped at 95% identity to the mouse genome. As *SB* has a tendency to 'hop' primarily within the vicinity of the original site of integration[6], we excluded insertions that mapped to the transposon donor chromosome (chromosome 15). Further elimination of insertions that did not map to the canonical TA insertion site required for *SB* integration[21–23] left a total of 68,782 sequences. We then combined all insertions that mapped to the same TA dinucleotide and originated from the same neoplastic nodule, leaving a final tally of the 8,060 nonredundant insertions.

We next looked for regions in the genome that had more *SB* insertions than predicted by random chance. These so-called common insertions sites (CISs) are most likely to harbor disease-related genes.

Based on Monte Carlo criteria for statistical significance (**Supplementary Methods**), we defined CISs as regions in the genome with six insertions located within 130 kb of each other, five insertions within 65 kb or four insertions within 20 kb. In total, 30 CISs were identified according to these criteria. Of these CISs, 11 appear to represent background events resulting from false priming at a specific site. This is because the T2/onc insertions either all begin at the same nucleotide, occur in loci with no annotated genes or are present among CISs defined by control insertion-site-mapping experiments using 3-week-old transgenic-mouse tail DNA carrying both the T2/onc and *Rosa26-SB11* transgenes (**Supplementary Methods**). The final list of CISs associated with mouse HCC is shown in **Table 1** and the 8,060 nonredundant sites of insertion are provided in **Supplementary Data** online. Notably, substantial overlap with this CIS list was seen in another set of liver tumors induced by a villin-Cre transgene (data not shown), further attesting to the significance of these genes for HCC. Villin is expressed in the microvilli of brush border epithelium lining of the gut and renal tubes in vertebrates. Importantly, the specific insertion sites associated with individual preneoplastic nodules during early tumorigenesis differed for each nodule, indicating that each nodule is a unique clone. In general, each preneoplasic nodule was characterized by a unique set of T2/onc insertions. Certain genes, such as *Egfr*, were reproducibly mutated by insertion mutations in nodules from the same mouse. However, these insertions are not in identical

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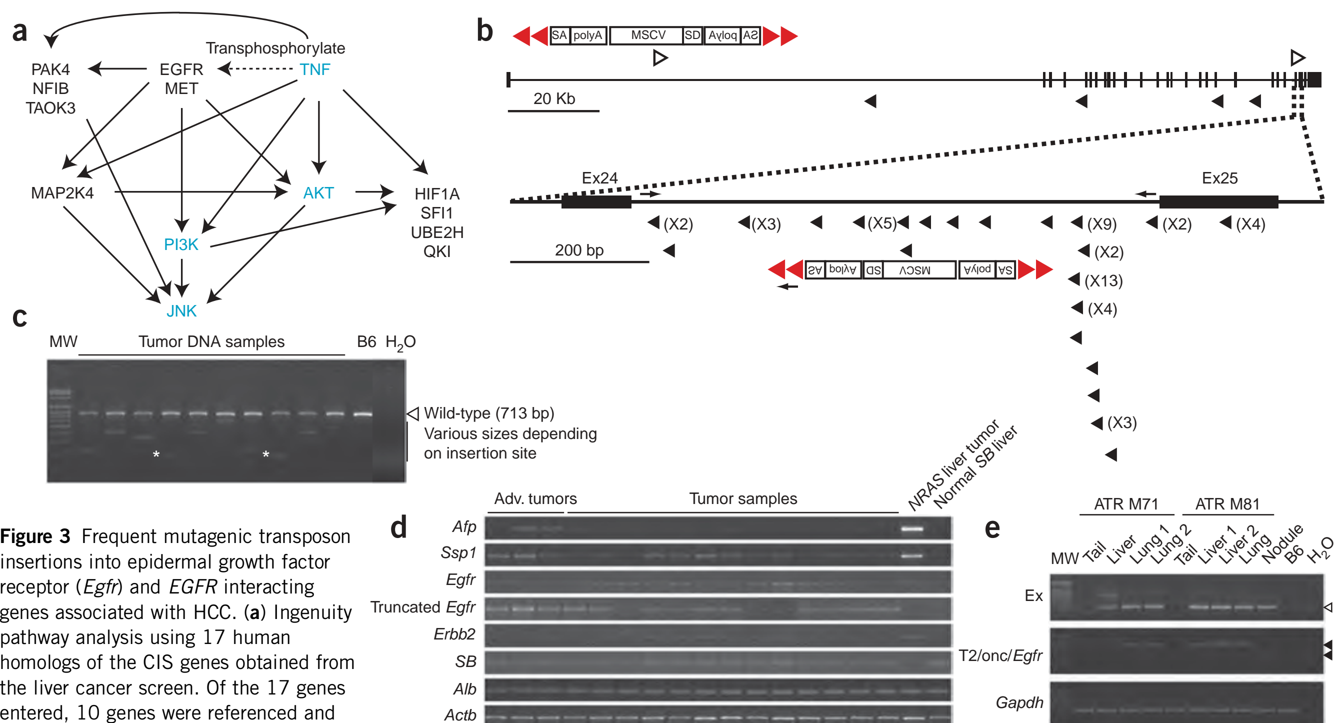

Figure 3 Frequent mutagenic transposon insertions into epidermal growth factor receptor (*Egfr*) and *EGFR* interacting genes associated with HCC. (**a**) Ingenuity pathway analysis using 17 human homologs of the CIS genes obtained from the liver cancer screen. Of the 17 genes entered, 10 genes were referenced and displayed in the network function pathways associated with post-translational modification, cancer and tumor morphology. The EGFR signaling pathway shows interactions with JNK, TNF and PI3K/AKT regulatory pathways. CIS genes are in black and other genes in this network are in blue. (**b**) Representation of insertions of the mutagenic transposon (T2/onc) into intron 24 of *Egfr*. Red triangles, inverted repeats/direct repeats (IR/DR) transposon flanking sequences; SA, splice acceptor; polyA, polyadenalytion signal; MSCV, long terminal repeat of the murine stem cell virus; SD, splice donor; open arrowhead, sense-orientated insertion of the T2/onc relative to the *Egfr* gene; arrowhead, anti-sense orientated insertion of the T2/onc relative to the *Egfr* gene; arrows, endogenous and vector primers used for the *Egfr* PCR genotyping shown in **c**. Numbers in parentheses indicate the frequency of transposon insertions at each particular site from different liver preneoplastic nodules. (**c**) Confirmation of transposon insertions in intron 24 of *Egfr*. PCR genotyping was performed using genomic DNA isolated from individual tumor nodules. A subset of these samples was subjected to *Egfr* PCR genotyping using endogenous and vector primers. Gel electrophoresis shows the endogenous *Egfr* (713 bp) band (open arrowhead), with transposon-integrated bands of varying sizes, depending on the insertion site within intron 24. Except for two insertion sites (asterisks) missed by pyrosequencing, all amplicons corresponded with the pyrosequencing data. MW, 100-bp molecular standard; B6, C57BL/6 tail genomic DNA; H_2O, double-distilled water (negative control). (**d**) RT-PCR analyses of tumor nodules. All neoplastic nodules were positive for *SB* transposase (*SB*) and albumin (*Alb*) transcripts, indicating transposition and that nodules were derived from hepatocytes, respectively. Most tumor nodules were positive for alpha-fetoprotein (*Afp*) transcripts, a clinical marker for HCC, and secreted phosphoprotein 1 (*Ssp1*), which is overexpressed in various cancers including HCC. Nodules taken from a 330-d-old triple transgenic male mouse with advanced tumors (shown in **Fig. 1c**) were strongly positive for *Afp* and *Ssp1*. All tumor nodules tested were positive for endogenous *Egfr* and for truncated-*Egfr* transcripts. *NRAS* liver tumor, HCC control taken from a tumorigenic liver overexpressing *NRAS G12V* oncogene[25]; *SB* normal liver, normal liver taken from a *SB*-expressing mouse; beta-actin (*Actb*), control to show equal loading of mRNA used for RT-PCR. RT-negative controls were also performed for each sample and no visible bands were seen for any of the markers tested (data not shown). (**e**) Confirmation of transposition events and transposon insertions in intron 24 of *Egfr* for HCCs and lung metastases. PCR genotyping was performed with genomic DNA isolated from the tails, livers and lung metastases of two triple transgenic male mice (ATR M71, 440 d old and ATR M81, 460 d old). Top panel, excision PCR assays (Ex) for transposition events in the lung metastases and HCCs (open arrowhead). No excision was detected in the tails of the triple transgenic male mice. Middle panel, PCR genotyping using only the endogenous *Egfr* forward and T/JB3 primers (T2/onc/*Egfr*) to confirm transposon insertion in intron 24 of *Egfr* for the lung metastases and HCCs. Gel electrophoresis demonstrates the transposon-integrated band (arrowhead) for both the lung metastases (lung) and HCCs (liver), but not in their tails. *Gapdh*, control to show equal use of genomic DNA template (100 ng) in PCR reactions. Nodule, a liver tumor nodule from a different animal was used to compare different transposon insertion sites; MW, 100-bp molecular standard; B6, C57BL/6 tail genomic DNA; H_2O, double-distilled water (negative control).

Table 1 Common insertion sites for HCC-associated genes

Gene	Chr	Position	Range	*n*	Nodules	Mouse
Egfr	11	16765887-16872714	107 kb	69	58	5
Novel EST gene cluster	9	3000138-3038047	38 kb	20	17	5
Sfi1	11	3046719-3136227	90 kb	13	13	4
Zbtb20	16	43349510-43460987	111 kb	8	7	4
ENSMUSESTG0000001569	10	52995507-53074648	79 kb	7	5	2
Nfib	4	82058117-82133086	75 kb	7	7	4
Taok3	5	117614813-117701538	87 kb	7	6	3
Slc25a13	6	6047524-6159681	112 kb	7	7	2
Qk	17	10379929-10457807	78 kb	6	6	3
Rnf13§	3	57552266-57663120	111 kb	6	6	3
Met	6	17449763-17545224	95 kb	6	6	3
March1	8	68422058-68551102	129 kb	6	5	2
Psd3	8	70451840-70580359	129 kb	6	6	3
Map2k4	11	65524193-65586089	62 kb	5	5	4
Trpm7	2	126659349-126720778	61 kb	5	5	3
Ube2h§	6	30181012-30207531	27 kb	5	4	3
Vrk2§	11	26373044-26373912	869 bp	4	3	3
Hif1a	12	75021346-75031073	10 kb	4	4	2
Pak4§	7	29367702-29371179	3 kb	4	4	3

Chr, chromosome; Range, chromosomal position of transposon insertions; *n*, frequency of transposon insertions; §, genes that did not have any transposon insertions from liver tumors generated with the villin-Cre transgenic mice used in the gastrointestinal cancer study (data not shown). Nodules, number of preneoplastic nodules from which the CIS was determined; Mouse, number of mice from which the nodules were isolated. Position based on the Ensembl NCBI m37 April 2007 mouse assembly.

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TA dinucleotides, with a few exceptions. We therefore conclude that each preneoplastic nodule was derived from an independent event resulting from random transposon insertional mutagenesis events. In some cases, identical *Egfr* gene insertions did occur in separate nodules, but as all other insertions were different in those samples, we concluded that the identical TA dinculeotide insertions into *Egfr* had occurred by chance owing to the strong selective pressure for insertions in intron 24 (see below). We previously observed T2/onc insertions into identical TA dinucleotides in *Braf* and *Notch1* in independent tumors in situations of strong positive selection for insertion into a specific part of an oncogene[7,8]. In contrast, our lung metastasis analysis, described below, demonstrates that clonal relationships can be detected between primary tumors and metastatic derivatives because identical T2/onc insertions occur in individual metastasis samples and a primary liver HCC tumor taken from the same mouse.

Pathway analysis of select CIS genes

Ingenuity Pathways Analysis (IPA) is a software application that enables network and functional analyses of gene sets of interest based on a repository of molecular interactions, regulatory events and gene-to-phenotype associations culled from the life sciences literature. The application can determine cellular and disease phenotypes most significant to a set of genes and can build molecular networks based on literature findings and pathways. Therefore, we used IPA to obtain a better understanding of the possible pathways and interactions between CIS genes. Of the 17 CIS genes analyzed, the three most significant signaling or disease functional annotations are post-translational modification ($P = 4.61E-09$), cancer ($P = 8.09E-06$) and tumor morphology ($P = 8.09E-06$) (**Supplementary Table 2** online). The CIS list includes homologs of several human genes that have been implicated in tumor formation and apoptosis of tumor cell lines: *EGFR*, *HIF1A*, *MAP2K4*, *MET*, *PAK4*, *VRK2*, *TRPM7* and *TAOK3*. IPA identified two network pathways overrepresented by human homologs of CIS genes. The first network includes two transcription factors (*NFIB* and *HIF1A*) and the second pathway involves genes that interact with *TNF*. The combined pathways from IPA are summarized in **Figure 3a**.

Frequent transposon insertions occur in *Egfr*

Transposon insertions in *Egfr* were detected in 85% ($n = 58$) of preneoplastic liver nodules isolated from experimental animals. These transposon insertions were most frequently detected in intron 24 of *Egfr* (**Table 1** and **Fig. 3b**) and in the antisense orientation, suggesting truncation of the gene product. Three-primer PCR genotyping using endogenous *Egfr* and transposon primers performed with genomic DNA isolated from individual tumor nodules confirmed the presence of transposon vectors in this locus (**Fig. 3c**). RT-PCR also confirmed the presence of the predicted truncated *Egfr* transcript in these preneoplastic nodules (**Fig. 3d**).

As *Egfr* insertions were also identified in preneoplastic nodules taken from a triple transgenic mouse, *Egfr* mutations also appear to contribute to tumorigenesis in a nonpredisposed genetic background. The insertions in this animal are predicted to result in a truncated Egfr protein (about 984 amino acids) containing the majority of the kinase domain but lacking the C-terminal domain. Indeed, this truncated Egfr was detected by western blot analysis in the liver tumors of older experimental triple transgenic male mice (**Supplementary Fig. 4b**).

Lung metastases derived from HCC

Analysis of genomic DNA taken from metastases of two triple transgenic male mice also demonstrated transposon insertion in intron 24 of *Egfr*, indicating that they were derived from the HCCs (**Fig. 3e**). Thirty-two additional lung metastatic nodules were isolated from a 432-d-old quadruple transgenic male. Insertion sites from these metastatic nodules were compared to three individual HCC nodules taken from the same animal to identify a clonal relationship between primary liver tumors and metastases, and between metastases. One of the liver HCCs (HCC3) seemed to share a common ancestor with a second HCC (HCC2) as both have identical *Egfr* gene insertions, which are distinct from the *Egfr* insertion in HCC1

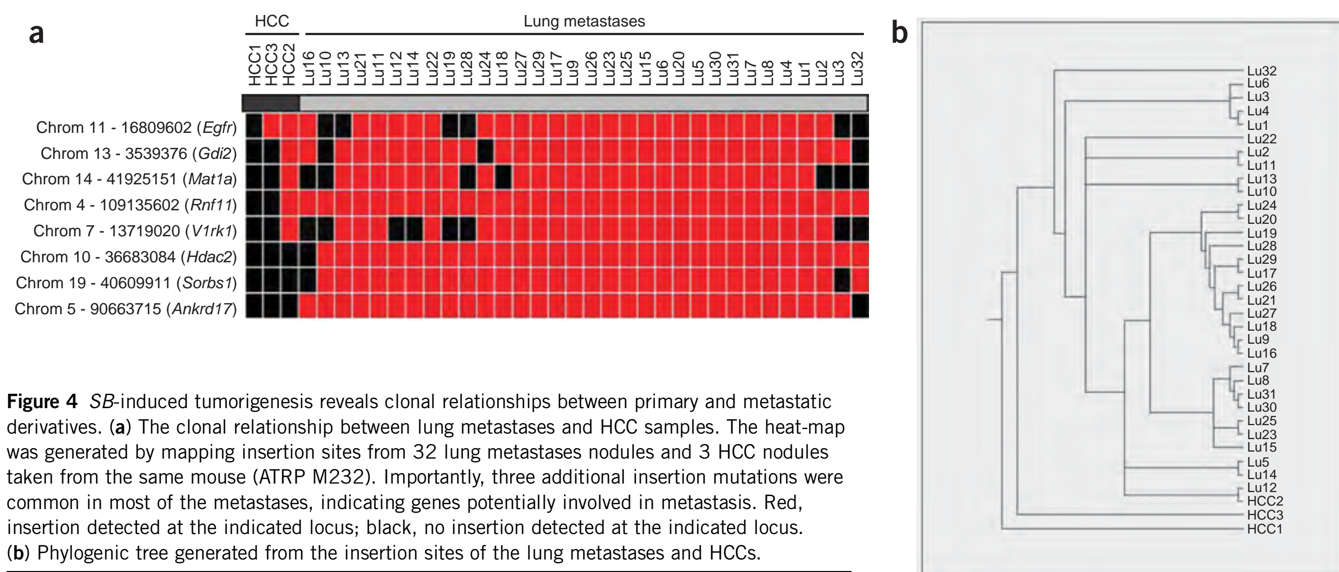

Figure 4 *SB*-induced tumorigenesis reveals clonal relationships between primary and metastatic derivatives. (**a**) The clonal relationship between lung metastases and HCC samples. The heat-map was generated by mapping insertion sites from 32 lung metastases nodules and 3 HCC nodules taken from the same mouse (ATRP M232). Importantly, three additional insertion mutations were common in most of the metastases, indicating genes potentially involved in metastasis. Red, insertion detected at the indicated locus; black, no insertion detected at the indicated locus. (**b**) Phylogenic tree generated from the insertion sites of the lung metastases and HCCs.

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(**Fig. 4a**). Most of the metastases share four additional insertions with HCC2, indicating that the metastases share a common ancestor with HCC2. Three additional insertion mutations were found in most of the metastases (**Fig. 4a**). From the phylogenetic tree generated from the insertion sites (**Fig. 4b** and **Supplementary Methods**), primary liver tumor HCC2 and all lung metastases have the closest common ancestor, suggesting that the lung metastases are actually derived from liver tumor HCC2. These preliminary data suggest that *SB*-induced tumorigenesis allows one to derive clonal relationships between primary and metastatic derivatives, and to discover metastases-specific insertion mutations that may drive this biological process.

Comparison with human hepatocellular carcinoma samples

Representative oligonucleotide microarray analysis (ROMA) of 100 human HCCs showed that increases or decreases in copy numbers of 17 human homologs of our CIS genes have been associated with human HCC (**Supplementary Table 3** online). We predict the effects of transposon insertions on CIS gene expression in **Supplementary Table 3** and **Supplementary Methods**. Genes with distinct copy number gains identified in human HCC samples ($n = 100$), homologs of which are also disrupted in our mouse model, include *EGFR*, *SLC25A13*, *MET* and *UBE2H*. Genes with distinct copy number losses in human HCC samples, homologs of which were also identified as mouse CIS genes in our analysis, include *MARCH1*, *PSD3*, *MAP2K4* and *NFIB*.

We also analyzed another cohort of 132 human samples spanning the whole spectrum of human hepatocarcinogenesis: normal liver ($n = 10$), cirrhotic liver ($n = 13$), low-grade dysplastic nodules ($n = 10$), high-grade dysplastic nodules ($n = 8$) and HCC ($n = 91$). Fifteen of the CIS genes were analyzed by combined single nucleotide polymorphism (SNP) and gene expression arrays. The most appealing candidates for clinical correlations were selected based on recurrent gene copy number changes, and correlated gene expression changes were compared with control samples (**Supplementary Methods**). Of the 15 genes, only three— *MAP2K4*, *QKI* and *UBE2H*—satisfy these criteria. *MAP2K4* and *QKI* have losses of DNA copy numbers with reduced mRNA levels, whereas *UBE2H* has DNA copy number gains with a substantial increase in mRNA levels (**Supplementary Fig. 5a** online). Associations between *MAP2K4*, *QKI* and *UBE2H* expression and clinicopathological variables were analyzed in 82 hepatitis C–related HCC patients treated with liver resection (**Supplementary Methods**). Although, owing to the small sample population, these genes did not display a significant difference in outcome measured by tumor recurrence or survival, high expression levels of *UBE2H* displayed a nonsignificant trend toward lower survival rates ($P = 0.09$) compared with low expression levels (**Supplementary Fig. 5c**). Studies involving the tyrosine kinase receptors *EGFR* and *MET*, both located on chromosome 7, recently showed that copy number gains of this chromosome are frequently associated with HCC and define a molecular class of HCC patients[24] (**Supplementary Fig. 5b**).

Functional validation of two CIS genes

As *UBE2H* seemed a strong candidate HCC oncogene, we used a cell proliferation assay to test its oncogenic potential. AML12 cells (adult mouse hepatocyte cell line, transgenic for human *TGFA*) stably transfected with a *Ube2h* expression vector have a higher proliferative rate than normal untransfected cells or AML12 cells transfected with an empty vector (**Supplementary Fig. 6** online).

We used the *Fah*-deficient mouse model[25] to test whether the truncated form of *EGFR* could contribute to neoplastic growth *in vivo*. In this assay, a test transgene is codelivered with an *Fah* expression vector to allow selective repopulation of genetically transformed hepatocytes *in vivo* under conditions that would normally kill hepatocytes. Two vectors were generated: one (pT2/FAHIL) co-expresses *Fah* and firefly luciferase, whereas the other (pT2/PGK-Truncated EGFR) expresses a truncated form of *EGFR* (exon 1 to exon 24) only (**Fig. 5a**). We used tail-vein hydrodynamic injection[26] to administer the vectors to *Fah*-deficient mice that express the *SB11* transposase knocked into the *Rosa26* locus (*Fah/SB11* mice). Upon withdrawal of NTBC (nitisinone, Orfadin), the mice underwent liver repopulation, as evidenced by stable weight gain and increasing luciferase expression (**Fig. 5b**). When a mouse injected with both pT2/FAHIL and pT2/PGK-Truncated EGFR was euthanized 43 d after injection, several patches of hyperplastic nodules were visible in the liver (**Fig. 5c**). RT-PCR revealed that these nodules express *Fah* and the truncated form of *EGFR* (**Fig. 5d**). Although immunohistochemistry confirmed the inability to detect EGFR in normal *Fah*-deficient liver (**Fig. 5e**), it also confirmed that induced hyperplastic liver nodules co-express Fah and EGFR

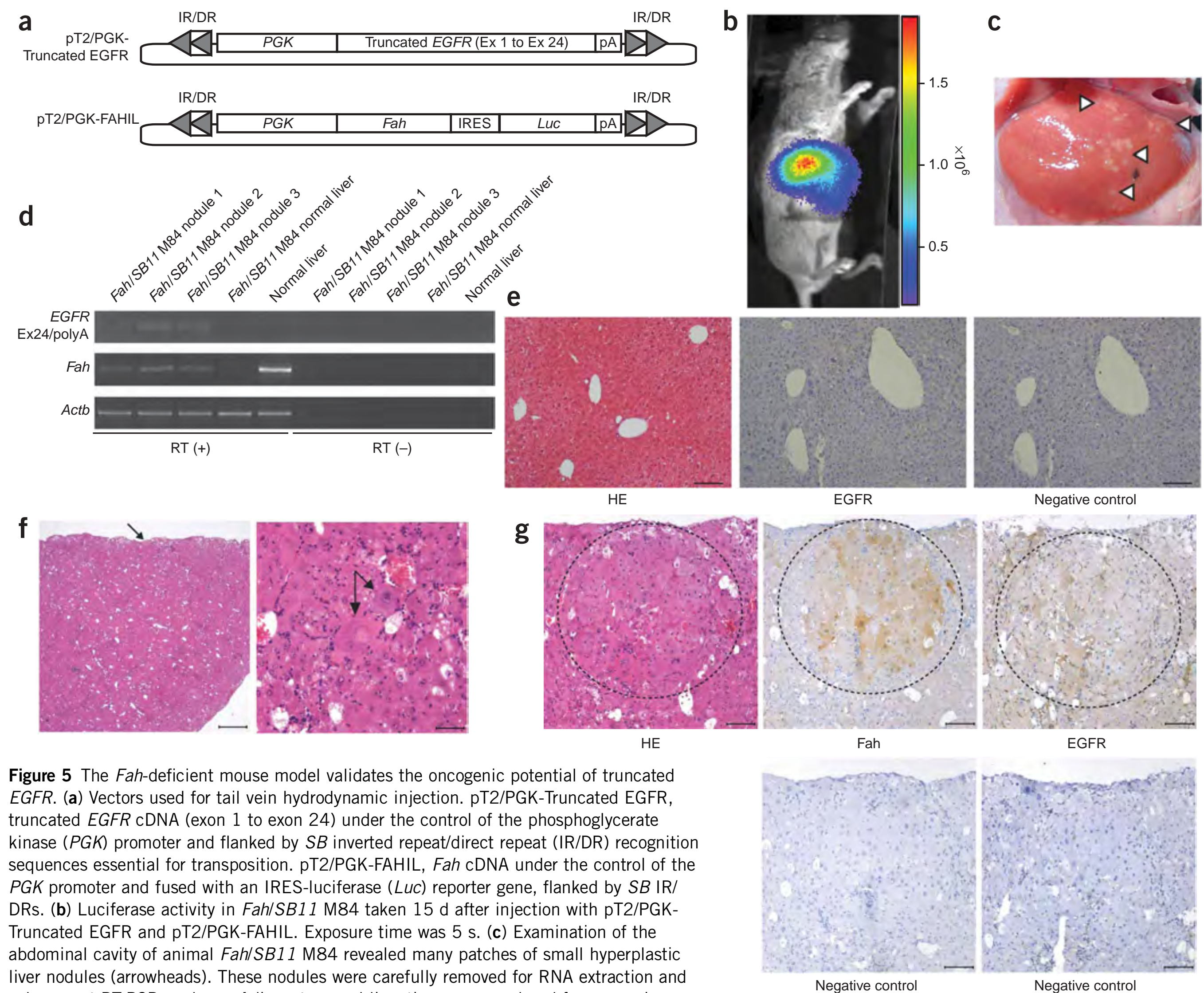

Figure 5 The *Fah*-deficient mouse model validates the oncogenic potential of truncated *EGFR*. (**a**) Vectors used for tail vein hydrodynamic injection. pT2/PGK-Truncated EGFR, truncated *EGFR* cDNA (exon 1 to exon 24) under the control of the phosphoglycerate kinase (*PGK*) promoter and flanked by *SB* inverted repeat/direct repeat (IR/DR) recognition sequences essential for transposition. pT2/PGK-FAHIL, *Fah* cDNA under the control of the *PGK* promoter and fused with an IRES-luciferase (*Luc*) reporter gene, flanked by *SB* IR/DRs. (**b**) Luciferase activity in *Fah*/*SB11* M84 taken 15 d after injection with pT2/PGK-Truncated EGFR and pT2/PGK-FAHIL. Exposure time was 5 s. (**c**) Examination of the abdominal cavity of animal *Fah*/*SB11* M84 revealed many patches of small hyperplastic liver nodules (arrowheads). These nodules were carefully removed for RNA extraction and subsequent RT-PCR analyses. Adjacent normal liver tissue was analyzed for comparison. (**d**) RT-PCR analyses of the liver nodules and adjacent normal tissue. Liver hyperplastic nodules expressed both *Fah* and the truncated form of *EGFR*, whereas the adjacent normal tissue was negative for both transcripts. RT (+), first strand cDNA synthesis with reverse transcriptase added; RT (–), first strand cDNA synthesis without reverse transcriptase. (**e**) Normal histology of *Fah*-deficient liver (hematoxylin-eosin stain, HE) and inability to detect EGFR by immunohistochemical staining. EGFR, treated with EGFR primary antibody; negative control, serial section not treated with the indicated primary antibody. Scale bars, 100 μm. (**f**) Histology of liver hyperplastic nodules induced by truncated form of *EGFR* using HE staining. Top panel, the capsular surface of the liver was irregularly nodular (arrow), but overall hepatic architecture was preserved with regularly spaced central veins and portal tracts. Scale bar, 500 μm. Bottom panel, a portion of hepatic lobule containing variably sized hepatocytes with two cytomegalic and karyomegalic hepatocytes in the center, one of which is binucleated (arrows). Occasional hepatocytes have vacuolated cytoplasm. Hepatic cords are not evident due to cellular crowding. Scale bar, 50 μm. (**g**) Representative hyperplastic nodule (enclosed within dashed circular line) within hepatic parenchyma comprising closely packed sheets of variably sized hepatocytes, including a karyomegalic cell. Note the mild compression of the surrounding hepatic parenchyma. Scattered neutrophils and lymphocytes, and mild extramedullary hematopoiesis suggested a low degree of inflammation. Immunohistochemical analyses of serial liver sections treated with the indicated primary antibody confirmed the co-expression of Fah and EGFR in liver nodules. Most of the hepatocytes within the hyperplastic nodule (enclosed within dashed circular line) expressed Fah. Hepatocytes within the hyperplastic nodule (enclosed within dashed circular line) and within surrounding parenchyma stained weakly for EGFR. EGFR staining is also prominent in the cytoplasmic membranes of cells bordering sinusoids. Negative control, serial sections not treated with the indicated primary antibody. Scale bars, 100 μm.

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(**Fig. 5f,g**). Notably, adjacent liver tissue, which appeared healthy, was negative for both transcripts (**Fig. 5d**).

DISCUSSION

The recent development of target-based therapeutics for treating cancer has sparked a worldwide effort to identify all of the genes and signaling pathways that cause it. But despite the potential of transposon-based insertional mutagenesis for identifying cancer genes, it has been impossible to control transposition in a manner that allows different types of cancer to be modeled. We used a conditional *SB* allele and a hepatocyte-specific Cre recombinase to screen for HCC-associated genes in mice. As expected, quadruple transgenic mice displayed more numerous and larger tumor nodules than triple transgenic animals, as a result of the *Trp53* mutant background,

which predisposes animals to cancer. Our conditional *SB* liver-tumor model is useful in elucidating genetic mechanisms for all stages of HCC tumorigenesis—from early hepatic adenoma to fully developed HCC, including metastasis.

Pyrosequencing technology facilitates the use of transposons for cancer gene identification by enabling amplification and sequencing of tens of thousands of *SB* insertion sites from a mixture of tumors in a single sequencing run. From 8,060 nonredundant insertions subsequently cloned from 68 tumor nodules, we identify 19 loci that seem strongly implicated in HCC. Our list of genes with multiple examples of CISs includes several homologs of human genes (e.g., *EGFR*, *HIF1A*, *MAP2K4*, *MET*, *PAK4*, *VRK2*, *TRPM7* and *TAOK3*) that have been implicated in tumor formation and apoptosis of tumor cell lines. IPA identified two network pathways overrepresented by these homologs of CIS genes. The first network includes two transcription factors, *NFIB* and *HIF1A*, which are capable of transducing *EGFR*-initiated phosphorylation-signaling cascades. *HIF1A* has also been suggested to play a role in tumor vascularization[27]. The second pathway involves genes that interact with *TNF*. *TNF* can induce tyrosine phosphorylation and internalization of *EGFR*, playing a critical role in NF-κB activation[28]. NF-κB, in turn, plays an important role in regulating apoptosis during liver tumorigenesis[29].

Transposon insertions in mouse *Egfr* that cause truncations in the C-terminal half of the gene product were common in *SB*-induced liver tumors. Deletions of the C-terminal domain of human EGFR (966–1006) have been shown to increase both autokinase activity and transforming ability *in vitro* and *in vivo*[30]. Internal deletions in the C terminus of EGFR have also been detected in naturally occurring EGFR mutants displaying tumorigenic properties[30–32], probably resulting in constitutively active forms of the protein owing to the destabilization of the inactive EGFR monomeric complex[33]. It has been suggested that truncated Egfr can form a heterodimer with Erbb2 and transphosphorylate the tyrosine sites[34]. Tyrosine-phosphorylated Erbb2 could then lead to the activation of other signaling pathways by different mechanisms and may play a role in HCC tumorigenesis[35]. Besides HCC, *EGFR* overexpression has also been associated with human breast and gut cancers[36–39]. *EGFR* is overexpressed in 15–40% of human HCCs and EGF signaling is activated in ~50% of human HCCs[39,40]. Although extra copies of *EGFR* were seen in 17 out of 38 (45%) HCC tumors, increased expression did not correlate with the increase in *EGFR* copy number[36]. Recent findings suggest that *EGF* signaling could even be related to HCC development based on significant differences in *EGF* genotype prevalence according to the risk of developing HCC[41]. In addition, use of erlotinib (Tarceva) to specifically target *EGFR* has shown interesting preliminary results in phase 2 clinical trials in human HCC[15,42].

The genes *EGFR*, *SLC25A13*, *MET* and *UBE2H* identified using CISs from our mouse model all showed distinct increases in copy number in human HCC samples. *EGFR* and *MET* are known proto-oncogenes, whereas *SLC25A13* and *UBE2H* may have novel oncogenic activities in HCC. *MET* encodes the tyrosine kinase receptor for *HGF* and is overexpressed in HCC[43]. Although our algorithm predicted a *Met* gene disruption in our *SB*-induced tumors (**Supplementary Table 3**), we suspect that these insertions actually activate the oncogenic activity of *Met*; five of six insertions could produce a kinase domain–containing truncated protein or activate the gene by enhancer insertion. It is also possible that loss of function of *Met* contributes to tumor development as *Met* knockout mice are more prone to developing liver tumors[44]. Genes with distinct copy number losses in human HCC samples whose homologs were also identified as CIS genes by our mouse model, include *MARCH1*, *PSD3*, *MAP2K4* and *NFIB*. *MAP2K4* has been identified as a putative tumor-suppressor gene in human solid tumors of breast, prostate and pancreas, and may have a similar function in the liver[45–47]. Although *PSD3* and *MARCH1* have not been shown to be involved in cancer, based on data presented here, they may have tumor-suppressor activity in HCC. Interestingly, the transcription factor *NFIB* is known to be upregulated in hepatitis-induced HCC[48]. Another interesting finding is that a large number of the CIS genes have human homologs that map to chromosome 7, which has copy number amplifications in >15% of human HCCs[49,50]. Moreover, when another cohort of 132 human samples spanning the whole spectrum of human hepatocarcinogenesis was compared with 15 human homologs of the CIS genes by combined SNP and gene expression arrays, preliminary results indicated a nonsignificant trend to higher tumor recurrence and poorer survival rates associated with higher expression levels of *UBE2H*. Our validation experiments and human comparative studies suggest a role for *UBE2H* in liver tumorigenesis. Furthermore, validation experiments confirmed the contribution of truncated *EFGR* to neoplastic growth *in vivo*.

A molecular classification of HCC based on gene copy number alteration and expression profiling was recently proposed[24]. The five classes, based on hierarchical clustering of gene expression data, are β-catenin, proliferation- and interferon-related neoplasms, a novel class of neoplasm defined by polysomy of chromosome 7 and an unannotated category. Although we did not recover recurrent insertions in *Ctnnb1* or homologs of any of the several human genes known to be implicated in HCC, we did observe an increase in β-catenin protein expression. We plan to use mRNA microarray profiling of *SB*-induced HCC to clarify whether our system models one or more of the non-CTNNB1 subclasses of HCC. Regardless, based on our comparison of CIS genes to gene copy number and expression changes in human HCC, it appears that homologs of three of the genes on our list—*UBE2H, QKI* and *MAP2K4*—are strong candidates for driving HCC. Moreover, homologs of several of the other CIS genes—including *MET*, *EGFR* and *HIF1A*—have been studied specifically in the context of human HCC and are likely to play a role in the development of this disease. Taken together, this indicates that the *SB* screen yields a high fraction of relevant events in human HCC.

These studies, combined with others showing that conditional transposon-based insertional mutagenesis can be used to model solid tumors in other organ sites such as brain and gastrointestinal tract (data not shown), define a powerful new method for dissecting the cancer genome and for developing better treatments for cancer. Future research directions include using this technology for further validation of both the HCC- and metastasis-associated genes identified here.

METHODS

Generation of transgenic animals. Alb-Cre transgenic animals were purchased from Jackson Laboratory[16]. They were initially bred with T2/onc homozygotes to obtain doubly transgenic animals carrying both *Alb*-Cre and T2/onc. The T2/onc transgenic line with the donor concatemer on chromosome 15, generated as previously described[8], was used in this study. Simultaneously, transgenic animals heterozygous for Rosa26-lsl-SB11 and p53-lsl-R270H (purchased from NCI, Frederick Mouse Repository) were interbred to obtain doubly transgenic animals. The two doubly transgenic lines were finally interbred to generate the required triple (*Alb*-Cre/T2/onc/*Rosa26*-lsl-*SB11*), quadruple (*Alb*-Cre/T2/onc/*Rosa26*-lsl-*SB11*/*p53*-lsl-*R270H*) and control animals of various transgene combinations. The genetic background of these animals was mixed, allowing for a diverse genetic population analysis.

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PCR genotyping. Identification of the various genotypes from both adult transgenic animal and pups was performed as follows. First, genomic DNA was isolated from tail clippings using standard proteinase-K treatment, phenol-chloroform extraction and ethanol precipitation. Genomic DNA was then dissolved in sterile TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8)) and quantified using a Nanodrop spectrophotometer. PCR genotyping was performed using 100 ng of diluted genomic DNA as template. PCR primers used for *Alb*-Cre were forward 5′-CACACTGAAATGCTCAAATGGGAGA-3′ and reverse 5′-GGCAAATTTTGGTGTACGGTCAGTA-3′ (amplicon 456 bp); T2/onc forward 5′-CGCTTCTCGCTTCTGTTCGC-3′ and reverse 5′-CCACCCCCAGCATTCTAGTT-3′ (amplicon 264 bp); *Rosa26*-lsl-*SB11* were *Rosa26* wild-type forward 5′-CTGTTTTGGAGGCAGGAA-3′, *Rosa26* wild-type reverse 5′-CCCCAGATGACTACCTATCCTCCC-3′, *SB* reverse 5′-CTAAAAGGCCTATCACAAAC-3′ (*Rosa26* wild-type and *Rosa26*-lsl-*SB11* amplicons are 420 bp and 266 bp, respectively); *p53*-lsl-*R270H* were *p53* wild-type forward 5′-TTACACATCCAGCCTCTGTGG-3′, *p53* wild-type reverse 5′-CTTGGAGACATAGCCACACTG-3′, *p53*-lsl-*R270H* conditional forward 5′-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3′ (*p53* wild-type and *p53*-lsl-*R270H* conditional allele amplicons are 170 bp and 270 bp, respectively); glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were forward 5′-GGAGCCAAACGGGTCATCATCTC-3′ and reverse 5′-GAGGGGCCATCCACAGTCTTCT-3′ (amplicon 233 bp). PCR conditions for *Taq* polymerase (CLP) were used according to the manufacturer's instructions with an initial denaturing step of 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 7 min. PCR products were separated on a 2% agarose gel and genotype determined by the absence or presence of expected amplicons.

Liver tumor analysis. The whole liver was carefully removed from the euthanized animal, washed and placed in cold PBS. The number of surface liver tumor nodules was counted for all liver lobes. All reasonably sized tumor nodules (>2 mm in diameter) were carefully removed from the liver lobes using fine forceps and placed in fresh cold PBS. These separated nodules were then halved using a sterile razor blade and split into samples for DNA and RNA extraction. Tissue samples for RNA were stored at –80 °C in RNAlater (Sigma) to prevent RNase contamination and degradation. Histological sections were taken only for larger tumor nodules (>2 mm in diameter), in addition to the samples for DNA and RNA extraction. DNA extraction was done as previously described in the PCR genotyping section. Extraction of RNA was done using the Trizol reagent (Invitrogen) using protocols described by the manufacturer. Formalin fixed-paraffin embedded sections from various tissues were sectioned at 5 μm using a standard microtome (Leica), mounted and heat-fixed onto glass slides. Tissue section slides were either processed and stained with hematoxylin-eosin (HE) using standard protocols, or used for immunohistochemistry as described in the next section.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections from various tissues were sectioned at 5 μm, mounted and heat-fixed onto glass slides to be used for immunohistochemical analyses. Briefly, the glass section slides were dewaxed and rehydrated through a gradual decrease in ethanol concentration. The antigen epitopes on the tissue sections were then unmasked using a commercially available unmasking solution (Vector Laboratories) according to the manufacturer's instructions. The tissue section slides were then treated with 3% hydrogen peroxide to remove any endogenous peroxidases. Blocking was performed at 4 °C using a M.O.M. mouse immunoglobulin-blocking reagent (Vector Laboratories) in a humidified chamber for several hours. The sections were then incubated overnight at 4 °C in a humidified chamber using various primary antibodies: SB transposase (1:100) (R&D Systems), Alb (1:200) (Abcam), Afp (1:100) (GeneTex), Ki67 (1:200) (Novocastra), β-catenin (1:500) (BD) and Fah (1:250) (AbboMax). After primary incubation, sections were washed thoroughly in PBS before incubating with horseradish peroxidase-secondary antibody raised against the primary antibody initially used. After thorough washes with PBS, the sections were treated with freshly prepared DAB substrate (Vector Laboratories) and allowed to develop adequate signal before stopping the reaction in water. For EGFR immunohistochemistry, EGFr Kit (Clone 31G7) (Zymed Laboratories, Invitrogen) was used according to the manufacturer's instructions, except for the following modification. An additional overnight blocking step using the M.O.M. mouse immunoglobulin-blocking reagent was incorporated after proteinase K treatment to reduce background staining. Finally, sections were then lightly counter-stained with hematoxylin, dehydrated through gradual increase in ethanol concentration, cleared in Citrosol and mounted in Permount (Fisher).

Pyrosequencing. Protocol for amplicon sequencing using the GS20 Flex pyrosequencing machine was as previously described by Roche. Briefly, 100 ng of genomic DNA isolated from individual tumors was digested with either *Bfa*I or *Nla*III, for left or right transposon IR/DR, respectively. A small volume of this enzyme digest was used for splinkerette linker attachment using the appropriate linker. To make the *Bfa*I linker, the following oligonucleotide sequences were annealed together using standard protocols, top strand 5′-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC-3′ and bottom strand 5′-TAGTCCCTTAAGCGGAG-3′. As for the *Nla*III linker, the following oligonucleotide sequences were annealed together using standard protocols, top strand 5′-GTAATACGACTCACTATAGGGCTCCGCTTAAGGGACCATG-3′ and bottom strand 5′- GTCCCTTAAGCGGAGCC-3′. Linker ligations were performed overnight at 16 °C using T4 DNA ligase (New England Biolabs). The ligation reaction was cleaned using MinElute 96-well plates (Qiagen) in a vacuum manifold and resuspended in 40 μl of sterile double-distilled water (DDW). This resuspended solution was then digested with either *Bam*HI or *Xho*I, for left or right transposon IR/DR, respectively. A small volume was then used for primary PCR using the following primers. Left IR/DR primer (*Bfa*I), 5′-CTGGAATTTTCCAAGCTGTTTAAAGGCACAGTCAAC-3′; right IR/DR primer (*Nla*III), 5′-GCTTGTGGAAGGCTACTCGAAATGTTTGACCC-3′ and common splinkerette primer was used for both IR/DRs, 5′-GTAATACGACTCACTATAGGGC-3′. PCR conditions for *Taq* polymerase (CLP) were used according to the manufacturer's instructions of an initial denaturing step of 94 °C for 5 min; 30 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1.5 min; followed by a final extension at 72 °C for 5 min. One microliter of the diluted first PCR product sample (1:75) was used as a template for the secondary PCR under the following conditions. Nested versions of the above primers carrying the required fusion sequences for GS20 Flex pyrosequencing (Fusion A and Fusion B), as well as a unique 10-bp barcode recognition sequence for each tumor sample. Primers were designed as such that the nested transposon primer have the Fusion A and barcode attached (Fusion A – barcode – nested primer) and the nested linker primer has the Fusion B sequence attached (linker nested – Fusion B). PCR conditions for Taq polymerase (Roche FastStart High Fidelity) were used according to the manufacturer's instructions of an initial denaturing step of 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1.5 min; followed by a final extension at 72 °C for 5 min. After the secondary PCR, the reaction was purified using MinElute 96-well plates in a vacuum manifold and resuspended in 30 μl of sterile TE. The amount of DNA in each PCR sample was quantified using the QuantIT picogreen kit (Invitrogen) and the samples were diluted to a final concentration of 2×10^5 molecules/μl for pyrosequencing.

Selection criteria for common insertion sites (CISs). See **Supplementary Methods**.

Ingenuity Pathways Analysis (IPA). Ingenuity Systems at http://www.ingenuity.com/.

***Egfr* PCR genotyping.** PCR genotyping was used to confirm the presence of the T2/onc transposon insertion in intron 24 of the *Egfr* gene. Briefly, genomic DNA was isolated from individual tumor nodules using protocols already described in the PCR genotyping section. PCR genotyping was performed using 100 ng of diluted genomic DNA as template. PCR primers used for *Egfr* intron 24 were forward, 5′-TACATGGTCAAAATCTCTCCAATAGGTC-3′ and reverse, 5′-ATTAGAAAGGGCAACGAAGCTTGC-3′, with an expected amplicon of 713 bp. A third primer specific for the IR/DR-R (T/JB3) of the T2/onc transposon vector was also included, 5′-AGGGAATTTTTACTAGGATTAAATGTCAGG-3′. PCR conditions were as described previously in the PCR genotyping section. The amplicon sizes varied depending on the position of the T2/onc transposon vector insertion site.

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When the T2/onc/*Egfr* amplicon is expected to overlap the endogenous *Egfr* product, a PCR genotyping using only the T/JB3 and *Egfr* intron 24 forward primers is used instead with the same PCR conditions.

RT-PCR. Extraction of RNA from tumor nodules was done using the Trizol reagent using protocols described by the manufacturer. First strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) as described by the manufacturer using 1 μg total RNA as template. Both reactions using with (RT+) and without (RT–) the reverse transcriptase were performed for all the samples. Subsequent PCR was performed using 1 μl of the cDNA as template with various primer pairs. Primer sequences for alpha-fetoprotein (*Afp*) were forward 5′-CCTGTGAACTCTGGTATCAG-3′ and reverse 5′-GCTCACACCAAAGCGTCAAC-3′ (amplicon 410 bp); secreted phosphoprotein 1 (*Ssp1*) forward 5′-CTTTCACTCCAATCGTCCCTAC-3′ and reverse 5′-GCTCTCTTTGGAATGCTCAAGT-3′ (amplicon 305 bp); Sleeping Beauty (*SB*) transposase forward 5′-ATGGGAAAATCAAAAGAAATCAGCC-3′ and reverse 5′-CGCACCAAAGTACGTTCATCTCTA-3′ (amplicon 221 bp); albumin (*Alb*) forward 5′-CCCCACTAGCCTCTGGCAAAAT-3′ and reverse 5′-CTTAAACCGATGGGCGATCTCACT-3′ (amplicon 127 bp); epidermal growth factor receptor (*Egfr*) forward 5′-GATAGATGCTGATAGCCGCCC AAAG-3′ and reverse 5′-TCATGCTCCAATAAACTCACTGCTT-3′ (amplicon 772 bp); truncated-*Egfr* forward (same forward primer used for *Egfr*) and reverse (specific for the T2/onc SV40-polyA) 5′-TGCTTTATTTGTGA AATTTGTGATGCTATTG-3′ (amplicon 320 bp); receptor tyrosine-protein kinase erbb2 (*Erbb2*) forward 5′-CCCAGATCTCCACTGGCTCC-3′ and reverse 5′-TTCAGGGTTCTCCACAGCACC-3′ (amplicon 376 bp); beta-actin (*Actb*) forward 5′-GTGACGAGGCCCAGAGCAAGAG-3′ and reverse 5′-AGGG GCCGGACTCATCGTACTC-3′ (amplicon 938 bp); neomycin (*Neo*) forward 5′-ATGATTGAACAAGATGGATTGCACG-3′ and reverse 5′-AAGGTGAGATG ACAGGAGATCCTG-3′ (amplicon 321 bp); ubiquitin-conjugating enzyme E2H (*Ube2h*) forward 5′-CTGAGCGGACCCCACGGGAC-3′ and reverse 5′-CAG CAACTGGGGCAGGAAGG-3′ (amplicon 505 bp); fumarylacetoacetate hydrolase (*Fah*) forward 5′-ATGAGCTTTATTCCAGTGGCC-3′ and reverse 5′-ACCACAATGGAGGAAGCTCG-3′ (amplicon 503 bp); truncated *EGFR* forward 5′-GACCCCCAGCGCTACCTTGTCATTCAG-3′ and reverse (specific for the rabbit β-globin polyA) 5′-GCCACACCAGCCACCACCTTCTG-3′ (amplicon 140 bp). PCR conditions are similar to PCR genotyping described previously except 25 to 30 cycles were performed to avoid amplicon saturation.

Representational oligonucleotide microarray analysis (ROMA). Microarray analysis was performed on human HCC samples as previously described[51].

Cell proliferation assay. AML12 (CRL-2254) was obtained from America Type Culture Collection (ATCC) and maintained according to the recommended culture conditions. An expression vector for *Ube2h* (MC200579) mouse cDNA was obtained from Origene. The empty vector (pcDNA) purchased from Invitrogen, was used as a negative control. Cell transfections were performed using Lipofectamine LTX (Invitrogen) with PLUS (Invitrogen) according to the manufacturer's recommendation. Transfected cell lines were grown in medium containing neomycin (0.5 mg/ml) for 2 weeks to select for stable cell populations. Stable cell populations for each expression vector were obtained from three individual transfections. Cell proliferation rate of the stable cell populations was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocols.

Hydrodynamic injection. Hydrodynamic injections were performed as previously described[25]. Briefly, fumarylacetoacetate hydrolase (*Fah*)-null mice carrying the *Rosa26-SB11* transgene were generated. Truncated-*EGFR* (exon 1 to exon 24) was PCR amplified from pBabe-Puro-LTR-EGFR (a kind gift from Heidi Gruelich) using the following primers: exon 1 forward 5′-ATGC GACCCTCCGGGACGGC-3′ and exon 24 reverse 5′-CTGAATGACAAGG TAGCGCTGGGGGTC-3′ was placed under the control of a phosphoglycerate kinase (*PGK*) promoter and cloned into the pT2 vector containing the *SB* flanking IR/DR recognition sequences to obtain pT2/PGK-Truncated EGFR. Two other constructs were also prepared: pT2/PGK-FAHIL, vector containing the *Fah* and luciferase gene under the control of the *PGK* promoter[52]. Twenty micrograms of each construct was hydrodynamically injected into 6-week-old *Fah*-null/*Rosa26-SB11* male mice (*Fah*/*SB11*) using previously established conditions[26]. These mice are normally maintained with 7.5 μg/ml 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) drinking water but replaced with normal drinking water after hydrodynamic injection of transposon vectors. These experimental animals were observed for weight changes and luciferase activity as previously described[25].

Note: Supplementary information is available on the Nature Biotechnology website.

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Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling

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Current neural induction protocols for human embryonic stem (hES) cells rely on embryoid body formation, stromal feeder co-culture or selective survival conditions. Each strategy has considerable drawbacks, such as poorly defined culture conditions, protracted differentiation and low yield. Here we report that the synergistic action of two inhibitors of SMAD signaling, Noggin and SB431542, is sufficient to induce rapid and complete neural conversion of >80% of hES cells under adherent culture conditions. Temporal fate analysis reveals the appearance of a transient FGF5+ epiblast-like stage followed by PAX6+ neural cells competent to form rosettes. Initial cell density determines the ratio of central nervous system and neural crest progeny. Directed differentiation of human induced pluripotent stem (hiPS) cells into midbrain dopamine and spinal motoneurons confirms the robustness and general applicability of the induction protocol. Noggin/SB431542-based neural induction should facilitate the use of hES and hiPS cells in regenerative medicine and disease modeling and obviate the need for protocols based on stromal feeders or embryoid bodies.

HES cells offer great promise for cell-replacement therapies, and recent advances in somatic cell reprogramming to iPS cells have opened the door to generating patient-specific cells for regenerative medicine and disease modeling[1]. To realize the full potential of these approaches for the production of neural cells, improved differentiation protocols are required that eliminate the use of undefined factors (such as neural-inducing stroma PA6 or MS5 cells[2,3]), the heterogeneous nature of embryoid body differentiation and the poor yield of protocols based on selective survival of neural progeny. Understanding and triggering the signaling pathways necessary and sufficient for neural induction in hES cells is a critical goal in this effort.

Several lines of evidence demonstrate a crucial role for SMAD signaling during neural induction. Elegant studies in frog identified bone morphogenic protein (BMP) inhibitors, including chordin[4], follistatin[5] and noggin[6], as the critical neural-inducing factors in the Spemann organizer. Mammalian noggin[7] has similar neural-inducing properties, and treatment with recombinant Noggin has been used in several hES-cell neural induction protocols[3,8]. More recently, the drug SB431542 was shown to enhance neural induction in an embryoid body–based hES-cell neural induction protocol[9]. SB431542 inhibits the Lefty/Activin/TGFβ pathways by blocking phosphorylation of the ALK4, ALK5 and ALK7 receptors. Although Noggin or SB431542 treatment improve the efficiency of neural induction, neither treatment alone is sufficient to neurally convert hES cells under defined or adherent conditions. Here we set out to test whether combined blockade of SMAD signaling using Noggin and SB431542 is sufficient to achieve full neural conversion and to avoid the use of embryoid bodies or stromal feeders.

We postulated that establishing an even cell distribution is critical for inducing homogeneous neural differentiation of hES cells. Therefore, undifferentiated hES cells were dissociated into single cells and replated onto Matrigel-coated dishes in conditioned medium supplemented with the ROCK inhibitor Y-27632[10], which promotes survival of hES cells as single cells. After 72 h, cells were switched from hES-cell conditions to knockout serum replacement medium containing either Noggin, SB431542 or both factors and allowed to differentiate for a total of 11 d (**Fig. 1a**). Perinuclear redistribution of SMAD4, the obligate co-SMAD, was observed after 24 h when both Noggin and SB431542 were present (**Supplementary Fig. 1** online). Neural induction was monitored by expression of PAX6, an early marker of neuroectodermal differentiation[11]. Combined treatment with Noggin and SB431542 greatly increased the efficiency of neural induction to >80% of total cells, compared with <10% PAX6+ cells when Noggin or SB431542 were used alone (**Fig. 1b**).

There are several potential mechanisms that could contribute to the synergistic action of Noggin and SB431542. These include destabilizing the activin- and Nanog-mediated pluripotency network[12], suppression of BMP-induced differentiation toward trophoblast lineage[13], suppression of mesodermal and endodermal fates by inhibiting endogenous activin and BMP signals[14,15], and promoting neuralization of primitive ectoderm by BMP inhibition[16]. Temporal analysis of gene expression revealed that treatment with SB431542 induced a rapid loss of Nanog expression (**Supplementary Fig. 2** online) and a large increase in the expression of CDX2 (**Fig. 1c**). These data suggest that SB431542-mediated loss of pluripotency is associated with differentiation toward the trophoblast lineage. Suppression of CDX2 in the presence of Noggin or Noggin/SB431542 demonstrates that one

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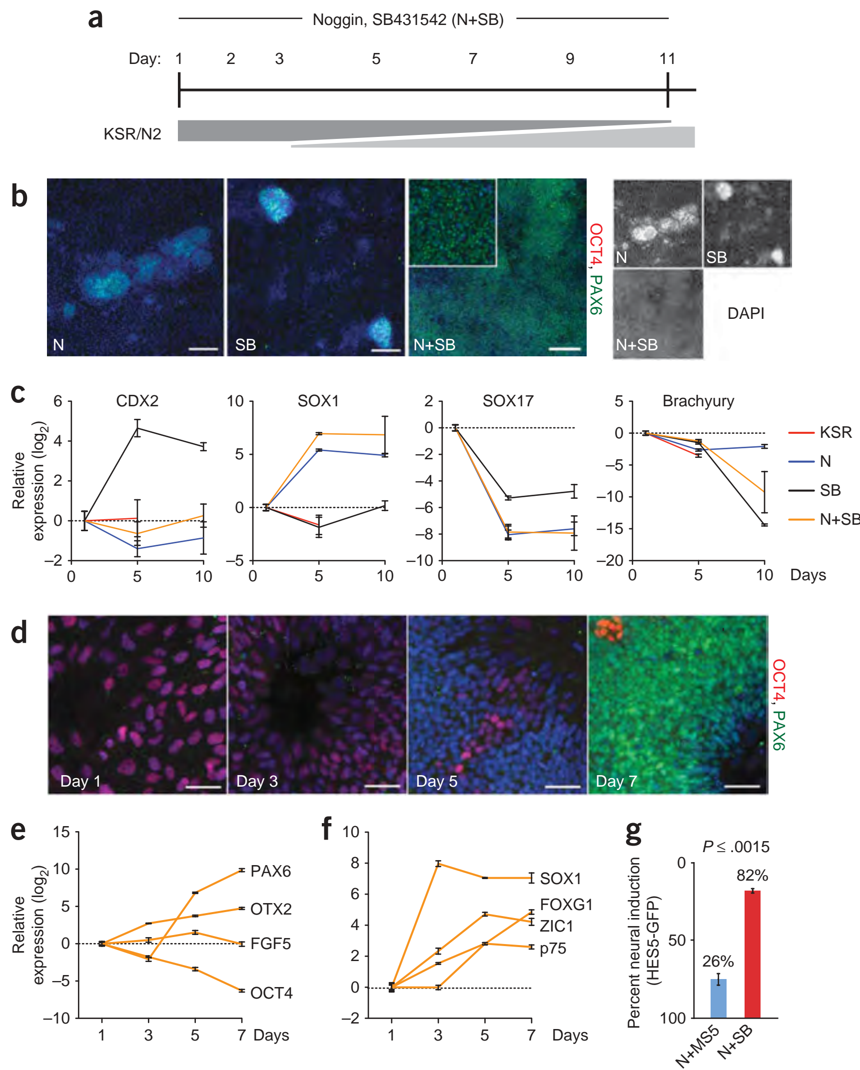

Figure 1 Dual-SMAD inhibition allows for highly efficient feeder-free neural induction in adherent cultures in 7 d. (**a**) Differentiation scheme using the combination of SB431542, an ALK inhibitor, and Noggin, a BMP inhibitor. (**b**) Dual-SMAD inhibition greatly improves neural differentiation (PAX6 expression, green) to >80% at day 11. Infrequent neural differentiation (<10% PAX6$^+$ cells) can be observed when the single factors are used. (**c**) Real-time PCR for early germ layer markers CDX2, SOX1, SOX17 and Brachyury. (**d**) Immunofluorescence for OCT4 (red) and PAX6 (green) expression indicates that rapid neuralization occurs by day 7. (**e**) Real-time PCR for PAX6, OTX2, FGF5 and OCT4 during dual-SMAD inhibition reveals an epi-stem cell intermediate at day 5. (**f**) Real-time PCR for neural and neuronal markers during dual-SMAD-inhibition differentiation toward neurectoderm. (**g**) A BAC reporter line (HES5-GFP) was used to quantify the percentage of neural induction for the method using MS5 stromal cells (with Noggin) or dual-SMAD inhibition (SB431542 and Noggin). All error bars represent s.e.m. and the *P* value was determined using the Student's *t*-test. N, Noggin; SB, SB431542; KSR, knockout serum replacement medium; N2, N2 medium. Scale bars, –200 μm (**b**); –50 μm (**d**).

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key role of Noggin is the repression of endogenous BMP signals that drive trophoblast fates upon differentiation. The pronounced induction of SOX1 in Noggin/SB431542-treated cultures confirmed a strong bias toward neuroectodermal lineage in the dual-SMAD-inhibition protocol. There is also evidence for suppression of alternative embryonic germ layers, such as Noggin-mediated suppression of SOX17 (endodermal lineage) and SB431542-mediated suppression of Brachyury (mesodermal lineage) (**Fig. 1c**). Taken together, these results indicate that SB431542 and Noggin work synergistically at multiple stages of differentiation to achieve efficient neural conversion of hES cells.

We next sought to characterize lineage progression of hES-cell progeny after the addition of the two inhibitors. Loss of OCT4 expression by day 5 and strong expression of PAX6 by day 7 (**Fig. 1d**) pointed to the presence of an intermediate cell type at day 5 of differentiation that was negative for both OCT4 and PAX6. Gene expression analysis revealed peak expression of the epiblast marker FGF5 at day 5 of differentiation, concomitant with high expression of OTX2, another epiblast marker whose expression is maintained during neural fate commitment (**Fig. 1e**). The expression of OTX2 and FGF5 before neuralization suggests the presence of epiblast-stage cells in our cultures[17]. Notably, the earliest neural marker expressed in our culture system was SOX1 (**Fig. 1f**), preceding induction of other neuroepithelial markers such as ZIC1 or PAX6 and preceding expression of anterior central nervous system (CNS; FOXG1) and neural crest (p75) markers. Whereas previous studies had suggested that PAX6 preceded SOX1 expression[18], early induction of SOX1 has been observed in mouse cultures[19]. One interesting possibility to explain early SOX1 expression could be direct modulation of SOX1 transcription by SMAD signaling in our culture system.

Our laboratory has recently described methods for establishing stable mouse[20] and hES-cell[21] transgenic reporter lines carrying bacterial artificial chromosomes (BACs) engineered to express GFP under the control of cell-type specific promoters. Here we used the *HES5:eGFP* BAC transgenic hES-cell reporter line, marking neural stem and precursor cell progeny[20,21], to measure the efficiency of neural induction. The dual-SMAD-inhibition protocol was compared to the standard MS5 protocol in the presence of Noggin[22]. To this end, *HES5:eGFP* cells were plated in medium supplemented with Noggin either in the presence of MS5 feeder cells or SB431542 and allowed to differentiate for 13 d, a stage when the GFP$^+$ cells were readily observed under both conditions (**Supplementary Fig. 3** online). GFP expression was quantified by flow cytometry. Nonmodified H9 cells were used as negative controls. MS5 cells were excluded from the analysis based on negative selection for the cell-surface molecule CD105 (**Supplementary. Fig. 4** online). Dual-SMAD inhibition yielded 82% GFP$^+$ cells at day 13, a more than threefold increase compared with the MS5/Noggin protocol (**Fig. 1g**). In contrast to the MS5 protocol, which requires plating of hES-cell colonies at low density in the presence of an MS5 mouse stromal cell line[22], the Noggin/SB431542 condition allowed for high plating densities. Therefore, in addition to higher percentages, the dual-SMAD-inhibition protocol also resulted in larger absolute numbers of *Hes5:eGFP*$^+$ cells per culture plate (data not shown).

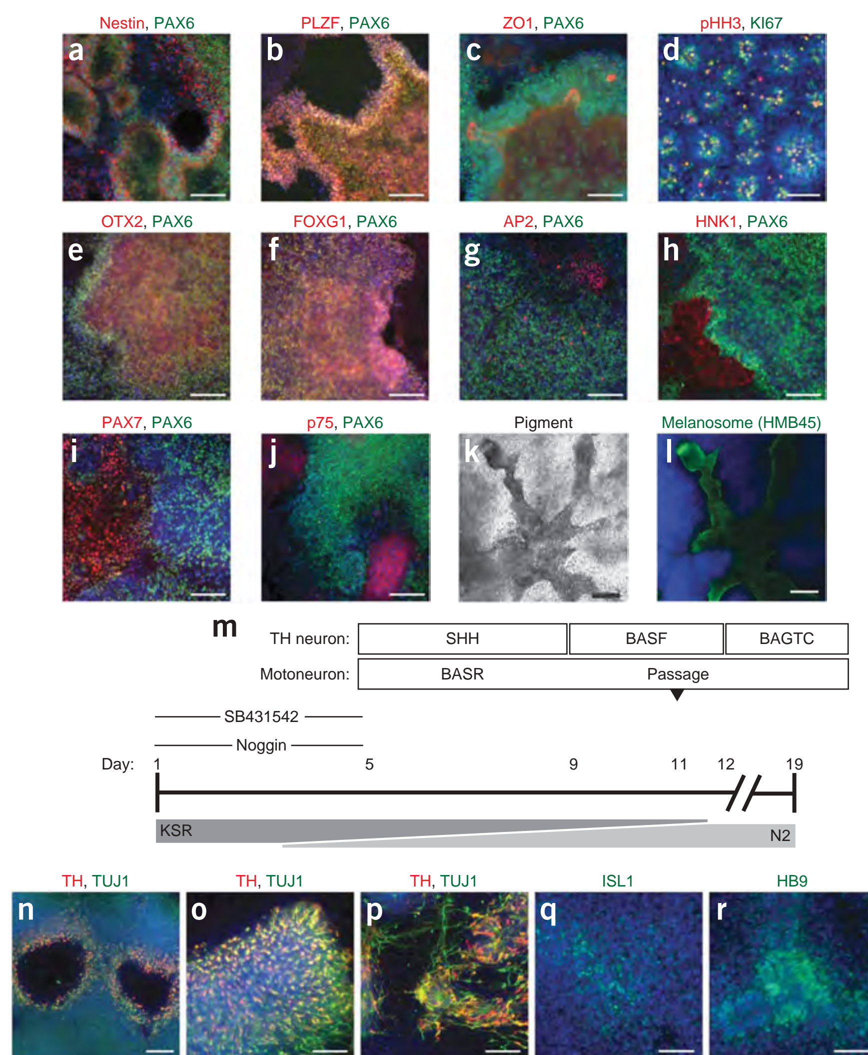

Figure 2 Neuralization of hES cells by dual-SMAD inhibition permits a pre-rosette, neural stem cell with dopaminergic and motoneuronal potential. (**a–c**) The PAX6+ neural tissue (green) expressed rosette markers (red) Nestin (**a**), PLZF (**b**), ZO1 (**c**). (**d**) Rosettes are formed when PAX6+ tissue is passaged to conditions promoting rosettes (BASF) confirmed by KI67 (green) and luminal phospho-histone H3 (red) expression, evidence of interkinetic nuclear migration. (**e,f**) In the absence of factors that confer regional neuronal specificity, the PAX6+ neural tissue (green) expressed OTX2 (**e**) and FOXG1 (**f**), indicating that the tissue defaults to forebrain specification. (**g–j**) Neural crest could be identified on the periphery of the PAX6+ tissue (green) based on AP2 (**g**), HNK1 (**h**), PAX7 (**i**), and p75 expression (**j**) (red). (**k,l**) Upon passage, the neural crest cells gave rise to pigmented cells (**k**) that expressed HMB45 (**l**; green), indicating melanosome synthesis. (**m**) Dopaminergic neuronal patterning was initiated with the addition of super sonic on days 5–9, followed by the addition of BDNF, ascorbic acid, sonic hedgehog and FGF8 on days 9–12. Dopaminergic cells were matured on days 12–19 with BDNF, ascorbic acid, GDNF, TGFb3 and cAMP. Motoneuronal patterning was initiated at day 5 with the addition of BDNF, ascorbic acid, sonic hedgehog, and retinoic acid. Cells were passaged on day 11. (**n,o**) Without passage, TH+ cells could be observed by day 19. (**p**) When passaged *en bloc* on day 12, more mature processes from TH+ cells were observed. (**q,r**) For motoneuron induction, nuclear expression of the motoneuron markers ISL1 (**q**) and HB9 (**r**) were observed within a total of 19 d of differentiation from hES cells. Scale bars, 100 μm (**a–c,e–j,o–r**); 50 μm (**d**), 200 μm (**k,l,n**).

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We previously reported the isolation of rosette neural stem (R-NS) cells[8] and neural crest stem (NCS) cells[3] from hES cells. We next sought to determine the lineage relationship of the early PAX6+ neuroectodermal cells observed in the dual-SMAD-inhibition protocol to the R-NS and NCS cell populations described previously. Immunocytochemical analysis showed that, similar to R-NS cells, PAX6+ neuroectodermal cells express general neural stem cell markers, such as Nestin, and R-NS cell markers, including promyelocytic leukemia zinc finger (PLZF; **Fig. 2a,b**; day 11 of differentiation). However, cytoarchitecture and ZO1 expression indicated that neuroepithelial cells under these conditions were nonpolarized, exhibiting a more primitive ES cell–like cytoarchitecture. These nonpolarized areas were interspersed with R-NS cell–like areas composed of polarized columnar epithelial cells (**Fig. 2c**). The developmental hierarchy of these two cell populations was further explored upon subsequent passage. Early neuroepithelial cells spontaneously converted into rosette structures with apical ZO1 expression and evidence of interkinetic nuclear migration after cell passage (**Fig. 2d**). These data suggest that the Noggin/SB431542 protocol yields an early PAX6+ neuroepithelial population capable of rosette formation. The early PAX6+ cells may therefore represent the most primitive hES cell–derived neural precursor stage isolated to date. R-NS cells have been shown to acquire anterior CNS markers by default[8].

PAX6+ neuroepithelial cells generated by means of the dual-SMAD-inhibition protocol exhibited an anterior CNS character, as evidenced by expression of Otx2 and FoxG1B (**Fig. 2e,f**), similar to R-NS cells[8]. Notably, PAX6– cells under these conditions co-expressed markers of neural crest, including AP2, HNK1, PAX7 and p75 (NGFR) (**Fig. 2g–j**). Manipulation of the initial hES-cell plating density skewed the ratio of PAX6+ CNS and PAX6– neural crest–like cells. High plating densities resulted in near-exclusive differentiation toward PAX6+ cells, whereas low densities promoted neural crest–like differentiation (**Supplementary Fig. 5** online). The presence of large numbers of neural crest–like cells before rosette formation suggested that dual-SMAD inhibition yields an early neural crest population distinct from R-NS cell–derived NCS cells[3]. Supporting the notion of an early neural crest population with distinct lineage potential, cells could be readily enriched for pigmented cells co-expresssing the melanosome marker, HMB45 (**Fig. 2k,l**). In contrast, R-NS cell–derived NCS cells typically do not yield pigmented cells under similar conditions[3]. However, not all HMB45+ cells co-expressed the neural crest marker SOX10, suggesting the presence of other pigmented cell populations, including retinal pigment epithelial cells arising from the PAX6+ domain (data not shown). It will be interesting in the future to compare differential fate potential of early versus R-NS cell–derived neural crest precursors in our hES-cell system with the fate potential of different neural crest precursor populations *in vivo*.

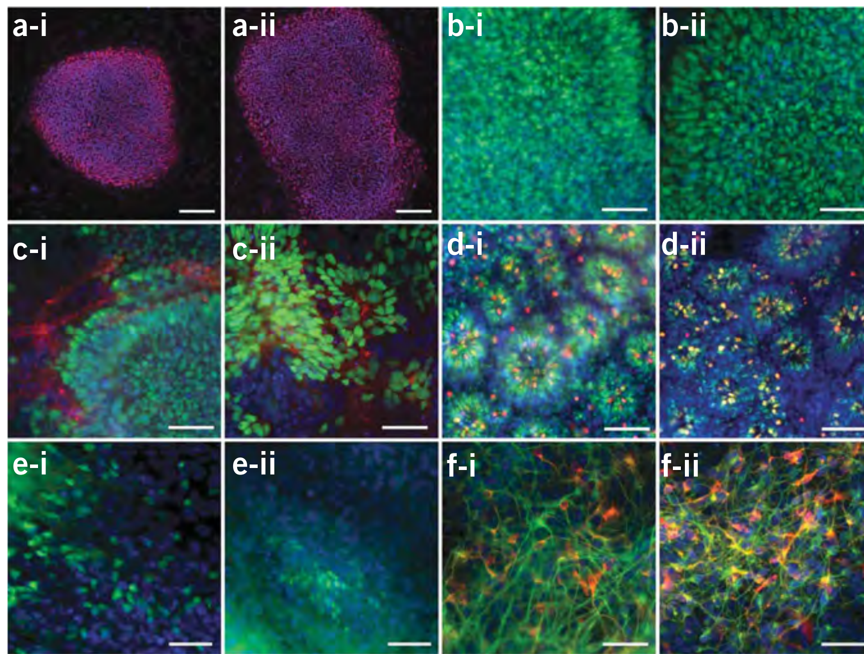

Figure 3 IPS cells can be differentiated to neural tissue using dual-SMAD inhibition and are patternable to dopaminergic neurons and motoneurons. (**a**) Two iPS clones (iPS-14 (i), iPS-27 (ii)) were generated and screened for OCT4 (red) as well as additional pluripotency factors (Tra-1-81, Tra-1-60, SSEA-4 and Nanog, data not shown). (**b**,**c**) The two clones were neuralized by dual-SMAD inhibition (PAX6 expression, green) (**b**), and neural crest could be observed by HNK1 staining (**c**). (**d**–**f**) Neural tissue from the iPS clones could be induced to form rosette-NS cells (**d**), shown by KI-67 (red) and phospho-histone H3 (green) expression, motoneurons (**e**), shown by HB9 expression (green), and dopaminergic neurons (**f**), shown by TUJ1 (green) and TH (red) co-expression. Scale bars, 200 μm (**a**); 50 μm (**b**–**f**).

Anterior-posterior and dorso-ventral identity and neuronal subtype potential are dependent on early exposure to morphogenic factors such as retinoic acid, FGF8 and SHH. We next explored the patterning potential of cells generated via the dual-SMAD-inhibition protocol. We postulated that day 5 of differentiation may present an appropriate developmental window for neural patterning as Oct4 expression is silenced between day 3 and 5, and the neural marker PAX6 is activated in the majority of cells between day 5 and 7 (**Fig. 1d**,**e**).

Derivation of cells expressing markers of dopamine neurons was observed after exposure to SHH and FGF8 (ref. 22) starting at day 5 and day 9 of differentiation, respectively (**Fig. 2m**). One week after SHH exposure, both FGF8 and SHH were withdrawn, and cells further differentiated in medium containing BDNF, ascorbic acid, GDNF, TGF-β3 and cyclic-AMP (BAGTC[22], see **Fig. 2m**). At day 19 of differentiation, a large proportion of Tuj1$^+$ neurons co-expressed tyrosine hydroxylase (TH) (**Fig. 2n**,**o**), the rate-limiting enzyme in the synthesis of dopamine. TH$^+$ neurons emerged under these conditions spontaneously even in the absence of cell passaging. However, derivation of more mature TH$^+$ cells with long neural processes was promoted after mechanical isolation and *en bloc* passage at day 12 of differentiation (**Fig. 2p**).

Nuclear expression of the motoneuron markers ISL1 and HB9 was observed after 2 weeks of exposure to BDNF, ascorbic acid, SHH and retinoic acid (BASR; day 19 of differentiation), confirming the derivation of somatic type motoneurons (**Fig. 2q**,**r**). Motoneuron derivation was limited to cultures passaged at about day 11 of differentiation (data not shown), suggesting a reduced patterning response at very high cell densities, as observed for hES cell–derived R-NS cells[8]. These data demonstrate a robust patterning response in Noggin/SB431542-treated neural progeny and derivation of

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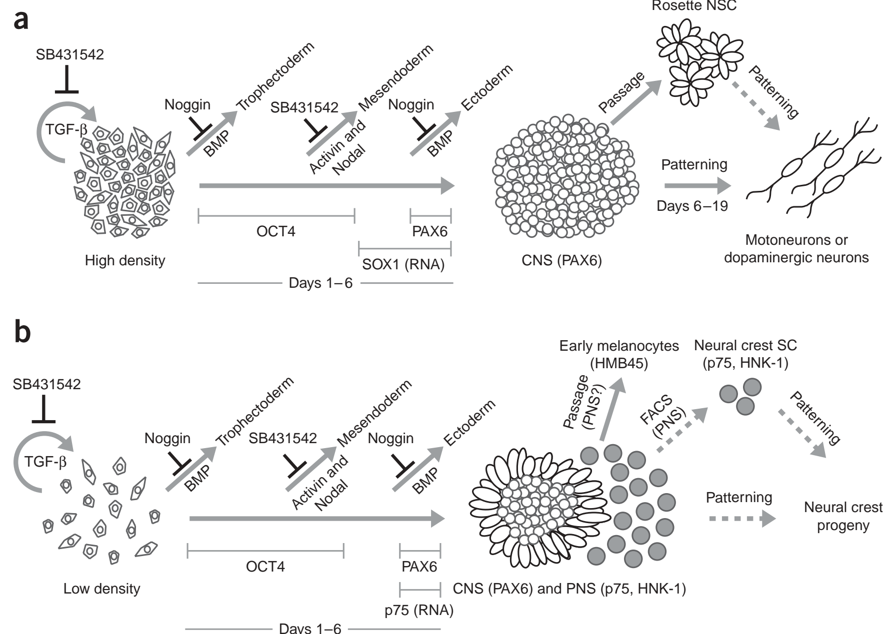

Figure 4 Model of proposed mechanisms that contribute to the action of Noggin and SB431542. These include destabilizing the TGF/activin- and Nanog-mediated pluripotency network, suppression of mesendodermal fates by inhibiting endogenous activin and nodal signals, and promoting neuralization of primitive ectoderm through BMP inhibition. (**a**) At high density, primarily CNS cells that are PAX6$^+$ are formed, which are capable of giving rise to R-NS cells and patternable neuronal populations of motoneurons and dopaminergic neurons within 19 d of differentiation. (**b**) At lower densities, both CNS fates with the properties described in **a** and neural crest fates are observed. Neural crest lineages include melanocytes and neural crest precursor cells amenable to patterning and subtype specification responses. In addition to cell density, it is likely that further manipulation of signaling pathways, including BMP pathways, will skew that ratio of CNS versus neural crest fates. Solid arrows indicate demonstrated cell fate potential; dashed arrows indicate proposed cell fates on the basis of current literature.

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relevant neuron subtypes after short differentiation periods (∼19 d), compared with 30–50 d when using stromal feeder–mediated induction protocols[3,22].

Recent publications have reported the reprogramming of human somatic cells into hiPS cells[1,23,24]. We wanted to next determine whether dual-SMAD inhibition could be used to reliably generate a broad repertoire of hiPS cell–derived neural cell types. Given the expected intrinsic variability among hiPS cell clones, reproducible differentiation results would confirm the robustness of our differentiation protocol. Two hiPS cell clones (iPS-14, iPS-27; **Fig. 3a**) were generated using lentiviral transduction of human fetal lung fibroblasts with cMYC, KLF4, OCT4 and SOX2. Both clones express the pluripotency markers including Nanog, Tra-1-60 and SSEA-3 at the undifferentiated state and are capable of differentiating into derivatives of the three germ layers (data not shown). Upon neural induction by means of the Noggin/SB431542 protocol, both clones yielded nearly homogeneous populations of $PAX6^+$ cells by day 11 of differentiation (**Fig. 3b**). Using the strategies described above to manipulate cell density, passage and patterning factors, both hiPS cell clones could be readily biased toward generating $HNK1^+$ putative neural crest progeny (**Fig. 3c**), hiPS cell–derived R-NS cells (**Fig. 3d**) and specific hiPS cell–derived neuron subtypes, including somatic motoneurons (**Fig. 3e**) and dopamine neurons (**Fig. 3f**). These data demonstrate robustness and extension of the dual-SMAD-inhibition strategy beyond hES-cell differentiation. The protocol offers an efficient, defined and robust platform for the rapid generation of hiPS cell–derived neural cell types.

In this report, we describe a method of neural differentiation that combines the inhibitors Noggin and SB431542 to block SMAD signaling. Noggin and SB431542 act on pluripotent cells at multiple stages of differentiation and provide access to an early intermediate progenitor capable of giving rise to known populations of R-NS and NCS cells (**Fig. 4**). Whereas for most of the studies presented here an 11-d treatment period was used, preliminary studies indicate that similar levels of neural induction can be achieved when the treatment is shortened to the first 5 d of differentiation (**Supplementary. Fig. 6** online; and data not shown). This should further reduce complexity and cost, particularly of recombinant Noggin. Noggin/SB431542 treatment greatly improves on current methods of generating neural tissue by inducing rapid and uniform neural conversion of human pluripotent cells under adherent culture conditions without the need for embryoid body formation or MS5 stromal feeder co-culture. The protocol allows for the derivation of relevant neuron subtypes after much shorter differentiation periods (∼19 d) compared with 30–50 d of differentiation for stromal feeder–mediated induction protocols[3,22]. Given the need for defined protocols that induce rapid and complete neural conversion, this technique may become the standard strategy for driving differentiation of human pluripotent cells.

METHODS

Cells and culture conditions. hES cell (WA-09; passages 35-45) and iPS lines (iPS-14, iPS-27; passages 4–10) were cultured on mouse embryonic fibroblasts plated at 12–15,000 cells/cm^2 (MEFs, Globalstem). A medium of DMEM/F12, 20% knockout serum replacement (Gibco), 0.1 mM b-mercaptoethanol, 6 ng/ml FGF-2 was changed daily. Cells were passaged using 6 U/ml of dispase in hES-cell media, washed and replated at a dilution of 1:5 to 1:10.

IPS cell generation. The cDNAs encoding hOct4, hSox2, hKlf4 and c-myc (Open Biosystems) were subcloned into self-inactivating lentiviral vectors driven by the human phosphoglycerate kinase (PGK) promoter. Lentiviral vector supernatants were produced by triple co-transfection of the plasmid DNA encoding the vector, pCMVΔR8.91 and pUCMD.G into 293T cells. Human fetal lung fibroblasts (MRC-5) purchased from ATCC (CCL-171) were seeded at 1.5×10^4 cells/cm^2 in Eagle's Minimum Essential Medium supplemented with 10% FBS (FBS). The following day the fibroblasts were transduced with equal amounts of supernatants of the four lentiviral vectors in the presence of 4 μg/ml polybrene for ∼16 h. Six days after transduction, fibroblasts were harvested by trypsinization and plated at 2×10^4 cells per 60 mm dish on a feeder layer of mytomycin C-treated mouse embryonic fibroblasts (CF-1). The next day, the medium was switched to hES-cell medium. The iPS lines were confirmed positive for Tra-1-81, Tra-1-60, SSEA-4 and Nanog by immunofluorescence and flow cytometry. In both hiPS cell clones, all four vector-encoded transgenes were found to be silenced.

Neural induction. hES-cell cultures were disaggregated using accutase for 20 min, washed using hES-cell media and pre-plated on gelatin for 1 h at 37 °C in the presence of ROCK inhibitor to remove MEFs. The non-adherent hES cells were washed and plated on Matrigel at a density of 10,000–25,000 cells/cm^2 on Matrigel (BD)-coated dishes in MEF conditioned hES-cell medium spiked with 10 ng/ml of FGF-2 and ROCK-inhibitor. Ideal cell density was found to be 18,000 cells/cm^2. The ROCK inhibitor was withdrawn, and hES cells were allowed to expand in cell medium for 3 d or until they were nearly confluent. The initial differentiation media conditions included knockout serum replacement media with 10 nM TGF-b inhibitor (Tocris) and 500 ng/ml of Noggin (R&D). Upon day 5 of differentiation, the TGF-b inhibitor was withdrawn and increasing amounts of N2 media (25%, 50%, 75%) was added to the knockout serum replacement medium media every 2 d while maintaining 500 ng/ml of Noggin. For MS5 induction, established methods previously reported were used[22].

Quantitative real-time PCR. Total RNA was extracted using an RNeasy kit (Qiagen). For each sample, 1 μg of total RNA was treated for DNA contamination and reverse transcribed using the Quantitect RT kit (Qiagen). Amplified material was detected using Quantitect SYBR green probes and PCR kit (Qiagen) on a Mastercycler RealPlex2 (Eppendorf). All results were normalized to a HPRT control and are from 4–6 technical replicates of 2–3 independent biological samples at each data point.

Neuronal patterning and differentiation. Dopaminergic patterning was initiated using BDNF, ascorbic acid, sonic hedgehog and FGF8 in N2 media as previously reported[22], and maturation was performed in the presence of BDNF, ascorbic acid, GDNF, TGFb-1 and cyclic-AMP. Motoneuron patterning was performed using BDNF, ascorbic acid, sonic hedgehog and retinoic acid in N2 media as previously reported[18].

Microscopy, antibodies and flow cytometry. Tissue was fixed using 4% paraformaldehyde for 20 min, washed with PBS, permeablized using 0.5% Triton X in PBS and blocked using 1% BSA in PBS. Primary antibodies used for microscopy included PAX6 (Covance), Oct4 (Biovision), AP2 (Novus Biologicals), GBX2 (Sigma), HNK1 (Sigma), HOXB4 (Developmental Studies Hybridoma Bank (DSHB)), Nestin (R&D), NKX6.1 (DSHB), OTX2 (gift), p75 (Advanced Target Systems), PAX7 (DSHB), PLZF (Calbiochem), TUJ1 (Covance), ZO1 (Zymed), BF1 (FOXG1, gift Esseng Lai), TH (Sigma), HB9 (DSHB) and ISL1 (DSHB). CD105-PE (eBioscience) was used for excluding MS5 stromal cells for flow cytometry on a FACScan (BD).

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS
S.M.C. and L.S. designed the study. E.P.P., M.T., L.S. and M.S. designed and generated the hiPS clones. S.M.C. and L.S. analyzed the data and wrote the manuscript. S.M.C. and C.A.F. performed the experiments.

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The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells

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Teratomas derived from human embryonic stem (hES) cells are unique among oncogenic phenomena as they are polyclonal and develop from apparently normal cells[1,2]. A deeper understanding of this process should aid in the development of safer cell therapies and may help elucidate the basic principles of tumor initiation. We find that transplantation of diploid hES cells from four independent cell lines generates benign teratomas with no sign of malignancy or persisting embryonal carcinoma-like cells. In contrast, mouse embryonic stem (mES) cells from four cell lines consistently generate malignant teratocarcinomas. Global gene expression analysis shows that survivin (*BIRC5*), an anti-apoptotic oncofetal gene, is highly expressed in hES cells and teratomas but not in embryoid bodies. Genetic and pharmacological ablation of survivin induces apoptosis in hES cells and in teratomas both *in vitro* and *in vivo*. We suggest that continued expression of survivin upon differentiation *in vivo* may contribute to teratoma formation by hES cells.

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The pluripotency and self-renewal capacity of hES cells make them a promising source of cells in regenerative medicine for the treatment of conditions such as Parkinson's disease, diabetes and heart failure. However, clinical development of therapies based on hES cells must address safety concerns arising from the capacity of these cells to form tumors. HES cell–derived tumors are usually referred to as benign teratomas[2], whereas mouse (m)ES cells have been reported to generate malignant teratocarcinomas[3]. By definition, teratomas are composed of somatic differentiated tissues only. Teratocarcinomas, in contrast, contain a core of malignant undifferentiated cells named embryonal carcinoma (EC) cells[4]. It has been suggested that hES cells may adapt to growth *in vitro* by acquiring genetic aberrations similar to those of malignant EC cells[5]. However, it is well established that even normal, nonadapted hES cells form teratomas[6,7]. In this study, we set out to investigate molecular mechanisms underlying hES-cell teratoma formation.

We transplanted 3×10^6–1×10^7 hES cells from eight lines (TE06, WA09, WA13, HUES12, HUES13, HUES14, BG01, CSES7) or mES cells from four lines (CCE, E14, J1, R1) under the kidney capsule of immunodeficient mice and analyzed the resulting tumors after 3 to 4 weeks (**Fig. 1**). All transplantations yielded tumors (45 hES-cell tumors in 45 mice and 10 mES-cell tumors in 10 mice, respectively) (**Supplementary Table 1** online). Most hES and mES cells were karyotyped to ensure that injected cells were karyotypically normal (**Supplementary Fig. 1** online), and in-depth experiments were performed mainly on four hES cell lines (TE06, WA09, WA13, HUES13; see below).

HES-cell tumors harvested after 30 d were small teratomas with occasional cystic morphology (small tissue mass and large fluid-filled cysts) (**Fig. 1a**) and contained differentiated cells representative of the three embryonic germ layers (**Fig. 1b**). They ranged in size from 0.31 cm^3 to 0.95 cm^3, with a median of 0.6 cm^3 (**Fig. 1f**). HES-cell tumors were always confined to the periphery of the injected kidney and were easily removed from the kidney. The overall appearance of the injected kidneys was normal, and the tumors did not penetrate the kidney tissue. HES-cell tumors could be allowed to develop for at least 10 weeks without an apparent additional burden to the host mouse. Previously, we showed that teratomas continue to grow at 8 weeks after transplantation, as measured by BrdU incorporation into the DNA of proliferating differentiated cells[1].

In marked contrast, the tumors generated from injection of the same number of mES cells were extremely large, ranging from 5.05 cm^3 to 12.29 cm^3, with a median of 5.59 cm^3 (**Fig. 1f**). In most cases, the recipient mouse had to be euthanized after $\sim$3 weeks as the tumor burden would have otherwise killed the host. Moreover, mES-cell tumors filled the host abdominal cavity, could not be easily separated from the host tissues in most cases (**Fig. 1c**) and contained undifferentiated malignant components together with differentiated cells representative of the three germ layers (**Fig. 1d,e**).

Teratocarcinomas are identified by the presence of EC cells, which are the malignant stem cells of the tumor. EC cells are absent from benign teratomas[4]. EC cells are typically detected by expression of OCT4 and Nanog[8,9]. Whereas OCT4 or Nanog were not detected in six hES-cell tumors from two different hES cell lines tested (WA09 and HUES13), both markers were detected in a representative mES-cell tumor (from the CCE cell line) (**Fig. 1g,h**). EC cells from either mouse or human teratocarcinomas can be grown *in vitro* after dissection of the tumor, forming colonies of tightly packed undifferentiated cells[10,11]. We dissected cells from six hES-cell tumors generated from three different hES cell lines (TE06, WA09 and CSES7) and seeded them *in vitro* on tissue-culture plates containing 10% FCS-supplemented DMEM medium. The growing cells resembled fibroblasts (not undifferentiated cells) (**Fig. 1i**) and could be propagated in culture for up to 13–15 passages only, after which they ceased to

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proliferate. When the tumor-derived cells were transplanted to a secondary mouse, they did not form tumors, indicating that they had lost their tumor-forming capacity (ten transplantations with five different cultures; data not shown). The same results were obtained when the teratomas were dissected in ES cell medium or mouse embryonic fibroblast (MEF)-conditioned medium.

MES-cell tumors (CCE, R1) seeded on tissue culture plates also generated some fibroblast-like cells. In marked contrast, however, many colonies of undifferentiated cells, resembling EC or undifferentiated mES cells, rapidly formed in these cultures (**Fig. 1j**). These EC-like cells generated fast-growing tumors upon transplantation to secondary recipient mice (**Fig. 1k**), and EC-like cells could be generated from those tumors (**Fig. 1l**).

EC cells harbor typical chromosomal abnormalities[5]. Analysis of three different cell cultures established from three teratomas generated from the hES cell line TE06 showed that they had a normal karyotype, similar to that of undifferentiated TE06 cells (**Fig. 1m** and **Supplementary Fig. 1**). Furthermore, high-resolution copy-number variation analysis of cells from one of these teratomas and of TE06 cells demonstrated that no genetic alterations, such as micro-deletions, occurred during tumor development (**Supplementary Fig. 2a** online).

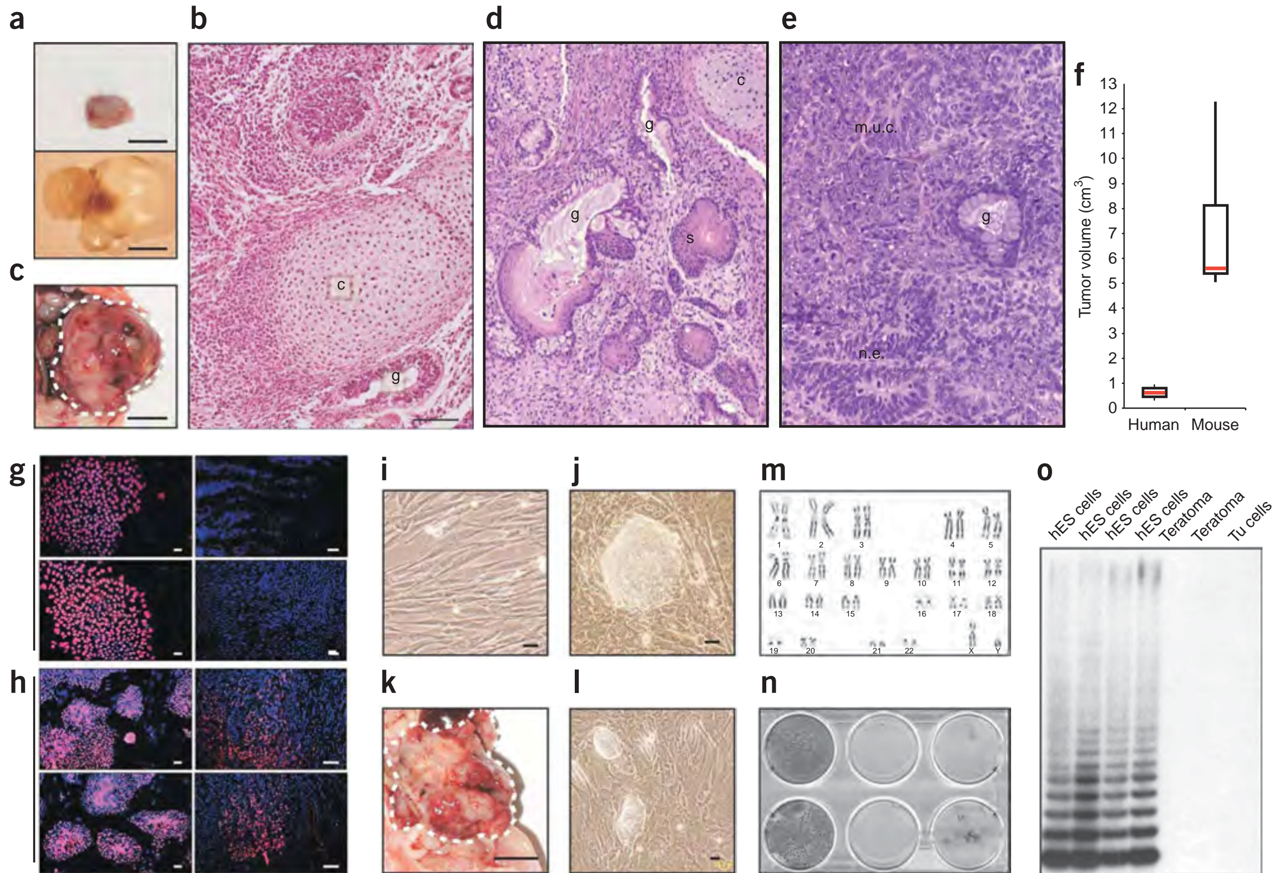

Figure 1 HES cells induce teratomas that are smaller than mES-cell tumors, do not contain EC-like cells, are karyotypically normal and are nonaggressive. (**a**) Morphology of hES-cell tumors. A small noncystic tumor and a large cystic tumor are shown. Scale bars, 1 cm. (**b**) Histology of an hES-cell tumor stained with hematoxylin and eosin. Mature cartilage (c) and glandular epithelium (g) can be observed. Scale bar, 50 μm. (**c**) Morphology of an mES-cell tumor. A large noncystic tumor tissue (circled with a dashed line) is shown. The tumor fills the abdominal cavity and is difficult to excise. Scale bar, 1 cm. (**d**,**e**) Histology of an mES-cell tumor stained with hematoxylin and eosin. Cartilage (c), glandular epithelium (g) and skin (s) components can be observed (**d**). (scale bar, 50 μm); glandular epithelium (g) and neuro-ectoderm (n.e.) components are embedded in malignant undifferentiated carcinoma tissue (m.u.c.) (**e**) (scale bar, 50 μm). (**f**) Comparison of hES-cell and mES-cell tumor volumes. Each box is composed of 1st and 3rd quartiles. Black bars represent min. and max. values. Red bars represent median value. For hES cells $n = 6$ tumors from two different lines. For mES cells $n = 5$ tumors from two different lines. (**g**,**h**) Immunofluorescence analysis of Oct4 and Nanog expression in undifferentiated hES cells (**g**) and mES cells (**h**) and their derived tumors (righthand panels). Cells were immunostained with Oct4 and Nanog (red). Nuclear DNA was stained with Hoechst (blue). Both proteins are detected in undifferentiated hES cells and mES cells and in mES-cell tumors, but not in hES-cell tumors. Scale bars, 20 μm. (**i**,**j**) Outgrowth of tumor-derived cells *in vitro*. Brightfield photographs of tumor-derived cells (Tu) from hES-cell (**i**) and mES-cell (**j**) tumors grown in culture. Undifferentiated colonies of EC-like cells are visible in the mES-cell tumor cells but not the hES-cell tumor cells. Scale bars, 20 μm. (**k**) Morphology of a secondary tumor derived from the EC-like mouse Tu cells shown in **j**. Scale bar, 1 cm. (**l**) Subsequent EC-like cells derived from the secondary tumor shown in **k**. Scale bar, 20 μm. (**m**) A representative normal 46XY karyotype of teratoma (Tu) cells derived from an hES-cell teratoma. (**n**) Soft agar assay. hES-cell teratomas (teratoma in agar; middle) were dissected directly into soft agar and were grown for 2 weeks. To verify that the dissection did not impair the viability of the cells, the same cells were also seeded on gelatin-coated wells in the same plate (teratoma on gelatin; right). The positive-control cells (left) are *Trp53*$^{-/-}$ MEFs transformed with *HRAS* and *E1A*. Teratoma cells grew well on gelatin but not in the agar. (**o**) Telomerase activity assay on hES cells and teratomas. Proteins extracted from undifferentiated hES cells, teratomas and teratoma-derived (Tu) cells were assayed for telomerase activity (TRAP assay), and the ^{32}P-labeled telomeric repeats were separated by acrylamide gel electrophoresis. Telomerase activity is demonstrated in the undifferentiated cells but not in the tumor or tumor-derived cells.

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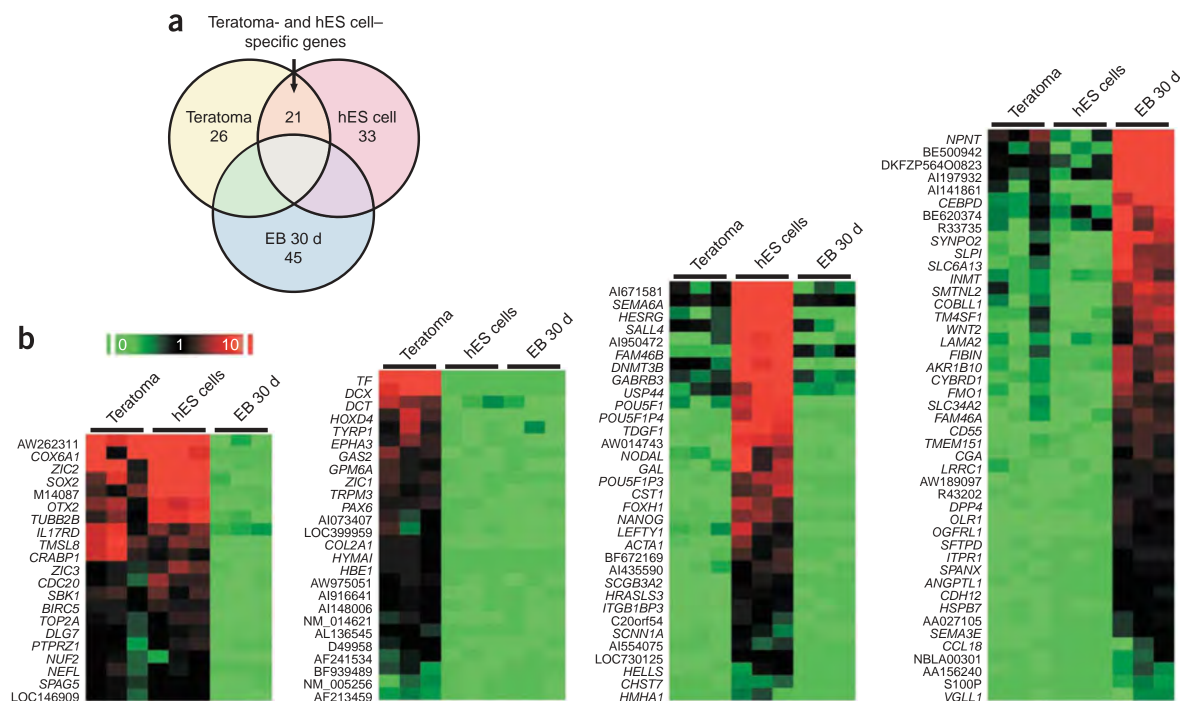

Figure 2 DNA microarray analysis to identify genes expressed in hES cells and teratomas but not mature embryoid bodies. (**a**) Venn diagram of the number of genes in each group that passed our selection criteria. The selection criteria were as follows: an expression level that is at least 10 times greater in a certain group compared to the other groups, even after setting the minimum expression level to 20% of the average normalized total expression in the microarray, and a "present" score in all three repeats of the group in which the gene is expressed, EB, embryoid bodies. (**b**) Hierarchical clustering and listing of the genes in the groups represented in **a**. Genes are listed by their gene symbol. Genes that did not have gene symbols are referred to by their Unigene entry number. Red represents high expression and green represents low expression.

Telomerase activity has been reported in human malignant teratocarcinomas[12] and undifferentiated hES cells[7] but not in benign mature teratomas[12]. We found that undifferentiated hES cells (WA13, HUES13) showed extensive telomerase activity, whereas extracts of teratomas and teratoma-derived cell cultures (WA09, HUES13) displayed no telomerase activity (**Fig. 1o**).

We examined the ability of hES-cell teratomas to form colonies in soft agar, an assay for an anchorage-independent growth of malignant cells. To reduce the likelihood that any EC cells present in the teratomas would be selected against by the culture conditions, we dissected the tumors (WA09, HUES12, HUES14) directly into the agar. MEFs from *Trp53*$^{-/-}$ mice, transformed with both *E1A* and *HRAS* viral oncogenes[13], served as a positive control. Whereas the transformed MEFs grew extensively in agar, no colonies were observed from the dissected teratoma. This lack of growth was not caused by damage to the cells during dissection, as the same cells grew well on gelatin-coated wells in the same plate (**Fig. 1n**).

Taken together, these results support the hypothesis that tumor formation by hES cells does not depend on the presence of EC-like cells and is an intrinsic property of normal (untransformed) hES cells. Next, we performed comparative DNA microarray analysis of the transcriptome of undifferentiated hES cells and teratomas (WA09) (**Fig. 2**). To exclude genes related to differentiation, we also studied mature (~30 d old) embryoid bodies (WA09) grown *in vitro*. In contrast to teratomas, which proliferate rapidly even after 2 months[1], embryoid bodies grown for ~30 d in suspension become cystic and

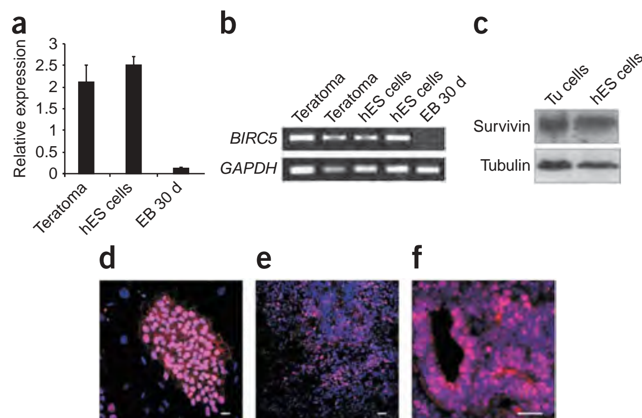

Figure 3 Survivin is highly expressed in hES cells and teratomas. (**a**) Expression of survivin in the DNA microarray. The expression of survivin relative to the average total expression level in the microarray is shown for teratomas (teratoma), undifferentiated hES cells and mature 30-d-old embryoid bodies (EB 30 d). The expression levels in each bar are the average of three independent repeat experiments. (**b**) RT-PCR showing the expression of survivin in teratomas, undifferentiated hES cells and 30-d-old mature embryoid bodies. *GAPDH* serves as a loading control for the PCR reaction. (**c**) Western blot showing the levels of survivin in teratoma-derived cells (Tu cells) and undifferentiated hES cells (hES cell). Tubulin is used as a loading control. (**d**–**f**) Immunofluorescence assays for the expression of survivin in undifferentiated hES cells and teratomas. Undifferentiated hES cell colony grown on MEFs; only the undifferentiated hES cells stain positive for survivin (**d**). Histological sections of a teratoma shown at two magnifications (**e**,**f**). Survivin protein is stained red; nuclear DNA is stained blue by Hoechst. Scale bars, 20 μm.

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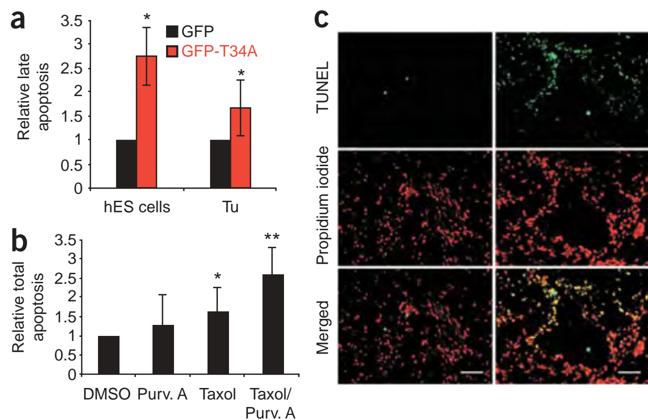

Figure 4 Genetic and pharmacological interruption of survivin activity induces apoptosis in hES cells and teratomas *in vitro* and *in vivo*. (**a**) Genetic interruption of survivin *in vitro*. Undifferentiated hES cells or teratoma-derived cells (Tu) were transfected with plasmids expressing a dominant-negative survivin fused to GFP (GFP-T34A) or GFP only. The cells were analyzed 24 h later for the percentage of Annexin V and PI positive cells by means of flow cytometry. The histogram summarizes the percentage of late apoptotic cells (Annexin V positive/PI positive). *, $P < 0.005$; $n = 4$. (**b**) Pharmacological interruption of survivin *in vitro*. Teratoma-derived cells were treated with DMSO, Taxol, Purvalanol A or a combination of Taxol and Purvalanol A. The cells were analyzed 16 h later for the percentage of Annexin V- and PI-positive cells by means of flow cytometry. The histogram summarizes the percentage of total apoptotic cells (Annexin V positive). *, $P < 0.05$; **, $P < 0.01$; $n = 5$. (**c**) Pharmacological interruption of survivin *in vivo*. Mice bearing hES-cell teratomas were injected with vehicle only (DMSO; lefthand column) or with a sequential treatment of Taxol and Purvalanol A (righthand column) over a 24 h interval. The mice were euthanized 18 h later, and teratoma sections were assayed by TUNEL. TUNEL assay–positive apoptotic cells are stained green. Nuclei are stained red with PI. Scale bars, 50 μm.

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cease to proliferate[14]. Our analysis was designed to identify genes that are expressed in both hES cells and teratomas but not in differentiated cells, as these genes may contribute to teratoma formation. By including only the most highly expressed genes in the three groups, we generated a list of 21 genes specific to hES cells and teratomas (leftmost column in **Fig. 2b**). We then scored these 21 genes according to their Gene Ontology database annotation. Thus, genes were credited if they were known oncogenes or if they were related to cell cycle progression, inhibition of apoptosis, signal transduction, transcription or translation, and discredited if they were housekeeping genes or related to differentiation of specific lineages. Finally, genes were also credited if their expression was enriched in ES cells according to the SOURCE database[15] and discredited if they were expressed in differentiated tissues at a similar level.

This ranking of the 21 genes showed that the strongest candidate gene was survivin (*BIRC5*). Survivin is the only member of the family of inhibitor of apoptosis proteins that also functions as a mitotic regulator[16]. Survivin is expressed in the great majority of cancers, including germ cell tumors, and is almost completely absent from normal tissues, including many primary cell lines[16,17]. Survivin is also expressed in early-stage embryos, and its deficiency results in lethality at the blastocyst stage[18,19].

Survivin was highly expressed in hES cells (WA09, HUES13) and teratomas (WA09, WA13, HUES12, HUES13) and downregulated in mature embryoid bodies (WA09) (**Fig. 3**). Copy-number variation analysis showed that WA09 and TE06 hES cells and teratoma cells had no gain in copy number of the survivin genomic locus (**Supplementary Fig. 2b**). Survivin was expressed in virtually all cells of undifferentiated WA09 hES cell (and E14 mES cell) colonies but not in the surrounding MEFs (**Fig. 3d** and **Supplementary Fig. 3** online). In teratoma sections of WA09, WA13 and HUES13 hES cells, survivin expression was observed throughout most of the tumor and was not confined to specific regions, suggesting that it is a general feature of this type of tumor (**Fig. 3e**,**f**).

We genetically disrupted survivin in hES cells and teratoma cells (WA09, WA13, TE06) *in vitro* by transfection of a plasmid containing the gene encoding the dominant negative survivin isoform survivinT34A fused in frame to *GFP*[20,21]. In this isoform, Thr34 of wild-type survivin is replaced by Ala ($Thr^{34} \rightarrow Ala$), thus abolishing a phosphorylation site of $p34^{cdc2}$-cyclin B1 that is required for survivin activity[20,21]. Notably, this dominant negative isoform was reported to induce apoptosis in cancer cells and to have no effect on normal, nontumorigenic cell lines[22,23]. An expression vector with only *GFP* was used as a control. The cells were analyzed for apoptosis 24 h after transfection by flow cytometry using Annexin V and propidium iodide (PI). To analyze only the cells that expressed the plasmid, we gated the GFP^{+} cell populations. Ectopic expression of survivinT34A increased the number of apoptotic cells in both hES cells and teratoma cells, with the most significant increase ($P < 0.005$) in the late apoptotic (Annexin V and PI double-positive cells) population (**Fig. 4a**). In contrast, the transfected cells showed no change in the cell cycle as measured by flow cytometry using Hoechst 33342 labeling (data not shown).

Sequential administration of the mitotic drug Taxol at very low doses followed by the $p34^{cdc2}$ inhibitor Purvalanol A has been shown to eliminate survivin activity in a $p34^{cdc2}$-dependent manner[21]. Inhibition of survivin resulted in increased tumor cell death by apoptosis, both *in vitro* and *in vivo*, without apparent systemic toxicity, whereas administration of Taxol or Purvalanol A alone or in reverse order gave negligible results[21]. To investigate the possibility of pharmacological inhibition of hES-cell teratomas, we treated tumor cells (WA09, TE06) *in vitro* with Taxol and Purvalanol A and analyzed the cells for apoptosis with Annexin V and PI (**Fig. 4b**). Purvalanol A alone had no significant effect and Taxol alone had some effect (1.6 ± 0.61-fold increase in apoptotic cells), whereas the combination of Taxol and Purvalanol A increased apoptotic cells by 2.59 ± 0.7-fold ($P < 0.01$).

We next examined pharmacological treatment *in vivo* on established teratomas. Teratomas (hES cell line WA09) were grown for 30 d, and Taxol was injected intra-peritoneally (7 mg/kg) followed by Purvalanol A (60 mg/kg) 24 h later. Control mice received vehicle injections at the same time points. The mice were euthanized 18 h after the last injection, and the teratomas were analyzed for apoptosis using the TdT-mediated dUTP nick end labeling (TUNEL) assay. Whereas only sporadic TUNEL assay–positive cells were observed in the control teratomas, massive apoptosis was detected within the teratomas of the drug-treated mice (**Fig. 4c**).

Our finding that tumors generated by hES cells are less aggressive than those from mES cells is consistent with some previous reports. For example, it has been suggested that the mechanisms leading to spontaneous testicular germ cell tumors are different in mouse and human[24]. Among human germ cell tumors, type I teratomas are benign, usually displaying normal karyotype, and are believed to arise from cells analogous to ES cells, whereas teratomas and teratocarcinomas categorized as type II germ cell tumors are malignant, usually aneuploid, and are thought to originate from transformed primordial germ cells[5]. At the molecular level, a particular murine *HRAS*-like gene, *Eras*, is expressed specifically

in mES cells and promotes their tumorigenicity[25]. The human homolog of *Eras*, *ERAS*, is expressed in hES cells but encodes a nonfunctional transcript[26]. Above all, the different tumor-forming potential of ES cells from the two species may reflect differences in the process of cellular transformation between mouse and man[27].

In a recent debate on the correct terminology for hES-cell tumors[28,29], it was suggested that teratoma formation by hES cells results from an embryonic-like differentiation process and is simply a manifestation of the expansion of developing primary tissues. We believe that it may be attributed to sustained expression of genes normally expressed in undifferentiated hES cells and downregulated during their differentiation *in vitro*[25]. Our analysis identified *BIRC5* as the most relevant candidate gene and suggested that persistent survivin expression contributes to teratoma formation by conferring increased resistance to apoptosis. *BIRC5* is categorized as a classical oncofetal gene because it is highly expressed in early embryonic stages and in the majority of cancers but is virtually absent from most normal adult tissues, including some proliferating cell types[17,18]. Our finding that survivin is expressed in both undifferentiated hES cells and hES-cell tumors but not in mature embryoid bodies suggests that other oncofetal genes normally expressed in hES cells, which were not identified using our stringent selection criteria, can promote tumor formation if they continue to be expressed upon differentiation *in vivo*.

We have also demonstrated that pharmacological inhibition of survivin induces apoptosis in hES cells and teratomas. Previous studies showed that survivin inhibition induces apoptosis in cancer cells but not normal cells[22,23,30]. Moreover, overexpression of the oncogene *MYC* in normal cells deficient in survivin induced apoptosis[31]. The chromosomal location of *BIRC5* on 17q25[17], a chromosomal region that can be amplified both in human germ cell tumors and during the culture adaptation of hES cells[6], suggests that culture-adapted hES cells with additional copies of survivin could be more tumorigenic than naive, karyotypically normal hES cells. Controlling teratoma formation is important for realizing the clinical potential of hES cells. Several methods for achieving this have been explored, such as clearing the grafted cells of residual undifferentiated cells and genetic insertion of suicide genes[32,33]. Pharmacological inhibition of teratoma-associated genes as described here represents another strategy. Molecularly targeted drugs, such as specific survivin antagonists[34], appear especially promising in this regard.

METHODS

Cell culture. hES and mES cell lines used in this study are listed in **Supplementary Table 1**. hES cells and mES cells were cultured on mitomycin-C–treated MEFs. hES cell culture medium was composed of KnockOut DMEM medium (GIBCO-BRL) supplemented with 15% KnockOut serum replacement (GIBCO-BRL), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma), 0.1 mM nonessential amino acid stock (GIBCO-BRL), 50 units/ml penicillin, 50 μg/ml streptomycin, 1:200 dilution of ITS (insulin-transferrin-selenium, GIBCO-Invitrogene) and 4 ng/ml basic fibroblast growth factor (bFGF). mES cell culture medium was composed of DMEM (Beit Haemek) supplemented with 15% FCS (Beit Haemek), 0.1 mM β-mercaptoethanol, 0.1 mM nonessential amino acids stock, 50 units/ml penicillin, 50 μg/ml streptomycin, 4 ng/ml bFGF and 1,000 units/ml LIF (Chemicon). Tumor-derived cells were isolated by manual dissociation of the tumor tissue into small cell clumps and further trypsinization for 20 min. Tumor-derived cells were then seeded and subsequently cultured on gelatin-coated dishes in DMEM supplemented with 10% FCS, 50 units/ml penicillin and 50 μg/ml streptomycin. Induction of embryoid bodies from hES cells was performed by withdrawing bFGF from hES cell growth medium and allowing the cells to aggregate in nonadherent Petri dishes as previously decribed[14].

Induction of tumors in mice. Induction of tumors by hES and mES cell xenotransplantation was performed on male severe combined immunodeficient (SCID)/beige or NUDE mice, as previously described[1]. Mice were kept in the specific pathogen-free unit of the Institute of Life Sciences at the Hebrew University. All experiments were performed according to approval of the Committee for Animal Care and Use of the Faculty of Sciences at the Hebrew University of Jerusalem.

Histology and immunofluorescence. For histology, tumors were fixed in 4% buffered formalin (BIO LAB) and embedded in paraffin. Histological slides were stained with hematoxylin and eosin and analyzed by a trained pathologist. For immunofluorescence, cultured cells were washed twice with PBS and tumors were embedded in OCT (Sakura), snap-frozen in liquid nitrogen and cut into 8-μm sections. Cells and tumor samples were then fixed for 15 min in 4% buffered formalin. Blocking and permeabilization was performed with 3% BSA, 10% low-fat milk and 0.1% Triton-X in PBS. hES cells, mES cells and hES-cell tumor sections were stained for Oct4 using mouse anti-Oct4 antibody (Santa Cruz Biotechnology) at 1:50–1:200 dilutions and Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) at 1:200 dilution. mES-cell tumor sections were stained for Oct4 using rabbit anti-Oct4 antibody (a kind gift from Yehudit Bergman) at 1:250 dilution and a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) at 1:100 dilution. hES cells and hES-cell tumor sections were stained for Nanog using a goat anti-human-Nanog (R&D Systems) at 1:50 dilution and Cy3-conjugated mouse anti-goat secondary antibody (Jackson ImmunoResearch) at 1:200 dilution. mES cells and mES-cell tumor sections were stained for Nanog using rabbit anti-Nanog (clone no. 76, a kind gift from Austin Smith) at 1:100 and Cy3-conjugated donkey anti-rabbit secondary antibody at 1:100 dilution. hES cells and hES-cell tumors were stained for survivin using a rabbit anti-survivin antibody (Santa Cruz Biotechnology) at 1:100 dilution and Cy3-conjugated donkey anti-rabbit secondary antibody at 1:200 dilution. Nuclear staining was performed using Hoechst 33258 (Sigma).

Karyotype and copy-number variation analyses. hES cells or tumor-derived cells in logarithmic growth phase were used for karyotyping. Cells were supplied with fresh growth medium overnight, and 100 ng/ml of colecemid (Beit Haemek) was added to the plated the next morning. The cells were then incubated for 30 min at 37 °C in a 5% CO_2 incubator, trypsinized, treated with hypotonic solution and fixed. Metaphases were spread on microscope slides, and by using G banding technique, the chromosomes were classified according to the International System for Human Cytogenetic Nomenclature. At least 20 metaphases were analyzed per sample. Copy-number variation analysis was done on genomic DNA using Affymetrix SNP 6 microarray according to the manufacturer's protocol. Copy-number variation results were analyzed using PARTEK and Genotyping Console 3.0.1 software against the 270 samples of the Human HapMap Project.

Telomerase activity assays. Cell and tissues lysates were extracted from samples by homogenization in a lysis buffer containing 10 mM Tris-HCl pH 7.2; 1 mM $MgCl_2$; 1 mM EGTA; 0.5% CHAPS; 10% glycerol. Protein concentration was determined using the Bradford method. Equal amounts of protein from each sample were incubated for 30 min at 30 °C in the presence of 0.1 μg TS primer (Synteza) and dNTPs in TRAP buffer (200 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 10 g/ml BSA and 0.005% Tween20) and subsequently inactivated at 95 °C for 5 min. PCR (29 cycles of 30 s at 94 °C, 30 s at 50° and 1 min at 72 °C) was performed using ACX primer (Synteza) and 0.2 μl of ^{32}P-dCTP. Samples were then separated on native 12.5% polyacrylamide gel electrophoresis and autoradiogrammed.

Soft agar assays. Wells of 6-well tissue culture plates were coated with a solid bottom agar layer of 1% agarose (GIBCO-BRL) in 2× concentrated growth medium and were let to solidify. Tumor-derived cells were produced as described above, suspended in 0.3% top agarose in 2× concentrated growth medium and seeded onto the wells. 1× concentrated growth medium was

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added after the top agar had cooled. Control tumor-derived cells in 1× concentrated growth medium were seeded directly on gelatin-coated wells in the same plate. As positive control, transformed MEFs were similarly trypsinized and seeded in the agar. The plates were incubated for 2 weeks at 37 °C in a 5% CO_2 incubator, and the medium was replaced twice weekly. At the end of the experiment, the cells were stained with MTT (Sigma) for viable colonies for 4 h and photographed.

DNA microarray analysis. DNA microarray analysis for gene expression was performed on Affymetrix U133 DNA microarray as previously described[35]. The hybridization signals in the DNA microarray were normalized by dividing the signal value for each probe by the average signal value of the hybridization in each experiment. We treated low signal values as noise and set the minimum expression level to 20% of the average normalized total expression in the microarray. To identify genes that are specific to each cell type, we compared the mean expression levels of each probe in this cell type to that of its mean expression in the other cell types. We then selected genes whose expression in all samples of the specific cell type were scored as "present" and were at least 10 times greater than that of the other cell types. Relative expression of selected genes in different tissues was examined using the SOURCE database[15] (Stanford University; http://smd.stanford.edu/cgi-bin/source/sourceSearch). The database presents relative expression of UniGene Clusters in different tissues according to the relative frequencies of their ESTs in the various tissues. The relative expression is then normalized for the number of clones from each tissue that are included in UniGene.

RT-PCR. RNA was extracted using TRI-reagent for total RNA isolation according to the manufacturer's instructions (Sigma). cDNA was synthesized using random hexamer primers. Amplification was performed on the cDNA using Takara Ex-Taq. PCR conditions include a first step of 3 min at 94 °C, a second step of 25–30 cycles of 30 s at 94 °C, a 1 min annealing step (60 °C for *BIRC5*; 62 °C for *GAPDH*), 30 s at 72 °C and a final step of 7 min at 72 °C. Primers for *BIRC5* were 5′-GGACCACCGCATCTCTACAT-3′ forward and 5′-GCACTTTCTTCGCAGTTTCC-3′ reverse. Primers for *GAPDH* were 5′-AGC CACATCGCTCAGACACC-3′ forward and 5′-GTACTCAGCGGCCAGCATCG-3′ reverse. Final products were examined by gel electrophoresis on 0.7% agarose ethidium bromide–stained gels.

Western blot analysis. Western blot analysis experiments were performed according to standard protocols. For survivin detection, a rabbit anti-survivin antibody (Santa Cruz Biotechnology) at 1:700 dilution and secondary HRP conjugated goat anti-rabbit (Jackson ImmunoResearch) at 1:20,000 dilution were used. As a loading control, mouse anti-tubulin (Sigma) at 1:80,000 dilution and a secondary HRP conjugated goat anti-mouse (Jackson ImmunoResearch) at 1:20,000 dilution were used.

FACS. Annexin V and PI staining for apoptosis detection was performed using the Annexin V-PE kit (Bender MedSystems) according to the manufacturer's instructions. FACS analysis was performed using FACSCaliber system (Becton Dickinson). Analysis was performed on CELLQUEST software (Becton Dickinson). Forward and side scatter plots were used to exclude debris from the histogram analysis.

Taxol and Purvalanol A treatments. Tumor cells were treated *in vitro* with 0.2 μM Taxol (Sigma) for 16 h. The medium was then replaced with a medium containing 10 μM Purvalanol A (Sigma), and the cells were cultured for an additional 16 h. Other wells were treated with either Taxol for 16 h followed by DMSO (the solution vehicle) for 16 h or DMSO for 16 h followed by Purvalanol A for 16 h. Control wells were treated twice with DMSO at the same time points. Mice bearing hES-cell teratomas for 30 d were injected intraperitoneally with 7 mg/kg Taxol and 24 h later were injected with 60 mg/kg Purvalanol A. Reagents were injected in a solution of DMSO/PEG400 at 1:1 ratio. Control mice received vehicle injections at the same time points. Mice were euthanized 18 h after the last injection. Fresh frozen 8-μm thick teratoma sections were subjected to TUNEL using the ApoAlert DNA Fragmentation Assay Kit (Clontech) according to the manufacturer's instructions.

Statistical analysis. Results are presented as means (± s.e.m). Crude results were transformed into log values, and relative means and *P*-values were calculated. *P*-values were calculated using two-tailed paired *t*-test. The figures are given after retransformation of the log values.

Microarray data. Microarray data are available in GEO (Gene Expression Omnibus) of NCBI with accession number GSE13586.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS
B.B. and N.B. conceived and designed experiments. B.B. performed all experiments and data analyses. O.B.-N. assisted in OCT4 and Nanog Immunofluorescence on undifferentiated mES. T.G.-L. assisted in karyotypyng analyses. B.B. and N.B. wrote the manuscript.

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Autocatalytic aptazymes enable ligand-dependent exponential amplification of RNA

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RNA enzymes have been developed that undergo self-sustained replication at a constant temperature in the absence of proteins[1]. These RNA molecules amplify exponentially through a cross-replicative process, whereby two enzymes catalyze each other's synthesis by joining component oligonucleotides. Other RNA enzymes have been made to operate in a ligand-dependent manner by combining a catalytic domain with a ligand-binding domain (aptamer) to produce an 'aptazyme'[2,3]. The principle of ligand-dependent RNA catalysis has now been extended to the cross-replicating RNA enzymes so that exponential amplification occurs in the presence, but not the absence, of the cognate ligand. The exponential growth rate of the RNA depends on the concentration of the ligand, allowing one to determine the concentration of ligand in a sample. This process is analogous to quantitative PCR (qPCR) but can be generalized to a wide variety of targets, including proteins and small molecules that are relevant to medical diagnostics and environmental monitoring.

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A well-studied class of RNA enzyme, the RNA ligases, catalyzes the RNA-templated joining of RNA molecules. Some RNA ligases have been made to operate as aptazymes, and some of these have been made to undergo ligand-dependent catalytic turnover to provide linear signal amplification with ongoing target recognition[4,5]. One of the RNA ligases is the "R3C" RNA enzyme, which was obtained using *in vitro* evolution[6]. This enzyme has been reconfigured so that it can self-replicate by joining two RNA molecules that result in formation of another copy of itself[7]. It also has been converted to a cross-catalytic format, whereby two RNA enzymes catalyze each other's synthesis from a total of four RNA substrates[8]. The cross-replication process is analogous to the ligase chain reaction[9], except that in cross-replication the nucleic acid being amplified is itself the ligase, and strand separation occurs spontaneously without requiring temperature cycling.

The original cross-replicating RNA enzymes were slow catalysts that amplified poorly[8]. Recently their activity was substantially improved so that they can undergo efficient exponential amplification, generating about a billion copies in 30 h at a constant temperature of 42 °C[1]. Exponential amplification can be continued indefinitely, so long as a supply of the four substrates is maintained. The reaction requires 5–25 mM Mg^{2+} but does not require any proteins or other biological materials.

Cross-replication involves a plus-strand RNA enzyme (E) that catalyzes the joining of two substrates (A′ and B′) to form a minus-strand enzyme (E′), which in turn catalyzes the joining of two substrates (A and B) to form a new plus-strand enzyme (E). The cross-replicating enzymes were converted to aptazymes by replacing the distal portion of the central stem-loop by an aptamer that binds a particular ligand (**Fig. 1**). The aptamer was installed in the substrates A and A′, and in the corresponding enzymes E and E′. Two different aptamers were chosen, one that binds theophylline (theo)[10] and another that binds flavin mononucleotide (FMN)[11]. In the absence of the ligand the aptamer domain is unstructured, resulting in destabilization of the adjacent catalytic domain, whereas in the presence of the ligand the catalytic domain becomes ordered so that exponential amplification can occur. The stability of the stem region connecting the aptamer and catalytic domains was adjusted to maximize the ratio of activity between the 'on' (ligand present) and 'off' (ligand absent) states. Unlike the linear amplification of conventional aptazymes, ligand-dependent activity is expressed exponentially in the growth rate of autocatalytic aptazymes, establishing sharp thresholds for ligand-dependent behavior.

The two theophylline-dependent aptazymes, E_{theo} and E'_{theo}, first were tested individually in a ligation reaction carried out under saturating conditions in the presence of 5 mM theophylline, exhibiting reaction rate constants of 1.4 and 0.6 min^{-1}, respectively (**Supplementary Fig. 1** online). Both enzymes had no detectable activity ($<10^{-4}$ min^{-1}) in the absence of theophylline or in the presence of 5 mM caffeine (which differs from theophylline by the presence of a methyl group at the N7 position of caffeine).

Cross-replication was initiated by adding 0.02 µM each of E_{theo} and E'_{theo} to a reaction mixture containing 5 µM each of A_{theo}, A'_{theo}, B, B′ and either 5 mM theophylline or 5 mM caffeine, which was maintained at a constant temperature of 42 °C. Brisk exponential amplification occurred in the presence of theophylline, but there was no detectable amplification in the presence of caffeine (**Fig. 2a**). Exponential amplification resulted in the formation of new copies of both E_{theo} and E'_{theo}, ultimately limited by the supply of substrates. A plot of enzyme concentration versus time exhibited a classic sigmoidal shape, indicative of

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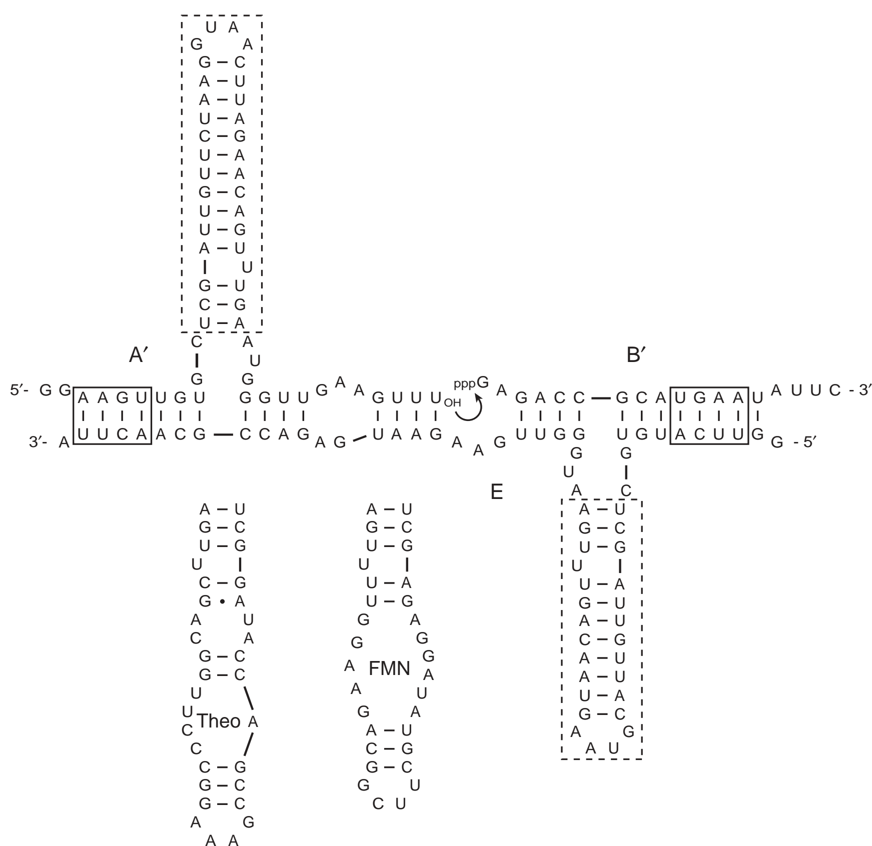

Figure 1 Sequence and secondary structure of autocatalytic aptazymes. The complex shown is that of the enzyme E and its substrates A′ and B′. Curved arrow indicates the site of ligation, resulting in formation of E′. The reciprocal reaction, involving the enzyme E′ and substrates A and B, is not shown. Dashed boxes indicate regions that were replaced by either the theophylline or FMN aptamer to form the corresponding aptazymes. Solid boxes indicate regions of Watson-Crick pairing that were replaced to allow multiplexed exponential amplification (the AAGU sequence in A′ was replaced by AGUA; the UGAA sequence in B′ was replaced by AUGA).

exponential growth subject to a fixed supply of materials. These data were fit to the logistic growth equation:

$$[E]_t = a/(1+be^{-ct})$$

where $[E]_t$ is the concentration of E (or E′) at time t, a is the maximum extent of growth, b is the degree of sigmoidicity and c is the exponential growth rate.

The exponential growth rates of E_{theo} and E'_{theo} were 0.78 and 0.97 h^{-1}, respectively, corresponding to a doubling time of about 50 min.

The maximum extents of synthesis of E_{theo} and E'_{theo} were 3.3 and 2.2 μM, respectively. Exponential growth can be continued indefinitely, however, if a portion of the completed reaction mixture is transferred to a new mixture that contains a fresh supply of substrates. This is analogous to reseeding the PCR, but unlike the PCR remains dependent on the presence of the ligand throughout the amplification process, thus avoiding target-independent amplification. After ~100-fold amplification, 1% of the reaction mixture was transferred to a new reaction vessel that contained 5 μM each of the four substrates but only those enzymes that were carried over in the transfer. Three successive incubations were carried out in this manner, resulting in 10^6-fold overall amplification after 15 h (**Supplementary Fig. 2** online).

The exponential growth rate of cross-replicating aptazymes is dependent on the concentration of the corresponding ligand. This allows one to construct standardized curves that can be used to determine the concentration of ligand in an unknown sample. The theophylline-dependent aptazymes were exposed to theophylline levels ranging from 0.2 to 5.0 mM, and the exponential growth rate of E_{theo} was determined. The growth rate as a function of theophylline concentration provided a saturation curve (**Fig. 2b**), which revealed that the aptazyme binds theophylline with a K_d of 0.51 mM. Thus, the aptazyme can be used to measure theophylline concentrations in the range of ~0.05–5 mM. The K_d for the theophylline aptamer in isolation is 0.1 μM[10], indicating that the aptamer is substantially destabilized in the context of the aptazyme. No attempt was made to optimize the aptamer in this context, as has been done for other aptazymes using *in vitro* selection[12,13].

The FMN-dependent aptazymes, E_{FMN} and E'_{FMN}, also underwent exponential amplification in the presence, but not the absence, of their cognate ligand. The exponential growth rates of E_{FMN} and E'_{FMN} in the presence of 1 mM FMN were 0.58 and 0.70 h^{-1}, respectively (**Fig. 2c**). The exponential growth rate of E_{FMN} was determined in the presence of various concentrations of FMN, which provided a saturation curve (**Fig. 2d**) and revealed that the aptazyme binds FMN with a K_d of 0.068 mM. The same FMN aptamer has been linked to the hammerhead ribozyme and exhibited a K_d of 5 μM in that context[12]. This compares with a K_d of 0.5 μM for the FMN aptamer in isolation[11].

Ligand-dependent exponential amplification can be performed using a pair of cross-replicating aptazymes that recognize two different ligands. As an example, a reaction was carried out using 0.02 μM each of E_{theo} and E'_{FMN} and 5 μM each of A_{theo}, A'_{FMN}, B and B′. There was no amplification in the absence of both ligands and only linear amplification in the presence of either theophylline or FMN, but robust exponential amplification in the presence of both ligands (**Supplementary Fig. 3** online). This system can be regarded as performing a logical AND operation, providing exponential signal amplification that is dependent on the presence of two different inputs.

It is straightforward to carry out multiplexed ligand-dependent exponential amplification, using two or more pairs of cross-replicating RNA enzymes that recognize their partners through distinct Watson-Crick pairing interactions (**Fig. 1**). Twelve pairs of cross-replicating RNA enzymes have been described[1], one of which was chosen to contain the theophylline aptamer and another to contain the FMN aptamer (installing the same aptamer in both members of a cross-replicating pair). In the presence of either 5 mM theophylline or 0.7 mM FMN, only the corresponding RNA enzymes amplified exponentially, with growth rates for E_{theo} or E_{FMN} of 0.35 or 0.43 h^{-1}, respectively (**Fig. 3**). In the presence of both ligands, both pairs of cross-replicating enzymes amplified exponentially, with growth rates for E_{theo} and E_{FMN} of 0.45 and 0.43 h^{-1}, respectively.

With each RNA-catalyzed ligation event, a 3′,5′-phosphodiester linkage is formed and one molecule of inorganic pyrophosphate is

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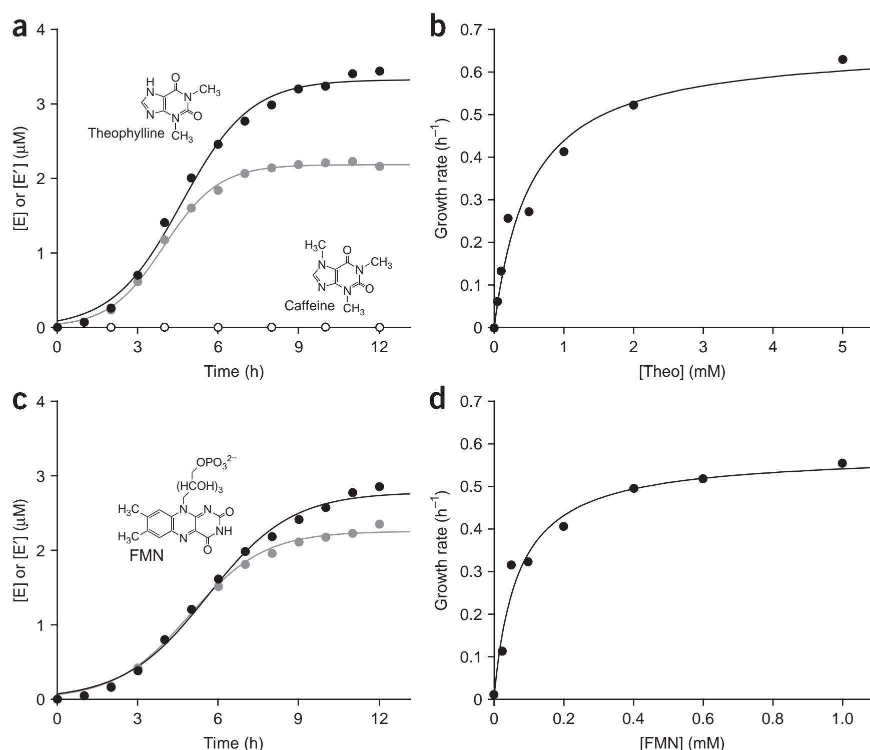

Figure 2 Ligand-dependent exponential amplification of RNA. (**a**) The theophylline-dependent aptazymes, E_{theo} (black) and E'_{theo} (gray), amplified exponentially in the presence of 5 mM theophylline (filled circles), but not in the presence of 5 mM caffeine (open circles). The structures of theophylline and caffeine are shown. (**b**) Exponential growth rate of E_{theo} in the presence of various concentrations of theophylline. (**c**) The FMN-dependent aptazymes, E_{FMN} (black) and E'_{FMN} (gray), amplified exponentially in the presence of 1 mM FMN. The structure of FMN is shown. (**d**) Exponential growth rate of E_{FMN} in the presence of various concentrations of FMN. Growth rates for reactions that did not proceed beyond 10% fraction reacted were determined by a linear rather than exponential fit.

released[6]. The released pyrophosphate can be used to generate a luminescent signal based on an ATP-regenerative luciferase assay[14]. A plot of light emission over the course of theophylline-dependent exponential amplification was nearly identical to that for formation of the ligated products (**Fig. 4**). The luminescent signal generated by various known concentrations of pyrophosphate was used to determine a conversion factor for relating light units to absolute concentrations of pyrophosphate (**Supplementary Fig. 4** online). These absolute concentrations were in close agreement with the absolute yield of ligated products over the course of exponential amplification (**Fig. 4**).

The major limitation of autocatalytic aptazymes as a quantitative method for ligand-dependent exponential amplification is the need for the aptamer domain to bind its ligand with some requisite affinity, while remaining compatible with efficient cross-replication. The desired binding affinity usually is dictated by the concentration of the ligand in its biological or environmental context. Methods for generating RNA aptamers that bind a target protein or small molecule with a particular affinity, often in the nanomolar range, are well established[15,16]. When these aptamers are placed in the context of an aptazyme, further optimization may be needed to regain the desired affinity. However, if the ligand concentration is very low, the concentration of RNA substrates required for efficient exponential amplification (typically micromolar) will exceed the desired K_d for the aptamer-ligand interaction. This would still allow ligand-dependent amplification, but at a reduced rate that is no longer dependent on the ligand concentration. One remedy would be to improve the K_m of the cross-replicating enzymes so that the enzyme-substrate interactions remain saturated even when the aptamer-ligand interaction is unsaturated. However, this approach would limit the amount of signal that could be generated for very low-abundance targets. Another approach, analogous to qPCR and other methods that link a rare recognition event to subsequent exponential amplification[17,18], would be to employ RNA replication as a reporter that is triggered by a recognition event. Unlike qPCR, such a process would be isothermal, but like qPCR, it would not benefit from ongoing sensing of the ligand during the course of exponential amplification.

Another limitation of autocatalytic aptazymes is that the molecules are composed of RNA, which is susceptible to degradation by ribonucleases or inhibition by nonspecific RNA-binding proteins. The theophylline-dependent aptazymes were rapidly degraded in the presence of 10% bovine calf serum but were able to undergo unimpeded ligand-dependent exponential amplification in the presence of serum that had been deproteinized by phenol extraction (**Supplementary Fig. 5** online). Clearly it will be necessary to develop nuclease-resistant forms of the aptazymes, as has been done for most aptamers that are used in a biological context[19,20].

Aptamers and aptazymes have emerged as powerful tools for detecting and generating biochemical responses to a wide variety of ligands[3]. Nature has exploited this mechanism in the operation of ‘riboswitches’[21], which are ligand-dependent riboregulators that occur widely in biology[22]. Scientists have engineered aptamers and aptazymes to sense proteins or small molecules[23–26], to control gene expression[27–28] and to perform molecular computation[29]. Autocatalytic aptazymes may be

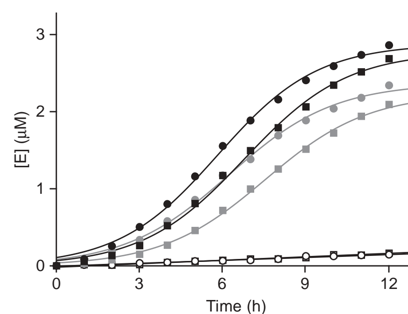

Figure 3 Multiplexed ligand-dependent exponential amplification of RNA. The theophylline- and FMN-dependent aptazymes were made to contain distinct regions of Watson-Crick pairing (**Fig. 1**). Exponential amplification of E_{theo} (circles) and E_{FMN} (squares) occurred in the presence of both ligands (black) and in the presence of their cognate ligand alone (gray) but not in the presence of the noncognate ligand alone (open symbols). Reaction mixtures contained 0.1 μM E_{theo} and E'_{theo}, 0.02 μM E_{FMN} and E'_{FMN}, and 5 μM each of the eight corresponding RNA substrates.

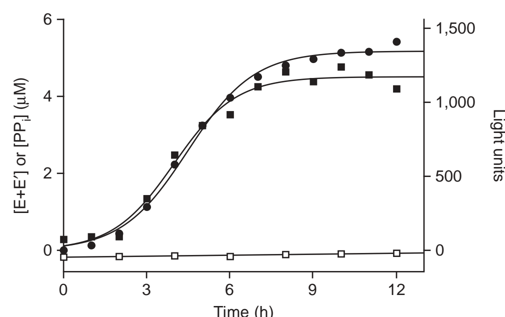

Figure 4 Monitoring the course of exponential amplification by a luciferase assay driven by the release of inorganic pyrophosphate that accompanies RNA ligation. Amplification was carried out in the presence of 5 mM theophylline, and the summed yields of E_{theo} and E'_{theo} were measured both by separating the ligated products in a denaturing polyacrylamide gel (filled circles) and based on the luminescent signal generated by an ATP-regenerative luciferase assay[14] (filled squares). Light units were converted to absolute concentrations of inorganic pyrophosphate based on comparison to known standards (**Supplementary Fig. 4** online). There was no light signal above background in the absence of theophylline (open squares); slightly negative values are due to imprecision in determining the conversion factor.

useful in some of these applications because they provide both specificity through dynamic sensing of the ligand and sensitivity due to ligand-dependent exponential amplification. Although several practical concerns still must be addressed, the ability to perform quantitative analysis of a variety of ligands under isothermal conditions may have utility in medical diagnostics and environmental monitoring.

METHODS

Materials. Oligonucleotides were synthesized on an Expedite automated DNA/RNA synthesizer (Applied Biosystems) using nucleoside phosphoramidites purchased from Glen Research. All oligonucleotides were purified by denaturing PAGE and desalted using a C18 SEP-Pak cartridge (Waters). Histidine-tagged T7 RNA polymerase was purified from *E. coli* strain BL21 containing plasmid pBH161 (kindly provided by William McAllister). *Thermus aquaticus* DNA polymerase was cloned from total genomic DNA and purified as described previously[30]. M1 RNA, the catalytic subunit of RNAse P, was obtained from *E. coli* genomic DNA (Sigma-Aldrich) by PCR amplification and subsequent *in vitro* transcription, as described previously[1]. Calf intestine phosphatase and T4 polynucleotide kinase were purchased from New England Biolabs, yeast inorganic pyrophosphatase was from Sigma-Aldrich and bovine pancreatic DNase I was from Roche Applied Science. Nucleoside and deoxynucleoside 5′-triphosphates, theophylline and FMN were purchased from Sigma-Aldrich, [γ-^{32}P]ATP (7 μCi/pmol) was from Perkin Elmer and caffeine was from MP Biomedicals. *Photinus pyralis* (firefly) luciferase, *Saccharomyces cerevisiae* ATP sulfurylase, adenosine 5′-phosphosulfate and D-luciferin were from Sigma-Aldrich. Bovine calf serum was from Omega Scientific, and Superasin (RNAse inhibitor) was from Ambion.

Preparation of aptazymes and substrates. All RNA enzymes and substrates were prepared by *in vitro* transcription in a reaction mixture containing 0.4 μM DNA template, 0.8 μM synthetic oligodeoxynucleotide having the sequence 5′-GGACTAATACGACTCACTATA-3′ (T7 RNA polymerase promoter sequence underlined), 2 mM each of the four NTPs, 15 U/μl T7 RNA polymerase, 0.001 U/μl inorganic pyrophosphatase, 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol and 50 mM Tris-HCl (pH 7.5). The mixture was incubated at 37 °C for 2 h, quenched by adding an equal volume of 15 mM Na_2EDTA, treated with 1 U/μl DNase I and extracted with a 1:1 mixture of phenol:chloroform. The RNA was precipitated, purified by PAGE and desalted. Transcription of M1 RNA was performed similarly, except employing a double-stranded DNA template that was generated by PCR.

The A and A′ substrates could not be obtained reliably by *in vitro* transcription due to heterogeneity at the 3′ end of the transcripts. Instead, these substrates were prepared from the corresponding E or E′ molecules by cleaving off the B or B′ portion using *E. coli* M1 RNA, as described previously[1]. The external guide sequence RNA for cleavage of E_{theo} and E_{FMN} had the sequence 5′-CGUAAGUUGCGGUCUCACCA-3′, and for E'_{theo} and E'_{FMN} had the sequence 5′-AUAUUCAUGCGGUCUCACCA-3′ (nucleotides complementary to the target RNA underlined). For the second pair of E_{theo} and E'_{theo} molecules used in the multiplex experiments, the external guide sequence RNAs had the sequence 5′-CGUAGUAUGCGGUCUACCA-3′ and 5′-GAAUAUCAUUGCGGUCUCACCA-3′, respectively. The A and A′ substrates were [5′-^{32}P]-labeled by first dephosphorylating using calf intestine alkaline phosphatase, then phosphorylating using T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled substrates were purified by PAGE and desalted using a Nensorb 20 cartridge (NEN Life Sciences).

Individual RNA-catalyzed reactions. RNA-catalyzed RNA ligation was performed in a reaction mixture containing 5 μM E or E′, 0.1 μM [5′-^{32}P]-labeled A′ or A, 6 μM B′ or B, 25 mM $MgCl_2$ and 50 mM EPPS (pH 8.5), which was incubated at 42 °C. Aliquots were taken at various times and quenched by adding an equal volume of gel-loading buffer containing 50 mM Na_2EDTA and 18 M urea. The products were separated by PAGE and quantified using a PharosFX molecular imager (Bio-Rad). The data were fit to the equation:

$$F_t = F_{max} - (a1\, e^{-k1 \cdot t}) - (a2\, e^{-k2 \cdot t}),$$

where F_t is the fraction reacted at time t, F_{max} is the overall maximum extent of the reaction, $a1$ and $k1$ are the amplitude and rate of the initial fast phase and $a2$ and $k2$ are the amplitude and rate of the subsequent slow phase, respectively.

In the presence of 5 mM theophylline, the reaction catalyzed by E_{theo} exhibited a fast phase with an amplitude of 0.57 and rate constant of 1.4 min^{-1}, followed by a slow phase with an amplitude of 0.24 and rate constant of 0.044 min^{-1}; the reaction catalyzed by E'_{theo} had an amplitude of 0.52 and rate constant of 0.59 min^{-1} in the fast phase and an amplitude of 0.26 and rate constant of 0.045 min^{-1} in the slow phase.

Cross-replication reactions. Cross-catalytic exponential amplification was performed in a reaction mixture containing 0.02 μM each of E and E′, 5 μM each of [5′-^{32}P]-labeled A and A′, 5 μM each of B and B′, 25 mM $MgCl_2$ and 50 mM EPPS (pH 8.5), which was incubated at 42 °C. The reaction was initiated by mixing equal volumes of two solutions, one containing the enzymes and substrates and the other containing the $MgCl_2$ and EPPS buffer. Aliquots were taken at various times, quenched, and the amounts of newly synthesized E and E′ were quantified as described above. The data were fit to the logistic growth equation, as described in the main text.

Luciferase assays. Known concentrations of inorganic pyrophosphate or samples taken from the cross-replication reaction were diluted tenfold into a reaction mixture containing 0.15 μg/μl luciferase, 0.00045 U/μl ATP sulfurylase, 10 μM adenosine 5′-phosphosulfate, 0.5 mM D-luciferin, 25 mM magnesium acetate, 0.1% bovine serum albumin, 1 mM dithiothreitol, 0.4 μg/μl polyvinylpyrrolidone (MW 360,000) and 100 mM Tris-acetate (pH 7.75). The pyrophosphate standards were prepared in a solution identical to that employed in cross-replication but lacking the RNA enzymes and substrates. Luminescence was detected using a Perkin Elmer LS55 luminescence spectrometer operating in bioluminescence mode, with a PMT voltage of 900 V, cycle time of 200 ms, gate time of 180 ms and delay time of 0. The flash count was set to 1, the emission filter was fully open and the emission slit width was 12 nm. After addition of the sample to the luciferase mixture, luminescence was monitored for 5 min with a 0.1 s integration time. The amount of light generated was linear over a pyrophosphate concentration range of 0.1–10 μM.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/

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Biotech scientists bank on big pharma's biologics push

Grace Wong

Biologics represent a new hope for big pharma and new dreams for biotech scientists.

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Why have biologics become a hot area of research for many large pharmaceutical companies? There are many reasons, most of which are related to risk and costs. Developing new drugs is an expensive, drawn-out proposition, with no guarantee of producing a safe, marketable product. Even if a drug does reach the market, there are always potential liability issues. Expiring patents and a flood of generic drugs entering the market have also led to decreasing revenues for big pharma's small-molecule chemical drugs.

As the pipeline of potential drugs has slowed, pharma companies are increasingly looking to biologics as an area of growth—investing heavily in biologics programs, buying companies and building facilities. Recent acquisitions include the purchases of Domantis by GlaxoSmithKline; MedImmune and Cambridge Antibody Technology by AstraZeneca; Rinat by Pfizer; Adnexus Therapeutics by Bristol-Myers Squibb; Chiron by Novartis; GlycoFi, Abmaxis and Insmed's follow-on biologic business by Merck; and CoGenesys by Teva. While pharmas may be laying off their staff in droves, they are still actively pursuing biologists with skills and experience in biologics. I recently asked some experts about pharma's interest in biologics and what their advice was for future scientists. Their answers follow.

The new frontier

First, pharmas are turning to biologics to avoid, or at least flatten, the 'revenue cliff' due to the patent expirations of their current small-molecule blockbusters, according to Jingsong Wang, a director at Bristol-Myers Squibb (New York). Adds Honghui Zhou, senior director at Centocor (Horsham, PA, USA), "big pharma wants to avoid putting all their eggs in one basket; that is, having a diverse portfolio of both small molecules and biologics certainly can decrease the overall risks."

Second, biologics do not have the same competition from generics. According to Reinhard Ebner, vice president at ActoKine Therapeutics (Chestnut Hill, MA, USA), "because proteins are large, complex molecules, development of generic versions is more difficult than for small-molecule drugs. However, the underlying science has progressed far enough to approve follow-ons for some of the smaller biologics." He adds, "and synthetic substitutes (mimetics) will some day be available for many biologics."

A deep and broad knowledge of biology is important for a career in biologics R&D, says Reinhard Ebner of ActoKine.

Joe McCracken of Genentech (S. San Francisco, CA, USA) explains, "FDA has become very conservative in approving drugs for broad use in non-life threatening diseases. Big pharmas now view biologics as being less susceptive to generic competition and more likely to be approved if they are being developed for life-threatening diseases." Morten Sogaard, executive director at Boehringer-Ingelheim, says, "what makes biologics great therapeutics is that they harness the same principles employed by endogenous proteins: exquisite specificity at the receptor level resulting in a well-defined biological effect with non-mechanism related adverse events being relatively rare." Peter Kiener, MedImmune's (Gaithersburg, MD, USA) executive vice president adds, "biologics' advantages include faster discovery times and more predictable activity" and "approaches with macromolecules provide the opportunity to engineer the therapeutic to be able to more effectively intervene in the disease pathway."

According to John Maraganore, CEO of Alnylam (Cambridge, MA, USA): "The challenge for pharma is discovery of new innovative medicines. This has been increasingly difficult for small molecule discovery over the last 20 years. At the same time, the number of biologic drug approvals has increased. Most of big pharma feel that they have missed out on the opportunity."

Finally, a higher percentage of biologics are blockbusters, according to Sheldon Fan, senior external research manager at Pfizer Asia Research (Shanghai). One reason is that "biotherapeutics can hit targets that a small molecule cannot, and generally they do not have compound-related toxicity," says Zhijian Lu, director at Wyeth (Cambridge, MA, USA). "Therefore, biologics provide rich opportunities for safely treating diseases that are not easily treatable with current small-molecule approach. Yet biologics are very complex molecules, which constitutes a much higher scientific and regulatory threshold for imitation." Another reason is their use in specialty areas of high unmet need, which offer high cost/benefit ratios, says Mervyn Turner, chief strategy officer and senior vice president of worldwide licensing and external research at Merck (Whitehouse Sta., NJ, USA).

Innovative thinking is key

"The skills required for a career in biologics R&D are not that different from those required for traditional drug discovery," says ActoKine's Ebner, "though a deep and broad knowledge of biology is important. Enthusiasm and creative thinking are more important than experience or expertise." However, Seng Cheng, group vice president

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at Genzyme (Cambridge, MA, USA) says, "skill set and knowledge base of scientists for biologics are very different than for small molecule drugs," and adds, "innovative thinking is always a desired trait irrespective of where you work."

The choice between innovative thinking or experience ultimately depends upon what the company is hiring the individuals to do, according to Robert Lewis, formerly of Aventis.

According to Jaume Pons, chief scientific officer at Rinat (S. San Francisco, CA, USA), "teams are built around diverse skills and experience: some will bring creativity and innovation, others very solid execution, others experience to move the program from idea to reality. These attributes are not usually found in one person. The most important criteria is a fit with the team needs and sense of urgency."

Other experts agree. John Birch, chief scientific officer for Lonza Biopharmaceuticals (Basel, Switzerland), states: "Innovative thinking and an ability to adapt are key to working with pharmaceutical and biotech companies. It is important for biologists to understand that their greatest asset is not just their experience but their ingenuity." And according to Abraxis BioScience senior vice president Lex Van der Ploeg (Los Angeles), "while many organizations will aim for specific skill sets, which can give a program a running start, personally I search for candidates with outstanding intellect, and experience in an area that projects rapid learning and the acquisition of new skills."

For Robert Lewis, a former senior vice president at Aventis, it depends upon what the biotech or pharma is hiring the individuals to do. "If a few innovators are needed, that could rebalance the criteria. However, hiring entities often look for technological abilities and experience as the highest ranking criteria, since they probably have some innovative ideas already." Timothy Wells, chief scientific officer at Medicines for Malaria Venture and former head of research at Serono (Geneva), agrees. "Experience is everything in research. Innovative thinking is very hard to measure—and so most recruitment is done based on the surrogate of which innovative scientists you have worked with. The choice of mentor is crucial and is probably the most important decision that you will ever make."

Pharma training programs

Past experience from successful biotech companies such as Genentech coupled with the current state of big pharma argue in favor of establishing training programs as a way for pharmas to develop new scientific talent and harness innovation, says Pfizer's Sheldon Fan. And Wyeth's Zhijian Lu believes that "it is important to have different arrangements, such as a postdoctoral program for creative biomedical exploration not directly bound to therapeutic projects. Wyeth Research maintains an active postdoc program that matches most of the academic programs, and it has been mutually beneficial to the company and the trainees." Similarly, a broad postdoc program has been launched throughout the entire Roche pharma and diagnostics divisions, according to Klaus Strein, head of pharma research at Roche in Germany. And at the Abbott Bioresearch Center (Worcester, MA, USA), "some of our most innovative employees joined us as postdocs and have made significant contributions while obtaining their educational goals," maintains divisional vice president Jochen Salfeld. "We prefer a system where we pair up strong postdocs with some of our most experienced and creative scientists, and also have them join an ongoing discovery program while exploring new biology or technology. This allows them to publish while getting real-life discovery experience."

Morten Sogaard agrees: "Postdoctoral programs need to be win-win propositions benefiting both the student (high-quality projects with possibility to publish in leading journals) as well as contributing to the productivity of the company." And according to Genentech's McCracken, "postdocs bring the latest technical knowledge with them and they keep an organization fresh. Most scientists are also most productive at this point in their careers." John Matthias, associate director at Roche Kulmbach, Germany, says, "postdoctoral training programs are certainly one way to keep pharma scientists in touch with academia. Stay flexible and maintain high quality skills in the area you are best in."

Others, such as Abraxis BioScience's Van der Ploeg, prefer candidates with a genuine interest in advancing the development of therapeutics and advancing patient care, as a long term career commitment. And Robert Lewis adds, "in tough economic times like these, pharmas unfortunately are less inclined to start entirely new postdoctoral programs in almost any area." Merck's Turner thinks the best advice is to "focus on the best academic laboratory to hone skills in biology, and prepare the mind for inspiration as science unfolds. Postdoctoral fellows can still gain important experience of R&D in the pharma industry by working in a laboratory that has teamed up with a pharma partner."

Roche's Klaus Strein recommends getting a broad education at recognized academic institutions.

According to Joerg Reinhardt, COO at Novartis (Basel, Switzerland), "our Presidential Postdoctoral Fellowship program provides talented scientists with a unique opportunity to perform high-quality research in an environment with the resources of a large pharmaceutical company." He adds, "fellows receive guidance from two mentors: a senior researcher at NIBR and a faculty member from an academic institution." In addition, Novartis creates free seminars or intern programs to help inspire and educate the next generation of scientists. One example is the Novartis Biotechnology Leadership Camp (BioCamp), a seminar for entrepreneurial, graduate and postgraduate students interested in pursuing a career in biotechnology. Joe Jimenez, CEO of Novartis explains that "BioCamp can provide participants with the tools they need to become successful in the field of biotechnology." Novartis's internship program is another example of big pharmas giving back to the community.

Taking the leap

What advice can be offered to scientists hoping to get into biologics research in big pharma? Roche's Strein recommends getting a broad education at recognized academic institutions. "Don't feel the need to specialize too early in your career," agrees Merck's Turner. "A broad foundation in biomedical research provides a strong long-term advantage."

Pfizer's Fan counsels young scientists to "find a place where you can be innovative and productive, as the current landscape in biotech and pharma is undergoing a profound change in the way new drugs are being found." The important thing, says the project director for a new biotech production plant at Sanofi-Aventis, "independent of the company's size, is the permanent capability of R&D teams to find and develop products for unmet therapeutic benefits."

Medicines for Malaria Venture's Timothy Wells says "clearly, there is added value to understanding how biologics work in an industrial setting. The question is whether the

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big pharmaceutical companies are the places to learn these skills. If you want to learn manufacturing, work in industry for a while."

Genentech's McCracken advises: "Find a company with a critical mass of expertise in specific biological areas, and with a business model that is driven by the science and not commercial objectives."

Conclusions

The pharma industry has undergone much change in the last decade with the advent of globalization and market fluctuations. With the necessary shift toward biologic drugs, there is another big change underway. Students need to understand industry's changing needs if they plan on careers in that area, and companies need to develop intern and postdoctoral programs to help develop new industrial scientists, as well as retraining programs to teach established researchers new skills.

The future is open to those with the right skills, unique talents and innovative minds—and not just at smaller, more innovative companies but also at large pharmas with the financial muscle and resources to get through late-stage product development. There will be many jobs available for those who have the skills, even in the current economy.

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PEOPLE

Vertex Pharmaceuticals (Cambridge, MA, USA) founder, president and CEO **Joshua Boger** (left) has announced his retirement effective in May 2009. To succeed him, the board of directors has appointed **Matthew Emmens** (right) president and has announced that he will transition to president, CEO and chairman upon Boger's retirement. Emmens has been a Vertex director since 2004, and he previously served as CEO of Astra Merck and of Shire plc, where he continues as chairman. **Charles Sanders**, Vertex's chairman, will remain as lead director on the board following Emmens' appointment as chairman. Boger will also remain an active member of the board.

"I founded Vertex in 1989 with a vision to pursue a new approach in drug discovery and develop medicines that would fundamentally change the treatment of serious diseases," Boger says. "I am very proud of what Vertex has accomplished, and we now stand on the cusp of great clinical and medical success. I welcome Matt, and have great confidence in his extensive experience leading companies through launches of breakthrough products, to take the reins and guide Vertex as we bring our innovations to patients."

Lance Berman, most recently senior medical director and global medical team leader at Pfizer, has been named chief medical officer of CPEX Pharmaceuticals (Exeter, NH, USA). He succeeds **Robert M. Stote**, who served as CMO of CPEX since its spin-off from Bentley Pharmaceuticals in June 2008. Stote will continue as a clinical consultant for CPEX.

Venture capital firm Atlas Venture (Boston and London) has promoted **Bruce Booth** to partner in the life sciences group. Booth joined the firm in 2005 and currently sits on the boards of Stromedix and Zafgen and is chairman and cofounder of Miragen.

Personalized medicine company DxS (Manchester, UK) has announced the appointment of **Jeff Devlin** as chief operating officer. Prior to DxS, he held positions as executive vice president and executive committee member of Shire Pharmaceuticals.

Neurobiological Technologies (Emeryville, CA, USA) has named **William A. Fletcher** as the company's acting CEO. Fletcher has been a director since February 2007 and has served as chairman of Teva Pharmaceuticals North America since December 2004.

Inspire Pharmaceuticals (Durham, NC, USA) has named former president and CEO of the Pharmaceutical Research and Manufacturers of America (PhRMA) **Alan F. Holmer** to its board of directors. Holmer previously served for two years on Inspire's board, resigning in February 2007 to accept an appointment in the US Treasury Department. He recently completed service as Special Envoy for China and the Strategic Economic Dialogue.

Gen-Probe (San Diego) has announced the appointment of **Eric Lai** as senior vice president, R&D. Lai was most recently vice president, pharmacogenetics experimental project coordination and analysis, at GlaxoSmithKline.

Genocea Biosciences (Cambridge, MA, USA), in conjunction with a $23 million Series A financing, has named **Staph Leavenworth Bakali** as its new CEO. Leavenworth Bakali, formerly COO of both ID Biomedical and PowderJect Pharmaceuticals, replaces Genocea cofounder **Robert Paull**, managing general partner of Lux Capital Management. Paull will remain on Genocea's board of directors.

Trinity Biotech (Dublin) has announced the appointment of **James D. Merselis** to its board as a non-executive director. Merselis is currently president and CEO of Alverix.

Aerovance (Berkeley, CA, USA) has named **Babatunde A. Otulana** as senior vice president and chief medical officer. Prior to joining the company, Otulana was senior vice president of development and chief medical officer with Aradigm.

Hans Pauli will step down as chief financial officer of OctoPlus (Leiden, The Netherlands) effective on March 31, 2009. The company's executive board will aim to fill the vacancy left by Pauli as soon as possible.

Nabriva Therapeutics (Vienna) has appointed **William Prince** as chief medical officer. He joins Nabriva from Surface Logix, where he served as chief development officer.

Stephen B. Shrewsbury has been named chief medical officer and senior vice president of clinical and regulatory affairs of AVI BioPharma (Portland, OR, USA). Shrewsbury brings 30 years of research, clinical development and product commercialization experience to AVI. Most recently, he served as chief medical officer and senior vice president, clinical development and regulatory affairs for Adamas Pharmaceuticals.

3SBio (Shenyang, China) has appointed **Bo Tan** as chief financial officer. He joined the company in October 2008, acting as the financial advisor for the company. Previously, he served as the executive director and a member of the investment committee for Bohai Industrial Fund Management Company, a private equity fund in China. Earlier stops include Eli Lilly & Company, EMD Pharmaceuticals and Lehman Brothers Asia.

Vojo Vukovic has been appointed vice president, clinical research at Synta Pharmaceuticals (Lexington, MA, USA). He has over 15 years of experience in oncology drug development and joins Synta from Pfizer, where he served as global medical lead for Sutent and axitinib in a number of cancer indications.

Greg Weaver has been named CFO and senior vice president of Poniard Pharmaceuticals (S. San Francisco, CA, USA). He was most recently CFO of Talyst, and during 2006 he served as senior vice president and CFO of Sirna Therapeutics.

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