Regenerative Medicine in China

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preface:

Regenerative Medicine Research in China: Demands and Practice

With its large—and growing—population of over 1.3 billion, China has a significant and urgent demand for tissue repair and regeneration technologies to serve the health care needs of its citizens. According to a 2010 national health data report, injuries, accidents, and other external factors accounted for 9% of hospitalizations in urban areas, ranking third after only respiratory diseases (12%) and digestive diseases (10%). Wounds from these factors, plus tissue pathologies including those from hereditary, metabolic, and chronic diseases, creates a huge patient demand for tissue repair and regeneration treatments, estimated to be approximately 100 million annually.

In China and throughout the world, regenerative medicine is a time-honored medical topic, with modern implications. Over the past 20 years, significant progress has been made in genetics, developmental biology, stem cell biology, and tissue engineering, all of which have increased our understanding of regenerative medicine and benefited the field of clinical therapeutics. In China, the synergy of these disciplines has enabled the field of tissue repair and regeneration to become one of the most active in medical research and practice. Basic research findings are being translated into the clinics and are changing the way medicine is practiced.

In China, three areas are showing particularly fast development: (1) stem cell biology, (2) organ constitution and replication, and (3) research into tissue engineering products for trauma treatment. For example, important breakthroughs have been made in replicating tetraploid complementation competent iPS mice by using induced pluripotent stem cells (iPSCs). Meanwhile, advances in isolating and culturing somatic stem cells has enabled the regeneration of skin sweat glands after severe burns, or of blood vessels in diabeties or other vascular diseases. Advances have been made in tissue engineering, particularly in the use of seed cells, the development of custom matrices, and the understanding of the role of regulatory factors, allowing for the reconstitution of bones, cartilages, nerves, blood vessels, and tendons. Many active clinical trials are under way, some showing promising results. Achieving synchronized regeneration of various tissues in a wound is a pivotal research area, while researchers are also exploring methods to prevent scar tissue formation in wounds when inducing skin stem cells to differentiate directly into various tissue types without the need for an iPSC phase.

Research in China aligns with global trends in all areas of regeneration medicine, with a particular focus on the agile and efficient translation of ideas from basic research to clinical application and addressing the challenges this raises.

Government support of regenerative medicine is strong. In the “Roadmap to 2050,” published by Chinese Academy of Sciences and Study on the medium- and long-term Development Strategy of Engineering Technology in China, regenerative medicine was listed as a key direction. Further, stem cell technology was categorized as a Class III medical technology in the Management Guidelines for Therapeutic Tissue Transplantation Technology by the Chinese State Food and Drug Administration. Additionally, the Xiangshan Science Conference twice (in 2005 and 2010) thoroughly discussed the key areas in which breakthroughs and critical regulatory attention is needed in regenerative medicine, laying a solid foundation for continued support and government investment in regenerative medicine.

Within the next decade, substantial progress in regenerative medicine in China is expected in the following areas: the use of induced stem cells in the synchronized repair and regeneration of multiple tissue types following injury, reconstitution of large organs through tissue engineering, and the large-scale clinical application of tissue engineering products. These advances will provide hope for the improvement of health care and the creation of a healthier society.

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preface:

Meeting New Challenges Head On

In any new field of research, especially one with the broad potential impact of regenerative medicine, there are going to be challenges. It is how those challenges are dealt with that defines the success of the new endeavor. China today is managing not only swift growth in her economy, but also a rapid increase in the affluence of her populace. With this, comes changes in lifestyle and even culture as well as higher expectations of the level of medical treatment received.

In order to address these issues, China is intent on growing both its basic research prowess and providing better training for its clinicians, nurses, and other medical staff in the increasingly important field of regenerative medicine. This booklet outlines some of the areas of focus for China as it pushes the boundaries of research and treatment.

One of the greatest advances since the discovery of stem cells has been the recognition that the path from pluripotency to a fully differentiated, mature cell is not as unidirectional as previously thought. Add to this the recent identification of numerous multipotent and unipotent stem cells in various tissues and many more doors open up for potential therapies utilizing regenerative medicine techniques.Researchers in China are making full use of these discoveries by manipulating stem and stem-like cells in new ways in order to help treat burn victims, diabetics, and those suffering the ravages of neurodegenerative diseases like Alzheimer's and Parkinson's.

Additionally, so-called bioscaffolds—biological support structures from which the cells have been removed—are being used in unique and interesting ways to, for example, repair skin defects caused by traumatic injury and regenerate nerves in spinal cord injuries. This exciting application holds much promise for relieving pain and improving the standard of living for all people.

Many of the therapies being tested in China are already in the clinic and showing potential. As many authors in this supplement point out, though, there are still many challenges to overcome. Ethical issues and the fear of tumors developing from transplanted stem cells top that list. However, these are not insurmountable obstacles. Researchers, clinicians, and backers of science within the government are diligently seeking solutions and continue to work toward the common goal of providing better, faster, and more effective treatments to everyone in China, while at the same time providing the world with cutting-edge technologies and methodologies.

Alan Leshner, Ph.D.
CEO, AAAS
Executive Publisher, *Science*

preface:

The Why and What of Regenerative Medicine in China

Medical professionals and researchers alike are constantly striving to find better ways to treat patients and cure disease. Unfortunately, this is not always possible. Although over centuries of biomedical development there have been notable successes, we still lack many of the answers. In addition, China faces the same tissue and organ replacement crisis as the rest of the world, where victims of spinal cord injuries long to walk again, and patients in dire need of a heart, liver, or kidney must wait for the availability of organs, sometimes in vain. The need is clearly evident. Fortunately, within the past 20 years, rapid progress in regenerative medicine has started to utilize the patient's own tissue, as well as stem and precursor cells, to produce life-saving tissues and organs to replace dysfunctional ones.

Regenerative medicine has already improved the quality of life for many individuals. Further consolidation of knowledge is now evident and a broad vision and corresponding practice is being developed. It has begun to make the impossible, possible.

In recent decades, regenerative medicine in China, as judged by the increase in the number of peer-reviewed publications and the expansion of funding, has emerged as one of the leading fields in both basic research and clinical treatment. Compared with traditional approaches, the individual therapeutic solutions, scientific research, and clinical implementation seen with regenerative medicine hold great promise for benefiting not just a small group, but society as a whole. China is committed to nurturing the opportunity to contribute in this scientific arena, in part by applying their unique resources and talents.

As with many new approaches, controversies arise, and regenerative research in China is no exception. This is especially true regarding cell therapeutics and novel approaches translated into the clinic. It is therefore important to identify and advance the best research conducted by those disciplined scientists and physicians who work hard to carry out their research under the internationally accepted norms and ethics guidelines.

The long-term promise of regenerative medicine is that it offers a faster, more complete recovery with significantly fewer side effects or complications. Progress will come through the development of innovative new therapies and the encouragement of best practices. Since this is also the mission of GlobalMD, *"to improve global health care through professional development, research, and collaboration without borders,"* we are pleased to support this supplement.

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Generation and Evaluation of Pluripotent Stem Cells

Qi Zhou, Ph.D.

Stem cell therapy has tremendous treatment potential for replacing some damaged cells. However, the problem of immune incompatibility remains unresolved. Creation of a homologous embryonic stem cell line derived from patient autologous cells using somatic cell nuclear transfer (SCNT) technology is one feasible solution. The generation of such embryonic stem cells (NT-ESCs), and the use of their differentiated derivatives for disease treatment, has been termed therapeutic cloning. We could successfully generate mouse NT-ESCs and did not find any significant difference between NT-ESCs and normal ESCs. However, SCNT is a very inefficient process. To overcome this, we attempted to modify the culture conditions for reconstructed embryos. We found that a sequential culture method, in which M16 medium was used for the NT process and KSOM medium for the late two-cell stage, dramatically increase the blastocyst development rate (*1*). Also, we tried to improve cloning efficiency using small molecule additives. We found that m-carboxycinnamic acid bis-hydroxamide (CBHA), a histone deacetylase inhibitor, could significantly increase the blastocyst development rate in SCNT-reconstructed embryos (Table 1). Moreover, CBHA increased blastocyst quality compared with Trichostatin A, another prevalent histone deacetylase inhibitor (*2*).

Oocyte source has been a limitation for human therapeutic cloning. To tackle this problem, we explored the possibility of reprogramming fertilized embryos. We found that two-cell stage electrofused mouse embryos, arrested in mitosis, could support developmental reprogramming of nuclei from donor cells ranging from blastomeres to somatic cells. Live, full-term cloned pups from embryonic donors, as well as pluripotent ESC lines from embryonic or somatic donors, were successfully generated when using two-cell stage electrofused mouse embryos (*3*). This approach indicated that discarded preimplantation human embryos could be an important resource for human therapeutic cloning.

Treatment	No. of reconstructed embryos	No. of pronucleus formation embryos	No. of two-cell embryos	No. of four-cell embryos	No. of morula	No. of (%) 2-cell blastocysts
Without modification	230	186	185	164	108	60 (32.9±2.9)
Culture method modification	91	70	69	68	55	44 (62.3±7.2)
CBHA treatment	87	70	69	67	58	49 (71.2±2.1)

Table 1. Improvement in preimplantation development of SCNT embryos.

To extend therapeutic cloning from mice to human, we made a series of improvements on various aspects of the process. We separated metaphase II oocytes into four grades according to morphology assessment criteria. We noted that embryos from oocytes of Grades A and B could develop to the blastocyst stage with similar development efficiency for every developmental stage. However, embryos from Grade C and D failed to develop to the blastocyst stage; most of the embryos were blocked at the two- to four-cell stage (*4*). Most of the oocytes obtained from Chinese clinical centers were classified as Grade C and D, which may be the main reason that therapeutic cloning using these oocytes often failed. We were able to active and develop human oocytes to the blastocyst stage, and derived human parthenogenetic embryonic stem cells from these embryos using human foreskin cell feeders. The embryonic stem cells had normal morphology, expressed all expected stem cell surface markers, and could differentiate into derivatives of all three germ layers. The ability to improve the success rate of ESC generation should facilitate studies of therapeutic cloning for research and clinical applications (*5*).

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Cell line	4N-competent cell line efficiency (%)	Percentage live pup births after 4N injection	Tumorigenesis efficiency of adult 4n mice (%)
ESC	3/5 (60.0)	2.8%	0/18 (0)
MEF-IPSC	3/6 (50.0)	2.65%	3/13 (23.1)
TTF-IPSC	8/18 (44.4)	3.8%	ND
NSC-IPSC	4/5 (80.0)	3.5%	ND

Table 2. Pluripotency and tumorigenesis of different cell lines. MEF-IPSC, iPSC lines derived from mouse embryonic fibroblasts (MEFs); TTF-IPSC, iPSC lines from adult tail tip fibroblasts (TTF); NSC-IPSC, iPSC lines from neural stem cells (NSC)

In pioneering work in 2006, mouse embryonic stem cell-like induced pluripotent stem cells (iPSCs) were generated from mouse somatic cells by expression of defined transcriptional factors (*6*). Whether iPSCs attain true pluripotency identical to their ESC counterparts was unclear at the start. Additionally, the production of live animals by the tetraploid complementation assay had not been demonstrated; this is regarded as the most stringent test for pluripotency, with failure indicating that cells were not fully pluripotent. We improved the methods of iPSC induction and produced live animals through the tetraploid complementation assay. The method reduced the time needed for iPSC induction to under a month and generated several iPSC lines derived from mouse embryonic and adult fibroblasts with the ability to produce viable, fertile, live-born progeny. This demonstrated that iPSCs derived from somatic cells could achieve full pluripotency similar to that of ESCs (*7*). To find an effective and quick method to assess the pluripotency of iPSCs at early stages, we compared iPSCs and ESCs with different pluripotency levels. We identified the *Dlk1-Dio3* region, a conserved, imprinted region of the mouse genome that was activated in fully pluripotent mouse stem cells but repressed in partially pluripotent cells, and which may serve as a pluripotency marker (*8*).

Safety is a major concern in the clinical application of iPSCs. To evaluate the tumorigenic potential of iPSCs, we examined adult iPSC-derived mice through tetraploid complementation. Tumorigenic cells were identified in 23.1% of iPSC-derived F0 mice but not in ESC-derived mice (Table 2), demonstrating that these iPSCs were tumor prone. We used quantitative PCR to examine the endogenous and transgenic expression levels of the four iPSC-induction factors, c-Myc, Klf4, Oct4 and Sox2, in various tissues of F0 iPSC, ESC, and control mice. We found the expression of transgenic c-Myc was statistically higher in tumorigenic tissues than in nontumorigenic tissues in iPSC-derived mice ($p<0.01$), indicating that the overexpression of transgenic c-Myc may contribute to the tumor formation in F0 iPSC-derived mice (*9*). In an attempt to create iPSC-derived mice without c-Myc, we generated c-Myc-free iPSCs with and without synthetic small compounds and used these cells to generate live-born pups through tetraploid complementation (*10*). Further study of tumor generation in these animals will hopefully shed further light on the issue of unwanted tumor formation in iPSC-derived mice.

Taken together, our work suggests that pluripotent stem cells hold great promise for regenerative medicine and drug discovery. However, the application of these cells needs to be further improved before they are ready for clinical use.

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The Application of Interspecies Chimeras in Regenerative Medicine

Andy Peng Xiang, Ph.D.* and Bruce T. Lahn, Ph.D.

Pluripotent embryonic stem (ES) or induced pluripotent stem (iPS) cells have attracted enormous attention in recent years, largely due to their potential to differentiate into a variety of specialized cells that can be used in cell-replacement therapy (*1, 2*). However, in vitro differentiation of stem cells has several major limitations. First, it can only produce limited cell types, because conditions cannot mimic the highly complex in vivo environment. Second, cell types produced by in vitro differentiation may not be truly identical to their in vivo counterparts. Finally, it seems unlikely that highly complex tissues and organs can be generated in vitro.

A possible solution is to differentiate ES cells in an in vivo environment—for example, by placing them in blastocysts where they can differentiate through the normal course of embryonic development. However, due to various ethical and practical constraints, human ES/iPS cells cannot be routinely placed into human blastocysts. We therefore wished to explore methods for differentiating ES/iPS cells of one species in the blastocysts of an evolutionarily divergent species.

Several reports had previously demonstrated the feasibility of creating interspecies chimeras by aggregating early embryos from two different species. Of these, viable chimeras have been obtained for only three pairs of species: between *Mus musculus* and *Mus caroli* (two laboratory mouse species), *Ovis aries* (sheep) and *Capra hircus* (goat), and *Bos taurus* (cow) and *Bos indicus* (zebu) (*3–6*). For each of these, the two species in the pair are rather closely related in evolutionary distance, differing in genome sequence by no more than 3%. Thus, it was unclear whether viable chimeras from two species separated by a much greater evolutionary distance could be made by merging embryonic cells. Specifically, we wished to know if it was possible to create interspecies chimeras by injecting ES cells of one species into the blastocysts of another divergent species.

We addressed this question by attempting to construct interspecies chimeras between *Apodemus sylvaticus* (wood mouse) and *Mus musculus* (house mouse) by injecting *Apodemus* ES cells into *M. musculus* blastocysts. These two species are separated by a rather large evolutionary distance, with an ~18% genome sequence difference. (By comparison, humans and Old-World monkeys such as the macaque are separated by an ~8% sequence difference.)

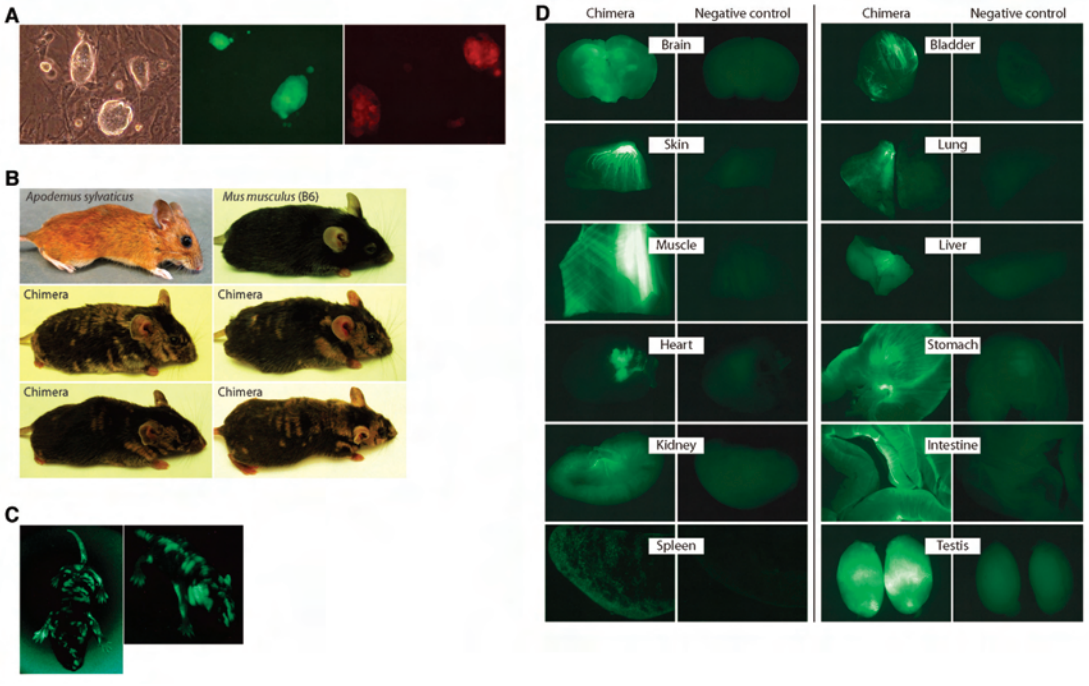

Figure 1. Images of *Apodemus sylvaticus* (AS) ES cells and *Apodemus–Mus* chimeras. (**A**) Left: phase-contrast image of AS-ES1 colonies; middle panel: green fluorescence image of AS-ES1 colonies expressing humanized Renilla GFP (hrGFP1); right: red fluorescence image of AS-ES1 colonies expressing DsRed fluorescent tag. (**B**) Photographs of *A. sylvaticus*, *M. musculus* B6, and four different chimeras. (**C**) Green fluorescence images of two different chimeras created with AS-ES1-hrGFP1 cells. (**D**) Green fluorescence images of unsectioned organs in a chimera. Two images are presented for each organ, the left from the chimera and the right from a negative control B6 animal.

We first derived ES cells from *Apodemus*, which remained undifferentiated after prolonged passage (Figure 1A), were karyotypically normal with 48 chromosomes, and expressed several pluripotency markers including alkaline phosphatase, Oct4, Nanog, and Rex1. We then showed that *Apodemus* ES cells could form embryoid bodies in vitro, which differentiated into all three germ layers (*7*). We further showed that *Apodemus* ES cells could also form teratomas when injected into nude mice. The teratomas contained all three germ layers based on histological examination (*8*).

We next introduced *Apodemus* ES cells into mouse blastocysts by microinjection and used female ICR outbred mice as surrogates to carry the blastocysts to term. About 7% of the animals born from injected blastocysts were chimeras based on coat color (Figure 1B). Chimerism can also be clearly visualized based on green fluorescent protein (GFP) fluorescence when we used GFP-positive *Apodemus* ES cells for the

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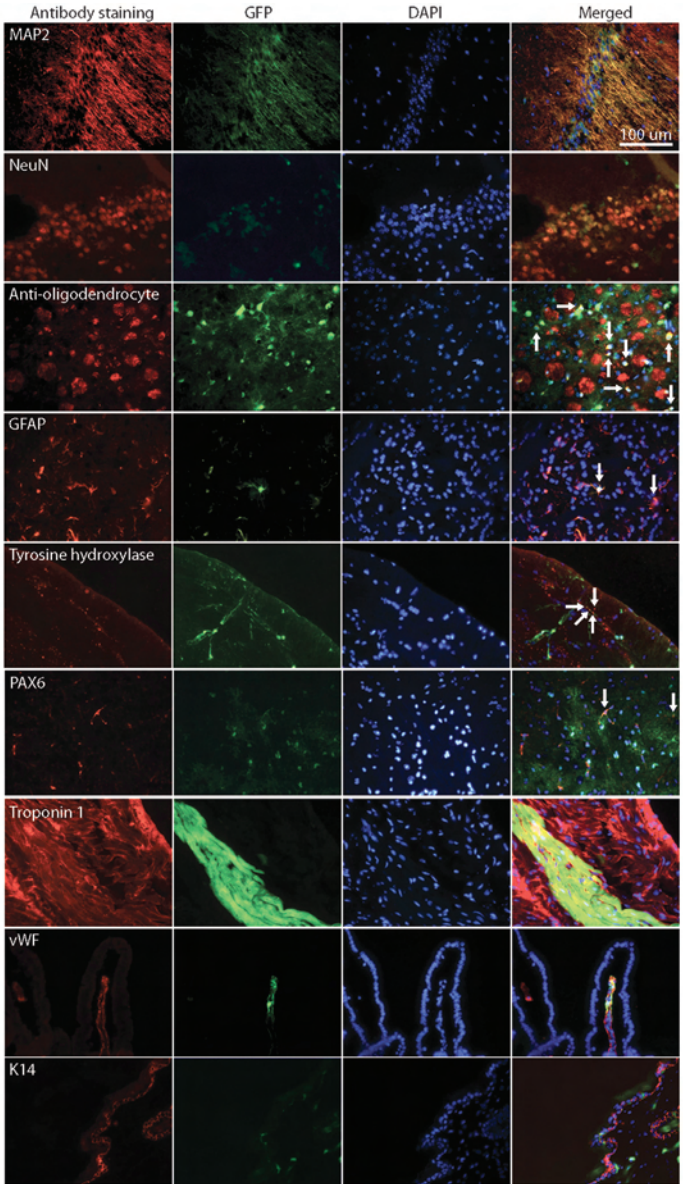

Figure 2. Immunofluorecence (IF) characterization of the cellular identity of *Apodemus*-derived cells in chimeras. Each section has four images in a row, which are (from left to right) IF staining using the antibody indicated, GFP fluorescence marking *Apodemus*-derived cells, DAPI stain marking nuclei, and merged image. The co-localization of IF signal and GFP indicates that *Apodemus*-derived cells have differentiated into cell types identified by IF. For merged images where co-localization between IF signal and GFP is not immediately obvious, arrows are used to indicate such co-localization. MAP2 and NeuN antibodies stain processes and cell bodies of neurons, respectively; anti-oligodendrocytes antibody stains oligodendrocytes and their myelin sheaths; GFAP antibody stains cell bodies of astrocytes; TH antibody stains axon terminals of dopaminergic neurons; PAX6 antibody stains cell bodies of adult neural stem cells; troponin 1 antibody stains cell bodies of cardiomyocytes; K14 stains cell bodies of epithelial cells; and vWF antibody stains cell bodies of endothelium. MAP2, NeuN, anti-oligodendrocytes, GFAP, and PAX6 staining was performed on cortical sections. TH, Troponin 1, K14, and vWF staining was performed on basal ganglia, heart, skin, and small intestine sections, respectively.

injection (Figure 1C). All chimeras appeared outwardly healthy, and thrived alongside their littermates. Based on GFP fluorescence, it is evident that all the internal organs of the chimeras carried significant contributions from *Apodemus* ES cells, including brain, skin, muscle, heart, kidney, spleen, bladder, lung, liver, stomach, intestine, and testis (Figure 1D). Using competitive PCR on a representative chimera, we demonstrated significant *Apodemus* contribution to most organs. The level of contribution ranged from a few percent to as high as 30–40% of cells in some organs, such as brain and muscle. The sperm of the chimera also had a 5% *Apodemus* contribution, indicating that *Apodemus* ES cells can contribute to the germline. Using markers for specific cell types, we showed that the *Apodemus*-derived GFP-positive cells have differentiated into a wide range of functional cell types, including neurons, oligodendrocytes, astrocytes, neural stem cells, cardiomyocytes, endothelial cells, and keratinocytes (Figure 2). Thus, *Apodemus* ES cells have contributed abundantly to all the tissues examined, where they differentiated into a wide range of cell types appropriate for the tissue (*8*).

Our results are notable in several regards. The first is the ability of *Apodemus* ES cells to fully integrate with the inner cell mass of mouse blastocysts to produce viable chimeras despite the considerable evolutionary distance separating these two species. The second is the extensiveness of chimerism in all the organs examined—as high as ~40% *Apodemus* contribution in some tissues. The third is the correct differentiation of the ES cells into a wide range of cell types intimately integrated into the host tissues. These results demonstrate the feasibility of differentiating ES cells into a wide range of cell types in vivo by introducing them into an evolutionarily divergent host.

Further genetic manipulation of either the ES cells or the host blastocysts could in theory increase or decrease the extent to which ES cells contribute to a particular tissue or organ. For example, if the host blastocysts are engineered to carry genetic defects that block the development of a particular tissue (e.g., a *Pdx1* mutation that leads to the agenesis of the pancreas), the percentage contribution of the ES cells to the affected tissue may increase dramatically. Recently, Kobayashi *et al.* indeed demonstrated the potential to generate functional rat pancreas in *Pdx1*$^{-/-}$ mice by injecting rat iPS cells into *Pdx1*$^{-/-}$ mouse blastocysts (*9*). Conversely, if the ES cells are engineered to carry genetic defects that prevent their differentiation down a particular lineage, then the ES cells won't contribute to that lineage in the chimeras. For example, contribution of ES cells to the brain could be minimized by knocking out a gene in ES cells essential for neural differentiation, which might help alleviate ethical concerns when placing human ES/iPS cells into nonhuman hosts.

The interspecies chimera system thus provides a proof of principle for the feasibility of differentiating pluripotent stem cells into a wide range of cell types—and perhaps even complex tissues—by allowing them to develop in vivo in an evolutionarily divergent host. This strategy might have important applications in regenerative medicine.

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Reprogramming MSCs Through In Vitro Differentiation and Dedifferentiation for Enhancing Therapeutic Potential In Vivo

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The overall effectiveness and clinical use of stem cells is largely limited by low levels of cell survival and desired differentiation in vivo (*1*). Our recent studies show that mesenchymal stem cells (MSCs) can be reprogrammed to enhance their in vivo therapeutic potential through in vitro differentiation and dedifferentiation.

Recent studies have shown that terminally differentiated mammalian cells can be manipulated in vitro to undergo dedifferentiation into induced pluripotent stem cells (iPSCs) through genetic reprogramming (*2*). While these reprogrammed cells offer promise for patient-specific regenerative therapy, the therapeutic potential of iPSCs is limited by low efficiency, immunogenicity, reprogramming errors, and genomic instability leading to cancer (*3*).

We investigated whether it was possible to induce dedifferentiation in culture without gene manipulation and obtain reprogrammed stem cells with improved therapeutic potential. We observed dedifferentiation in vitro when we studied the plasticity of adult rat bone marrow MSCs (*4*). After in vitro induction and differentiation into 5 hydroxytryptamine (5-HT)-sensitive neurons, these cells could revert back to a morphological and phenotypical state similar to that of MSCs upon withdrawal of neuronal induction medium. The dedifferentiated MSCs (DeMSCs) could undergo further expansion in vitro and could be induced to redifferentiate into 5-HT sensitive neurons again, indicating that these adult stem cells are more plastic than previously thought. This notion is further supported by later studies demonstrating that monoclonally derived MSCs could cross the oligo-lineage boundary and become cells of unrelated lineages (*5, 6*). While direct transdifferentiation has been proposed to be the major route of MSC plasticity, we have demonstrated that dedifferentiation is a prerequisite for changing cell fate and redifferentiating into a different lineage, i.e. from neurons to epithelial cells or vice versa (*6*). In manipulating cell fates of MSCs in vitro, we made an interesting observation that cells that had undergone dedifferentiation exhibited stronger differentiation potency, i.e. had higher neuronal differentiation rates and neuronal marker expression levels when induced to redifferentiate. This suggests that DeMSCs may not be identical to their original MSCs counterparts.

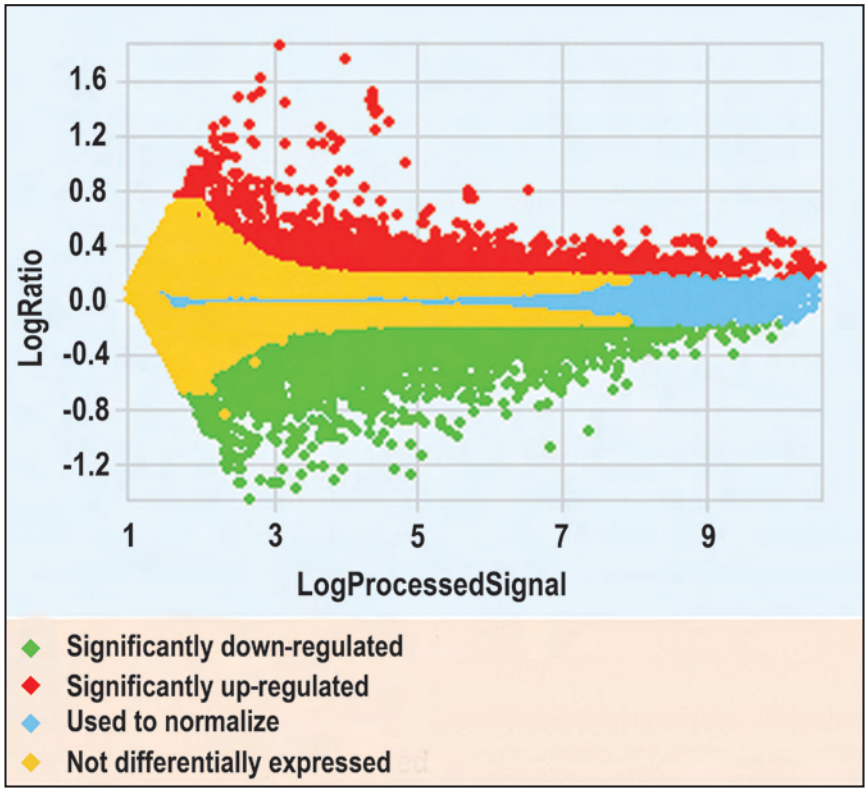

Figure 1. Scatterplot showing differential genes expressed in DeMSCs vs. MSCs as assessed by a rat whole genome microarray. Red: up-regulated genes (650/41012, 1.5%) in DeMSCs; Green: down-regulated genes (1240/41012, 3%); Yellow: genes not differentially expressed.

In a recent study, we set out to characterize DeMSCs and compare them to the undifferentiated MSCs (*7*). While DeMSCs were found to retain their immunophenotypic characteristics and mesodermal potential, detailed analysis of gene expression profiling showed 650 (1.5%) genes up-regulated and 1,240 (3.0%) genes down-regulated by more than twofold in DeMSCs compared to undifferentiated MSCs (Figure 1), indicating that DeMSCs had been reprogrammed. Further analysis showed that 13 out of 84 apoptosis-related genes examined were differentially expressed between MSCs and DeMSCs, with enhanced expression of bcl-2 family proteins in DeMSCs. Enhanced survival

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in DeMSCs was also demonstrated with a greater number of viable cells, compared to MSCs, observed under oxidative stress. We also noticed that the expression of neuronal markers in DeMSCs was much higher than that in undifferentiated MSCs, indicating that DeMSCs maintained some neuronal traits, and therefore possibly greater potential for redifferentiation into neurons. The enhanced survival and neuronal potency of DeMSCs, which can persist up to three to four passages, prompted us to investigate the therapeutic potential of DeMSCs in vivo using a rat model of neonatal hypoxic-ischemic brain damage (HIBD), a common cause of long-term neurological disability in children with no effective treatments currently available (*8*). Our results showed that while both transplanted GFP-tagged MSCs or DeMSCs were readily found near the injection sites three days after transplantation, only DeMSCs could be detected by day seven (Figure 2A, 2B), with some co-localization with neuronal markers NF-M and MAP2, indicating enhanced survival and neuronal differentiation in vivo. Importantly, a shuttle box test to evaluate cognitive function (*9*) showed significantly greater improvement in DeMSC-treated animals compared to MSC-treated rats one to two months after cell transplantation. Taken together, our results suggest that MSCs can be reprogrammed through culture manipulations with enhanced therapeutic potential in vivo.

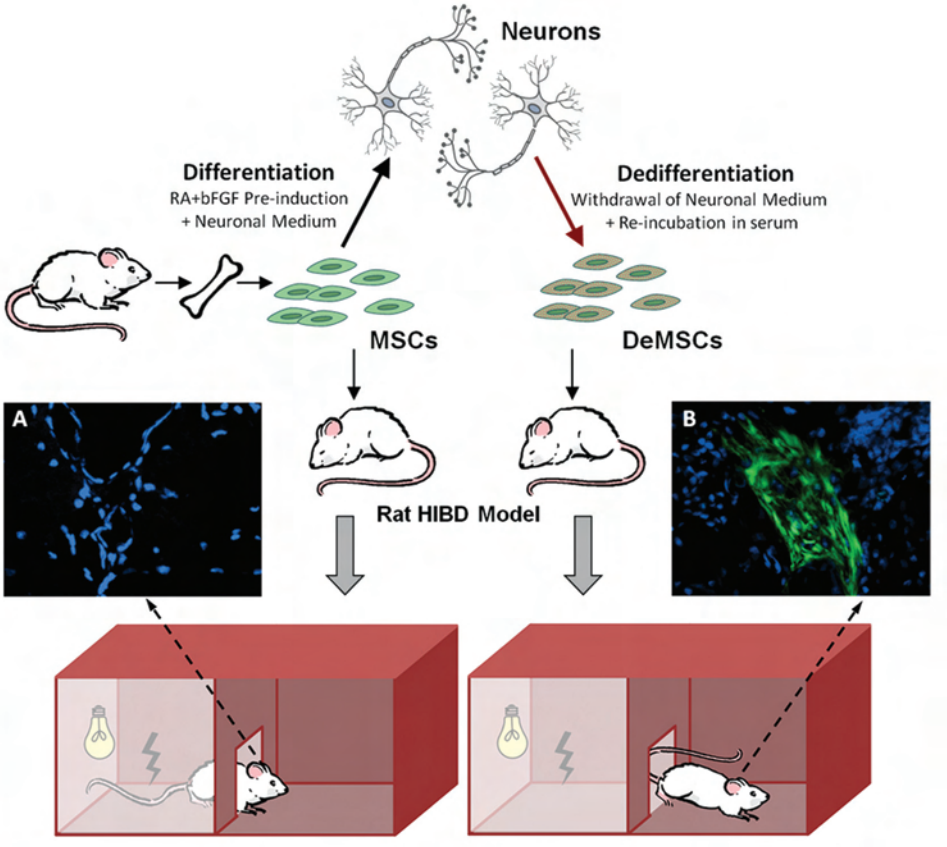

Figure 2. Schematic illustration of the experimental design and beneficial effects of DeMSCs in HIBD model (from reference *7*). To initiate neuronal differentiation, rat bone marrow derived monoclonal MSCs were first pre-induced with media consisting of DMEM/F12/10% FBS/10^{-7} M all-*trans*-retinoic acid (ATRA) and 10 ng/mL basic fibroblast growth factor (bFGF). Cells were washed with PBS and transferred to neuronal induction media composed of modified neuronal medium (MNM, DMEM/2% DMSO/200 µM butylated hydroxyanisole (BHA)/25 mM KCl/2 mM valproic acid/10 µM forskolin/1 µM hydrocortisone/5 µg/mL insulin) for 24–48 hours. Dedifferentiation was achieved by withdrawal of neuronal induction medium, and then reincubating cells in DMEM/F12 with 10% FBS for 24–48 hours. To determine the therapeutic effect of DeMSC in vivo, GFP-MSCs or GFP-DeMSCs were injected into the right lateral cerebral ventricle five days after the induction of hypoxic-ischemic brain damage (HIBD) rat model. Representative brain sections obtained seven days after transplantation show no GFP-expressing MSCs (**A**) but strong GFP signal from DeMSCs (**B**), indicating enhanced survival. Shuttle box test evaluating learning and memory ability was performed, demonstrating significantly greater improvement in DeMSC-treated animals as compared to the MSC-treated rats one to two months after cell transplantation.

Our findings provide a novel and clinically practical method to overcome the hurdles faced by current MSC-based therapy. The dedifferentiation-driven reprogramming in culture may prove to be a simpler, safer, and more effective approach in regenerative medicine compared to iPSCs. Interestingly, the genes known to be responsible for inducing iPSCs are not significantly altered in DeMSCs, suggesting different mechanism(s) involved. In particular, significant upregulation of miR-34a was observed in DeMSCs and was linked to enhanced cell survival and neural potentiality, hinting at the possible involvement of an epigenetic mechanism in the reprogramming. It remains unclear whether the dedifferentiation-driven reprogramming is limited to MSCs committed to neuronal lineage differentiation. Further investigation will undoubtedly improve in vitro reprogramming of MSCs to maximize the therapeutic efficacy of DeMSCs as a novel and practical treatment strategy in regenerative medicine.

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Nonhuman Primates: Important Animal Models for Regenerative Medicine

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There are more than 400 species of nonhuman primates (NHPs) in the world. China—one of the few areas with natural NHP habitats—is home to 24 of these species. As our closest relatives, NHPs are genetically more similar to humans than other model animals. They are also more similar to humans in both anatomy and physiological function, especially with respect to the central nervous system. Thus, NHPs make ideal animal models—and sometimes the only models—with sufficient validity to effectively study certain human disease. In the last two decades, advances in stem cell research have brought researchers closer to the therapeutic goal of replacing defective tissue with in vivo-engineered cells. However, a number of technical hurdles must be overcome before therapies based on pluripotent human stem cells can enter the clinic (*1*). NHPs provide an ideal model to test the safety and efficacy of stem cell therapies prior to clinical trials on humans and therefore can play a pivotal role in their success.

Currently, the largest human-raised NHP populations are in the United States and China. In China alone, the total exceeds 200,000, consisting mainly of rhesus and cynomolgus monkeys. Along with the fast growth in numbers, the breeding facilities in China have made significant progress in achieving international standards for animal care. For example, Kunming Biomed International (KBI), which specializes in both NHP disease model development and contract research operation, has obtained Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accreditation for its world-class facilities and professional management. Following are three recent examples of progress in NHP research in China.

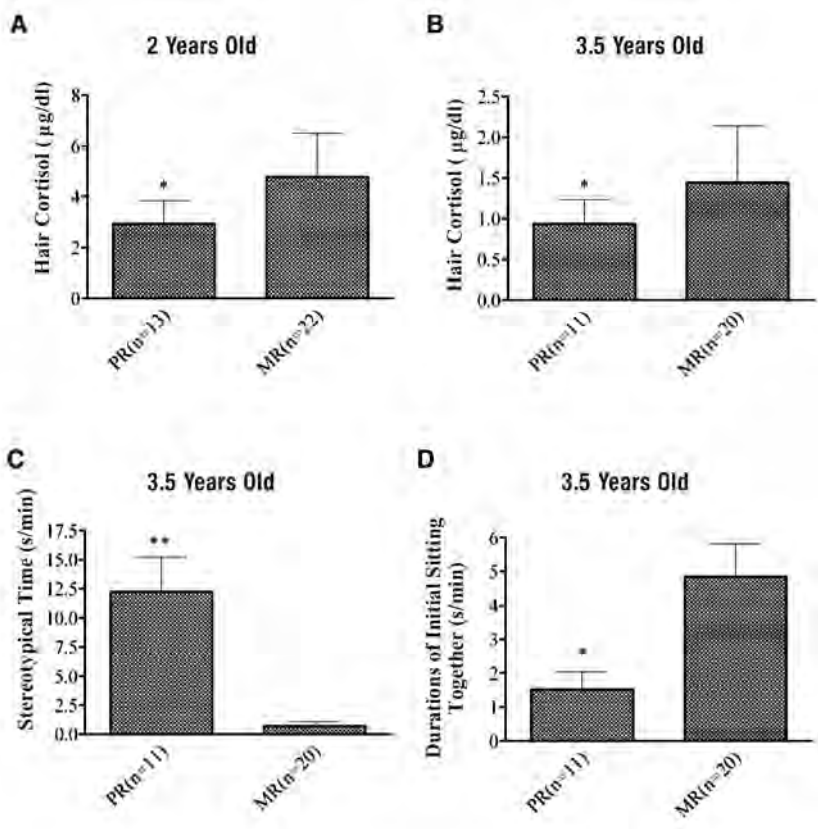

Figure 1. Comparison of hair cortisol levels (μg/dL; mean±SEM) between peer-reared (PR) (n=13, 11) and mother-reared (MR) monkeys (n=22, 20) at 2 years (**A**) and 3.5 years (**B**) of age respectively, and durations of stereotypical behavior (**C**) and sitting together (**D**) (mean±SEM) of the PR (n=11) and MR monkeys (n=20) at 3.5 years of age. These results demonstrate that the deleterious effects of MS on rhesus monkeys are long-lasting and cannot be compensated by a later normal social life. Note that 2 and 3.5 years of age are equivalent to 1.5 and 3 years of normal social life (starting at 0.5 years old), respectively.

Advances in Genomics

With the rapid advance of genome sequencing, several NHP genomes have been decoded. For example, whole genome deep sequencing of the Chinese rhesus macaque and cynomolgus monkey has recently been accomplished through the collaboration of several research groups from southern China (*2*). These two species are the most commonly used NHP models in biomedical research, especially neuroscience and drug discovery, and the identification of orthologs of human druggable genes as well as human putatively pathological missense alleles is extremely valuable in delineating the genetic basis of human diseases and for developing novel drugs. The similarity between humans and NHPs is also reflected at the cellular level. We have recently shown that the microRNA profile of embryonic stem cells from rhesus macaques is much closer to humans than that of mice, confirming the utility of developing preclinical models of cell therapy using NHPs (*3*).

Early Separation Studies

Another recent advance has been in the area of developing a model for human early adversity. Maternal separation (MS), which can lead to hypothalamic-pituitary-adrenal axis dysfunction and behavioral abnormalities in rhesus monkeys, is frequently used to model early adversity. Whether this deleterious effect could be reversed by a later normal living experience was unknown. In our study, we compared the cortisol levels in mother-reared (MR) and peer-reared (PR) rhesus monkeys after 1.5 and 3 years of normal social life (equivalent to 2 and 3.5 years old, respectively) following early MS. These results showed that PR monkeys had significantly lower basal hair cortisol levels than the MR

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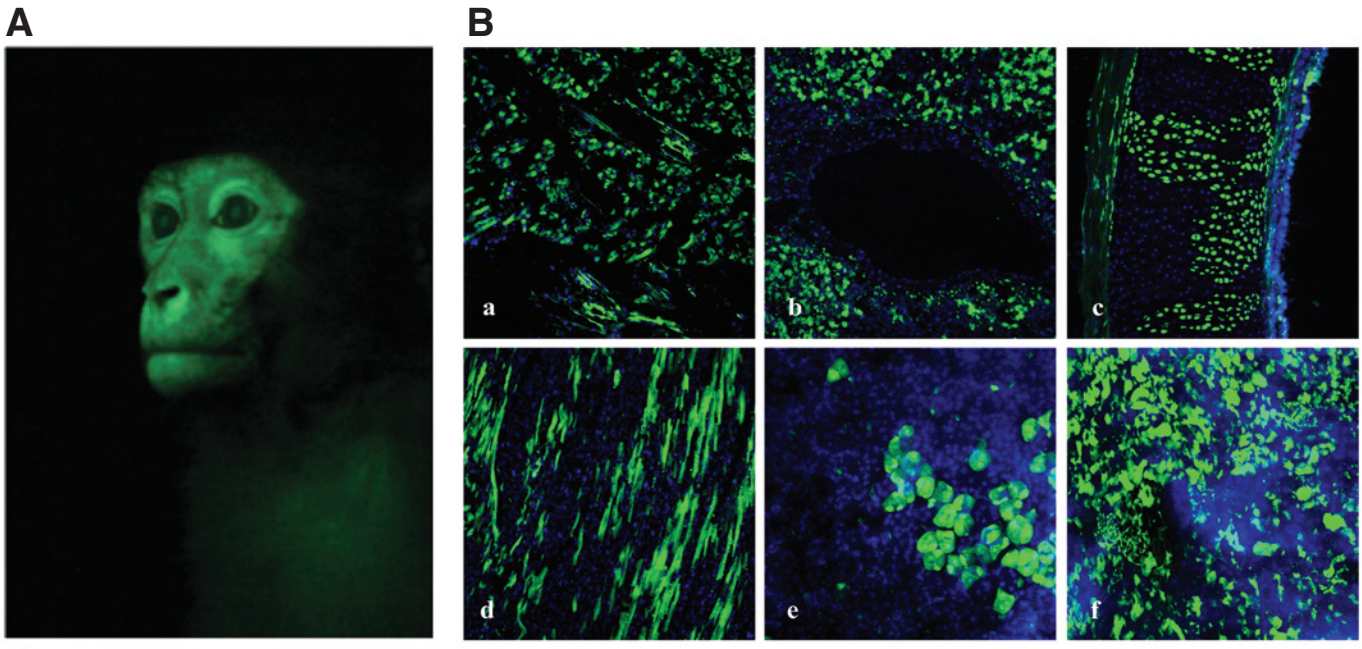

Figure 2. Transgenic rhesus monkey. (**A**) Transmission light image of transgenic-positive monkey (two years old); (**B**) Confocal microscope analysis of GFP expression (green) on frozen sections of brain (a), liver (b), windpipe (c), heart (d), stomach (e), and kidney (f) prepared from a GFP-positive fetus.

monkeys at both time points examined. The plasma cortisol was also assessed after 1.5 years of normal social life, and the results indicated that the peak PR cortisol response to acute stressors was substantially delayed. In addition, after 3 years of normal social life, abnormal behavioral patterns were identified in the PR monkeys. They showed decreases in locomotion and initiations of sitting together, as well as increases in stereotypical behaviors (repetitive, unvarying actions that appear to have no goal or function, including pacing, digit sucking, self-grasping, rocking, bouncing, and lip-smacking) compared with MR monkeys (Figure 1). These results demonstrate for the first time that, in contrast to findings from rodents (*4, 5*), the deleterious effects of MS on rhesus monkeys are long-lasting and cannot be compensated by a later normal social life (*6*). Therefore, MS in rhesus monkeys may provide a good animal system to model early adversity in humans and to investigate the development of psychiatric disorders induced by exposure to early adversity.

Transgenic NHPs

As another avenue for disease research, transgenic technology provides the opportunity to induce diseases or symptomatologies afflicting humans that do not occur naturally in NHPs. Such investigations may further our understanding of the genetic etiology of disease and assist in the development of new therapies. To date, there have been few reported successes in producing transgenic monkeys (*7–9*). The low-efficiency of monkey transgenesis at present hinders its application to clinically relevant disease studies. Recently, we developed an improved methodology for producing of transgenic rhesus monkeys, making use of a simian immunodeficiency virus (SIV)-based lentiviral vector that encodes enhanced green fluorescent protein (EGFP), and a protocol for infection of early-cleavage–stage embryos. Importantly, infection does not alter embryo development. Moreover, the timing of infection, either before or during embryonic genome activation, has no observable effect on the level and stability of transgene expression. One transgenic fetus was obtained from a fraternal triple pregnancy. In another case, four infant monkeys were produced from four singleton pregnancies, of which two expressed EGFP throughout the whole body (Figure 2) (*10*). These results demonstrate the usefulness of SIV-based vectors for generating transgenic monkeys and improving the efficiency of transgenic technology in nonhuman primates. Based on this progress, we are currently developing a transgenic monkey model for human neurodegenerative diseases, such as Parkinson's and Alzheimer's.

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Studying Stem Cell Biology Using In Utero Transplantation in Animal Models

Fanyi Zeng, M.D., Ph.D.

Mouse embryonic stem cells (ESCs) were first isolated in 1981 (*1, 2*) and human pluripotent stem cells were cultured from blastocysts in 1998 (*3*). Since then stem cell research has grown and thrived due to its great promise for disease treatment and regenerative medicine. The generation of induced pluripotent stem cells in 2006 (*4*) provided an especially promising tool for personalized medicine. While ESCs were derived from the inner cell mass originally to recapitulate the developmental potential of early embryos in in vitro systems, the in vivo differentiation, distribution, and migration of pluripotent stem cells remains unclear. Establishment of animal models by stem cell transplantation may offer a useful in vivo system to study these processes in stem cell biology.

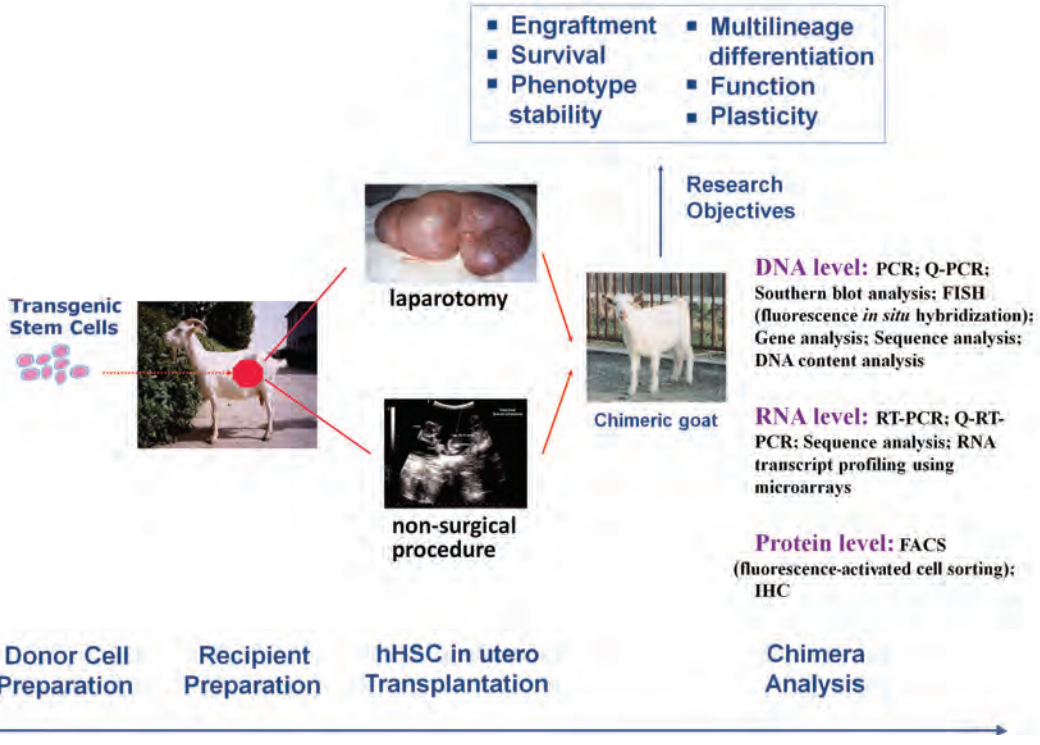

Figure 1. Experimental procedures for generating an in vivo stem cell model via in utero transplantation.

Multi-tissue engraftment and differentiated functions can be achieved with allogenic or xenogenic cells via in utero stem cell transplantation (IUSCT), providing a possible approach for establishing animal models to investigate the biological functions of transplanted stem cells in vivo. Briefly, allogenic or xenogenic stem cells were injected into the peritoneal cavities of fetal goats at 45–55 days of gestation (full-term gestation is 145 days) under sonographic guidance (displaying a transect of the peritoneal cavities) or laparotomy by using a #7 piercing needle (Figure 1). The former means of IUSCT has a significantly lower miscarriage rate than that observed for the latter open abdominal surgery technique for IUSCT (*5*). This was an efficient and safe method routinely used for our experiments. Several stem cell types, including human hematopoietic stem cells, mouse embryonic stem cells, and mouse induced pluripotent stem cells, were transplanted into fetal goats to establish chimeric models (*5–7*).

The following results were observed in an experiment to create chimeric goats by transplanting human cord blood cells. By transducing the cells with a GFP marker before transplantation, the engrafted GFP^+ cells could be detected by GFP fluorescence, fluorescence-activated cell sorting (FACS) analysis, and immunohistochemistry (IHC) in multiple organs, including kidney, muscle, liver, spleen, heart, and lung of the recipient goats (Figure 2). GFP^+ cells were observed to have clustered distributions within each tissue in all tissue types examined (Figure 2D). Polymerase chain reaction (PCR) of several human-specific genomic loci confirmed the existence of human cells in goat tissues (Figure 2A). Reverse transcription PCR (RT-PCR) and IHC analysis revealed human tissue-specific RNA and protein expression in multiple hematopoietic and nonhematopoietic organs, including lung, kidney, spleen, and liver (Figures 2B and 2C). Whole-genome RNA expression profiling in blood and liver samples further confirmed the expression of human genes in the chimeric animals.

Human/goat chimerism provides a large animal model for studying stem cell biology in a live organism. However, in this case the biology of engrafted stem cells was being evaluated in a xenogenic background. To avoid this, an allogenic stem cell transplantation mouse model was established. Because of their small size, short breeding cycle, and defined genetic background, mice provide an alternative way to study stem cell biology in vivo. Enriched populations of stem cells were usually injected mid-gestation (typically at E14.5) into the fetal abdomen using a surgical approach. By developing a new intrauterine transplantation instrument and modifying existing surgical procedures, we successfully performed stem cell injection as early as E12.5, a significant technical challenge, and improved the efficiency of generating chimeric mice (*8*).

Disease models can be generated using either the xenogenic or allogenic approach when artificially or naturally defective stem cells are transplanted into healthy fetuses. By transplanting fetal goats in utero with Lin^-CD34^+ human cord blood cells transduced with BCR-

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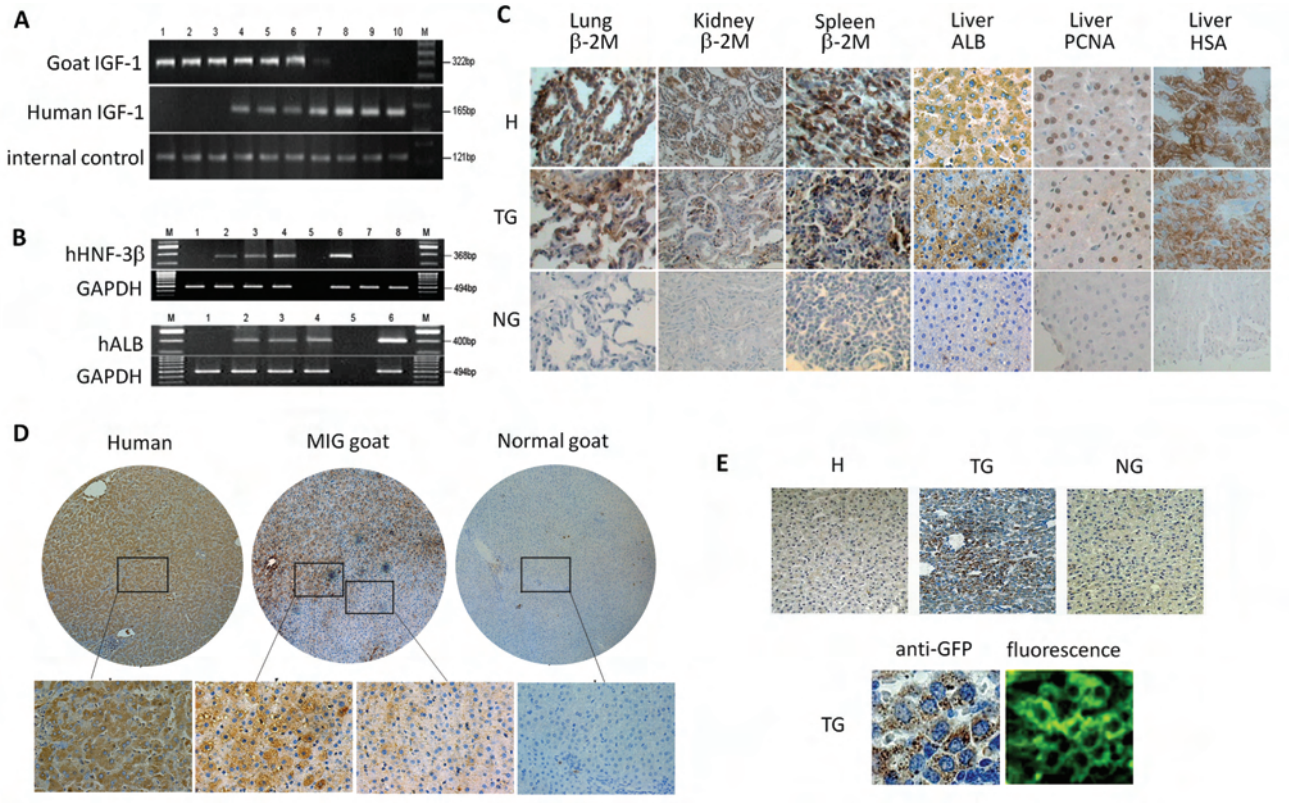

Figure 2. Molecular analysis of chimeric stem cell goat model (from reference *7*). (**A**) Species-specific PCR of IGF-1 DNA in liver samples from: Lanes 1–3, normal goats; lanes 4–6, transplant goats; lane 7, GFP+ goat liver cells; lanes 8–10, human samples. (**B**) RT-PCR analysis of human gene transcripts for hepatocyte nuclear factor 3β and serum albumin (hALB) expressed in the transplant liver tissue (lanes 2–4) or human liver tissue (lane 6) and CB cells (lanes 7–8), but not in the control goats (lane 1). Lane 5 is blank. (**C**) Immunohistochemical analysis for human β2 microglobulin antigen, hALB, proliferating cell nuclear antigen, and hepatocyte-specific antigen in various tissues of human (H) samples, transplant goat (TG) and normal goat (NG) (magnification: 400x). (**D**) Staining for hALB was performed on sections of human, transplant goat (MIG), and normal goat livers (magnification: 50x and 200x). (**E**) Upper panel shows anti-GFP staining (brown) was present in the liver cells (cytoplasm) of MIG-transplant goat (TG), but not in human or normal goat (NG) (magnification: 100x). (**E**) GFP+ cells in the liver of TG with immunohistochemistry staining, and corresponding images with fluorescence emission (magnification: 400x).

ABL retrovirus, a chronic myeloid leukemia (CML) animal model that mimics the initiating early chronic phase of CML was established (unpublished data), and may serve as a tool for research on CML pathogenesis and drug development.

When healthy stem cells are transplanted into a disease-prone fetus, the engrafted stem cells can protect the animal from disease onset or symptoms; this therapeutic effect makes IUSCT a potential tool for prenatal treatment of genetic diseases or protection against future cell or tissue injury. For example, in a mouse model using chemically induced liver injury using CCl_4, the differentiation of in utero-transplanted hematopoietic stem cells into human hepatocyte-like cells was demonstrated by detecting the expression of human hepatocyte-specific proteins and the partial repair of liver damage (*9*). This mouse model provides a useful tool to study the regeneration of human hepatocyte-like cells from transplanted stem cells. It also demonstrates the therapeutic potential of in utero transplantation for protecting individuals at high risk for liver disease or injury.

In summary, chimeric animal models established through IUSCT provide a unique system for studying immune tolerance and the kinetics of stem cell engraftment, homing, differentiation, gene expression, and plasticity under nondisease conditions. They may also help to evaluate the clinical potential of prenatal treatments for a number of human genetic diseases or prophylaxis for those likely to need future cell or tissue repair, or xenogeneic organ transplantation. In the near future and with in-depth studies of stem cell biology, IUSCT could become a routine regenerative medicine approach for clinical treatments of genetic defects and disease risks.

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Mesenchymal Stem Cells Hold Great Promise for Regenerative Medicine Treatments

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Mesenchymal stem cells (MSCs), a type of adult stem cell, have generated a great deal of interest over the past decade in the field of regenerative medicine (Table 1). We have isolated and culture medium-selected a subpopulation of cells with extensive proliferation ability and immunomodulatory function. To explore their potential application in disease treatment, we first evaluated their safety with both in vitro and in vivo experiments. Flk1$^+$ MSCs did not form teratomas in mice when injected in vivo and

First author reference	Description	No. of patients	Disease	HLA-matching locus	Follow-up period in months (median range)	Outcome
Kaiyan Liu *et al.* (*7*)	Open-label, randomized phase II clinical study to assess the outcome of MSC coinfusion during haploidentical HSC transplantation	Control group (n=28); Treatment group (n=27)	ALL,AML,CML	3 locus (Control 16; Treatment 17) 4 locus (Control 10; Treatment 6) 5 locus (Control 2; Treatment 4)	Control group 24.6 (0.7–33.5); Treatment group 23.5 (3.8–32.5)	Coinfusion of MSCs facilitates platelet recovery without increasing leukemia recurrence in haploidentical hematopoietic stem cell transplantation
Mei Guo *et al.* (*8*)	New haploidentical NST regimen that consisted of a haploidentical peripheral blood stem cell transplantation combined with IBMI of donor-derived MSCs, modified nonmyeloablative conditioning, and GVHD prophylaxis	Treatment group (n=33)	ALL,AML,CML-AP	3 locus (n=19) 4 locus (n=10) 5 locus (n=4)	8 (1.5–60)	Coinfusion of MSCs facilitates successful engraftment and mild GVHD in modified haploidentical non-myeloablative transplantation without T cell depletion for high-risk acute leukemia
Hong Zhou *et al.* (*9*)	Case report	Treatment group (n=4)	ScGVHD after HSC in ALL,AML,MM patients	-	14.4 (4.6–23)	MSCs infusion in the treatment of ScGVHD is therapeutically practicable, with no detectable side effects.

Table 1. Clinic applications of MSC. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CML-AP, chronic myelogenous leukemia in accelerate phases; HSC, hemopoietic stem cell; IBMI, intrabone marrow injection; MM, multiple myeloma; NST, nonmyeloablative stem cell transplantation; ScGVHD, sclerodermatous chronic GVHD.

MSCs (Flk1$^+$CD44$^+$CD29$^+$CD105$^+$CD166$^+$CD34$^-$CD31$^-$Lin$^-$) named Flk1$^+$ MSCs from a variety of human tissues. These are multipotent can even inhibit malignant cell proliferation by secreting DKK-1 (*1*). We carried out a preclinical trial to further evaluate the feasibility and safety of intravenous infusion of these cells after ex vivo expansion. Rhesus monkey and human Flk-1$^+$ MSCs were isolated from bone marrow, expanded, and transplanted into rhesus monkeys and human volunteers, respectively. During the infusion of these cells, vital signs of the recipients were all normal. Laboratory tests for blood, bone marrow, kidney, and liver function were conducted and no significant changes were observed after infusion of cells (*2*). Flk1$^+$ MSCs are the first

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stem cell product to receive approval for clinical trials from the Chinese State Food and Drug Administration (SFDA).

After confirming the safety of Flk1$^+$ MSCs for clinical application, we began to evaluate their efficacy for treating several diseases in two clinical trials. The first study found that a new transplantation strategy combining haploidentical peripheral blood stem cells (PBSCs) and Flk1$^+$ MSCs could improve donor engraftment and prevent severe graft-versus-host disease (GVHD). Thirty-three patients with high-risk acute leukemia underwent transplantation with PBSCs from HLA-haploidentical donors without T cell depletion. All of the patients achieved full donor chimerisms, including six (18.2%) who switched to full donor chimerisms from mixed chimerisms within one to two months after transplantations. Rapid hematological engraftment was observed with neutrophils >0.5x10^9/L at day 11 and platelets >20x10^9/L at day 14. Fifteen patients (45.5%) developed grade I–IV acute GVHD (aGVHD) and only two (6.1%) developed grade III to IV aGVHD. Nine (31%) of 29 evaluable patients experienced chronic GVHD (cGVHD). Upon follow-up for 1.5 to 60 months, 20 (60.6%) patients were alive and six (18.2%) had relapsed leukemia out of the original 33 patients. The probability of three-year survival was 57.2%.

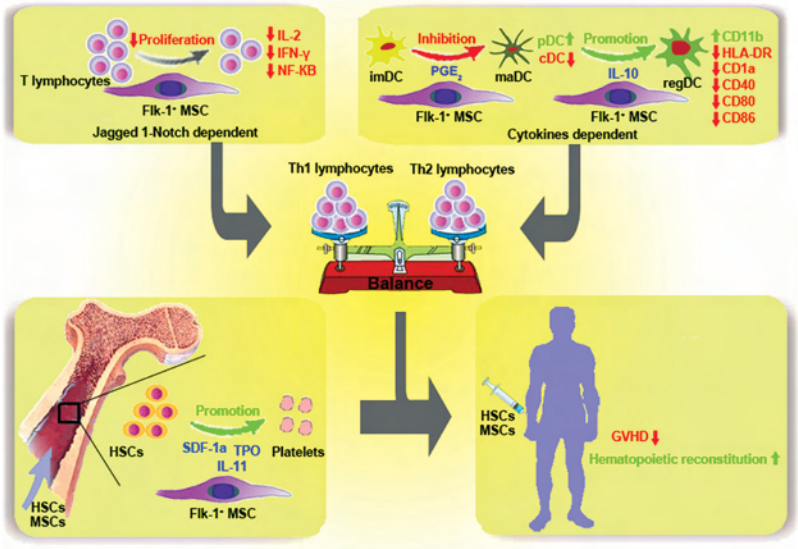

Figure 1. Coinfusion of MSCs improved outcomes of haploidentical hematopoietic stem cell transplantation. MSCs have the ability to inhibit T lymphocyte proliferation and inflammatory cytokine secretion through the Jagged-1-notch signaling pathway, and can also inhibit dendritic cell maturation by secreting cytokines such as PGE2 and IL-10. Hence, one of the possible mechanisms of MSCs ability to ameliorate aGVHD and cGVHD may be that they are involved in maintaining Th1/Th2 balance through immunomodulating T lymphocyte and dendritic cells. Another possible mechanism may be that MSCs can elevate concentrations of SDF-1α, thrombopoietin (TPO), and interleukin-11 in recipients and facilitate platelet recovery. MSC, mesenchymal stem cell; HSC, hematopoietic stem cell; DC, dendritic cell; imDC, immature DC; maDC, mature DC; regDC, regulatory DC; pDC, plasmacytoid DC; cDC, conventional DC; GVHD, graft-versus-host disease.

The second study was an open-label, randomized phase II clinical trial to assess the outcome of MSC coinfusion (3–5x10^5 cells/kg) during haploidentical hematopoietic stem cell transplantation. Over the course of a year, a total of 55 patients who were diagnosed with leukemia in complete remission were enrolled and randomized to either the treatment group (n=27) or the control group (n=28). No immediate side effects related to MSC infusion were noted, and the median times of white blood cell and platelet recovery were comparable between the two groups. However, within 100 days, the time to a platelet concentration of >50x10^9 cells/L was markedly faster in the treatment group compared with the control group (22 days vs. 28 days; P=0.036). Stromal-derived factor-1α (SDF-1α) reached a peak concentration more rapidly in the treatment group compared with the control group (8 vs. 16 days). The concentrations of thrombopoietin (TPO) and interleukin-11 were also elevated in the MSC-treated group compared with the control group. These data suggested that the MSCs may promote platelet survival by secreting hematopoietic stimulating factors, such as SDF-1α, TPO, and IL-11, which may recruit CD34t HSCs and provide a microenvironment that supports hematopoiesis. The accumulative occurrence rate of aGVHD greater than grade 2 was 51.8% and 38.9% in the treatment and control groups (P=0.422), respectively, whereas the incidences of cGVHD were 51.4% and 74.1% (P=0.261), respectively.

We also investigated the possible mechanisms underlying the immunomodulatory effects of Flk1$^+$ MSCs which are mediated through interacting with a wide range of immune cells or secreting bioactive molecules. Flk1$^+$ MSCs facilitate the immunosuppressive effect of cyclosporin A on T lymphocytes through Jagged-1-mediated inhibition of NF-κB signaling (*3*). In the presence of Flk1$^+$ MSCs, the percentage of cells with a conventional dendritic cell phenotype is significantly reduced whereas the percentage of plasmacytoid dendritic cells increased, thus biasing the immune system towards Th2 and away from Th1 responses (*4*). Interestingly, Flk1$^+$ MSCs could drive mature dendritic cells to differentiate into a novel Jagged-2-dependent regulatory dendritic cell population and escape their apoptotic fate, providing more evidence to support the role of Flk1$^+$ MSCs in rejection prevention during organ transplantation and treatment of autoimmune disease (*5*). In BXSB mice that were born with an immunologic deficiency, Flk1$^+$ MSCs can significantly down-regulate Th2 cells and further inhibit the abnormal activation of humoral immunity to maintain the original balance (*6*). Based on studies in our lab, we generated a schematic diagram of the immunomodulatory effects of Flk1$^+$ MSCs both in vitro and in vivo (Figure 1).

In conclusion, our studies demonstrate that Flk1+ MSCs represent a safe and effective treatment for some hematological disorders. We are now carrying out more clinical investigations to extend their potential applications in other conditions.

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Endogenous Regeneration of Pancreatic Islet Cells

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Diabetes is a global health problem and each year more than seven million people are newly diagnosed. In China, it is estimated that at least 3% of population suffers from diabetes. Currently, insulin replacement therapy is the gold standard for treatment, but can't always prevent the development of diabetic complications. Further, the transplantation of a new pancreas or islets has yet to overcome the issues of immune rejection. Through recent research, we have found that pancreatic duct ligation can induce pancreaticoduodenal ampulla, causing obstruction and dilation of the main pancreatic duct (Figure 1A). The head and uncinate process of the pancreas were removed, and samples were taken from the surrounding dilated main pancreatic duct. As a control, we used samples from 10 patients who had undergone pancreatic surgery without pancreatic duct obstruction or dilation. The specimens were cut into sequential 5 μm sections, and stained for insulin, Ki67 (a marker of cell proliferation), and with DAPI. Our results demonstrated evidence of β-cell

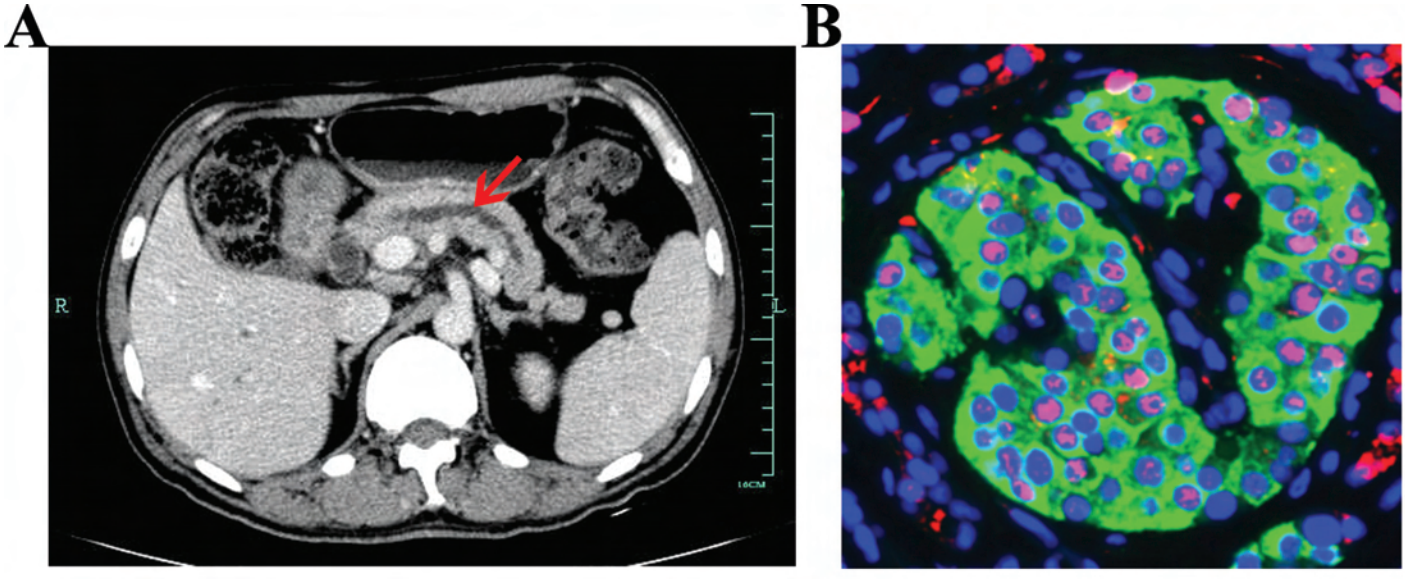

Figure 1. (**A**) Computed tomography scan showing dilated pancreatic duct (red arrow) in human study, identical to the pancreatic duct ligation model in rodents. (**B**) Immunofluorescent staining for Ki67 (red), insulin (green), and DAPI (blue) in human pancreas (magnification: 400x). The pink area indicates Ki67 and insulin double-stained cells, which demonstrates increased β-cell proliferation.

pancreatic regeneration, which could potentially engineer an islet organ serving diabetic management.

Though the number of pancreatic β-cells is fixed after birth, it has been observed that the endocrine component of the pancreas has a regenerative capacity under certain circumstances, including pancreatectomy and pregnancy. We attempted to investigate if pancreatic duct obstruction (similar to partial duct ligation) could prompt β-cell proliferation in humans. We collected pancreatic tissues from 60 patients who underwent radical pancreaticoduodenectomy using a Whipple resection. All patients received surgery due to a tumor located in the regeneration induced by pancreatic duct dilation in humans, as shown in Figure 1B. Eighteen of 60 patients with pancreatic duct obstruction and dilation showed significant β-cell proliferation as shown by an increase in cells that were positive for both Ki67 and insulin after pancreatic duct obstruction. Based on this study, similar experiments were carried out in eight-week-old male C57BL/6 mice to develop a surgical model for partial duct ligation. Ligation of the splenic portion of the pancreatic duct or a sham operation was performed, and pancreatic tissues from the splenic lobe distal to the ligated site were removed at 1, 3, 5, 7, 14, or 28 days, or 2, 4, or 8 months postoperation. We found β-cell regeneration and the presence of Ki67-positive β-cells was significantly increased in mice with partial duct ligation compared to the sham surgery group (Figure 2A). However, fasting glucose concentrations were similar before and after the operation in the two groups. These results indicate that partial duct ligation surgery in mice is able to induce pancreatic β-cell proliferation.

To further investigate if the induced pancreatic β-cells share a similar microenvironment to the original β-cells, we examined the tissue architecture of pancreatic tissue in mice from the above study. We found that the exocrine tissue of the pancreas was replaced by nonfunctioning

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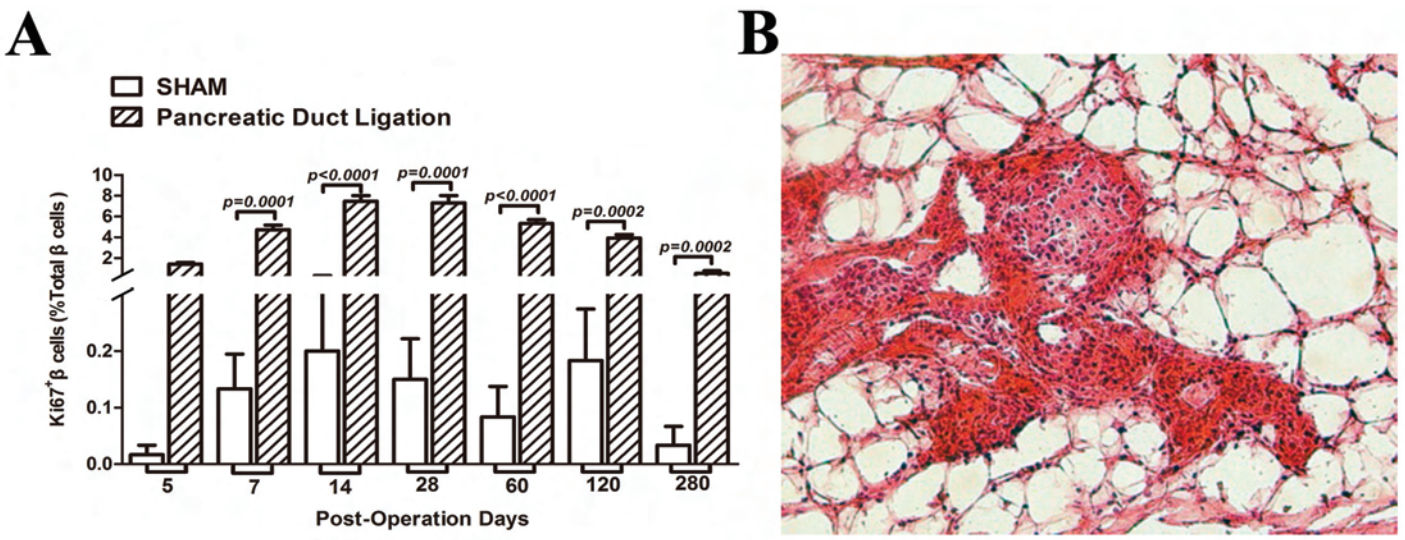

Figure 2. (**A**) Quantitative analysis of β-cells regeneration following pancreatic duct ligation in mice. A bar graph with a broken Y axis was used to allow compact display of extreme values. Results are expressed as Ki67 positive β-cells (% total β-cells) and represent means ± SEM (n=6 animals per group). (**B**) Histological staining of pancreas two months after pancreatic duct ligation (magnification: 200x). The exocrine tissue of the pancreas was completely replaced by nonfunctioning fat tissue, but with intact endocrinal function and active β-cell proliferation.

adipose tissue, which occupied the splenic lobe in a similar shape to the original by two months after partial duct ligation, leaving the islets separated from the extracellular matrix framework. We described this observation as "the persimmon tree in fall" based on histological observations (Figure 2B). Though all the leaves have fallen off, the surviving trunk is covered with vibrant fruits. In support of this observation several clinical studies demonstrated that as long as chronic pancreatitis followed by duct obstruction does not produce destructive damage to the pancreas, most of the islet functions are preserved even when exocrine tissue is lost (*1–5*).

Recently, so-called metabolic surgery has become increasingly popular. For some endocrine diseases, metabolic surgery represents a shift from drug- and behavior-based treatments to surgical intervention. There are three common surgical procedures: Roux-en-Y gastric bypass (RYGB), laparoscopic adjustable gastric banding (LAGB), and bilio-pancreatic diversion (BPD), all of which could affect food intake and/or nutrition absorption (*6–8*). Bariatric surgery was initially an operation intended only for obese patients, but it has been shown that this surgery induces not only drastic weight loss and decreased nutrient absorption, but also a series of metabolic changes, including dramatically improving glucose and lipid metabolism, and even remission of type 2 diabetes and hyperlipidemia (*6–8*). Although the observation and mechanisms are still controversial, some investigators claim that surgical modification of the gastrointestinal tract can alter hormonal regulation of the intestine—through the regulation of glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic peptide receptor (GIPR) (*9, 10*). Others believe that macrophages and T cells in the intestine play a critical role in normalizing metabolic homeostasis. In fact, some argue that type 2 diabetes is an inflammatory disease and components of the immune system are dysfunctional in type 2 diabetes patients, affecting levels of specific cytokines and chemokines (*11*). In our study, we have shown that surgically induced partial duct ligation of pancreas in mice and human can promote pancreatic β-cell proliferation. Although the actual mechanisms are still unknown and require further investigation, it is possible that the inflammatory process in the exocrine tissue environment in the pancreas may contribute to the process of β-cell proliferation.

In summary, we have provided evidence of pancreatic β-cell proliferation by induced partial duct ligation of pancreas. The study hints at the possibility that building an "islet organ" may depend on the microenvironment of the pancreas and that removal of exocrine cells can trigger islet β-cell regeneration.

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Epidermal Basal Cells: Support for More Than Epidermis Regeneration?

Weidong Han, M.D., Ji Lin, M.D., Meixia Chen, M.S., Cuiping Zhang, Ph.D., Xiaobing Fu, M.D.*

The skin is the largest organ in the human body and is composed of two layers, the epidermis and the dermis. Skin contains several different types of stem cells, including epidermal, melanoblast, mesenchymal, and neural-like stem cells (*1*). Emerging evidence suggests that epidermal stem cells exist in at least three distinct niches: the hair follicle bulge, the base of the sebaceous gland, and the basal layer of the epidermis (*2*). These different compartments undergo constant cellular turnover to replace dead or damaged cells.

Undifferentiated epidermal cells located in the basal layer of the epidermis not only contribute to the homeostasis of the interfollicular epidermis under physiological conditions, but also participate in the re-epidermalization of injured skin (*2, 3*). Differentiated epidermal cells can even dedifferentiate into epidermal stem cell-like cells under chronic wound conditions in the skin (*4*). They have also been shown to have similar phenotypic and functional characteristics to epidermal stem cells (*5*).

Compared with other types of stem cells in the skin, keratinocytes from the epidermal basal layer are readily accessible and easily cultured and expanded in vitro (*6, 7*). Because of their plasticity, somatic stem cells are considered to be a potential cell source for regenerating various tissues. Although some of the stem cells harbored in the skin can be induced to differentiate into other cell types and thus manifest potential applications for tissue regeneration, they are difficult to isolate and require long-term culture to achieve sufficiently large numbers.

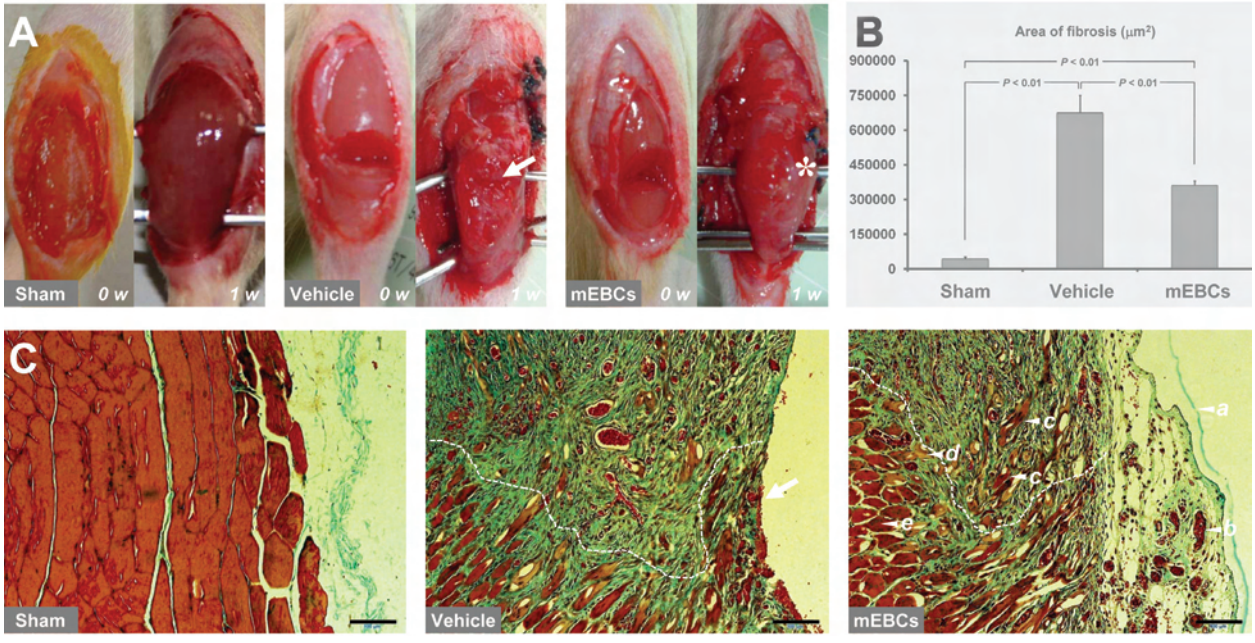

Figure 1. Macroscopic improvement in rats injected with mouse epidermal basal cells (mEBCs). (**A**) The lacerated tibialis anterior muscle was filled with well-formed tissues (star) in the mEBCs group at 1 week (1w) after injury, while there were no such signs of repair (arrow) in the Vehicle group. (**B**) The area of fibrosis in the indicated groups was calculated and expressed as mean±S.E.M. (**C**) The fibrotic area stained blue by Masson trichrome staining was significantly decreased and replaced with regenerated epimysium (*a*), enriched microvessels (*b*), scattered neonatal myofibers (*c*), and hypertrophic muscle fibers (*d*) in the mEBCs group, while larger fibrotic areas, unhealed defects (arrow) and thinner muscle fibers were found in the Vehicle group. Relatively yellow-stained muscle fibers (*e*) represent the fibers located at the original injury site (white dashed line, C). Sham: sham-operation; Vehicle: lacerated injury + vehicle injection; mEBCs: lacerated injury + 1×10^6 mEBCs injection; *0 w*: immediately after laceration; *1 w*: 1 week after laceration. Scale bars represent 100 μm.

Epidermal basal cells (EBCs), although abundant and enriched with undifferentiated cells, have been considered to be unipotent and thus unusable for tissue regeneration, except for epidermal reconstruction. However, this belief was challenged by our recent findings that these EBCs can be induced to differentiate into other cell types under appropriate conditions (*8*).

To exclude interference from hair-follicle-derived stem cells, hairless human foreskin epidermis was used to investigate the multipotency of EBCs. Isolated EBCs were cultured in EpiLife medium, which allows only the growth of keratin-positive keratinocytes, promotes robust proliferation of EBCs, and efficiently prevents their differentiation for at least five passages. EBCs transdifferentiation was induced using serum or lineage-committed medium. Unfortunately, EBCs rapidly underwent full epidermal differentiation under pure inductive conditions. We therefore developed an acclimatization induction strategy that involved

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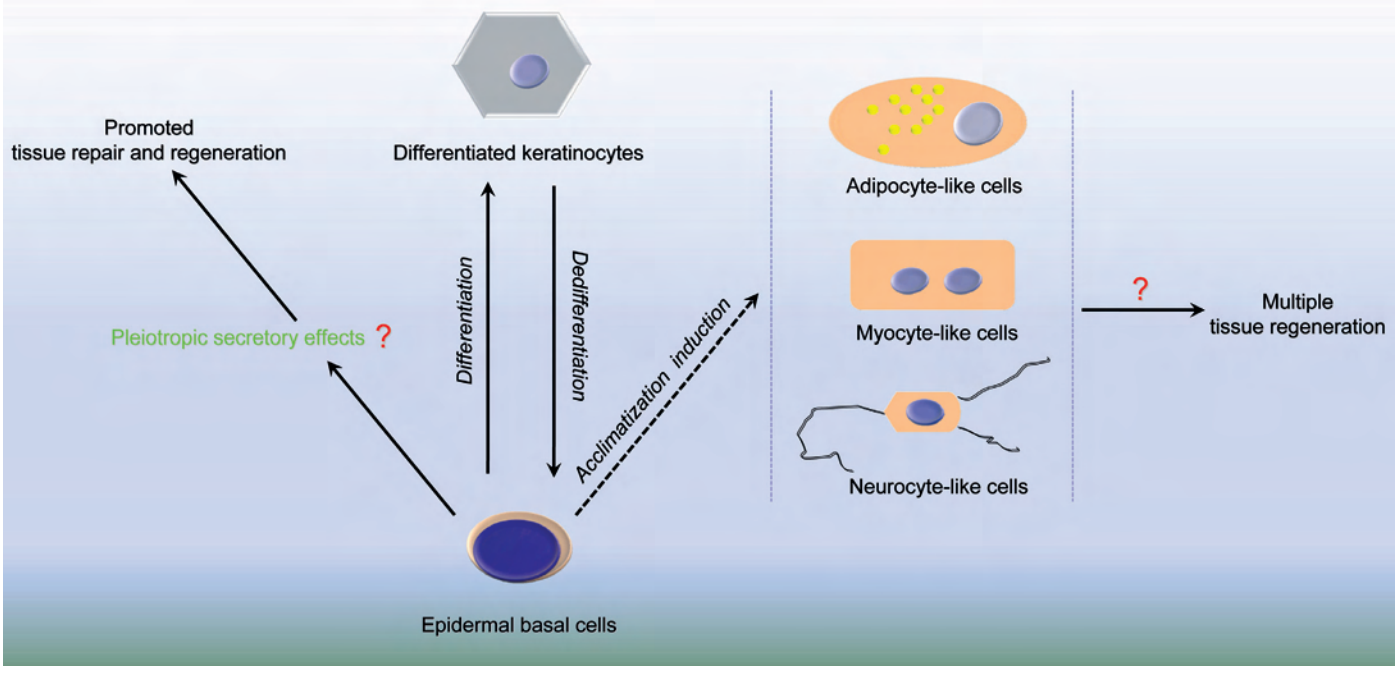

Figure 2. Schematic illustration of the potential application of epidermal basal cells in tissue repair and regeneration except for re-epidermalization of skin.

gradually introducing the induction medium into the EpiLife medium. The multipotent nature of EBCs could be expressed by adjusting the ratio between the two media. Three weeks of serum acclimatization induced EBCs to produce a large number of smooth-muscle-like cells and fewer adipocyte- and neurocyte-like cells. In contrast, except in terminally differentiated epidermal cells, lineage-specific acclimatization induced EBCs to differentiate exclusively into adipocytic, myogenic, or neurogenic lineages.

Given the potential plasticity of other multipotent adult stem cells, we investigated the suitability of EBCs for use in tissue regeneration other than epidermal reconstruction. An immunocompromised rat model of skeletal muscle injury was established using the left tibialis anterior muscle, as previously reported (*9*). This experiment involved a sham-operated group (Sham), a lacerated injury + vehicle injection (Vehicle) group and a lacerated injury + 1×10^6 mouse EBCs injection (mEBCs) group. Vehicle or cells were injected into the lacerated site as a single dose. After one week, the lacerated regions were significantly occupied with bridging tissue in rats in the mEBCs group, while the lacerated regions in the Vehicle group showed no such signs of repair (Figure 1A). However, further analysis showed that mEBCs had already disappeared from the lacerated regions and surrounding areas by this time point (data not shown). Pathological analysis with Masson trichrome staining (Figure 1B and 1C), in more than 10 rats per group, showed that the impaired epimysium had reappeared 1 week after mEBCs injection, under which enriched microvessels could be observed. The fibrostic area within the lacerated region was smaller and hypertrophic muscle fibers were observed at the bottom of the lacerated region in the mEBCs group, compared with the Vehicle group. Intriguingly, sparse myofibers within the fibrotic area were only consistently observed in the mEBCs group, suggesting that mEBCs injection promoted myogenesis. These results indicate that EBCs can efficiently improve the rate and extent of healing of skeletal muscle injuries. Further studies are underway to identify the mechanisms responsible for this process. The versatile improvement of the lacerated area induced by mEBCs and the apparent absence of surviving transplanted EBCs within the lacerated area after only 1 week suggest that these cells may play pleiotropic trophic roles in repair and even regeneration of injured skeletal muscles, and that these beneficial roles may rely on their paracrine potential. The role of cell secretary effects in promoting tissue repair and regeneration, e.g., by mesenchymal stem cells, is becoming an attractive topic of investigation (*10*).

Based on the above results from our laboratory, the potential application of EBCs in tissue repair and regeneration is summarized in Figure 2. The discovery of the plasticity and putative pleiotropic secretory effects of EBCs opens a window for future research into the application of these cells. However, several basic questions remain to be answered, such as the relationship between the functional characteristics of EBCs and the exact cell phenotype. If dedifferentiation-derived epidermal stem-like cells possess similar multipotency and potential paracrine effects to EBCs, they will provide a more plentiful and convenient source of cells than other types of adult stem cells for the repair and regeneration of tissues other than skin.

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Human Perinatal Stem Cell Banking: Experiences and Perspectives for Regenerative Medicine

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There is great promise for the role of stem cells in regenerative medicine. Since HLA-matched adult organ donors are not always available, stem cells derived from several birth-associated perinatal tissues—including cord blood, placenta, and umbilical cord—can be banked as a safeguard against future life-threatening conditions requiring replacement tissue (*1, 2*). Long-term storage of stem cells from different sources is a way to preserve multipotent tissue for possible future clinical applications. Since 2002, we have worked on banking cord blood hematopoietic stem cells (CB-HSCs) for clinical use (*3*) and at present the number of stored CB-HSC samples is about 150,000 units. Previously, the use of CB-HSC transplantation was limited to pediatric patients, but the recent discovery by several groups (*4*) that a large number of HSCs are present in placenta has allowed us to develop a placenta HSC bank—opened in 2010—that will increase the therapeutic utility of perinatal HSC in adult patients in the near future.

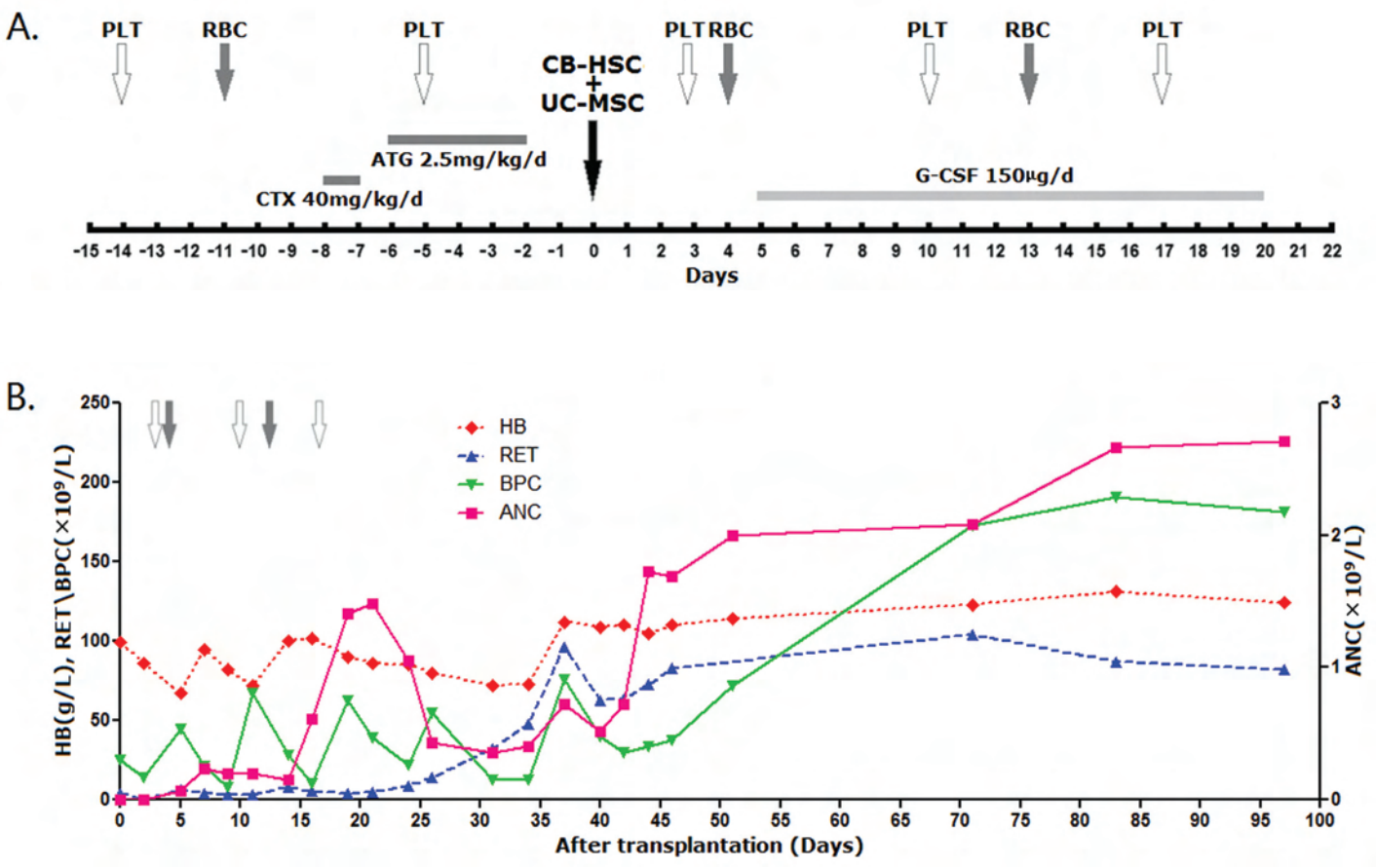

Figure 1. Treatment regimen for very severe aplastic anemia. (**A**) Treatment started with cyclophosphamide (CTX) 40mg/kg/d for 2 days (days -8 to -7), then rabbit thymoglobin (ATG) 2.5 mg/kg/d over 5 days (day -6 to -2). At day 0, the patient (weight 20 kg) received an infusion of 5.31 x 10^8 cord blood hematopoietic stem cells (CB-HSCs; 1.058 x 10^6 $CD34^+$cells) and 2 x 10^7 umbilical cord mesenchymal stem cells (UC-MSCs). The patient received several transfusions of platelets (PLT, white arrows) and red blood cells (RBC, grey arrows) before and after stem cell transplantation. Granulocyte-colony stimulating factor (G-CSF; 150 μg/d) injections were performed from days 5 through 20. (**B**) Hematopoietic recovery was complete 36 days after co-transplantation

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The Application of MSCs

Mesenchymal stem cells (MSCs) are multipotent stem cells present in all tissues, but particularly rich in perinatal tissues (*5, 6*). MSCs are being tested in a number of clinical trials and show great promise for clinical application. In 2006, we established a quality-controlled biobank of perinatal MSCs that now contains over 10,000 samples, providing an excellent source of MSCs for preclinical and clinical investigations (*2*). We have also developed standardized cell banking procedures in which we assay cell phenotype, purity, biological function, genetic stability, tumorigenicity, and microbial contamination. To determine the safety of isolated MSCs, several in vivo studies were performed in mouse and monkey models. In tumorigenicity studies, following subcutaneous transplantation of perinatal MSCs at various doses into NOD-SCID mice, no tumor formation was observed up to three months after treatment. In monkeys, multiple administrations of perinatal MSCs were done by intravenous injection once every two weeks for six weeks, with a dose of $2x10^6$ or $1x10^7$ cells/kg body weight. The study showed no toxicity of the transplanted cells in any of the animals (*7*). We also performed pilot clinical studies using banked perinatal MSCs for treatment of multiple sclerosis (*8*) and type 2 diabetes (*9*). The results indicated that the allogeneic perinatal MSCs were safe and effective. Further trials are in progress or planed for treatment of other refractory diseases.

Transplantation with HSCs

CB-HSC transplantation from HLA-identical siblings has shown promising results in children, supporting targeted efforts to bank autologous or family perinatal stem cells (*1*). Recently, we treated a four-year-old child with very severe aplastic anemia (VSAA) by co-transplanting autologous CB-HSCs and umbilical cord MSCs (UC-MSCs). The patient was admitted for skin hemorrhage spots, mouth ulcers, and fever. During hospitalization, his blood cell count progressively decreased and severe pancytopenia was seen 10 days later. He was diagnosed with VSAA based on bone marrow aspirate and biopsy results, as well as chromosome analysis, serology, and hemolysis. He was treated by co-transplanting autologous CB-HSCs and UC-MSCs previously isolated at the child's birth (*2*). The treatment regimen is shown in Figure 1A. The patient's clinical manifestations quickly improved and hematopoietic recovery was complete 36 days after cell transplantation (Figure 1B). To our knowledge, this is the first successful treatment of VSAA using autologous perinatal HSC and MSC co-transplantation.

In summary, our data suggest that banking of perinatal stem cells offers unique opportunities to speed up clinical translation of stem cells as "cell medicine" for both autologous and allogeneic therapies.

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Porcine Corneal Equivalent for Xenographs

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The severe shortage of donor corneal tissue is a significant problem throughout the world, and especially in China. There are only about 4,000 corneal transplantations performed annually in China, leaving more than four million patients on the waiting list. In the past decade, significant efforts have been made to develop substitutes for human donor corneal tissue. Biosynthetic corneal matrix, i.e., tissue-engineered cornea substitute, is believed to be a good solution (*1, 2*).

Corneal stroma composes more than 90% of the thickness of the cornea and contributes to optical transparency. Therefore, reconstruction of corneal stroma is the major challenge in corneal tissue engineering. Previous studies using synthetic polymers or natural collagen proteins have encountered difficulties in generating a corneal stromal equivalent with high transparency and strength. As a result, researchers tend to use corneal stromal matrices from other species. In recent years, porcine cornea has attracted much attention, due to its similarity to human cornea in thickness, topography, and stable refractive status (*3*).

The application of porcine tissue in humans, however, must overcome the obstacles of xenogenic rejection and the potential transfer of pathogens from pig to human. Because the cornea is a relatively immune-privileged tissue without blood vessels, xenotransplantation using fresh porcine corneas—which include live cells—will not cause hyper-acute rejection as with other, vascularized organs, such as the liver or kidney (*4*). Instead, rejection normally happens after two to four weeks. It has been proposed that removing corneal cells could reduce xenogenic rejection and the risk of pathogen transfer. To achieve this goal, different methods have been developed in recent years to generate an acellular corneal stromal matrix (*5–7*). Although some decellularization methods have shown high efficiency in removing stromal cell components, there are still concerns regarding the feasibility of the matrix generated from those procedures. First, the decellularization procedures can also remove part of the extracellular matrix, especially soluble matrix components such as glycosaminoglycan, including lumican and keratocan, which are important for keratocyte phenotype maintenance and corneal stroma remodelization. Second, the decellularization procedure may disorganize corneal stromal collagen fibers, compromise corneal transparency, and cause permanent opacity of the stromal tissue or a slow regaining of corneal transparency after transplantation. Third, most of the protocols introduce chemical reagents, such as detergents or enzymes, into the corneal tissue, the residues of which could damage the host tissue (*8*). Moreover, most decellularization procedures take two to five days, with multiple steps, making quality control in mass production difficult.

In order to keep the native structure of the corneal stromal matrix, it is generally accepted that the less manipulation required, the better the tissue reconstruction. Therefore, we proposed that it might not be necessary to remove the keratocytes from porcine cornea in the matrix preparation. Instead, devitalization of the keratocytes may be sufficient to generate a feasible matrix for corneal tissue engineering. The in situ dead keratocytes may not induce a strong immune reaction, and the dead cells will gradually degrade over a relatively long period of time after transplantation, which may reduce the immune reaction during tissue remodelization. A recent study using dehydrated porcine cornea in rhesus lamellar corneal transplantation showed a much lower rejection rate (*9*), which supports the notion that dead cells left in the corneal matrix may not be a significant issue for porcine-to-human xenotransplantation.

Based on this concept, we applied a simple method to process porcine corneas. Instead of dispase digestion, the epithelium and endothelium of the fresh porcine cornea were removed with a cell

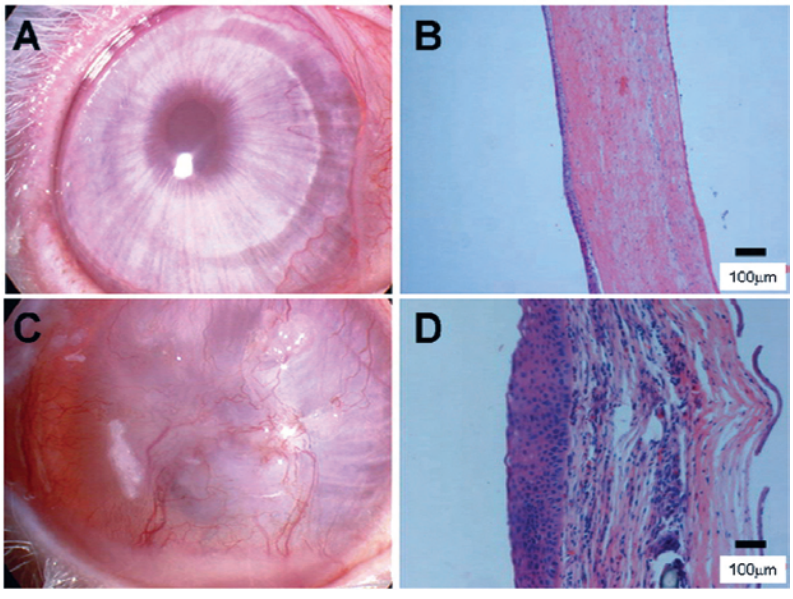

Figure 1. Slit lamp microscopic and histologic images of rabbit cornea with xenogenic porcine corneal lamellar keratoplasty. (**A**) Six months after rabbit lamellar keratoplasty, cornea transplanted with devitalized porcine corneal matrix showed high transparency without neovascularization. (**B**) H&E staining of the cornea with devitalized porcine corneal matrix showed normal corneal thickness, three to four layers of epithelial cells, relatively lower keratocyte density in the anterior stroma, and indistinguishable border between donor and recipient tissue. (**C**) Six months after rabbit lamellar keratoplasty, cornea transplanted with fresh porcine corneal matrix showed opacity of the entire cornea, with severe neovascularization. (**D**) H&E staining of the cornea with fresh porcine corneal matrix showed dramatically increased corneal thickness, highly stratified epithelium, and significant cell infiltration in the graft tissue.

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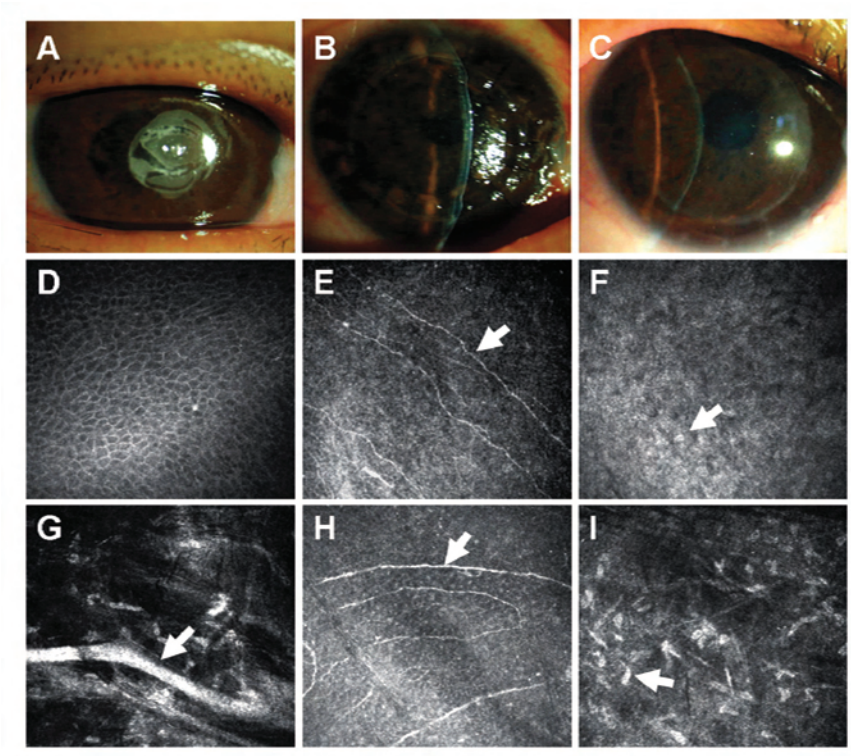

Figure 2. Slit lamp microscopic and in vivo confocal microscopic images of a patient's cornea. (**A**) The patient's right eye showed a calcified corneal epithelium and anterior stroma before keratoplasty. (**B**) Five days after surgery, the graft was re-epithelialized and showed mild edema. (**C**) Fifteen months after surgery, the graft was transparent. In vivo confocal microscopy showed (**D**) a regular epithelium at a depth of 35 µm, (**E,** arrow) subepithelial nerve plexus, and (**F,** arrow) few keratocytes in the central graft stroma three months after the surgery. Fifteen months after surgery, (**G,** arrow) the nerve plexus was present in the central graft stroma at a depth of 200 µm and (**H,** arrow) the subepithelial nerve plexus became denser. Moreover, (**I,** arrow) keratocytes dramatically increased in the central graft stroma at a depth of 110 µm.

scraper. Subsequently, the corneal stromal tissue was frozen with liquid nitrogen for 1 minute and thawed for 10 minutes; three rounds of this procedure were conducted to devitalize the keratocytes, followed by dehydration in a lyophilizer for 8 hours. After that, the porcine corneal tissue was sterilized by exposure to a radiation dose of 25 kGy from a cobalt 60 source. The corneal matrix was then ready for use.

We first tested the immunogenicity of the corneal matrix in rabbits by subcutaneous transplantation, and found that all porcine corneas matrices with live cells were rejected within four weeks, while there was no rejection of matrices with devitalized cells after eight months of observation. To further test the efficacy in corneal transplantation we set up a larger study. Porcine corneal matrices were rehydrated in Hank's Balanced Salt Solution for 10 min and the lamellar tissue was prepared, with a diameter of 5 mm and thickness of 200 µm. Rabbit corneal lamellar keratoplasty was performed using this tissue, with fresh porcine corneas containing live cells acting as a control. We followed 26 animals (half of which were controls) for six months. The results showed that all the fresh transplants were rejected in 2–12 weeks, while there was no rejection of the devitalized transplants. Furthermore, the devitalized corneal matrices maintained high transparency, while the fresh corneal transplants displayed severe opacity and neovascularization. Histology examination showed normal epithelium and stromal cell repopulation in the devitalized matrix, and the interface between the donor tissue and the recipient tissue was indistinguishable. In contrast, the fresh corneal transplants displayed hyper-proliferative epithelium, numerous cell infiltrations in the stroma, and dramatically increased stromal thickness (Figure 1).

We also used such a porcine corneal matrix for human lamellar keratoplasty in one case of corneal stromal dystrophy. We found that the graft was completely re-epithelialized in five days. The subepithelial nerve plexus was detected three months after surgery, and keratocytes were found in the graft stroma 15 months after surgery by in vivo confocal microscopy (Figure 2). The long-term graft survival, with high transparency, stromal cell repopulation, and nerve ingrowth in the graft tissue indicated that porcine cornea with minimal manipulation to devitalize resident cells may be sufficient for transplantation. Therefore, porcine cornea may serve as an ideal candidate in the constitution of tissue-engineered corneas.

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Tissue Engineering: An Important Component of Regenerative Medicine in China

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The field of tissue engineering has developed rapidly over the last three decades, becoming a challenging technological frontier in the life sciences and paving the way for the emergence of regenerative medicine as a viable mode of therapy (*1*). As its population has grown, China has seen an increased incidence of tissue and organ defects caused by accidental injury, natural disasters, and a variety of diseases. This increase is of concern and needs to be addressed urgently, in part through improvements in national medical and health services. However, these challenges also provide opportunities for the research and development of tissue engineering therapies in China. Since 2000, both the central government, as well as many local municipalities, in China have demonstrated significant support for research into stem cells and tissue engineering, establishing a number of tissue engineering laboratories and research centers where world-class work is now being performed.

Both policy and financial support have contributed to the rapid development of tissue engineering in China. The field has advanced through the initial stages of feasibility studies and the construction of tissue-engineered organs in nude mice. It is now entering the third stage with the construction of engineered organs and tissues in animals having normal immune function. As an indication of the progress, the number of articles published from Chinese laboratories related to tissue engineering present in the PubMed database has increased from 49 in 2000 to 654 in 2011. Additionally, of the 41,969 relevant articles published, 4,375 of them were from China, second in number only to the United States.

The growth of tissue engineering has led to interdisciplinary cooperation between a wide range of different research fields, including biology, molecular biology, materials science, computer science, and bioinformatics, and has also engendered the formation of a thriving biotechnology industry around translational tissue-engineered products (*2*). Recently, a type of tissue-engineered skin was approved for clinical application by the Chinese State Food and Drug Administration (SFDA). Furthermore, the efficacy of a variety of tissue-engineered corneas and nerves are being investigated in clinical trials, and some tissue-engineered cartilage, bone, and tendons are being produced or are in the process of product registration at the Chinese National Institutes for Food and Drug Control (NIFDC). In addition, many standards for producing tissue-engineered products have been or will be published. Indeed, the clinical translation of tissue-engineered products in China has become an important part of international industry.

However, many critical issues remain to be resolved before widespread acceptance in China of tissue engineering for clinical application. First, certain basic guidelines have not yet been agreed upon, such as a unified and mature platform with standard operation regulations for stem cell isolation, purification, and amplification. Moreover, critical questions still remain unanswered, including what is the optimal isolation method, what types of purified and cultured cells can be used for transplantation, what criteria should be used for the stem cell identification and classification, what training should be required to perform stem cell transplantations, and how results should be evaluated.

Second, the efficiency of translation from basic research to clinical implementation is not satisfactory and clinical results are uncertain; this is a worldwide problem for the tissue engineering industry (*1*). Historically, very few tissue-engineered products have made it through the rigorous clinical trial process; thus, tissue-engineered products are far from being regarded as mainstream treatments, and only a small proportion of the market share has been taken. Although over a hundred domestic patents and dozens of international patents related to tissue engineering have been filed in China, the first tissue-engineered product registration from the SFDA was not received until 2007. Thus, there exists a large gap between concept and realization of products coming out of tissue engineering research.

Third, a reliable safety evaluation system, necessary for the translation of research to clinical therapy, is still lacking and the relevant regulations and legal constraints are still being developed (*3*).

In response to these problems, strategies for product development should be reviewed (*4*). In China, early tissue engineering strategies focused on tissue construction and were directly used for tissue repair in large animals, resulting in a unique development path. However, certain fundamental issues need to be addressed first, such as characterizing the interaction between transplanted cells and their recipient microenvironment, determining how the differentiation of cells is controlled in vitro and in vivo, and investigating in more depth how seed cells function in vivo. Therefore, it is essential to continue to support and expand basic research in order to achieve the sustainable development of tissue-engineered products. Furthermore, expansion and intensification of interdisciplinary cooperation are essential. The tissue engineering industry should be expanded into different areas of medicine, and beyond, including the use of tissue-engineered products for the industrial production of high quality protein, as an alternative to animal testing for drug development, for toxicology screening, in pharmacogenetics research, and for the detection of biological or chemical hazards via a tissue sensor.

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Tissue Engineering for Myocardial Repair and Regeneration

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Heart failure is a leading cause of death worldwide (*1*), and current therapies only delay progression of the disease. Tissue engineering strategies present a new way of generating functional myocardium. There are two main strategies used in cardiac tissue engineering: the engineered heart tissue (EHT) approach and the injectable myocardial tissue engineering approach.

Embryonic stem cells (ESCs) can differentiate directly into cardiomyocytes, are capable of integrating with the host heart, and can improve electrical conduction. Therefore, ESCs could potentially provide an unlimited supply of cardiomyocytes for cell therapy to regenerate functional myocardium. However, clinically usable myocardial tissue generated from ESCs has yet to be produced. Therapeutic cloning, where the nucleus from a donor cell is transferred into an enucleated oocyte in order to extract pluripotent ESCs (nuclear transferred ESCs, nt-ESCs), offers a potentially limitless source of cells for tissue engineering applications. The use of transplantable tissue derived from therapeutic cloning may lead to the avoidance of immune responses that typically are associated with transplantation of non-autologous tissues. However, there are no published reports focusing on the ability of nt-ESCs to aid in engineered tissue construction and damaged tissue repair.

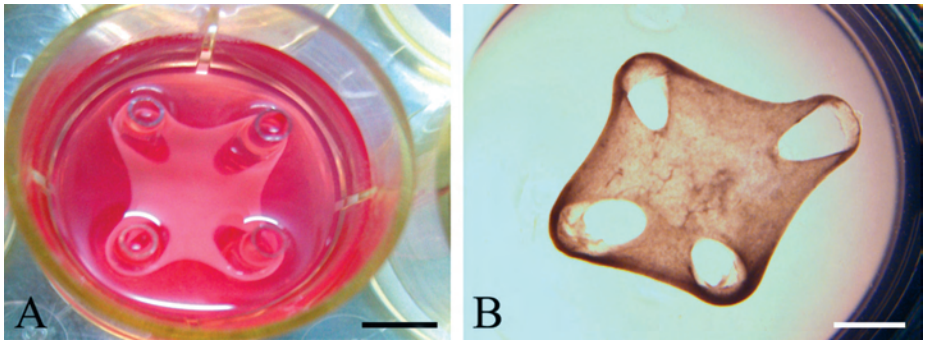

Figure 1. Macroscopic views of EHT (*6*). (**A**) EHT condensed and contracted gradually during cultivation in a self-made mold. (**B**) Spontaneously contracting EHT constructed by seeding cardiomyocytes derived from mouse nt-ESCs into liquid type I collagen. Upon removal from the stretching device, the EHT beat continuously at approximately 1.1 Hz for greater than 10 days. Scale bar, 5 mm.

Creating spontaneously contractile EHT by combining neonatal rat cardiomyocytes, collagen I/Matrigel scaffolds, and a mechanical stretching device is one of the most creative and promising approaches, first developed by the Zimmermann group (*2*). The feasibility of this approach has been documented in animal models (*3*). In the past few years, we have made extensive efforts to optimize our approaches and techniques to generate functional EHT. By using optimized cell seeding density of neonatal rat cardiomyocytes together with collagen and our own mechanical stretching system, we generated synchronously beating, three-dimensional EHT in vitro (*4*).

In an effort to explore whether EHT can be generated in vitro from ESCs, ESC-derived cardiomyocytes were obtained and cultured to produce embryoid bodies in a slow turning lateral vessel bioreactor, followed by cardiogenic differentiation and enrichment through a Percoll gradient. The enriched cardiomyocytes were then mixed with liquid type I collagen/Matrigel scaffold and stretched using a mechanical stretching device for seven days. Results showed that the EHT could beat synchronously and respond to physical and pharmaceutical stimulation. Histological, immunohistochemical, and transmission electron microscopic studies further indicated that the EHT both structurally and functionally resembled neonatal native cardiac muscle. No teratoma formation was observed for four weeks in the EHT implanted subcutaneously in nude mice (*5*). For the first time, we successfully produced engineered, spontaneously contracting cardiac tissue constructs in vitro from ESC-derived cardiomyocytes. This study illustrates the possibility of using ESCs for myocardial tissue engineering and cardiovascular disease therapy.

The task of generating EHT from ESCs is the beginning of a therapeutic cloning strategy for myocardial repair and regeneration. In theory, nt-ESCs carry the same genome as their donor somatic cells. After directed induction, the differentiated cells could rescue the damaged tissues without immune rejection. However, the persistence of abnormalities in cloned animals has introduced doubt as to whether nt-ESCs may pose risks in their therapeutic application. This perceived risk may lead one to question whether nt-ESCs can be used to generate EHT in vitro and whether the EHT can repair damaged myocardial tissue.

To explore the possibility of using nt-ESCs, we generated EHT from nt-ESC-derived cardiomyocytes in a self-made mold to simultaneously keep the EHT from contracting and provide static stretch. After seven days of static followed by seven days of mechanical stretching, the EHT was implanted in an infarcted rat heart. Four weeks after transplantation, the specimens were evaluated by histological examination, echocardiography, and multielectrode array measurement. The results showed that large (thickness/diameter, 2–4 mm/10 mm), spontaneously contracting EHT was generated successfully (Figure 1). EHT derived from nt-ESCs integrated and electrically coupled to host myocardium and exerted beneficial effects on the left ventricular function of the infarcted rat heart. No teratoma formation was observed up to four weeks after EHT implantation in the rat heart (*6*). This is the first study on regenerating a failing heart by combining myocardial tissue engineering and therapeutic cloning. The data suggest that EHT can be generated from nt-ESCs, and the resulting EHT has a strong potential for the therapeutic heart regeneration. Our findings also suggest that regenerative

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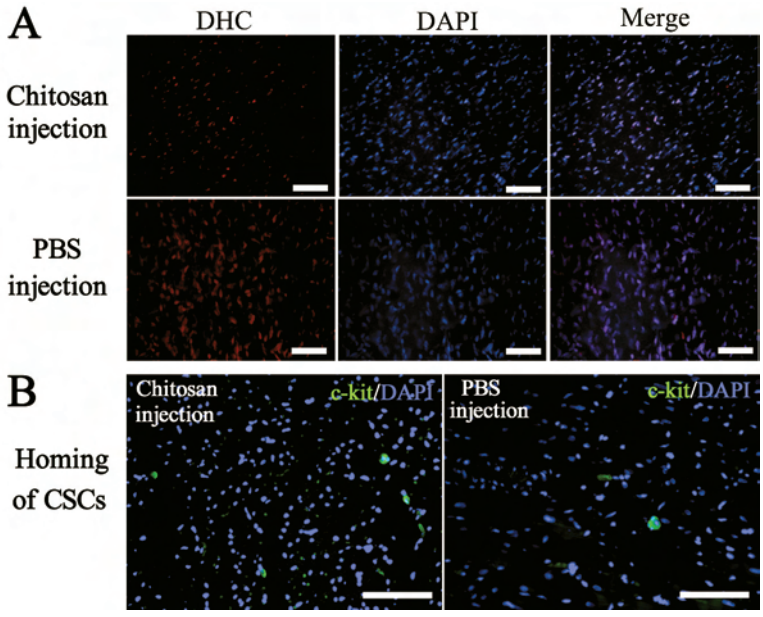

Figure 2. The effects of chitosan hydrogels on ischemic myocardial microenvironments. (**A**) 24 hours after injection, sections from chitosan hydrogel-injected hearts showed a lower fluorescent intensity (red) of DHC (a lipophilic dye used to measure superoxide levels of infarcted myocardium, mainly ROS) staining than sections from PBS-injected hearts. (**B**) 1 week post-injection, more c-kit-positive cells (a marker for cardiac stem cells) were observed in chitosan hydrogel-injected hearts than in PBS-injected hearts. Scale bar, 100 μm.

medicine strategies have the potential to benefit not only from nt-ESCs but also from induced pluripotent stem (iPS) cells since both cell types are reprogrammed.

We successfully generated EHT using other stem cell types (including induced pluripotnet stem cells (iPSCs) and mesenchymal stem cells) with different kinds of supporting biomaterials, including porous materials and decellularized heart scaffolds. Despite preserving the cardiac function in infarcted hearts and generating heart muscle, EHT could also serve as an ideal three-dimensional model to study the development of the heart in vitro. We used EHT to examine, for the first time, the formation and development of the intercalated disc, a functional unit between cardiomyocytes (unpublished data). We demonstrated that proteins making up the intercalated disc assembled in a strictly ordered fashion during formation and development. Perhaps no model system is more ideally suited for characterizing the stepwise assembly and functional integration of the intercalated disc. Additionally, EHT proved conducive to uncovering the characteristics and regularity in the intercellular communication of telocytes, a distinct cell population of the interstitial cells. We used EHT to investigate the distribution pattern and interaction between telocytes and cardiomyocytes and to provide insights to better understand the role of telocytes in the architectural organization of the myocardium (*7*).

In addition to the EHT approach, we have also made progress in the injectable cardiac tissue engineering approach. Since injectable biomaterials have the potential to improve cell retention and survival, and to regulate the ischemic myocardial microenvironment, injectable cardiac tissue engineering is now being explored as a novel approach to support cell-based therapies and enhance their efficacy for treating cardiac disease. Recently, we discovered that temperature-responsive chitosan hydrogels and oligo(poly(ethylene glycol) fumarate) hydrogels were effective injectable scaffolds to deliver stem cells (ESCs, nt-ESCs, adipose-derived stem cells, iPSCs) or therapeutic agents into ischemic myocardium for treating myocardial infarction (*8–11*). Results showed that chitosan hydrogels improved stem cell retention and graft size. After 4 weeks of implantation, heart function, wall thickness, and microvessel densities within the infarcted area were improved significantly (*8, 9*). We also showed that co-injection of basic fibroblast growth factor (bFGF) with temperature-responsive chitosan hydrogels enhanced the effects of bFGF on arteriogenesis, ventricular remodeling, and cardiac function (*10*). More interestingly, chitosan hydrogels alone also contributed to cardiac repair, including increased neovascularization and improved heart function. Investigation of the mechanism underlying the contribution of chitosan hydrogels indicated that they contribute to cardiac repair by exhibiting a regulatory effect on the ischemic myocardial microenvironment. This is achieved by protecting against reactive oxygen species-induced adhesion impairment, which interferes with transplanted cell integrity, and endogenous stem cell homing (Figure 2). As a cationic polymer, the in situ recruitment of growth factors is another benefit exerted by chitosan hydrogels (*12*). We further developed several novel biomaterials with the ability to regulate the ischemic myocardial microenvironment. In addition, by using iPSCs transduced with a tri-fusion reporter gene consisting of firefly luciferase-red fluorescent protein-truncated thymidine kinase (fluc-mrfp-tTK), we demonstrated that undifferentiated iPSCs possess a low immunogenicity, which increases once they reach a more differentiated state, providing a novel insight into the immunogenicity of iPSCs (unpublished data).

Taken together, our findings suggest that coupling tissue engineering approaches with advances in stem cell and biomaterial technologies can improve cardiac function of the failing heart. The field of myocardial tissue engineering is still in its infancy and important challenges remain. Future research designed to address these challenges will be required to achieve clinical impact.

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The Chitosan Scaffold Facilitates Regeneration of Adult Brain and Spinal Cord

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Brain or spinal cord injury can result in death or life-long disability for many patients. Certain therapies, such as using tissue or cell transplantation to stimulate regeneration, or gene therapy such as the knock out of Nogo-A/B, have been shown to facilitate the growth and extension of neuronal axons to some degree (*1–3*). However, their applications in the clinic have been restricted by issues including immune rejection, ethical concerns, and questions of safety related to gene therapy treatments.

Neural stem cells (NSCs) have recently been identified in many areas of the adult brain and spinal cord, but it appears that neurogenesis is too rare an event following injury in the adult central nervous system to reliably repair damaged nerves (*4*). Although researchers have tried various methods to improve neurogenesis efficiency, including injecting specific factors or implanting transgenic cells overexpressing certain factors into the lesioned area (*5, 6*), these treatments are not yet clinically applicable in humans owing to the dangers of infection and restrictions associated with using gene therapy.

Our study aimed to construct a biocompatible biomaterial scaffold that could provide for the slow release of growth factors. The scaffold would be tested by implanting it into a lesioned area of the brain or spinal cord and observing its effect on neurogenesis efficiency relative to a control. The hope was that neurons derived from the endogenous NSCs and stimulated to grow by the factors carried in the scaffold would integrate into the host neural circuit, aiding in the repair of the injured brain or spinal cord.

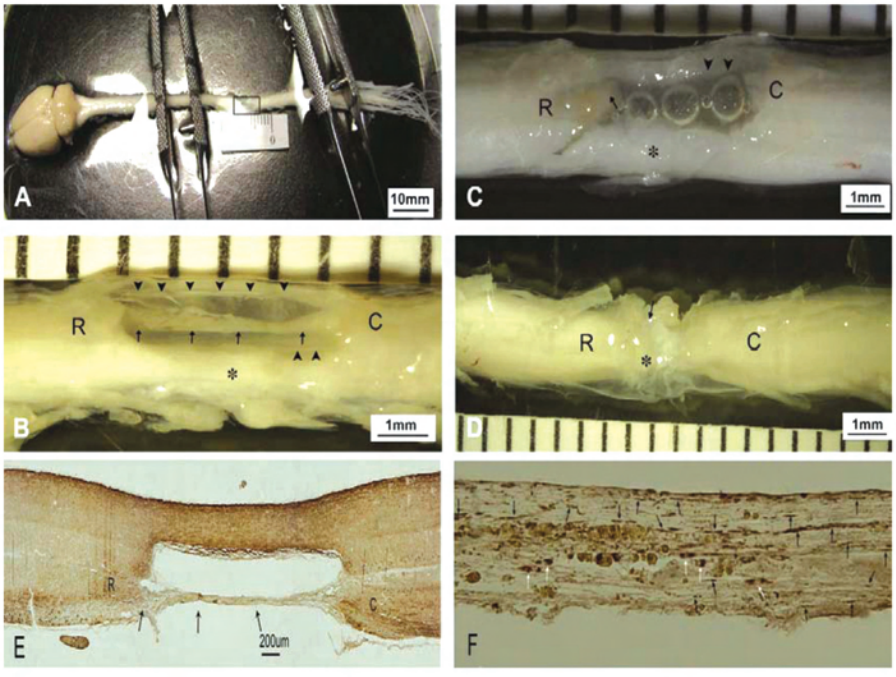

Figure 1. Dorsal view of rat spinal cord 12 months post-surgery. (**A**) Representative sample from the chitosan tube plus collagen group. Area within the black box indicates the operation site. (**B**) Higher magnification of the area in the box in panel A. Arrow indicates the regenerated spinal cord tissue. Tissue on the contralateral side (*) was not operated on. Outer tube wall is indicated by arrowheads. (**C**) Representative sample from the tube-alone group (a chitosan tube without collagen). Arrows show a small amount of regenerated tissue at the proximal end of the tube. (**D**) representative sample from the lesion-control group (sham operation was performed, without implantation). Twelve months post-surgery, the operated area was filled with dense connective tissue as shown by light microscopy. (**E**) Longitudinal tissue section from the T8-10 spinal cord segment in the chitosan plus collagen group 12 months after implantation. Regenerated nerve tissue in the lesion area connects the rostral and caudal ends (arrows). Tissue section was stained using an antibody against neurofilament (NF, brown). R, rostral end; C, caudal end. (**F**) Higher magnification of the newly regenerated nerve tissue shown in E. NF-positive neuron-like cells are indicated by white arrows, NF-positive fibers are indicated by black arrows.

First, using cell culture, we screened for the optimal biomaterial to support adherence, survival, migration, proliferation, and differentiation of spinal cord-derived NSCs into neurons. After modifying and combining the chitosan (a derivative of the polysaccharide, chitin) scaffold with collagen and neurotrophin 3 (NT-3), we tested the kinetics of NT-3 release at different dosages. The results showed NT-3 release over a period of up to 14 weeks. We further determined that a dosage of 100 ng/mL NT-3 was optimal for inducing a high percentage of differentiation of the NSCs into neurons, including γ-GABAergic and cholinergic neurons, which may mediate sensory and motor activities of the spinal cord (*7, 8*). We then implanted the chitosan scaffold into the damaged region of an adult rat with either a lesioned spinal cord or lesioned CA1 region of the hippocampus. Following the operation, using immunohistochemistry, nerve tract tracing, transmission electron microscopy, and nerve electrophysiology, we observed axonal regeneration and neural circuit reconstruction in the lesioned area. Using the BBB-21 method or Morris water maze (MWM) test, we evaluated motor function of the hind limbs of the spinal cord-injured rats or the spatial cognitive ability and memory function of the brain-injured rats. The results showed that the chitosan scaffold could induce axonal regeneration of adult rat spinal cord and brain tissue, as well as promote neural circuit reconstruction, leading to partial recovery of the injured motor or cognitive functions. Interestingly, endogenous neuron-

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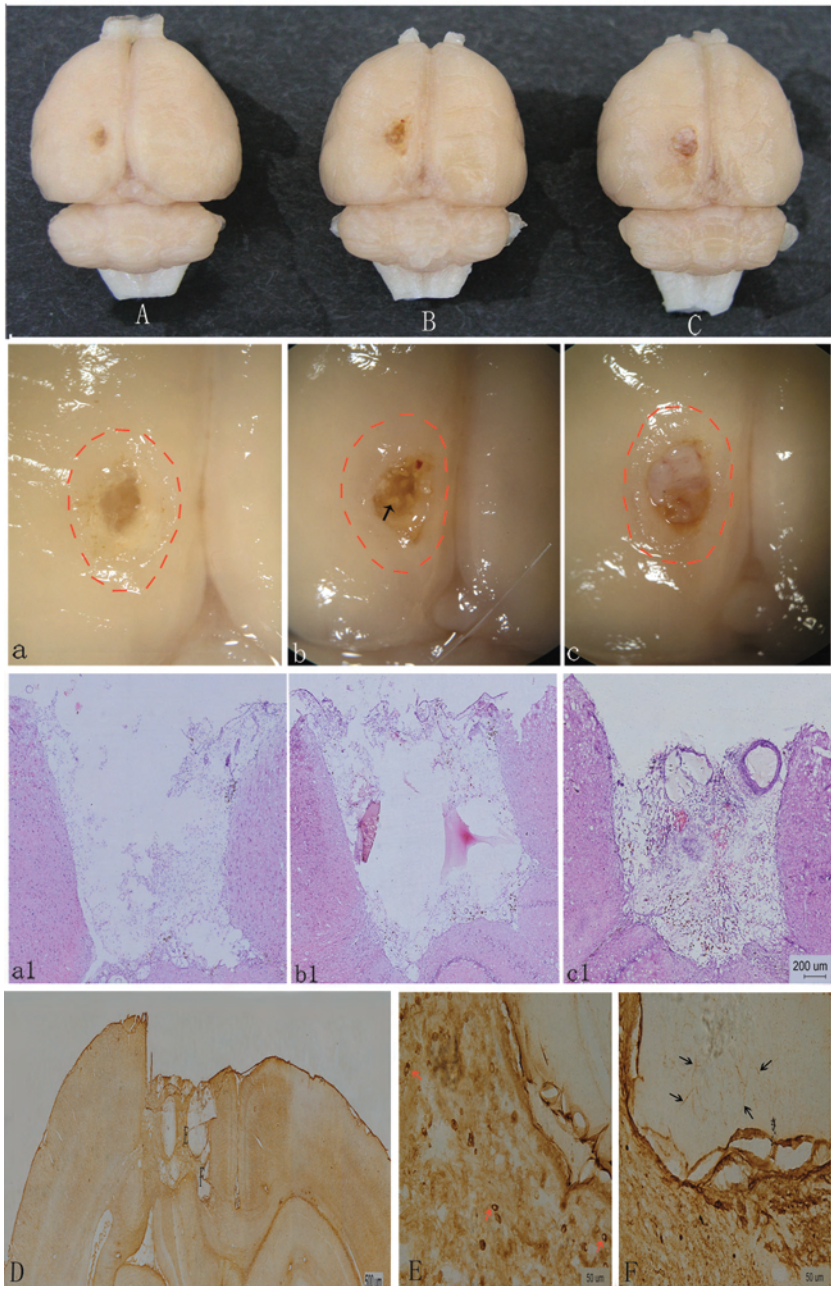

Figure 2. A, B, and **C** show brains from rat study one month postsurgery. a, b and c are higher magnification images of A, B and C showing the lesioned areas. **a1, b1** and **c1** are H&E-stained coronary sections of the rat brain for each group. A, a, and a1 are the lesion control group. One month postsurgery, a hole and glial scar were clearly observed. B, b, and b1 are the chitosan only (no NT-3) group. There was a small amount of newly produced tissue and undegraded chitosan carrier in the injured area (indicated by the arrow). The area of the hole was significantly reduced. C, c, and c1 are the NT-3-chitosan group. The newly produced tissue occupied almost the whole injured area, with no obvious hole (a, b, c, magnification: 20x). (**D**) One month post-surgery, the coronal sections of the rat brain for the NT-3-chitosan group were immunohistochemically stained for neurofilament (NF, brown). **E, F** are higher magnification images of D. One month post-surgery, a large number of NF-positive neuron-like cells (red arrows) and axons (black arrows) were observed in the coronal sections of the injured area for the NT-3-chitosan group.

like cells participated in the regeneration of spinal cord tissue (Figure 1) and restructuring of brain tissue (Figure 2) (*9, 10*). Moreover, intraperitoneal injection of BrdU, nerve tract tracing, and immunoelectron microscopy demonstrated that these neuron-like cells originated from NSCs in the ependyma of the spinal cord or in the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus. Compared with the lesion-control group (in which the same operation was performed, but without implantation) and the chitosan scaffold alone group (in which a chitosan scaffold combining with collagen, but without NT-3, was used), the NT-3-chitosan scaffold significantly increased the neurogenesis efficiency of the adult rat spinal cord or brain. NT-3 could activate and recruit a large number of endogenous neural stem cells in the spinal cord or brain, and facilitate their proliferation and migration to the lesioned area, where they differentiated into neurons and established functional connections with the host spinal cord, partially restoring lost function (unpublished data). Meanwhile, gene chip analysis indicated that a group of genes associated with endogenous neurogenesis, axonal regeneration, and neuroactive ligand-receptor interaction and cell adhesion were greatly up-regulated during the regeneration stage after spinal cord injury. This trend was also demonstrated by qRT-PCR and Western blotting.

In summary, the chitosan scaffold has been shown to induce axonal regeneration of adult spinal cord or brain and reconstruction of the neural circuitry, resulting in the partial restoration of motor function or cognitive ability. The use of chitosan scaffolds has been approved by the Chinese State Food and Drug Administration and will enter clinical trials in the near future. We are optimistic that this technology will bring new hope to patients suffering from brain and spinal cord injury.

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A Tissue Engineering Strategy for Peripheral Nerve Regeneration

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Peripheral nerve injuries are a common and difficult clinical problem worldwide with a steadily increasing incidence in recent years, affecting about 3% of trauma patients. These injuries result in partial or total impairment of motor, sensory, and autonomic functions in the involved segments of the body. Severe injuries that form a large nerve gap between the proximal and distal nerve stumps may cause life-long disability, impact the patient's quality of life, and constitute significant social and economic burdens. The current gold standard for surgical treatments is autografting, namely bridging the nerve gap with autologous nerve grafts which are harvested from another site in the body. Because of inevitable drawbacks associated with autografting—mainly the limited supply of donor nerves, donor site morbidity, and secondary deformities—the development of artificial substitutes for autologous nerve grafts is a pressing need in the field of regenerative medicine. Tissue engineering has proven to be helpful at fulfilling this important task (*1*).

We previously demonstrated that a natural and biodegradable polysaccharide, chitosan, was biocompatible with neuroglial cells by virtue of its positive effects on the adhesion, survival, migration, and proliferation of Schwann cells (*2*). We also demonstrated that the in vivo biodegradation product of chitosan, chitooligosaccharide, could support neural cell adhesion and encourage neuronal differentiation and neurite outgrowth through the up-regulation of neurofilament and N-cadherin expression. We fabricated chitosan-based nerve grafts, using an injection molding method, which were composed of a chitosan conduit filled with polyglycolic acid (PGA) filaments. These nerve grafts were used to bridge a 30 mm long sciatic nerve gap in dogs. Six months later, the results showed that the injured nerve trunk was reconstructed with restoration of nerve continuity and axonal functions such as electrical conduction and axoplasmic transport. Further, the target muscles were re-innervated with improvements in the locomotor activity of the injured limb, while the nerve graft had been completely degraded and absorbed by the body (*3*). Other nerve grafts constructed with the same methods were also used to bridge a long-term delayed sciatic nerve gap in rats, and experimental observations confirmed the possibility of using chitosan-based nerve grafts for such repairs (*4*). Our chitosan-based nerve graft invention has been patented in China.

With approval from the Chinese State Food and Drug Administration (SFDA) for the use of chitosan-based nerve grafts in clinical trials, we have launched a prospective randomized controlled multicenter study in four Chinese public hospitals. Two human case studies have been reported, in which 30 and 35 mm long median nerve gaps were repaired with chitosan-based nerve grafts. The three-year follow-up indicated that overall motor and sensory function of both patient's injured nerves had recovered to M_4 or S_3+ levels, according to British Medical Research Council (MRC) grading scale (Figure 1) (*5*).

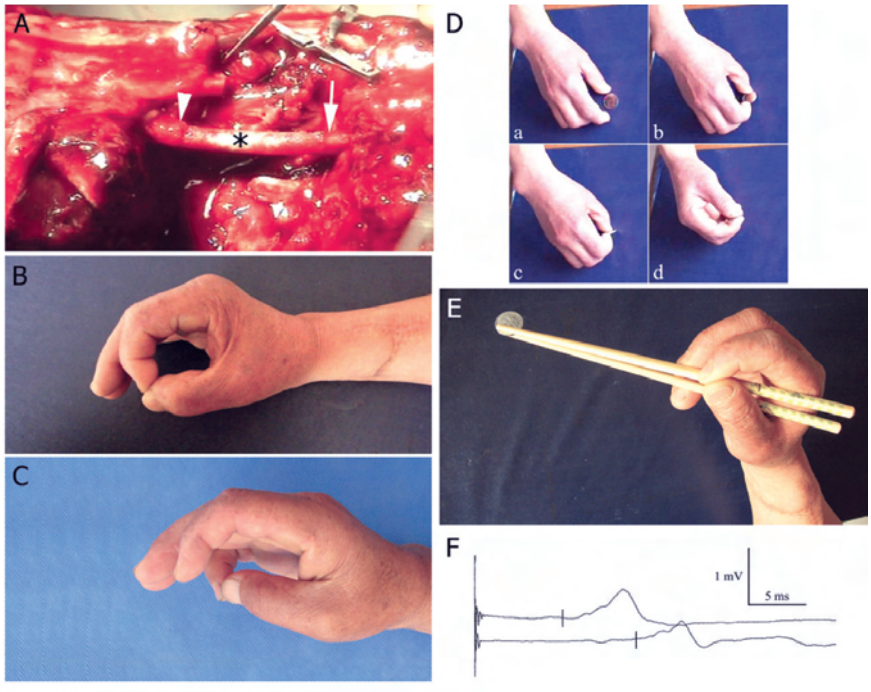

Figure 1. Bridging a 30 mm long human median nerve gap in the distal forearm by a chitosan-based (chitosan/PGA) nerve graft. Intra-operative view (**A**) showing that the nerve graft (labeled with *) was implanted across a nerve gap between two nerve stumps (labeled with an arrow and arrowhead). Photographs (B–E) showing that at 36 months after grafting, the functional recovery of target thenar muscles enabled the injured hand to do many actions such as digital opposition of the thumb to the index and little fingers (**B** and **C**), coin picking (**D**) and chopsticks handling (**E**). Representative electromyographic data depicting the compound muscle action potentials recorded on the right abductor pollicis of the patient at 36 months after grafting (**F**). Adapted from reference *5*.

As another promising natural biomaterial, silk fibroin proteins have attracted our further research interest owing to their favorable physicochemical and biological properties. After completing in vitro biocompatibility evaluation of silk fibroin with peripheral nerve tissues and cells (*6*), we adopted a unique processing method to create a biomimetic design of silk fibroin-based nerve grafts, which features an eggshell-like tubular structure with appropriate mechanical property, permeability, and biodegradability. These nerve grafts were implanted to bridge a 10 mm long sciatic nerve gap in rats. The morphological and functional outcomes verified the effectiveness of silk fibroin-based nerve grafts for peripheral nerve repair (*7*). We have obtained a patent in China for these grafts and are now applying for international patents; thus far, a patent has been approved in the European Union.

The introduction of support cells and/or growth factors into scaffolds

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represents an optimal way for generating typical tissue engineered nerve grafts that have an enhanced ability for repairing extended peripheral nerve gaps. We observed that bone marrow mesenchymal stem cells (MSCs) could be induced to transdifferentiate into Schwann cell-like cells in vitro and in vivo, and, more importantly, might directly release growth factors or indirectly modulate the behavior of Schwann cells to relay and magnify neurotrophic actions (*8*). Driven by this finding, we engineered two types of nerve grafts by incorporating MSCs, as support cells, into a silk fibroin- or chitosan-based scaffold for bridging 10 mm long sciatic nerve gaps in rats (*9*) or 50 and 60 mm long sciatic nerve gaps in dogs (*10, 11*), respectively. Comprehensive evaluation using multiple measurements at different times postgrafting revealed that the regenerative outcomes of both types of tissue engineered nerve grafts were close to those of autologous nerve grafts (Figure 2). The experimental variables involving larger animals (dogs) were deliberately selected to be clinically relevant and suitable for scaling up to humans. To further accelerate translation from experimental studies to clinical applications, we have attempted the use of chitosan-based, MSCs-containing tissue engineered nerve grafts for bridging 50 mm long median nerve gaps in rhesus monkeys. It is worth mentioning that for dog- and monkey-related preclinical studies, we completed safety evaluations of MSCs-based therapies using clinical chemistry and histopathological assessments during at least a one-year follow-up period. In addition, we prepared a neural scaffold based on immobilization of nerve growth factor (NGF) onto the modified chitosan with genipin, a natural and low toxicity cross-linking agent, and investigated the controlled release of NGF from the scaffold. This in vitro study will provide a solid basis for the in vivo application of commercially available growth factors for peripheral nerve repair (*12*).

Revealing the molecular basis of peripheral nerve regeneration will open the possibility of improving the functional outcomes of peripheral nerve repair. Tissue engineered nerve grafts are likely to increase the intrinsic growth capacity of neurons, modulate the interactions between neurons and Schwann cells, and eventually orchestrate a regenerative process. All these cellular events are regulated by multiple signaling pathways and complex gene networks. Some key molecules are responsible for the distinct regulatory programs, and microRNAs also contribute to the successful regeneration by tempospatial changes, as evidenced by our observations (*13, 14*). Further insights into the molecular mechanisms underlying peripheral nerve regeneration might provide more tissue engineering-based therapeutic strategies for peripheral nerve injuries.

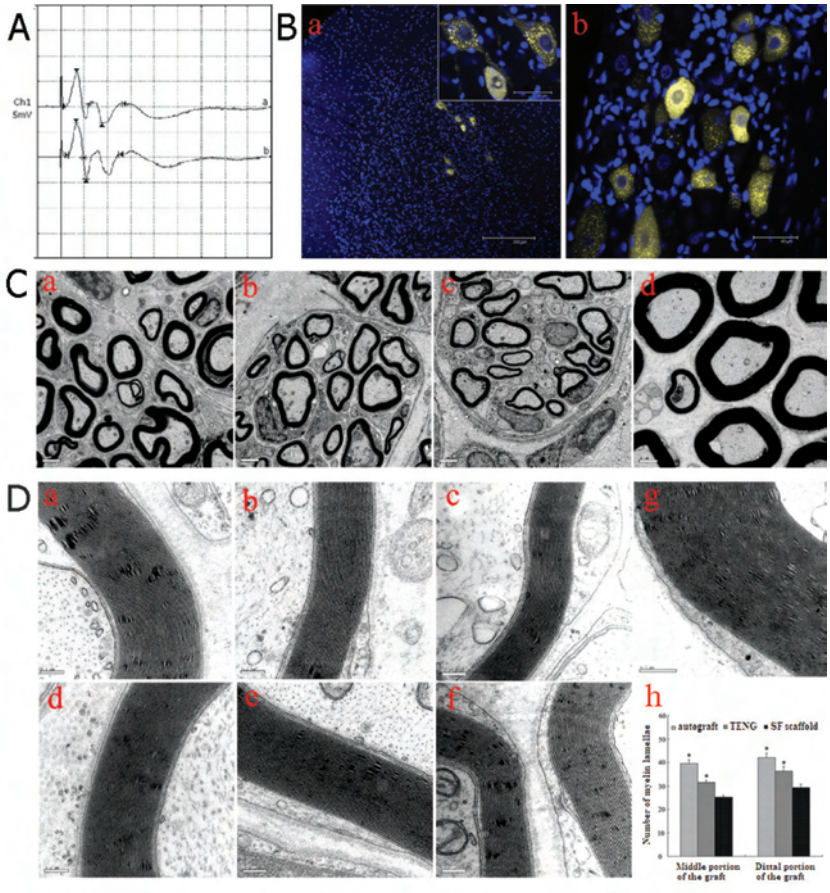

Figure 2. Comprehensive evaluation of regeneration outcomes at 12 weeks after bridging 10 mm long sciatic nerve gaps in rats by silk fibroin-based, bone marrow mesenchymal stem cells (MSCs)-containing nerve grafts. (**A**) Representative electromyographic data depicting the compound muscle action potential recorded at the injured side. (**B**) FluoroGold retrograde tracing showing that the labeled motor neurons occurred in spinal cord (a) and the labeled sensory neurons occurred in dorsal root ganglia (b). (**C**) Transmission electron micrographs comparing the regenerated nerves formed at the middle portion of the autologous nerve graft (a), silk fibroin-based, MSCs-containing nerve graft (TENG, b), and silk fibroin-based (SF) scaffold (c), respectively, as well as the contralateral, uninjured nerve (d). Scale bar, 2 μm. (**D**) Transmission electron micrographs comparing the lamellar structure of regenerated myelin sheaths formed at the middle portion (a–c) or distal portion (d–f) of the autologous nerve graft (a, d), TENG (b, e), and SF scaffold (c, f), respectively, as well as the contralateral, uninjured nerve (g). Scale bar, 0.2 μm. Also shown in (h) is a histogram comparing the number of regenerated myelin lamellae for autograft, TENG, and SF scaffold groups, respectively. *p<0.05 vs. silk fibroin-based scaffold group. Adapted from reference *9*.

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Tissue Engineering for Soft Tissue Regeneration

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Soft tissues—such as cartilage and tendon—are frequently injured, particularly in sports, but they have limited regenerative capacity. They seldom spontaneously heal after trauma or diseases, causing significant loss of performance in sport and decreased functional capacity in daily life. More than 30 million tendon and ligament injures occur annually worldwide, and these continue to rise. Since current therapies have had limited success, the development of stem cell and tissue engineering solutions for cartilage and tendon regeneration have been receiving increasing attention. There are two basic strategies for tissue regeneration: to recruit and differentiate the stem cells of host tissue or to implant exogenous stem cells.

Recruiting and Inducing Endogenous Stem Cells

Bone marrow cells released by microfracture or full-thickness cartilage defect can initiate in situ cartilage repair, but with limited efficiency. We found that transplantation of autologous platelet-rich plasma (PRP) in a collagen matrix improved in situ bone marrow-initiated cartilage repair, restoring a larger surface area of the cartilage defects than controls (*1*). Moreover, the PRP treatment group had higher histological scores and more glycosaminoglycan (GAG) content as well as a higher Young's modulus of the repaired tissue when compared with the control group. Besides the study of PRP, we also investigated using bone morphogenetic proteins (BMPs) as modulators for in situ hyaline cartilage regeneration (*1*). It was found that BMP-4 was more effective in stimulating cartilage differentiation of messenchymal stem cells in vitro and the formation of joint surface cartilage tissue in vivo than BMP-7 (*2*).

For tendon regeneration studies, we developed a bioactive knitted silk-collagen sponge scaffold incorporating exogenous stromal cell-derived factor-1 α (SDF-1α), which selectively increased the recruitment of fibroblast-like cells, enhanced local, endogenous SDF-1α and tendon extracellular matrix production, and reduced the accumulation of inflammatory cells. This bioactive scaffold therefore enhances selective migration and homing of those cells participating in situ tendon regeneration (*3*).

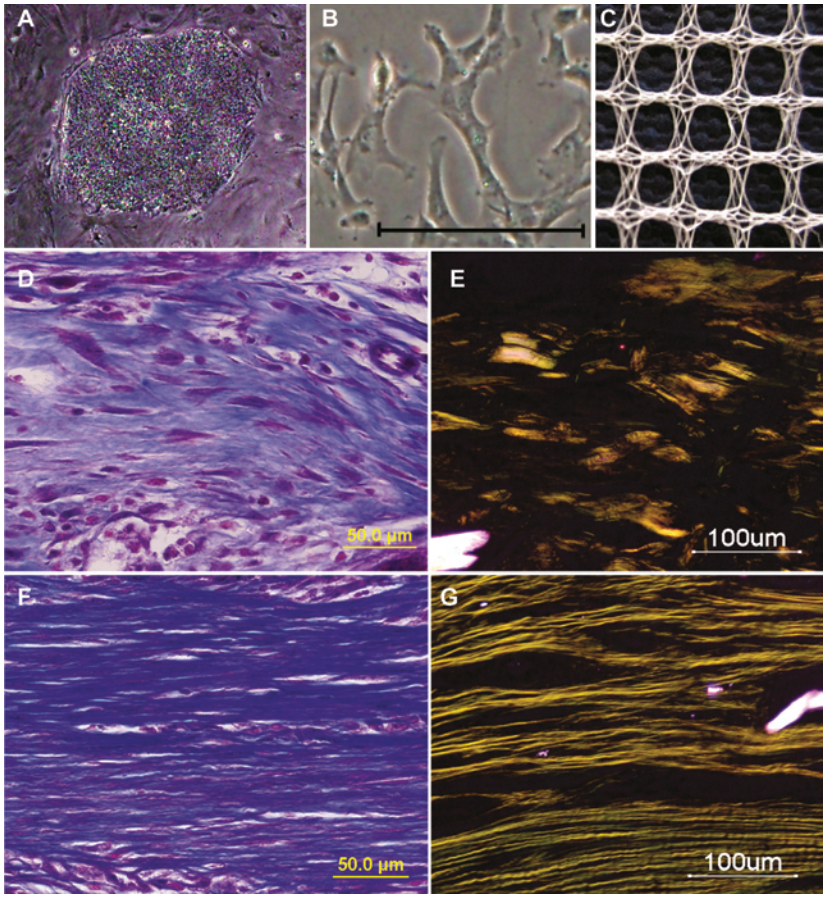

Figure 1. hESC combined with silk scaffold for tendon regeneration. (**A**) Morphology of hESC clones in culture. (**B**) Morphology of MSCs derived from ESCs (hESC-MSC). (**C**) Structure of knitted silk scaffold. (**D**–**G**) ECM deposited at the repair site at four weeks postsurgery in rat Achilles tendon repair model. Shown are representative examples of the collagen deposition of tendon repaired with silk scaffold alone (**D**), maturation of collagen fiber in the tendon repaired with silk scaffold alone (**E**), improved collagen deposition of tendon repaired with hESC-MSC-containing silk scaffold (**F**), and better maturation of collagen fiber in the tendon repaired with hESC-MSCs-containing silk scaffold (**G**). (D and F are Masson's Trichrome stain, E and G are polarized light microscope photographs of collagen fiber).

Stem Cell Implantation

Several potential sources of stem cells can be utilized for cartilage and tendon regeneration. Mesenchymal stem cells (MSCs) in particular have been extensively investigated, so we decided to examine the fate of allogeneic MSCs after implantation using a tendon defect rabbit model. The implanted MSCs remained viable, as shown by the trace fluorescence eight weeks after surgery. Moreover, the morphology of MSCs changed from a round shape to a tenocyte-like spindle shape at approximately five weeks after implantation (*4*). The tensile stiffness of neo-tendon tissue in the allogeneic MSCs-treated group reached 87% of normal (*5*) and restoration of the fibrocartilage zone at tendon-

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to-bone insertion point was observed (*6*). Although extensively studied, problems still exist with the use of MSCs, including finding standard identification markers, limited cell proliferation potential, and ectopic bone formation. It is known that embryonic stem cells (ESCs) are a good source of cells for tissue regeneration and therefore present an alternative to MSCs. Our group is the first, to our knowledge, to provide evidence that human ESCs may represent a potentially powerful cell source for tendon tissue engineering. To demonstrate this, ESCs were stepwise differentiated to tenocytes through an MSC transition stage. Human ESC-derived mesenchymal stem cells (hESC-MSCs) expressed a comprehensive panel of adult MSCs surface markers and differentiated into the tenocyte lineage under mechanical stimulus and addition of the transcription factor, Scleraxis, a marker of tendon progenitor cells. In a patellar tendon repair model, hESC-MSCs improved tendon regeneration both structurally and functionally, possibly through the secretion of growth factors that initiate endogenous tendon regeneration (*7*). A similar positive effect using hESCs was observed in a joint cartilage repair study (unpublished data). A stepwise differentiation approach, first inducing hESCs to differentiate into MSCs and then allowing the MSCs to form specific tissues, successfully avoids the risk of teratoma formation. It therefore provides a potentially safe strategy for utilizing ESCs in cartilage and tendon regeneration.

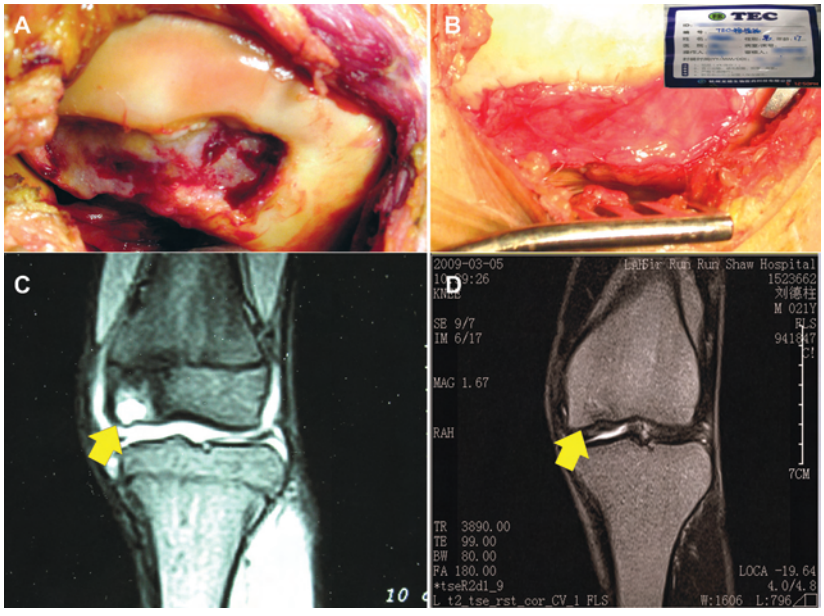

Figure 2. Large chondral defects of the knee were treated by tissue engineered cartilage (TEC) transplantation. (**A**) A 19-year-old male with 4.8 x 2.5 cm knee cartilage defect. (**B**) TEC transplantation. (**C**) Presurgery MRI image of knee. (**D**) 14 months postsurgery MRI image of knee after TEC transplantation. (The arrows indicate the cartilage defect area.)

Delivering Stem Cells Using Scaffolds

We carried out a series of experiments to optimize the design of functional silk scaffolds for tendon regeneration, as well as bilayer collagen scaffolds for cartilage regeneration. A new type of tendon scaffold was developed by incorporating silk fibers with a collagen matrix in a knitted structure. This structure provides sufficient internal space for seeding of cells and subsequent formation of connective tissue bundles. The collagen matrix preserves the internal space under physical loading and plays an important role in tendon matrix organization (*8*).

We fabricated three-dimensional cell sheets in which cells were held within the synthesized matrix. Three-dimensional sheets of MSCs were easily fabricated and cells maintained their differentiation potential within the scaffold, suggesting that this is a convenient strategy for efficient cell seeding onto physical scaffolds (*9*). When subjecting a knitted silk-collagen sponge scaffold seeded with hESC-MSCs to mechanical stimulation, the hESC-MSCs sheets exhibited tenocyte-like morphology and cells expressed tendon-related gene markers (e.g., Collagen type I & III, Epha4, and Scleraxis), as well as other mechanosensory structures (e.g., cilia) and proteins (integrins and myosin). Microscopy and gene expression assays showed that the transplanted hESC-MSCs not only contributed directly to tendon regeneration, but also exerted a modifying effect on the implantation site environment (Figure 1) (*10*).

Clinical Trial of Tissue-Specific Progenitor Cells for Cartilage Regeneration

A promising candidate for cartilage regeneration is the cartilage progenitor cell (CPC). It has been hypothesized that a population of progenitor cells resides within the articular cartilage in order to maintain tissue homeostasis. The implantation of CPCs provides a practical solution for repairing damaged joint cartilage. Following new regulations from the Ministry of Public Health in China, we established a standard approach for CPC-based tissue engineered cartilage (TEC) transplantation and successfully implanted TEC for large chondral defects of the knee (over 12 cm^2) (*11*). A group of 12 patients with articular cartilage injury was treated with TEC composed of autologous cartilage cells within a 3-D scaffold. The TEC was engineered at the Longhill Bio-med Tech. Co. Ltd., a GMP-certified facility. Eleven of 12 patients achieved excellent or good outcomes based on MRI evaluation and International Knee Documentation Committee (IKDC) scores two years after surgery (Figure 2). This pilot study demonstrates the promise of using TEC for cartilage regeneration. Further investigation and confirmation is warranted, using a larger group of test subjects.

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Tissue Engineering: Hope for Tendon Regeneration

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Tendon injuries and defects are common clinical problems. Due to their specialized anatomy, tendons are difficult to repair. Conventional materials, including autologous or allogeneic tendon grafts and artificial materials, have thus far proved unsatisfactory and have significant limitations. Since 1986, we have been studying tendon anatomy, nutrition, healing models, and growth modulation of tendon cells (*1*). Our results suggested that tissue engineered tendons (TETs) derived from co-culturing of seed cells and scaffold materials in vitro may provide an attractive option for tendon replacement and repair.

We have shown that the proliferative potential of cultured human tendon cells (hTCs) declines substantially after the 13th passage. In order to produce TETs, we attempted to establish an immortalized standard hTC line that retains its normal functional characteristics. Using electroporation, we transfected a tsA58H plasmid into hTCs (ThTCs) allowing cell growth to be controlled by altering temperature or serum supplements. Such ThTCs could be subcultured up to the 75th passage without apparent alteration in their morphology and ability to secrete type I collagen. No tumorigenicity was detected. We also constructed a human telomerase reverse transcriptase eukaryotic expression plasmid, pGRN145, and transfected it into ThTCs and hTCs. The transfected cells showed telomerase activity with a concomitant extension of life span and maintenance of original biological characteristics, but no tendency for malignant transformation (*2*), which enabled us to establish a standard cell line for TETs.

To identify an ideal support for the construction of TETs, we tested a variety of synthetic and bio-derived scaffolds. We found that synthetic scaffolds degraded quickly in vivo and lacked sufficient strength to allow earlier motion and rehabilitation after implantation, which can result in adhesion and poor functional outcomes (*3*). By contrast, in vitro experiments indicated that bio-derived scaffolds, predominantly composed of extracellular matrix components, could promote adhesion of tendon cells to the scaffold (*4*). Proliferation and morphology of tendon cells seemed to be dependent on collagen concentrations and the micropatterned surface of the substrates (*5*). Based on these observations, we have developed a new type of bio-derived scaffold with natural tendon, which facilitates cell attachment and infiltration (*6*). The architecturally optimized scaffold was used to form a multilayer support for TETs (Figure 1). This 3-D architecture provides a temporary mechanical support as well as a bio-derived scaffold which can be incorporated and remodeled by cells during the healing process. The bio-derived scaffold with its native extracellular matrix, can modulate host processes such as cell migration, proliferation and orientation, and angiogenesis in a manner that can enhance and accelerate the process of tendon repair.

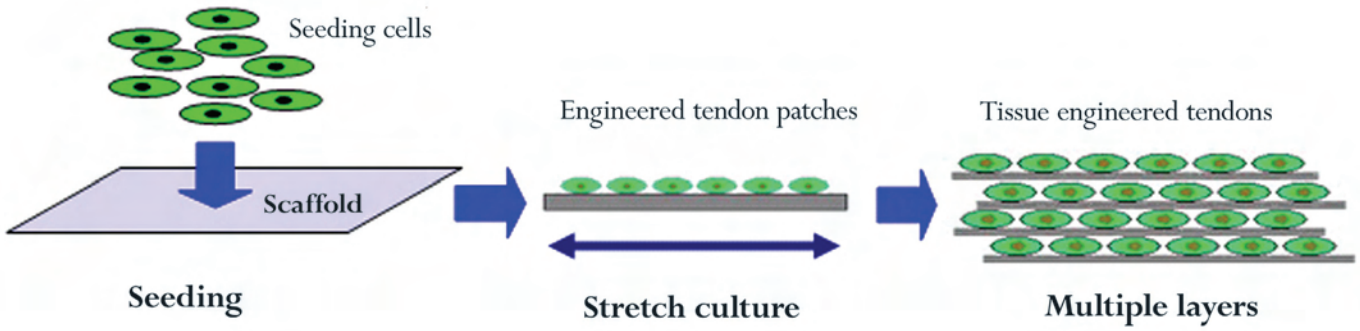

Figure 1. Graphical representation of TETs construction using multiple layers of engineered tendon patches.

To formulate the desired biological properties, stem cells must be cultured in a tendon extracellular matrix microenvironment with both biochemical and biomechanical stimulation. Bone marrow stromal cells (BMSCs) have the potential to differentiate along various mesenchymal lineages. Nonetheless, our studies suggested that their differentiation into tendon-like cells relies largely on uniaxial mechanical stimulation (*7*). TETs can also benefit from mechanical stimulation prior to implantation. Using our tailor-made bioreactor, uniaxial tensile strain was applied to BMSCs-seeded bio-derived scaffolds in culture. The strain directed cellular differentiation and promoted extracellular matrix development (*8*) and these constructs could then be used as TETs.

The biocompatibility, healing process, biomechanical strength, rejection reaction, and overall efficacy of TETs were tested in nude mice, rats, rabbits, chickens, and rhesus monkeys. Implantation experiments confirmed that TETs could efficiently repair tendon defects as well as facilitate the healing process. Cell-based assays and genotype analysis confirmed the survival of TETs in vivo (Figure 2).

Based on the above results and after preclinical safety evaluation, we treated seven volunteers with old defects of the Achilles tendon and 11 volunteers with coracoclavicular ligament injury using TETs produced by a GMP-certified facility. No adverse effects were reported

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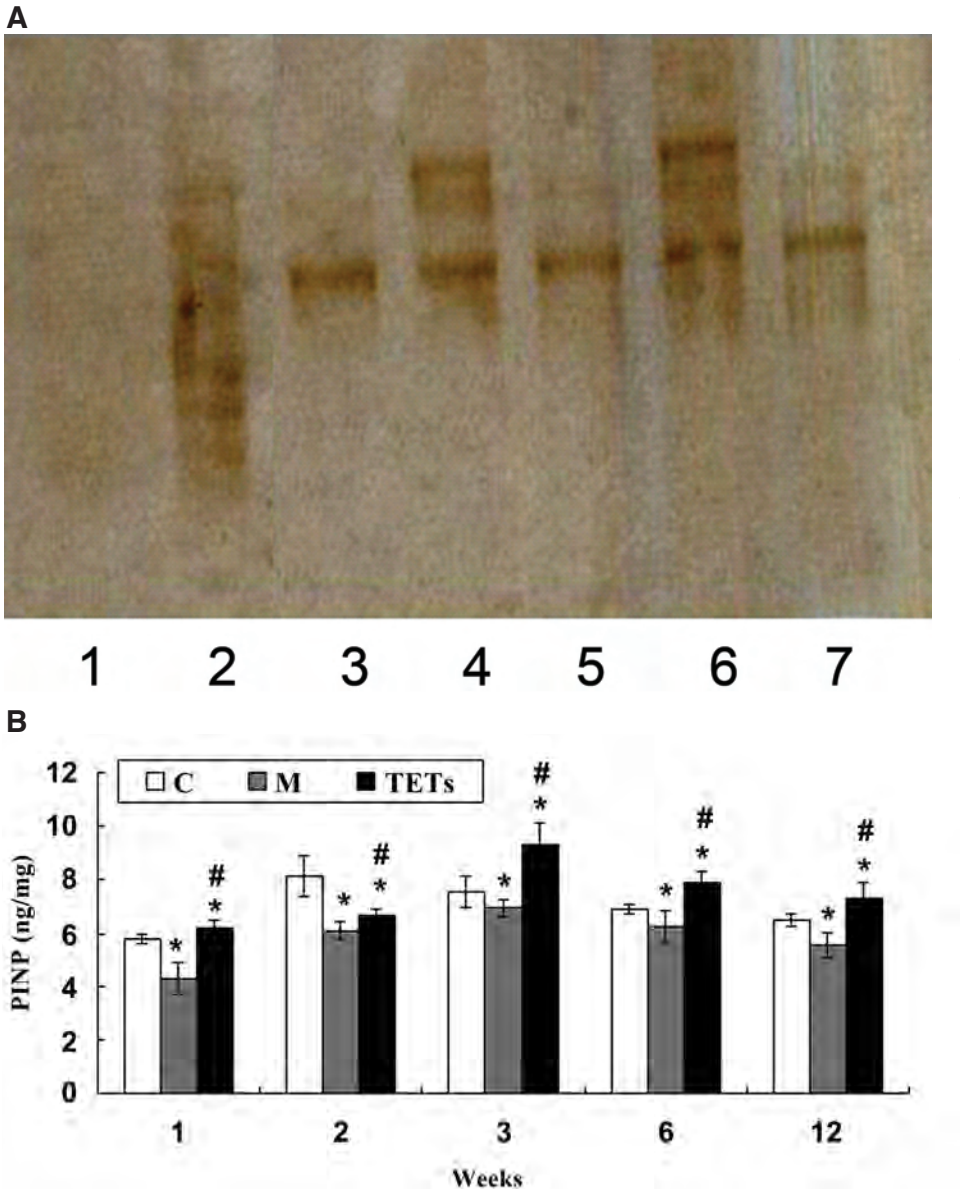

Figure 2. (**A**) The tendon sheath of *flexor digitorum profundus* was repaired with TETs constructed with allogeneic tendon cells seeded on scaffold material in rhesus monkey. Genotyping of a short tandem repeat locus, CSF1PO, confirmed that there was a non-autogeneic allele at the site until 12th week after implantation. Lane 1: negative control; 2: allele ladder; 3: autograft; 4 and 6: TETs implant; 5 and 7: scaffold material implant. (**B**) Radioimmunoassay of ammonia terminal peptide of type I procollagen content indicated that collagen synthesis in TETs and autograft groups was more active than materials-only group at various time points. C, autograft; M, scaffold material implant; TETs, tissue engineered tendon implants. *, $p<0.01$ vs. C. #, $p<0.01$ vs. M.

during a follow-up period of more than 35 months. Clinical outcomes were satisfactory and recovery was close to normal (*9*). MRI examination revealed that the repaired tissues showed a low signal on T1W1 screens, similar to the preexisting healthy tissues. Tissue samples obtained from two patients who were reoperated to remove the steel wire fixation showed by genotyping analysis of short tandem repeat loci that the TETs have survived in vivo for at least six months.

In summary, our research suggests that TETs are a feasible solution for tendon repair. Although preliminary clinical trials has shown that the TETs can restore tendon function to some extent, more studies are required to fully demonstrate safety and effectiveness as well as to formulate an efficient scale-up and manufacturing process which can generate TETs with the desired specifications in a reproducible and consistent manner. With the completion of multicenter clinical trials, we expect that TETs will soon bring benefits to patients in the clinic.

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Biodegradable Conduit Small Gap Tubulization for Peripheral Nerve Mutilation: A Substitute for Traditional Epineurial Neurorrhaphy

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Functional recovery after peripheral nerve injury (PNI) is hindered by a number of factors, including the formation of the correct sensory and motor nerve fiber connections, difficulties in distal target organ reinnervation and painful neuromas (*1*). Promoting the effective docking of different fibers and target organ reinnervation remains a challenge for surgeons (*2*). Inspired by the phenomenon of peripheral nerve selective regeneration put forth by Cajal (*3*), we proposed that the repair of PNI could be greatly enhanced using a biodegradable conduit where a small gap remains between the stumps for selective regeneration. The method and materials used in this technique for PNI repair are protected by the Chinese State Intellectual Property Office (ZL 01136314.2).

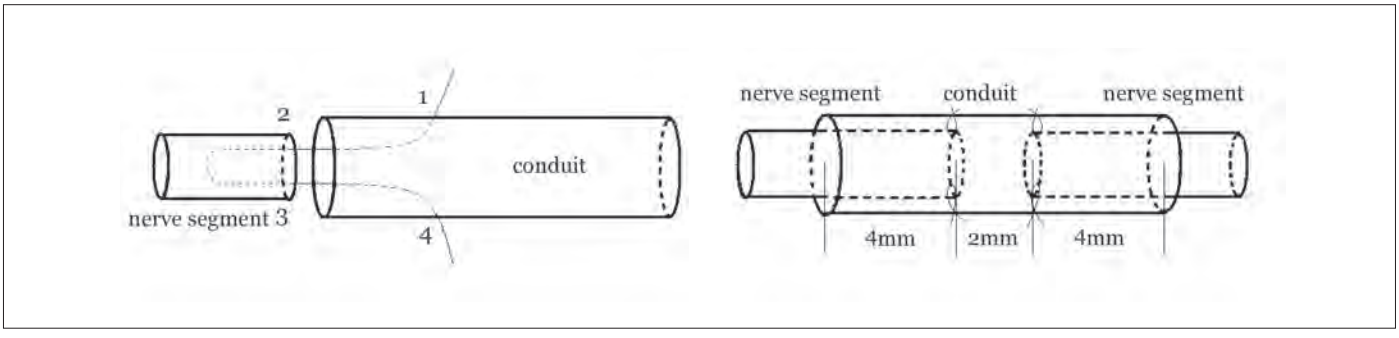

Figure 1. Graphical representation of the suture methods for biodegradable conduit small gap (2 mm) tubulization. Numbers 1, 2, 3 and 4 denote the suture sequence. A 2 mm gap remained between the two ruptured nerve stumps in the biodegradable conduit.

A model of peripheral nerve mutilation, simulated in Sprague-Dawley rats and rhesus monkeys, was repaired with a biodegradable conduit and had the following features: Good biocompatibility and degradation characteristics, displaying tubulization and conductivity during the regeneration process; the gap between two ruptured stumps that was suitable for regeneration was 2 mm; the extent of regeneration, as assessed by histology, electrophysiology and functional assessment, was better for small gap tubulization with a biodegradable conduit than traditional epineural neurorrhaphy (*4–6*).

Based on experiments in animals, a multiple centers human clinical trial of biodegradable conduit small gap tubulization for peripheral mutilation was performed in Peking University People's Hospital, Beijing Jishuitan Hospital, and Jilin University First Hospital from November 2008 to February 2010. Permission was obtained from the Institutional Review Boards of the Chinese National Drug Administration and the Scientific Ethics Committees of each hospital. This clinical trial was registered in the Chinese Clinical Trial Registry Center (ChiCTR-TNRC-10000801).

After obtaining informed consent, patients were randomized into two groups, with 25 cases of peripheral mutilation being repaired by epineurial neurorrhaphy and a further 25 cases repaired by biodegradable conduit small gap (2 mm) tubulization. All patients received follow-up examinations at one, two, four, and six months postsurgery and were assessed for functional recovery by British Medical Research Council standards and a revised version by Zhu Jiakai *et al.* (*7*), which includes motor function grading (M), sensory function grading (S), and autonomic nerve function grading (A). The combined evaluation of both groups was compared after four and six months. The electrophysiological examination was performed six months postsurgery and the recovery rate of both groups was compared.

The multiple center clinical trial results were as follows: (1) Good biocompatibility and degradation was observed for the biodegradable conduit for tubulization and conduction. (2) The suture time for biodegradable conduit small gap tubulization was 8.33 ± 1.59 min, which is approximately 20% faster than traditional epineural neurorrhaphy, which is 10.71 ± 2.89 min. (3) The occurrence of painful neuroma rate (visual analogue scales, VAS) of the biodegradable conduit small gap tubulization and epineural neurorrhaphy groups were 9.0% and 77.50% at one month; 3.00% and 45.20% at two months; 0.00% and 29.00% at four months; and 0.0% and 23.60% at six months, respectively. Differences were statistically significant at all four time-points. (4) The overall functional assessment of the biodegradable conduit small gap tubulization group at four and six months postsurgery was 18.93% and 36.19% higher than the epineural neurorrhaphy group, respectively. Both differences were statistically significant. (5) The sensory

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conduction rate comparison of the biodegradable conduit small gap tubulization and epineurial neurorrhaphy groups at six months postsurgery was 74.70% and 70.20%, respectively, while the motor conduction rate comparison was 65.20% and 59.60%, respectively. Both results were statistically significant at the four- and six-month time-points. (6) The combined functional recovery effects and the electrophysiological examination results of the biodegradable conduit small gap (2 mm) tubulization group were much higher than that of the traditional epineurial neurorrhaphy group, especially in cases with rotated ruptured peripheral nerve stumps.

The suture method used for biodegradable conduit small gap (2 mm) tubulization is shown in Figure 1. The use of biodegradable conduit small gap (2 mm) tubulization in the repair of peripheral nerve mutilation allows for nerve stumps to be sutured directly, without tension. This technique is a revolutionary methodological reformation and was not originally described for peripheral nerve defect repair, as reported by Lundborg *et al.* (*8, 9*). The regeneration space provided by the biodegradable conduit and the 2 mm gap between the ruptured stumps allows for selective regeneration of peripheral nerves, while preventing the escape of ruptured regenerating fibers, and reducing the occurrence of painful neuroma (*10*). We also found multiple regenerating axons in the 2 mm gap, namely one proximal axon regenerated more than three to four axons, which also matured and innervated distal axons and target myoceptors. This may be responsible for the restoration of mobility and functional recovery in early animal experiments.

Animal experiments and human clinical trials have shown that the effect of biodegradable conduit small gap (2 mm) tubulization on the regeneration of peripheral nerves was better than traditional epineural neurorrhaphy for peripheral nerve mutilation, especially in cases where the stumps of the peripheral nerves were rotated. Advantages of this procedure, such as the simplicity of the surgery and reduced operative time, indicate that biodegradable conduit small gap (2 mm) tubulization can be used to repair peripheral nerve mutilation, thereby replacing the less effective traditional epineurial neurorrhaphy which has been used for almost a century.

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Repairing Bone Defects by Allogeneic, Gene-Modified, Adipose-Derived Stem Cell and Heparin-Chitosan–Coated Acellular Bone Matrix

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Tissue-engineered bone could provide an effective therapy for large bone defects. However, there are hurdles that limit its therapeutic application in patients (*1, 2*), including the fact that stem cells need to be isolated from the recipient to obtain autologous tissue, thus limiting the amount that can be collected. Another challenge is ensuring a sufficient blood supply to the tissue-engineered bone posttransplantation. Our research has focused on these areas in an attempt to make tissue-engineered bone more applicable in clinic practice (*3*).

Autologous stem cells are regarded as the most ideal for cell therapy; however, the extended in vitro culture time required for amplification, induction, and differentiation, as well as limited cell availability, have restricted their widespread application (*4*). Recently, we investigated the possibility of creating a "universal" stem cell. Previous studies have shown that immune rejection, a critical issue in allotransplantation, is caused mostly by major histocompatibility complex (MHC)-I cell surface proteins (stem cells express very little MHC-II) (*5*). Indeed, low expression of MHC-I in tumor cells could explain how they evade immune surveillance (*6*).

The products of human cytomegaloviruses US2 and US3 have been shown to decrease MHC-I expression. Therefore, in the first experiment, two short sequences of US2 and US3 were transfected into adipose-derived stem cells (ADSCs) to test whether they could downregulate the MHC-I expression of ADSCs. The biology and biosafety

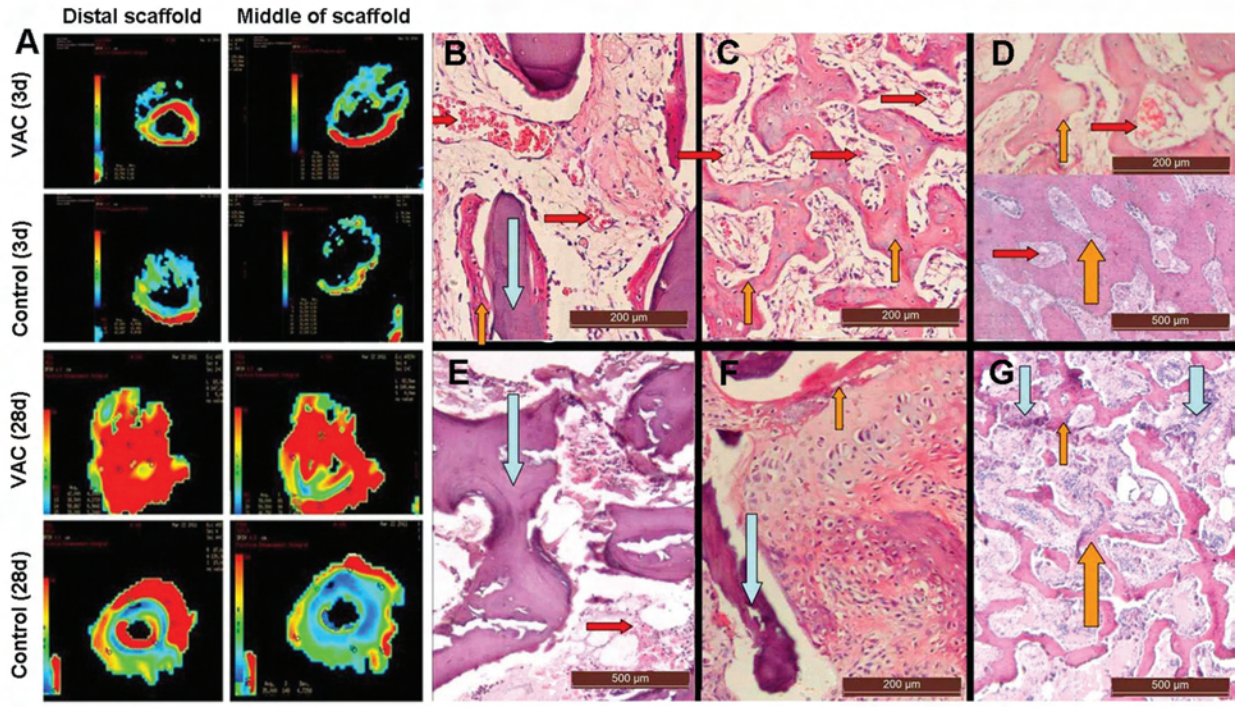

Figure 1. CT perfusion and histological examination showed that vascularization of tissue-engineered bone was improved by the vacuum-assisted closure (VAC) technology. (**A**) The blood volume of the CT perfusion was increased in the VAC group at different time points. The histological changes showed that the amount of vasculature and bone matrix synthesis increased in the VAC group (**B–D**) compared to the control group (**E–G**). (Red arrows: blood vessels, Orange arrows: new bone, Blue arrows: scaffold).

of the US2/US3-ADSCs was determined by assays for proliferation and multidifferentiation capacity, morphology, toxicity, tumorigenicity, teratology, and histocompatibility. The results showed that while the biological characteristics of the US2/US3-ADSCs were similar to those of normal ADSCs, the level of MHC-I expression was much lower. The US2/US3-ADSCs were used to construct tissue-engineered bone that was then transplanted into a pig animal model carrying a bone defect. No significant differences were observed in the efficacy of the allogeneic US2/US3-ADSCs versus the autologous ADSCs in this experiment. These results indicate that US2/US3-ADSCs could serve as "universal" stem cells to treat bone defects and for other cell-based therapies in the clinic.

Another critical issue faced in bone tissue engineering is ensuring an adequate blood supply, especially when treating large bone defects. Following implantation of tissue-engineered bone in the injured area, coagulation could obstruct fresh blood from perfusing the transplantation site, potentially causing the bone reconstruction to fail. To inhibit the activation of coagulation, the scaffold (acellular bone matrix, ACBM) was coated with heparin/chitosan (HC/ACBM) using a layer-by-layer technology (*7*). The amount of heparin released from the acellular bone matrix was tested using a spectrophotometer and showed persistent heparin release for at least one week in vitro. In addition, the activated partial thromboplastin time (APTT) was significantly longer than that in the control scaffold, which suggests that the released heparin was having an anticoagulation effect (*7*). In a pig animal model, a special negative pressure tube with vacuum-assisted closure (VAC) was placed in the center of the ACBM scaffold in an attempt to improve blood perfusion of the tissue-engineered bone. Computed tomography (CT) perfusion and histological observations showed a higher rate of blood perfusion, more granulation tissue formation indicating better healing, and less bacterial colonization (Figure 1). Furthermore, histological examination showed increased vascularization in the HC/ACBM group at the scaffold implanting sites for large defect bone repair.

In summary, US2/US3-ADSCs had lower immunogenicity and could be ideal stem cells for application in allotransplantation clinical therapeutics. The use of tissue-engineered bone constructed with HC/ACBM together with VAC technology and US2/US3-ADSCs may provide a novel experimental methodology for correcting the large bone defects in the future.

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Promoting Wound Healing in Burn Patients: Pig-Derived Tissues

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How to treat severe burn injuries has been a great challenge for centuries. Although the clinical outcomes for burn patients are still far from ideal, some progress has been made over the last few decades. Two of the main issues for treating burns are finding materials, such as cadaver skin, pig skin, or artificial skin, to cover the extensive wounds, and to reconstruct the cutaneous appendages—such as sweat glands, hair follicles—using stem cells. However, treating burn wounds with allo- or xenogeneic materials can induce an immune response and decrease the survival of the skin graft. Current immune suppression strategies in solid organ transplantation have not been considered as the optimum methods for skin transplantation in burn patients because these patients are immune suppressed. Additionally, scars formed following deep burn injury lack skin appendages, such as rete ridges, sweat glands, and hair follicles, leading to epidermal fragility and anhidrosis (inability to sweat normally). Limited progress has been made using either tissue engineering or stem cell transplantation to reconstruct lost skin appendages. Our research has therefore focused on strategies to induce immune tolerance to pig skin without requiring systemic immune suppression, and on reconstructing normal skin tissue with rete ridges, sweat glands, sebaceous glands, and hair follicles using pig-derived stem cells.

Since the discovery that cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) can block the co-stimulatory pathway between antigen presenting cells and T cells and thus inhibit full T cell activation (*1*), we and others have demonstrated that CTLA4Ig (a fusion protein containing the extracellular domain from CTLA4 and modified CH2-CH3 domains of IgG) is able to induce T cell anergy and prolong the survival of allografts, such as heart, bone marrow, kidney, and skin, in an antigen specific manner (*2–4*). Systemic administration of CTLA4Ig is more likely to induce unwanted side effects than local administration, particularly in severely burnt patients who have compromised immune competence. Therefore, we investigated whether CTLA4Ig gene transfer into skin grafts using an adenovirus vector could achieve localized expression of CTLA4Ig and subsequent immunosuppression. Our data showed that pig skin grafts transfected with the CTLA4Ig gene and grafted onto Balb/c mice survived longer compared to grafts transfected with empty vector (Figure 1A). Safety is a primary concern when using pig skin grafts in humans because of the potential for the transfer of pig retrovirus. However, based on results from a retrospective study of patients who were grafted with living pig tissues (*5*) and after careful evaluation of the abovementioned adenovirus-mediated CTLA4Ig gene transfer model, we felt that the risk was minimal and therefore instituted a multi-center, randomized parallel control clinical trial. Two hundred patients with deep second degree burn wounds, approximately 10 x 10 cm in size, received pig skin grafts transfected with either the CTLA4Ig gene or empty adenovirus vector. The results demonstrated that CTLA4Ig gene-transfected pig skin grafts survived significantly longer than empty vector grafts (18.6 days for CTLA4Ig-gene transfected grafts versus 13.6 days for control) (Figure 1B) without any detectable systemic side effects (data not shown). This gene transfer strategy utilizing pig skin has been approved by the Chinese State Food and Drug Administration (SFDA) and is currently being used in selected

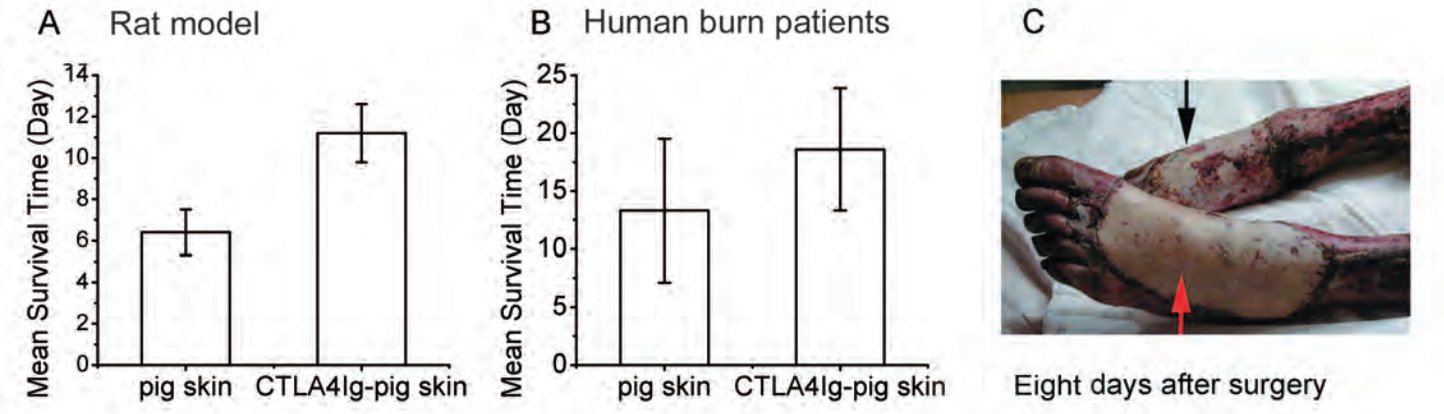

Figure 1. Survival of CTLA4Ig-transfected pig skin in vivo. (**A**) The mean survival time of CTLA4Ig gene transfected pig skin was significantly longer than that of the control group (empty vector) in rat ($p<0.001$). (**B**) The mean survival time of CTLA4Ig gene-transfected pig skin was significantly longer than that of control group on the burn patients (18.8 days vs. 13.6 days) ($p<0.001$). (**C**) Photograph of both CTLA4Ig gene-transfected pig skin and control on a second degree burn wound eight days posttransplantation. Right foot (black arrow) was covered by control pig skin. Left foot (red arrow) was covered by CTLA4Ig gene-transfected pig skin.

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burn units in China (Figure 1C.). Further evaluation of the effectiveness and safety of using CTLA4Ig gene-transfected pig skin grafts is currently being carried out. To improve the effectiveness of gene transfer and remove the need for continually creating transfected pig skin, a skin-specific CTLA4Ig transgenic pig is currently in development.

Previous studies have demonstrated the potential for using in situ stem cells to reconstruct hair follicles (*6, 7*) and bone marrow mesenchymal stem cells cultured in vitro appear to show a sweat gland cell phenotype (*8*). However, fully reconstructing skin appendages in deeply burned areas is still a challenge. A key reason for the lack of success is a poor understanding of, and limited ability to control, stem cell differentiation. Moreover, in vitro construction of the complex three-dimensional structure of skin presents another big challenge. However, one promising way to address these problems is to utilize stems cells that are already committed to differentiate into a particular tissue or organ, called organ-committed precursors, to grow skin grafts. This has already been achieved with organs such as the liver, lung, spleen, and kidney by Reisner *et al.* (*9*). We investigated whether embryonic pig skin precursors (EPSPs) could grow and differentiate into fully functional skin. Our data clearly demonstrate, for the first time, that EPSPs will gradually form full-thickness skin of 1 or 2 mm^2 up to 47 mm^2 (Figure 2A) with all appendages, including epidermis with melanocytes, rete ridges, hair follicles, sebaceous glands, and sweat glands (Figure 2B) in a nude mouse model (*10*). We determined that these neoregenerative tissues were of pig origin using antibodies against either pig epithelial tissue (cytokeratin MNF116) or dermal tissue (Vimentin 9) (Figure 2D) (*11*). Further, we found EPSPs needed to be harvested after embryonic day 56 in order to prevent teratoma formation, as demonstrated by pathology (data not shown) (*10*). Although EPSPs were obtained from embryos and should be less immunogenic, these cells did not grow when grafted into immune-competent mice (data not shown), implying that they were rejected by the host. Induction of immune tolerance to pig-originated tissue is currently being studied in order to be able to potentially use EPSPs directly for wound treatment. In summary, our work provides a novel strategy for using pig-derived materials to treat severe burns wounds and promote cutaneous wound repair.

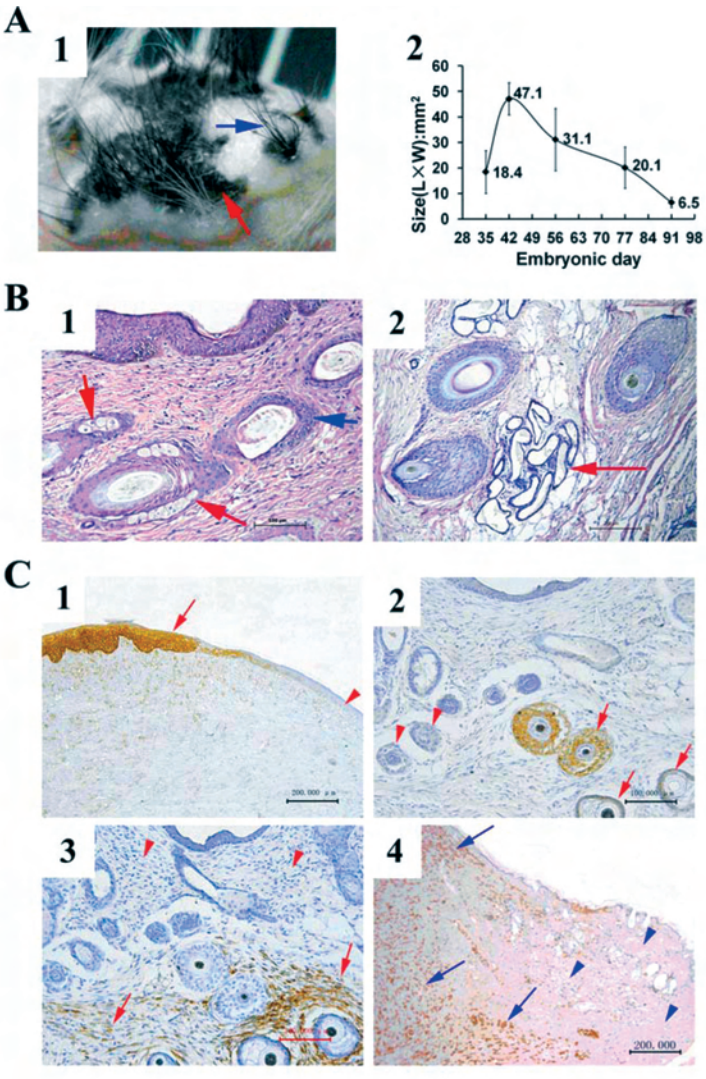

Figure 2. Growth and differentiation of embryonic pig skin precursors (EPSPs). (**A**) Gross observation of EPSPs growth. Four weeks after implantation (panel A1), black coat color (i.e., neoregenerative pig skin) became visible (red arrow). After an average of 4–6 weeks postimplantation, black hair was noted (blue arrow). EPSPs at embryonic day 42 (E42) showed the maximal growth potential (panel A2). There were significant differences between E42 and E35 (p=0.001), E42 and E77 (p=0.001), and E42 and E91 (p=0.001). There was no significant difference between E42 and E56 (p=0.21). (**B**) Developmental histology of embryonic day 56 (E56). Sebaceous glands are indicated by red arrows and the hair follicle by a blue arrow (panel B1). Sweat gland ducts are indicated by the red arrow (panel B2). (**C**) Immunohistological staining of neoregenerative tissues 6–12 weeks after implantation of E56 EPSPs. Cytokeratin MNF116, a marker for pig epithelium and nonreactive with nude mouse epithelia, was used to detect pig skin epithelium. Vimentin V9, a marker for pig mesenchyma tissue and nonreactive with nude mouse mesenchymal cells, was used to detect pig-origin fibroblasts, adipocytes, muscle cells, and blood vessel endothelium. Cytokeratin MNF116-positive cells derived from EPSPs were observed in the epithelium at 12 weeks postimplantation (panel C1, red arrow) and in the hair follicle sheet at six weeks postimplantation (panel C2, red arrows). Vimentin 9-positive cells could be seen in the dermis, around hair follicles, at six weeks postimplantation (panel C3, red arrows), as well as in deep dermis tissue under the host tissue by 12 weeks postimplantation (panel C4, blue arrows). MNF116-negative and Vimentin 9-negative cells from derived from the host nude mouse are indicated by arrowheads in panels C1 and C2, and panels C3 and C4, respectively.

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Artificial Skin as a Sweat Gland Regeneration Matrix

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The advent of artificial skin brings great promise for the treatment of burns, donor sites, chronic skin ulcers (e.g., venous, pressure, and diabetic foot ulcers), and various other dermatological conditions. However, despite the therapeutic efficacy that skin substitutes have shown in vivo (*1*), artificial skin grafts have yet to replace the current gold standard of autologous skin grafts or achieve widespread clinical use. This may be explained, at least in part, by the fact that no artificial skin product has been able to completely replicate the anatomy, physiology, or biological stability of natural skin. From a therapeutic standpoint, restoring skin appendages, including sweat glands, sebacious glands, and hair follicles, is important for maintaining the skin's homeostasis and physiological functions. Sweat glands, for example, play a key role in regulating body temperature and their restoration offers the hope of improving the comfort level and living quality of severe skin injury survivors. Though regenerating skin appendages has proven difficult thus far, our studies have focused on regenerating sweat glands because the recent development of three-dimensional (3-D) bioengineering-based techniques could offer a solution to the reconstruction of the complex temporal-spatial pattern of sweat gland structures.

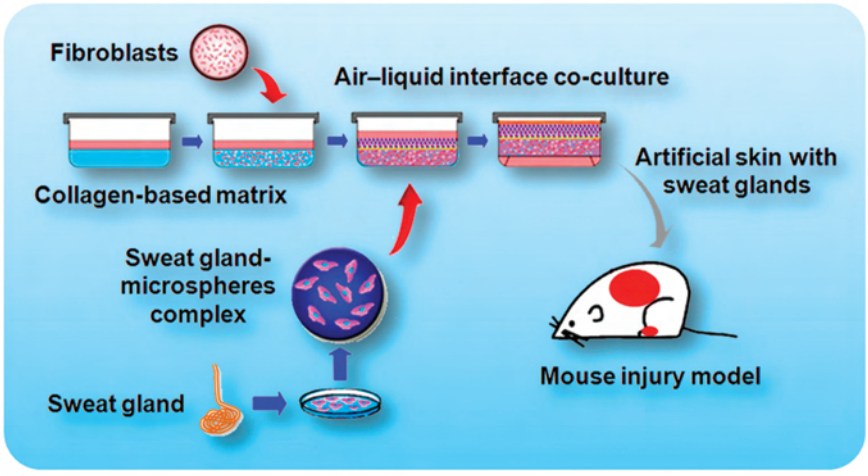

Figure 1. Construction of artificial skin that incorporates sweat glands. The construct consists of a "dermis" with fibroblasts embedded in a collagen-based matrix and an "epidermis" with sweat gland cells and the epidermal growth factor (EGF)-loaded microspheres complex. The efficacy of wound healing and sweat gland regeneration was examined by implanting the artificial skin into excisional wounds on both the back and hind leg paws in a murine model.

Stem cells residing in skin appendages have been found to play a role in the repair and regeneration of injured skin (*2–5*). However, no evidence was found for the creation of new sweat glands, which are derived from the epithelial compartment during development and the regeneration of defective skin tissues. Recently, Reichmann proposed using human sweat gland cells to generate a stratified epidermis on collagen hydrogels. However, sweat gland-derived cells only exhibited the properties of epidermal keratinocytes and no sweat glands were found in the generated epidermis (*6*). These observations impy that not only the appropriate cell type, but also the proper microenvironment, is essential for the recreation of such complex structures. These indispensable elements for in vivo tissue regeneration are also demonstrated to be important for tissue morphogenesis and homeostatic maintenance in engineered tissue models (*7*). From a tissue-engineering point of view, this limitation can be overcome through bioengineered 3-D models, which have the potential to mimic the natural skin's microenvironment (*8*).

Although a number of tissue engineering-based therapies have been investigated for both research and clinical applications, using 3-D models to help restore functional sweat glands in artificial skin has been overlooked thus far. Therefore, we have proposed a multistep tissue engineering process to construct skin in vitro that incorporates sweat glands and can improve the quality of skin repair and sweat gland regeneration during the wound healing process (Figure 1). To ensure that sweat gland cells survive, proliferate, and function after their introduction, we engineered a 3-D human skin construct that replicates the natural sweat gland growth matrix. The construct consists of a "dermis" with fibroblasts embedded in a collagen-based matrix, which provides scaffolding, nutrient delivery, and the potential for cell-to-cell interaction, and an "epidermis," which is comprised of sweat gland cells and microspheres loaded with epidermal growth factor (EGF), a critical inducer for sweat glands development (*9*). Naturally derived materials-based microsphere technology has been attracting growing interest from researchers for its potential use in cell transplantation and tissue-engineering applications. Since growth factors contained in microspheres can be released slowly, reducing loss through diffusion, enzyme digestion, or deactivation, a more potent and consistent effect induced by these factors can be seen. Moreover, microspheres also represent a vehicle for persistent cell growth to circumvent toxic trypsinization and maintain denser aggregation of these cells during tissue formation.

For the preparation of EGF-releasing microspheres, we first immersed gelatin microspheres with diameter of 50–200 μm in an EGF solution in order to load the miscroshperes with EGF molecules. We then analyzed the kinetics of EGF release from the microspheres to determine the rate and longevity of EGF diffusion into surrounding media. We found that EGF was released in a biphasic fashion, characterized by approximately 60% cumulative release in the initial five days, followed by the remaining 30% released in the following two weeks. Sweat gland cells were inoculated onto the EGF-containing microspheres and cultured for three days. Meanwhile, we prepared a 3-D matrix by seeding fibroblasts into cold type I collagen. The cell-microsphere aggregations were then delivered into the uppermost layer of above-mentioned matrix via injection, and keratinocytes were also inoculated at their outermost surface. After incubation for one day, these compounds were raised to the air-liquid interface for an additional week. After two weeks, bud-like sweat gland structures could be observed in this artificial skin. Additionally, we found clear evidence that markers of sweat gland cells were sporadically expressed within the artificial skin models (*10*). More importantly,

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by implanting this engineered skin construct, we demonstrated successful regeneration of sweat glands in the cutaneous wounds of mice (*10*). Except for the indispensible role of sweat gland cells, the regeneration process hinged on the coexistence of EGF and microspheres, which was demonstrated by the deteriorative effects of their absence in the control group (*10*). These results support our contention that 3-D bioengineering of tissue can support sweat gland reconstruction and shows great promise for future therapeutic application to severe skin injury. Enhanced healing was evident in the group treated with engineered skin incorporating sweat gland cells, reflected by more rapid re-epithelialization and organized collagen deposition. This finding is consistent with the abovementioned report that suggests the constructive and supportive role of sweat gland cells in the formation of stratified epidermis (*6*). Although these results are encouraging, extrapolating these findings to humans is still premature. Investigation of the mechanisms underlying the regeneration process and enhanced healing benefits of this model is needed, as well as further characterization of the interaction between sweat gland cells and other skin cells.

Other challenges remain to be addressed before this artificial skin model can be translated into the clinic, such as finding a more ideal cell source. Because of poor cell yield and low efficiency of successful culture, sweat gland cells cannot fulfill the requirement of large-scale applications. We are currently investigating whether bone marrow-derived mesenchymal stem cells (BM-MSCs) can be used to construct an artificial skin that could regenerate both skin tissue and sweat glands during skin repair, because considerable evidence suggests that BM-MSCs have a strong propensity to ameliorate cutaneous damage in response to injury, and they are currently the preferred cell type for clinical use. However, for stem cells to be used, a safe and effective delivery system needs to be designed. The engineered skin model we designed meets these criteria since it allows cells to remain in contact with the wound bed and to be kept viable in the often-hostile wound microenvironment.

To investigate whether BM-MSCs are a good source for sweat gland repair, we constructed a similar artificial skin model in vitro, but used BM-MSCs from C57-green fluorescent protein (GFP) transgenic mice instead of sweat gland cells. This ongoing study has thus far indicated that EGF-loaded BM-MSCs-engineered skin treatment significantly increases the number of regenerating sweat gland-like structures. However, the lack of co-localization of GFP and sweat gland markers suggests that paracrine effects of BM-MSCs might be the causative agent in the induction and development of sweat glands. The cellular origin of the newly formed sweat glands remains to be identified. Importantly, this model highlights the need to find ways to identify the mechanisms that govern cell fate in vivo, as well as the need for practical and relevant biomarkers that can be used to monitor the activity of MSCs after administration. Because we lack these necessities, BM-MSCs and their related functions during wound healing appear more complex than initially envisioned. Despite these drawbacks, this strategy has shown potential application for the recovery of functional sweat glands in vivo. We hope this novel skin construction model will serve as a feasible and effective delivery system for stem cells in regenerative skin applications.

While our ultimate goal is to build a functional artificial skin that provides a healthy environment for sweat gland regeneration, these studies also show promise for the regeneration of other spatially organized organs that can be reconstituted through tissue-engineering methods. Although building a structurally and functionally complete tissue model is practically difficult, researchers in multiple disciplines including biologists, clinicians, and engineers need to work together to address this challenge.

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Collagen-Based Functional Biomaterials for Tissue Regeneration

Bing Chen, Ph.D., Zhifeng Xiao, Ph.D., Chunying Shi, M.Sc., and Jianwu Dai, Ph.D.*

The use of functional biomaterials as scaffolding material, together with biologically active molecules or cells incorporated into that structure, show great promise for promoting tissue regeneration. Among those biomaterials suitable for tissue engineering, collagen is widely used because it is the most abundant protein in the human body and therefore biocompatible with many current applications. Here, we review our recent work on collagen scaffolds loaded with collagen-binding growth factors and stem-cell–capturing functional collagen scaffolds and their effects on tissue regeneration.

Growth factors stimulate cell proliferation, migration, and differentiation, as well as accelerate tissue regeneration. However, in clinical practice it is difficult to maintain the therapeutic concentrations of growth factors at wound sites because they rapidly diffuse away from the application site. We have developed an immobilization strategy that prevents the rapid diffusion of growth factors at the target sites. By fusing growth factors to a collagen-binding peptide using genetic engineering, the proteins acquire the ability to bind specifically to collagen (see Figure 1A). Functional biomaterials are subsequently

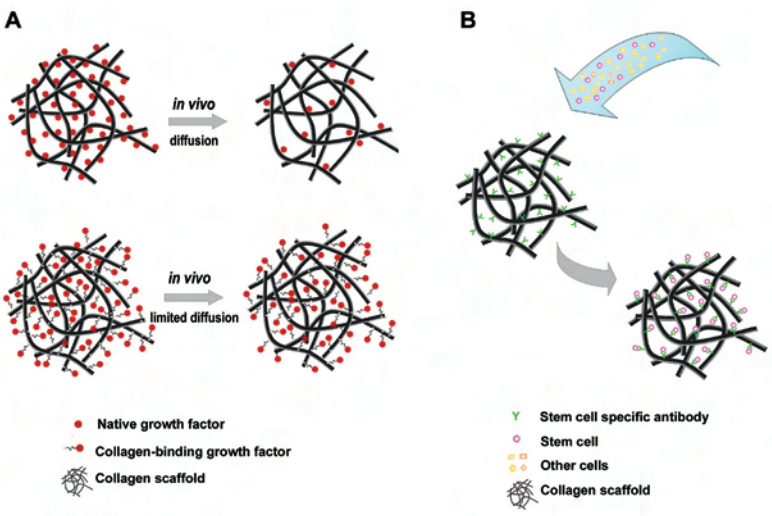

Figure 1. Diagram explaining collagen-based functional biomaterials. (**A**) Schematic drawing of collagen-binding growth factors binding to collagen, thereby limiting their diffusion. (**B**) Collagen scaffold with stem cell surface-specific antibodies to capture autologous stem cells from circulating blood.

produced by combining collagen scaffolds with collagen-binding growth factors. We have tested a variety of such constructed functional biomaterials in animal models with skin, bone, abdominal wall, bladder, uterine endometrium, and heart tissue defects as well as several nerve injuries, and found significant tissue regeneration (see Table). The findings suggest that this immobilization strategy could be employed for many important areas of regenerative medicine.

Bone Formation

Segmental bone loss or non-union results in a demand for new tissue to replace and restore the function of the lost bone. Active bone filling materials are needed to promote new bone formation. Demineralized bone matrix (DBM) is a bone-graft substitute that preserves the natural structure of collagen, but lacks the factors needed to induce bone formation. We tested whether infusing a genetically engineered collagen-binding growth factor into a DBM could be an effective functional biomaterial to induce bone growth in the rabbit mandible defect model. The bone defects measuring 12 mm x 5 mm x 4 mm—a so-called critical size defect—were created on both mandibles. After fusing bone morphogenetic protein-2 (BMP-2), a potent osteoinductive factor, to a collagen-binding domain (CBD-BMP2) and loading on the DBM, the functional biomaterials were grafted to the mandible defect sites. Remarkable osteoinduction and homogeneous bone formation were observed, with X-rays indicating healing percentages four weeks after surgery of 80%, 60%, and 30% in CBD-BMP2 group, native BMP2 group, and no treatment group, respectively. By 12 weeks, the healing percentages were 98%, 90%, and 60%, respectively. Histological analysis showed the CBD-BMP2 group formed more homogeneous bone than the other groups (*1*).

Skin and Muscle Regeneration

Skin and muscle tissues have layered structures with anisotropic and elastic properties. We tested whether porous collagen membranous materials with collagen-binding growth factors could be effective for regenerating these complex tissues.

Cellularization and vascularization of the scaffold are the important steps for tissue regeneration. In 2 cm x 2 cm full-thickness rectangular skin defects on the backs of rats, the implanted collagen membranes activated by collagen-binding platelet-derived growth factor BB (CBD-PDGF-BB) were uniformly cellularized and vascularized (*2*). In a model of rabbit ear dermal ischemic ulcer, made by dissection and ligation of the rostral and central arteries of the ear after full-thickness excision of dermal, the activated collagen membranes promoted the healing of the dermal ulcer wound (*3*).

Additionally, we tested collagen membranes with collagen-binding basic fibroblast growth factor (CBD-bFGF) in abdominal wall, bladder, and uterine tissue repair rat models. In the abdominal wall defect model, the functional collagen membrane enhanced the integration of skeletal myofibers into the scaffold and improved the mechanical strength of the regenerative skeletal muscle (*4*). In the bladder partial cystectomy model made by transecting the upper part of the bladder, a CBD-bFGF–loaded collagen membrane improved vascularization, and smooth muscle cell ingrowth in the scaffold. Urodynamic tests revealed that the reconstructed bladder tissue was functional (*5*). Finally, in the model with a severely damaged uterine endometrium, CBD-bFGF–activated collagen membranes promoted endometrial regeneration and improved pregnancy outcomes (*6*).

Myocardial Tissue Regeneration

Ischemic heart disease causes permanent myocardial injuries. Collagen constitutes the bulk of the cardiac extracellular matrix and its expression is up-regulated during the injury. We hypothesized that endogenous collagen could act as the target of collagen-binding vascular endothelial growth factor (CBD-VEGF) to improve myocardial regeneration. We tested the effects of injecting CBD-VEGF into the infarcted area of rats with acute myocardial infarction. The CBD-VEGF was mainly retained in the cardiac extracellular matrix. Four weeks after treatment, we observed reduced scar size and increased proliferation of capillary vessels in the infarcted areas. Echocardiography and hemodynamics evaluations confirmed that cardiac functions were improved (*7*). A functional cardiac patch consisting of the collagen membrane and CBD-VEGF was also assessed in a rabbit myocardial infarction model. After four weeks, it had effectively induced cells to penetrate the collagen membrane and had promoted vascularization in the infarcted myocardium by 12 weeks. Echocardiography and hemodynamic assessments indicated that cardiac function was improved (*8*).

Nerve Regeneration

Nerve regeneration is important for the repair of injuries to peripheral and central nervous systems, but regeneration under natural conditions in these systems is rare. It is important that nerve repair provide two things: an ordered scaffold to guide the orientation of the regenerative nerve, and neurotrophic factors (essential for nerve cell survival and growth) to induce regeneration of the neurons.

In the peripheral nerve repair, we functionalized linear ordered collagen scaffolds (LOCS) with collagen-binding neurotrophic factors to test whether this could promote nerve regeneration. LOCS have an ordered collagen structure and can guide the orientation of the regenerating nerve. LOCS loaded with collagen-binding ciliary neurotrophic factor (CBD-CNTF) were shown to improve neuronal survival and regeneration in the rat sciatic nerve transection model. Moreover, they

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Collagen scaffold	Collagen-binding growth factor	Animal model	Regenerated tissue (*reference*)
Demineralized bone matrix (DBM)	BMP-2	Rabbit mandible bone defect model	Bone (*1*)
Collagen membrane	PDGF-BB	Rat full-thickness skin defect model	Skin (*2*)
	PDGF-BB	Rabbit ear dermal ischemic skin ulcer model	Skin (*3*)
	bFGF	Rat abdominal wall defect model	Abdominal wall (*4*)
	bFGF	Rat bladder partial cystectomy model	Bladder wall (*5*)
	bFGF	Rat uterine endometrium defect model	Uterine horn (*6*)
	VEGF	Rabbit acute myocardial infarction model	Heart (*8*)
Linear ordered collagen	BDNF	Rat hemisected spinal cord injury model	Spinal cord (*10*)
	BDNF	Rat transected spinal cord injury model	Spinal cord (*12*)
	CNTF	Rat sciatic nerve transection model	Sciatic nerve (*unpublished data*)
Endogenous collagen	VEGF	Rat acute myocardial infarction model	Heart (*7*)
	BDNF	Rat crushed spinal cord injury model	Spinal cord (*11*)
	NGF	Rat sciatic nerve crush model	Sciatic nerve (*9*)

Table 1. Functional collagen-based biomaterials for tissue regeneration.

significantly enhanced nerve regeneration as well as the recovery of nerve function, as assessed by behavioral, electrophysiological, and histological analysis (unpublished data). Further, because collagen itself is abundant in the nerve extracellular matrix, we tested whether it could serve as an endogenous scaffold for nerve regeneration by injecting collagen-binding nerve growth factor (CBD-NGF) into the injured area of rats with a crushed sciatic nerve. CBD-NGF was mainly retained at the injection site by binding to the endogenous collagen and promoted nerve repair and function recovery, as assessed by western blotting analysis, animal behavioral, electrophysiological, and histological examination (*9*).

Spinal cord injuries (SCIs) are a serious central nervous system (CNS) trauma that often results in permanent damage and severe disabilities. Brain-derived neurotrophic factor (BDNF) is important in neural survival and axonal regeneration in the CNS. Therefore we tested whether LOCS combined with collagen-binding brain-derived neurotrophic factor (CBD-BDNF) could help promote nerve regeneration in the rat hemisected spinal cord model. The results showed that the functional biomaterials significantly improved nerve recovery evaluated by the Basso, Beattie, Bresnahan scale, and by immunohistochemical staining (*10*). Further, high levels of endogenous collagen at the wound area after spinal cord injury led us to hypothesize that this could serve as the in situ binding site for CBD-BDNF. A one-time intrathecal injection of CBD-BDNF into the injury site of a rat spinal cord crushed injury model showed retention of CBD-BDNF, as well as neural regeneration and locomotion recovery assessed by western blotting analysis, behavioral, electrophysiological, and histological analysis (*11*).

During CNS regeneration, the lack of axon regeneration has been partially attributed to specific inhibitor molecules such as myelin associated proteins and chondroitin sulfate proteoglycans (CSPGs). Because these inhibitors are downstream of EGFR signaling, we attempted to antagonize these molecules by neutralizing EGFR using an antibody. We cross-linked the LOCS with the anti-EGFR antibody, followed by CBD-BDNF addition. The triple-functional biomaterial was implanted into the injury site in a 6 mm spinal cord transected injury model, and it provided effective bridging and stimulation effects for neural regeneration. Further, it promoted the recovery of electrical transmission of synapses and prevented the formation of glial scars, which also contributes to a lack of axon regeneration in CNS regeneration (*12*).

Stem Cell Capturing

Another strategy for constructing functional biomaterials is to recruit native autologous stem cells (or other cell types) to collagen scaffolds at a wound site. In a mouse model, we attempted to recruit stem cells using an anti-Sca-1 antibody crosslinked to a collagen scaffold, since Sca-1 is a known surface antigen on hematopoietic, cardiac, and skeletal muscle stem/progenitor cells. We found that Sca-1–positive stem cells could be enriched to the functional collagen scaffold (see Figure 1B). When the same collagen scaffold was transplanted as a patch to repair a surgical heart defect in the mouse model, it enriched autologous stem cells from the circulation and promoted myocardial regeneration (*13*).

In summary, collagen scaffolds with collagen-binding growth factors or stem cell-capturing antibodies are functional biomaterials which can effectively promote tissue regeneration in various animal models. However, tissue regeneration is a complex process, involving many endogenous factors. We would therefore like to test functional collagen materials with multiple growth factors in future studies to determine whether the collaborative effects could further improve tissues regeneration. The ultimate goal is to use these functional biomaterials to develop medical device products that can reconstruct damaged human tissues or organs.

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Functional Sweat Gland Regeneration: Preliminary Success but Still a Long Way to Go

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The survival rate for burn patients in China is almost 98% due to advances in burn treatments. However, these survivors still face the loss of skin function due to the formation of hypertrophic scars during healing, which do not regenerate skin appendages such as hair follicles, sweat glands, and sebaceous glands. Humans normally expend about 25% of their body heat through perspiration. However, burn survivors cannot perspire because of the loss of skin sweat glands, which harms their body's ability to regulate temperature. Thus, promoting the recovery of skin function during wound healing presents an important challenge for physicians and medical scientists. Over the past few years, we have been investigating the theories and technologies behind sweat gland regeneration to help restore sweat gland function for burn patients. We have developed an innovative method to recover sweat gland function in transplanted skin, which can potentially be used to establish tissue-engineered skin to treat both burn victims and those with heritable skin diseases that impair their ability to perspire.

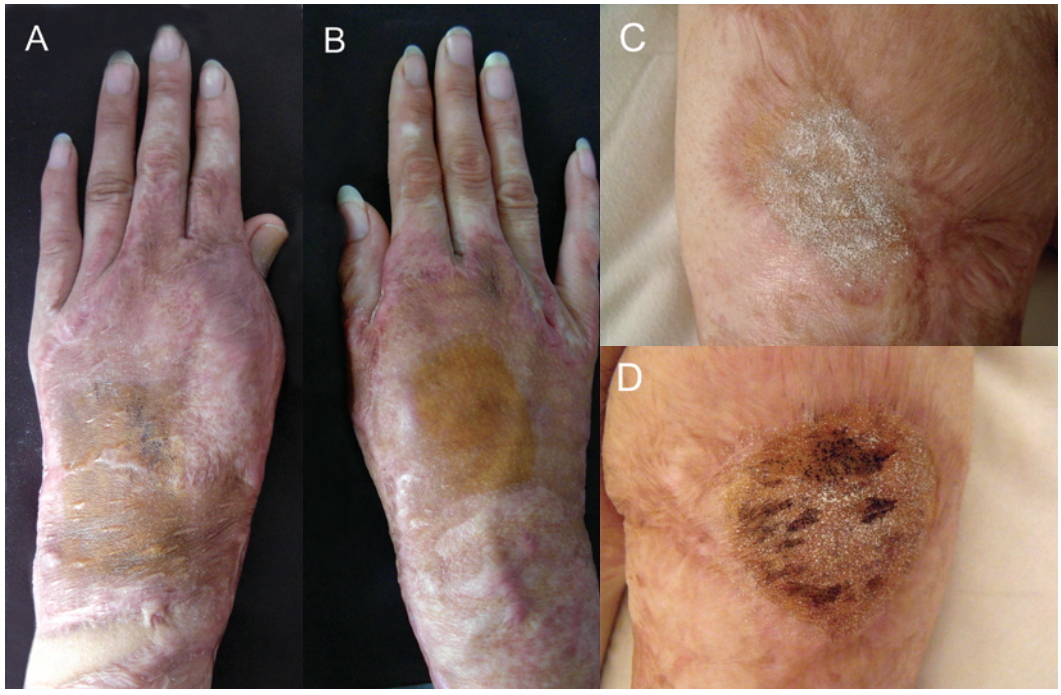

Figure 1. One-year and three months follow-up of a burn case treated with induced mesenchymal stem cells (MSCs) and its control. Iodine-starch perspiration test confirmed a positive reaction in wounds implanted with sweat gland-like cells (**A**) and a negative reaction in the control (**B**). Two-year follow-up in a burn case treated with induced MSCs and its control. Iodine-starch perspiration test confirmed a negative reaction in the control (C), but a positive reaction in wounds implanted with sweat-gland–like cells (**D**).

Our first goal was to understand the developmental characteristics of sweat glands in embryos and adult skin. Sweat gland development is a complicated biological process with three embryonic stages, beginning in week 12 and going through approximately week 36 (*1, 2*). In the earliest stage, around week 12, basal cells gradually enlarge and form cell clusters, which become cell buds and epidermal ridges. These cell buds then migrate down into the dermis as cords and form the anlagen of skin appendages. In the second stage, these anlagens form sweat glands with coil-like structures and primitive intracutaneous ducts. In the final stage, the sweat glands continue to mature and become vascularized. Sweat-gland development requires the synergetic interaction of stem cells, growth factors, and matrix metalloproteinases. In human fetal skin, epidermal stem cells serve as the stem-cell source for sweat gland development. Growth factors, cytokines, and extracellular matrix components are all required for these cells to proliferate and differentiate into mature epidermal cells. In particular, epidermal growth factors may serve as autocrine or paracrine modulators that signal epidermal cells to form the sweat-gland cell clusters (*1, 2*). Matrix metalloproteinases also help sweat gland bud formation and migration from epidermis to dermis. However, adult skin can no longer naturally regenerate sweat glands in this way. Moreover, it lacks a postdamage regeneration process. Therefore, sweat gland regeneration must be stimulated by exogenous stem cell therapy.

In order to find a stem cell-like source to use for adult skin regeneration, we attempted to induce epidermal cells to dedifferentiate into epidermal stem-like cells. In 2001, we found and reported in *The Lancet* that epidermal cells could be dedifferentiated into epidermal stem cells via growth-factor stimulation both in vivo and in vitro (*3*). Since then, we have evaluated the possibility of using epidermal stem cells, mesenchymal stem cells (MSCs), and adipose stem cells as seed cells for regenerating sweat-glands in severely burned skin. We proposed MSCs as the best choice because they exist in marrow and cannot be easily damaged in case of burn, they are abundant, and they can be easily obtained (*3–5*).

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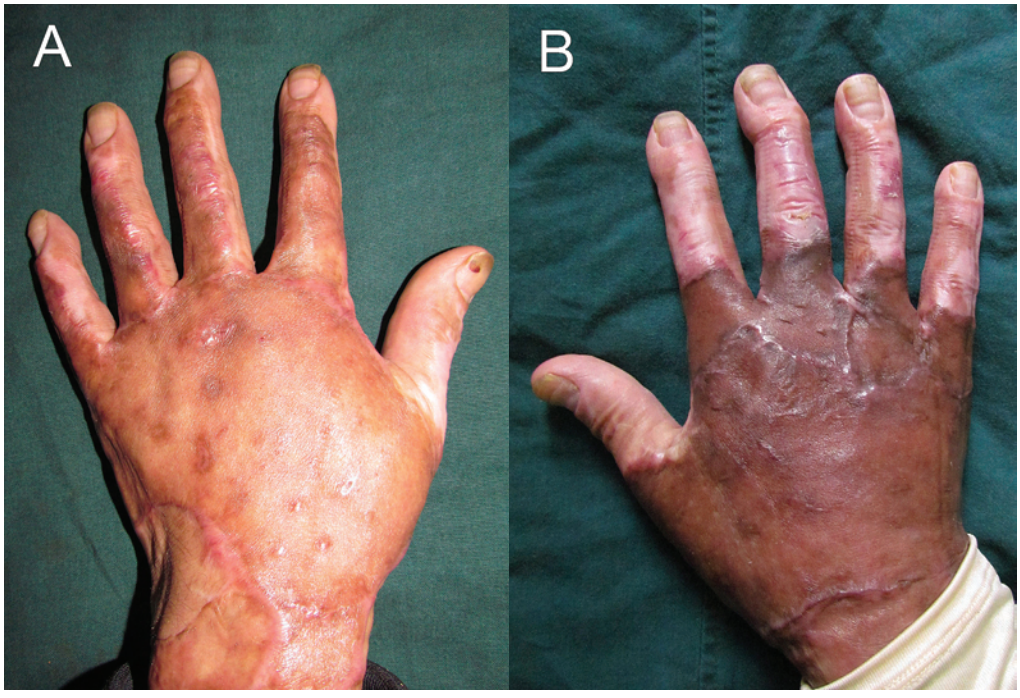

Figure 2. Three-month follow-up in a burn case treated with induced MSCs. Scar formation in wounds treated with induced MSCs (**A**) is reduced compared to the control (**B**).

We next attempted to induce the differentiation of human bone-marrow MSCs (hMSCs) into sweat-gland–like cells (SGCs) using in vitro co-culture methods. We found that the key variables to induce this phenotype shift were heat shock stimulation and temperature control. Subjecting SGCs to 47ºC for about 40 minutes and then 37ºC for one to two hours was most effective at stimulating the disruption of intercellular junctions via the retraction of the cytoplasm, which resulted in the release of an unknown cytoplasmic factor that stimulated the phenotype change of co-cultured MSCs. This coculture method can produce a normal SGC phenotype for about 37% of the MSCs cultured (*6–9*). Subsequently, we labeled induced SGCs with BrdU to detect proliferation in these cells. The labeled cells were transplanted onto the paws of BALB/c nude mice following a full-thickness scald injury. Two weeks later, we used an iodine-starch perspiration test to confirm the presence of sweat in SGC-implanted wounds. Further, a histological examination confirmed that the perspiring cells came from the BrdU-labeled SGCs (*6–9*).

Following these cell and animal experiments, we tested SGCs as a burn treatment for patients in a clinical trial. We obtained autologus bone-marrow MSCs from patients and co-cultured them using the method described above. We then transplanted these induced SGCs onto the skin wounds of the burn patients. As the skin healed, it appeared to regenerate sweat gland-like structures. We confirmed the presence of such structures in biopsies of the treated wounds, and demonstrated that the sweat gland-like structures showed normal sweating function using the iodine-starch perspiration test (Figure 1) (*8, 9*). The pH and levels of biochemical components, such as Na^+, K^+, Cl^-, and $iCa2^+$, in the sweat obtained from the transplantation area were similar to that in normal sweat. To date, 23 patients with severe burns have undergone this innovative sweat gland regeneration process and were found to have satisfactory sweating results. A three-year follow-up study of some typical cases has confirmed a continued sweating ability. In addition, scar formation was found to be less severe in the induced MSC-treated wounds compared to controls (Figure 2).

These results led us to investigate whether induced SGCs could be used as seed cells for establishing a new generation of tissue-engineered skin that contains sweat glands. Because different pH and cell culture conditions are needed for SGCs and tissue-engineered skin, a key step was finding a medium that was be suitable for both. Our preliminary studies confirmed that tissue-engineered skin containing sweat glands could indeed be established with the method (*10*).

Though our preliminary success with sweat gland regeneration brings new hope for small organ repair and regeneration using stem cell dedifferentiation or transdifferentiation, we still have a long way to go. We would still like to explore whether there are better co-culture media and/or conditions for the transdifferentiation of MSCs into SGCs. Moreover, questions remain regarding the underlying mechanisms of sweat gland regeneration and their viability for clinical application. For example, a histological evaluation of the sweat gland-like structures in the induced MSC-treated wound biopsies differed from normal sweat-gland structures. Thus, whether these structures have the same perspiration mechanisms and function as normal sweat glands still needs to be investigated. Another issue is whether our method can address all of the perspiration anomalies that occur with massive and deep skin burns, since we only observed perspiration in small areas of the regenerated skin (*9*). We believe that our preliminary work investigating engineering skin constructs with sweat glands provides new hope for burn patients (*10*).

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Functional Tooth Regeneration

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Tooth loss is one of the most common disorders in humans and leads to physical and mental dysfunction (*1*). Current tooth replacement therapies do not involve biological restorations and have unsatisfactory outcomes. For example, dental implants, one of the preferred artificial prostheses, can fail to meet bionic requirements due to a lack of physiological periodontal tissue support. Tooth regeneration, a strategy providing living, functional, and biocompatible tissues that is more in line with the human desires for real third dentition, can represent an attractive alternative to classical prostheses-based therapies. Studying tooth regeneration can contribute to understanding developmental processes that are applicable to regenerative medicine. Recently, significant progress has been made using various methods to tissue-engineer regenerated tooth tissue (*1–3*). Two strategies for tooth regeneration have been established: stem cell-based tissue engineering and mimicking tooth development through epithelial-mesenchymal cell interactions.

We have proposed the concept of generating a "bio-root" using stem cell-based tissue engineering. The strategy is to implant preshaped root-like scaffolds embedded with seed stem cells into the alveolar bone in order to form a functional bio-root capable of supporting post crown prostheses (*4*). We also identified a new population of stem cells isolated from the root apical papilla of human teeth (SCAP) that demonstrated a high capacity for tissue regeneration (*4*). Using a large animal model, the miniature pig, we introduced a protocol for bio-root implantation: a root-shaped hydroxyapatite/tricalcium phosphate scaffold loaded in the SCAP and coated with periodontal ligament stem cells (PDLSCs) was implanted into the jawbone. After three to six months, a porcelain tooth crown was inserted into the bio-root and the regenerated tooth performed physiological activities for four weeks. Newly formed bio-roots capable of supporting a physiological-shaped tooth crown demonstrated significantly improved biological integration, compressive strength, and functional restoration. Importantly, our studies demonstrated that dental stem cells maintain low immunogenicity, thus it is feasible to bank dental stem cells for accessibility in dental clinical practice for future autologous and allogeneic use in tooth regeneration (*5, 6*). In addition, autologous and allogeneic PDLSC-mediated periodontal tissue regeneration was successfully achieved in a miniature pig periodontitis model (*5, 7*). Furthermore, we found that prostaglandin E2 plays a crucial role in allogeneic PDLSC-mediated immunomodulation and periodontal tissue regeneration in vitro and in vivo (*5*). A long-term follow-up study of regenerated bio-root based functional tooth using allogeneic dental stem cells is now being performed in miniature pigs by our group. Taken together, these results suggest that the bio-root is a promising alternative for dental implants used clinically today.

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Next, we tested a strategy for whole tooth regeneration taking advantage of epithelial and mesenchymal cell interactions to mimic tooth development. When single dental epithelial cells reassociated with dissociated dental mesenchymal cells from tooth germs, the compartments of cell reassociation retain odontogenic potential and faithfully recapitulate typical dental histogenesis and morphogenesis in vitro and in vivo in mice (*2*). These results showed that the method for bioengineered tooth germ through cell aggregation can be used for tooth regeneration. Thus, a two-step implantation–transplantation strategy was proposed for whole tooth regeneration, in which the tooth crown is constructed in vitro, followed by ectopic implantation in vivo to achieve root development and vascularization, resulting in a final in situ implantation to replace missing teeth. Initial temporary ectopic implantation under the skin of the host not only reduces immunoreaction but also allows further development of the bioengineered tooth (*2*). Similar feasibility of regenerating a functional tooth in the jawbone was also validated in rodents by other groups (*3, 8*). The source of seed cells and the control of tooth development are the main challenges of tooth regeneration based on cell reassociations. In addition to the above-mentioned dental stem cells as a promising source for mesenchymal cells, we showed that c-Kit$^+$ bone marrow cells can give rise to ameloblast-like and odontoblast-like cells, providing a novel cell source for tooth regeneration (*9*).

The miniature pig has both deciduous and permanent dentitions and tooth development and eruptions in this model are similar to those in humans, which provides plausible advantages for investigating tooth development and regeneration (*10*). On the basis of previous work, we investigated the regeneration potential of tooth germs from miniature pigs. The cap stage germs of the first mandibular deciduous molar teeth isolated from miniature pigs on embryonic day (ED) 43 were processed using four strategies (Figure 1). A miniature pig's tooth was regenerated using a pellet made up of only dental epithelial cells, reassociated with a pellet of reaggregated dental mesenchymal cells, maintained in a compartmentalized format. After culturing for three days, the reassociated cells were transplanted into a nude mouse kidney capsule, and were able to give rise to a correct tooth structure, including development of the crown, root, and supporting tissues (periodontium, cementum and attached bone) 16 weeks after transplantation. In contrast to reassociation, reaggregation of dissociated dental mesenchymal cells alone from cap stage tooth germs could form only bone-like tissue and failed to generate a tooth structure. We also confirmed that reaggregation of mixed epithelial and mesenchymal cells from miniature pig cap stage tooth germ cell pellets, without compartmentalization, can maintain the ability to self-sort and develop into a tooth germ structure. In addition, a nondissociated cap stage tooth germ transplanted into a kidney capsule could continue to develop and form a tooth with surrounding bone. These studies provide a useful model system for analyzing the development and organogenic molecular mechanisms of tooth regeneration in a large mammal model. An advanced strategy for transplanting a regenerated tooth in the jawbone is currently being developed by our group.

In conclusion, our studies provide potential translational strategies for stem cell-mediated bio-root and whole tooth regeneration, with a final goal of achieving functional tooth regeneration in humans.

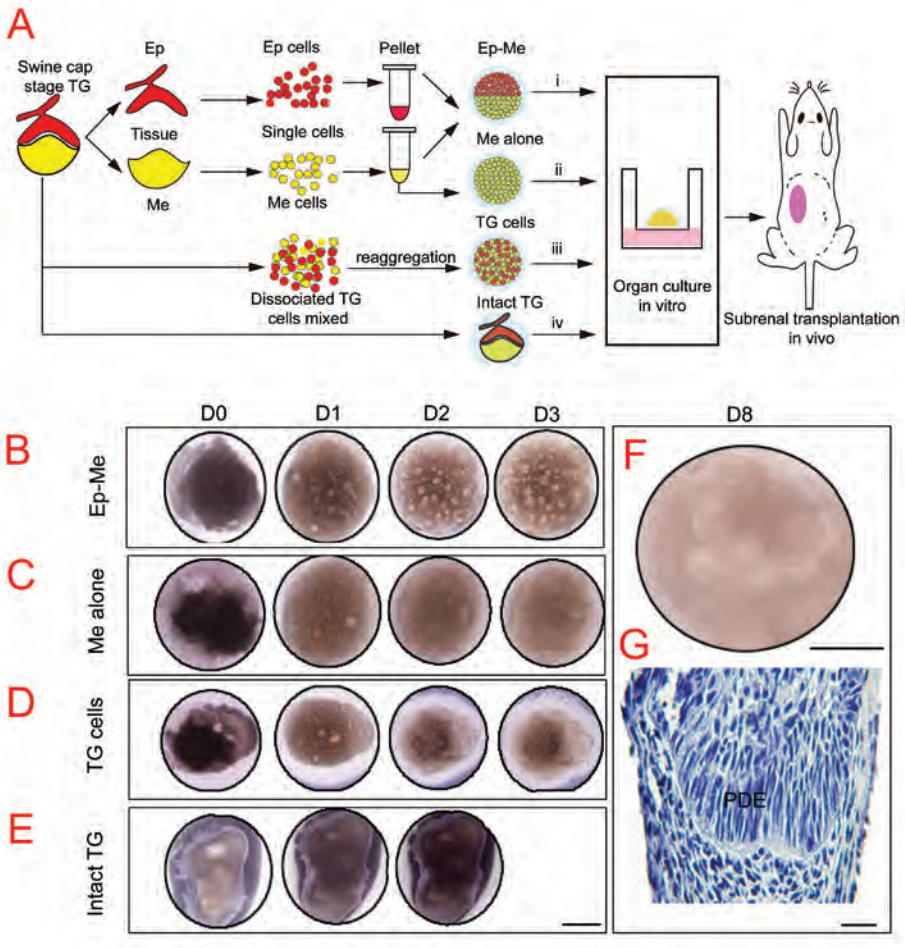

Figure 1. Strategies and studies for tooth regeneration in a large animal model. (**A**) Schematic representation of cap stage (TGs) at embryonic day 43 from a miniature pig treated with four different techniques for the regeneration in vitro and in vivo. In technique i (Ep-Me), after separating epithelium and mesenchyme, the two tissues were dissociated into individual cells and pelleted separately, then reassociated to form explants of artificial TG, allowing the cells to remain compartmentalized. In technique ii (Me alone), the explants were composed only of mesenchymal cells without epithelial cells from TG. For technique iii (TG cells), cap stage TGs were dissociated into mixed single cells, including both epithelial and mesenchymal cells, and allowed to reaggregate without compartmentalization. In technique iv (intact TG), the cap stage TGs were used without disassociation. All explants were cultured for three days in vitro on Transwell inserts in 12-well cell culture plates and then transplanted into the kidney capsules of nude mice for 8 or 16 weeks. (**B** to **E**) Phase contrast images of explants using the four techniques, cultured in vitro for three days. (**F**) Phase contrast image of Ep-Me cultured in vitro for eight days, showing morphologic changes of reassociated explants. (**G**) Histology of Ep-Me explants cultured for eight days in vitro, showing a bud stage-like epithelial structure formed with two histological distinctive cell populations: peripheral elongated cells and central stellate cells. Ep, epithelium; Me, mesenchyme; PDE, polarized dental epithelium; TG, tooth germ. Scale bars: 500 μm in B to F and 50 μm in G.

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Therapeutic Implications of Dermal Multipotent Cells for Wound Repair

Chunmeng Shi, Ph.D.*, Li Tan, M.S., Yongping Su, Ph.D., Tianmin Cheng, M.D.

It has recently come to light that many mesenchymal/connective tissues harbor multipotent stem/progenitor cell populations that hold great promise for regenerative medicine. It is expected that the skin will be a larger reservoir for adult stem/progenitor cells compared to other tissues because it is the largest organ in the body and can be accessed easily with minimal invasiveness. However, the characterization of skin stem/progenitor cells has only recently begun to gain attention. Here we review our studies investigating the characteristics and therapeutic potential of dermal multipotent cell populations.

In 2001, we and others first described the existence of previously unidentified dermal multipotent cell populations in the skin of adult mammals, such as mice, rat, and human, which are similar to bone marrow mesenchymal stem cells (*1*). We provided direct evidence that these dermal cell populations have a wide differentiation potential by intravenously transplanting dermal cells from green fluorescent protein (GFP) transgenic mice into lethally irradiated recipient mice. These GFP cells gave rise to cells in many different tissue and organs, such as bone marrow, lung, liver, and kidney (Figure 1). Stem cell therapy is expected to have significant impact on future regenerative medicine therapies and a sufficient supply of stem cells is critical for clinical applications. We also established a unique clonal population of dermal multipotent cells (DMCs) from neonatal rat skin (*2*). DMCs were isolated based on whether they quickly adhered to tissue-culture plastic and possessed the characteristic ability to differentiate in vitro into multiple mesenchymal and neural lineages, and insulin-producing cells, suggesting a high potential for multilineage differentiation. Their surface antigenic profile has been shown to be positive for CD44 and CD90 expression, but negative for CD34, similar to marrow mesenchymal stem cells. These cells exhibit a much higher proliferation potential than bone marrow-derived mesenchymal stem cells under ex vivo culture conditions. We have proposed that the hair follicle papilla is the likely anatomical niche for these multipotent dermal cells (*1*).

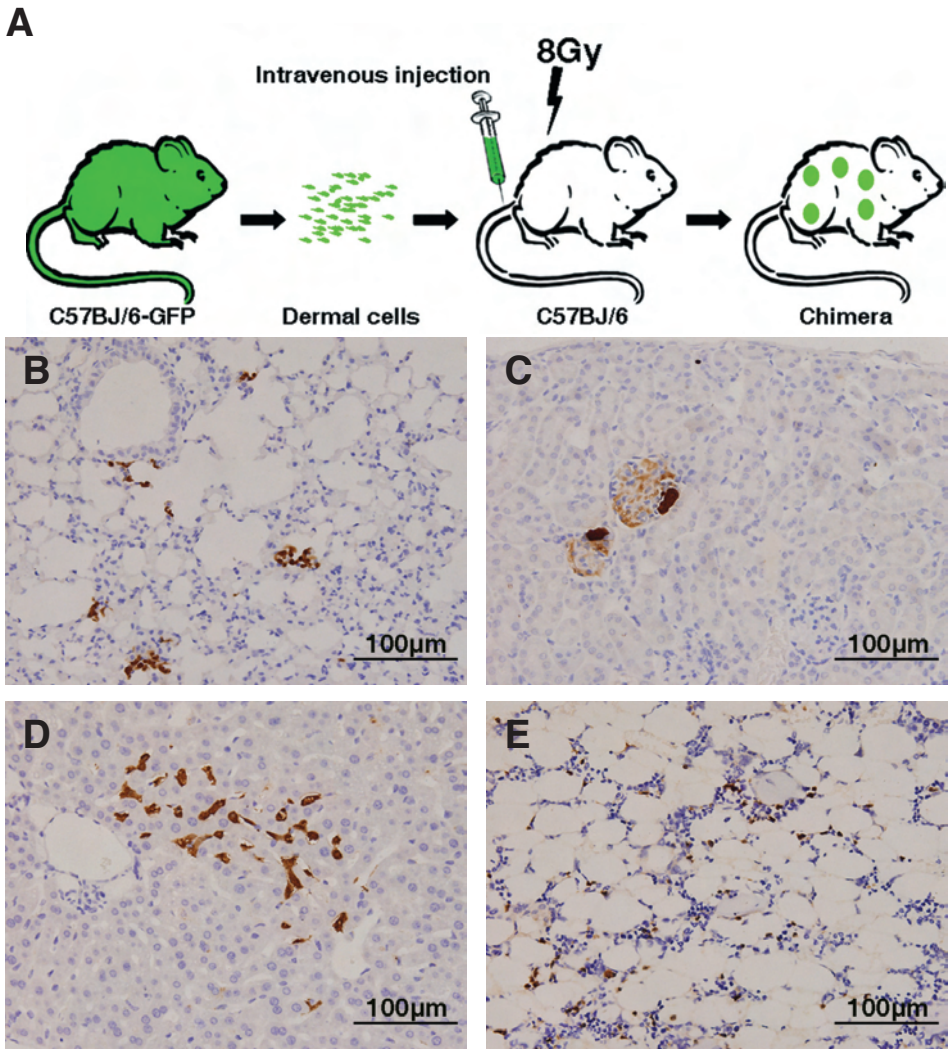

Figure 1. Dermal-derived cells incorporated into multiple tissues in mice receiving lethal doses of irradiation (8Gy). The lethally irradiated mice received transplanted dermal cells from newborn C57BL/6-GFP mice and the resulting chimerism was evaluated using immunohistochemical staining with GFP-specific antibody (**A**). GFP-positive donor cells were observed in multiple recipient tissues, such as lung (**B**), liver (**C**), kidney (**D**), and bone marrow (**E**) two weeks after transplantation.

However, the number of stem cells in the adult tissue is very low, and there is a need to identify ways to expand the supply of undifferentiated cells ex vivo. We have established that β2-microglobulin, a MHC class I subunit, could act as a novel growth factor to stimulate the ex vivo expansion of undifferentiated multipotent stem cells (such as dermal multipotent cells) to reduce the use of fetal bovine serum, which can elicit possible contamination or immunological reaction for clinical application (*3*).

The existence of multipotent cell populations within dermal tissues strongly suggests they may play a role in the repair of wounded tissues. Therefore, we investigated the therapeutic effects of applying exoge-

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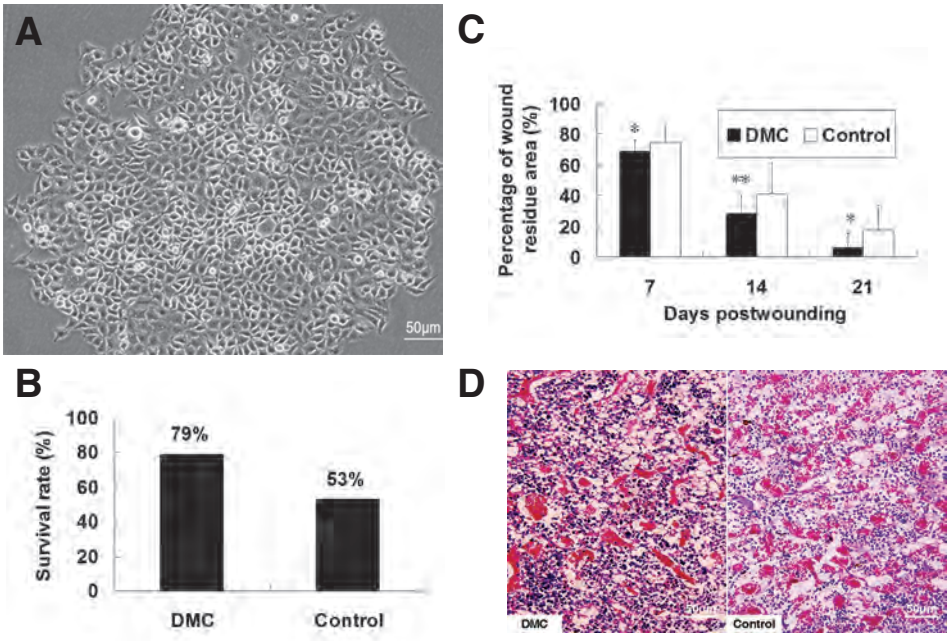

Figure 2. Multipotent dermal cells have multiple therapeutic effects in rats with radiation-induced and skin wound injuries. Systemic transplantation of $5x10^6$ DMCs (**A**) into rats that received 5 Gy total body irradiation of γ rays and skin wounds corresponding 2.5% of total surface area led to an increase in the survival rate of 26% (**B**), accelerated wound healing (**C**, *, $p<0.05$; **, $p<0.01$), and improved bone marrow hematopoiesis (**D**) in comparison with the control group without cell transplantation.

nous DMCs to skin wounds and showed that both topical and systemic transplantation of DMCs accelerates the healing of simple wounds in rats. However, we noted that topical transplantation promoted the healing effects earlier than systemic transplantation (*4*). Additionally, we showed that engrafted DMCs could differentiate into cell types that are important for tissue repair, indicating they may be a potential source of wound-healing fibroblasts (*2*). DMCs also produce many growth factors—such as hepatocyte growth factor (HGF) to improve wound healing (*4*). HGF not only induces mitogenic and antiapoptotic activity, but also has an immumodulatory action to create an appropriate inflammatory response for DMCs at the wound. Furthermore, we also observed that DMCs have the capacity to induce the formation of hair follicle-like structures at subcutaneous sites in nude mice when implanted along with follicle epithelial cells, suggesting that these multipotent dermal cells are also involved in skin regeneration (*5*). In addition to skin wound healing, DMCs are also capable of promoting the hematopoietic recovery in sublethally irradiated rats. These cells can act as an alternative source of marrow stromal cells, which restore the microenvironment and promote hematopoietic recovery (*6*). There is a preferential recruitment of implanted DMCs to wounded tissues that is mediated, at least in part, by an elevated expression of stroma-derived factor (SDF-1) after an injury. SDF-1 is a ligand for the CXC chemokine receptor 4 (CXCR-4) on DMCs and SDF-1/CXCR4 signaling is critical for the recruitment of stem cells to the site of injury. DMCs were further applied through tail vein in rats that received radiation and have skin wound injuries, and the results were the first to indicate that systemic transplantation of dermal stem cells can simultaneously promote wound healing, hematopoietic recovery, and increase the survival rate (by 26%) (Figure 2). This study suggests that stem cell therapy can achieve multiple therapeutic effects and provides a potential new strategy for the treatment of severe traumatic injuries with multiple tissue/organ damage, such as radiation combined injuries (*7*).

Given that DMCs play an important role in wound repair and are now emerging as potential therapeutic agents (*5*), we propose that the dermis may represent one of the best autologous sources of stem/progenitor cells for therapeutic applications, not only in the replacement of skin, but also for the tissue repair of other organs. However, one of the current challenges in studying stem/progenitor cells within the dermis is a lack of specific markers for histological localization. In this regard, there is a need for specific markers and methodologies to be developed that can trace and dissect the distinct and functional subpopulations of resident or implanted DMCs to help understand the role of DMCs within wounded tissues. Additionally, there is a risk that cell therapies will produce malignancies, an idea that has been highlighted by our and other recent studies (*8*). We have observed that dermal multipotent cells can undergo spontaneous transformation after long-term ex vivo culture and that the Ras/Raf/MEK signaling pathway, protein phosphatase 2A (PP2A), and transcriptional coactivator PC4 may play a role in the malignant transformation of these cells (*8, 9*). Further, we have identified a class of lipophilic heptamethine cyanine dyes that preferentially accumulate in a variety of viable tumorigenic cells via an energy-dependent pathway. These dyes have superior optical, biocompatible, and pharmacokinetic properties for tumor targeting and for imaging with a superb contrast index value that make them attractive for detecting tumorigenic cells (*10*). Considering this safety issue, the long-term risk for tumor occurrences that result from the use of adult multipotent stem/progenitor cell in different pathological conditions must be further investigated. Moreover, there is an urgent need to develop more sensitive methods to detect tumorigenic cells at early stages.

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Regeneration and Repair of Intervertebral Disc Degeneration

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Lower-back pain is a serious medical and social problem, and one of the most common causes of disability. It is estimated that about 80% of the population will experience low back pain at some period during their lifetime and about 18% is experiencing low back pain at any given moment. Intervertebral disc degeneration is considered to be one of the major causes of low back pain (*1*). Current treatment options are limited to relieving symptoms, with no attempt to restore the disc's structure. Recently, there has been a growing interest in developing strategies that aim to biologically repair or regenerate the degenerated disc.

Disc cells actively modulate their metabolic activities by a variety of substances including cytokines, enzymes, enzyme inhibitors, and growth factors in a paracrine and/or autocrine fashion. Disc degeneration is characterized by loss of proteoglycans, so the ability to maintain or reconstitute the content of proteoglycans, by altering the balance between synthesis and degradation, is an emerging strategy for treatment.

Treatments for degenerated discs have two main objectives: restoration of the disc's structure and elimination of pain. Depending on the phase of disc degeneration they target, treatments can be classified as regenerative or reparative (Figures 1A to 1D) (*2*). In general, regenerative strategies, such as cell, gene, and protein therapy, target the early stage of degeneration and focus on the degenerating nucleus pulposus. Repair strategies, however, are more appropriate for more advanced stages of degeneration that are characterized by structural degradation of both the nucleus pulposus and annulus fibrosus.

During early degeneration (Figure 1B), when a disc is still structurally intact, endogenous nucleus pulposus cells can be used to increase extracellular matrix synthesis. Several growth factors, such as bone morphogenetic protein-2 (BMP-2), BMP-7 (also known as osteogenic protein-1; OP-1), growth differentiation factor-5 (GDF-5), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), and others have been found to stimulate matrix production, while interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) inhibit the synthesis of matrix by enhancing its catabolism (*3–5*). This is the reasoning behind the administration of growth factors or cytokine antagonists to degenerated discs. Our previous studies have found that OP-1 can stimulate proteoglycan synthesis of nucleus pulposus cells (Figure 2). GDF-5 was also found to stimulate proteoglycan and type II collagen expression in mouse disc cells, and enhance cell proliferation and matrix synthesis and accumulation in bovine disc cells. Based on these studies, the U.S. Food and Drug Administration has recently granted the initiation of clinical trials for OP-1 and GDF-5 (*5*). The therapeutic effectiveness of growth factors might be enhanced by the use of additional, synergistic growth factors and/or catabolic cytokine receptor antagonists (such as IL-1Ra).

The potential success of anabolic factors being injected directly into the disc might be limited due to their short biological half-life and rapid diffusion away from the delivery site. In a degenerated disc, prolonged exposure to anabolic factors is needed for them to be effective. This can be achieved by introducing disc cells carrying specific transgenes encoding the anabolic factors. Genes may be transferred to the disc by in vivo or ex vivo strategies by way of viral or nonviral vectors. Transgenes shown to increase the accumulation of matrix by disc cells include those that encode OP-1, BMP-2, TGF-β, GDF-5, transcription factor Sox-9, and tissue inhibitor of metalloproteinase-1 (*5, 6*). Indeed, intradiscal gene therapy looks like a promising future treatment for disc degeneration, but safety concerns are a major barrier to its clinical use.

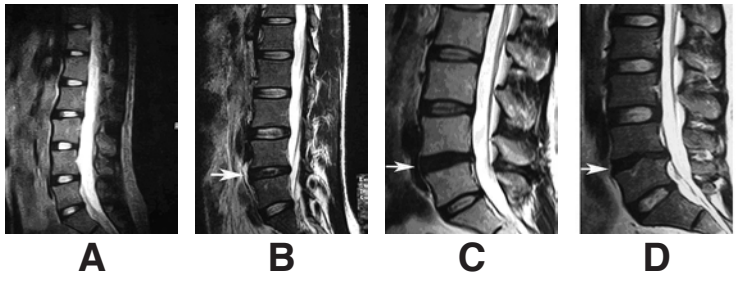

Figure. 1. Magnetic resonance imaging showing normal and degenerate discs. (**A**) Normal lumbar intervertebral disc, with a bright, hyperintense white signal intensity (L4/5). (**B**) Early stages of a degenerating disc, with an intermediate gray signal intensity (L4/5 disc, arrow). (**C**) Later stages of a degenerating disc, with a hypointense dark gray signal intensity (L4/5 disc, arrow). (**D**) Advanced stage of degenerating disc, with a hypointense black signal intensity and a narrowing disc space (L4/5 disc, arrow).

During the later stages of degeneration (Figure 1C), it may be necessary to supplement the disc with additional cells. Transplantation of various cell types, such as autologous disc cells, chondrocytes, or mesenchymal stem cells (MSCs), have been studied in animal models (*7*). In contrast to stem cells, which have to be collected from fat tissue or disc tissue, MSCs can be readily obtained from autologous sources such as bone marrow. This makes MSCs better candidates for transplantation. Recently, Yoshikawa *et al.* (*8*) carried out two clinical case studies in which MSC transplantation restored disc height and function and improved symptoms. More recently, Orozco *et al.* (*9*) reported a pilot study in which 10 patients with discogenic back pain underwent injection of autologous expanded MSCs into the nucleus pulposus and showed rapid improvement in pain and disability. Both animal and clinical studies indicate that MSC therapy is a promising treatment for disc degeneration. Moreover, there are encouraging results reported concerning stem cells obtained from other sources, such as human umbilical cord blood, that also are capable of differentiating toward mesenchymal cell lineages (*6, 9*).

Reparative strategies are focused on either augmenting or replacing a degenerated disc's tissue to reestablish healthy disc function. A recent focus for this strategy is using tissue engineering, particularly for advanced stages of disc degeneration (Figure 1D). Although nucleus pulposus tissue engineering has been a popular technique, more atten-

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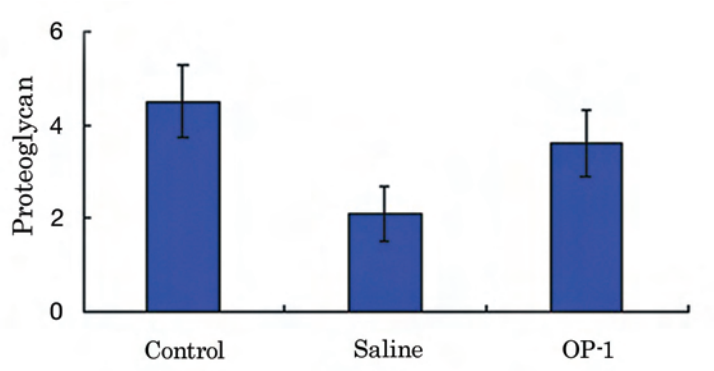

Figure. 2. Proteoglycan contents (% of wet weight) of nucleus pulposus in control, saline injection, and OP-1 injection discs in the rabbit annular puncture model. Adolescent New Zealand White rabbits received an annular puncture with an 18-gauge needle to induce lumbar disc degeneration. Four weeks later, either saline or 100 μg OP-1 was injected into the center of nucleus pulposus. Four weeks after OP-1 injection, the mean proteoglycan content of discs with the injection of OP-1 was significantly higher than that with saline injection (p<0.001).

tion has recently been given to the annulus and whole disc composite tissue. If a mechanically stable whole disc that contains a composite structure of the nucleus and annulus can be engineered, repair of an advanced degenerated disc might be possible. Recently, Bowles *et al.* (*10*) reported that transplantation of a tissue-engineered whole disc into the caudal spine of athymic rat produced a new matrix, maintained disc height, and restored biomechanical function, providing a critical step forward in developing biological therapies for disc degeneration.

The benefits of biologically based treatments appear to be limited to restoring disc structure; whether disc regeneration would result in pain relief remains unclear. That said, recent data from animal studies have shown changes in cytokine expression following growth factor injection, indicating a possible mechanism for pain relief. In the rabbit annular puncture model, the injection of OP-1 suppressed cytokine expression (IL-1β, TNF-α, and IL-6) by the tissues of nucleus pulposus and annulus fibrosus (*4*). Because these pro-inflammatory factors can induce a variety of pain markers, such as nerve growth factor, one can hypothesize that this therapeutic approach may have an effect on pain generation (*4*). Further, the first human clinical trial for growth factor injection therapy is currently underway and may shed light on the clinical outcome. MSCs may also help relieve pain by reducing inflammation (*9*). A recent study indicates that MSCs also induce the production of anti-inflammatory cytokines (*11*). In a noncontact coculture system, human MSCs and nucleus pulposus cells could express RNA for BMP-2, 4, 6, 7, TGF-β1, IGF-1, and EGF. Through secreted soluble factors in a paracrine fashion, a small number of MSCs significantly promoted proliferation of nucleus pulposus cells (*11*). However, additional studies are needed to elucidate the underlying mechanisms of pain relief.

In the advanced stage of disc degeneration, the nutrient-deprived disc environment will affect its capacity for regeneration and repair. A combination of the treatments for disc regeneration and pain relief may optimize treatment. More research is needed to further define the effects of these potential therapeutic agents in different models and eventually in humans.

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Electrical Fields Initiate Epidermal Stem Cell Migration and Enhance Wound Healing

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Growing evidence suggests that endogenous electric fields (EFs), which occur naturally in and around wounds, provide an essential regulatory cue for wound healing in addition to chemical and cellular factors, such as various cytokines and inflammatory cells, which play important roles in anti-infection, debridement, angiogenesis, and fibrogenesis. Our studies have investigated the involvement of direct current (DC) fields in the regulation of migration and differentiation of reparative cells that regulate wound healing (*1*). Our results revealed that modulating EFs at wound sites, by local administration of prostaglandin E2 or furosemide, significantly enhanced or inhibited the wound healing process, respectively. Here we review our studies on the effects of physiological EFs on the migration of epidermal stem cells (ESCs), the relationship of EFs to wound healing, and possible mechanisms of action of EFs.

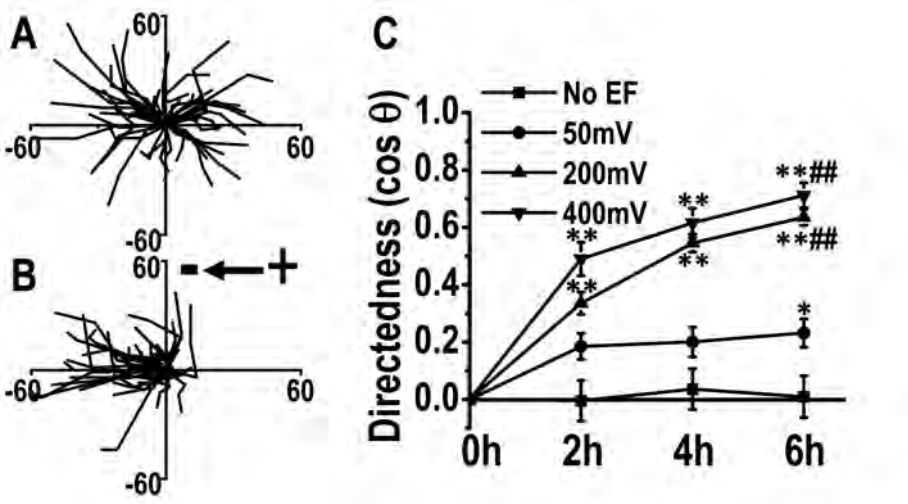

Figure 1. Directional migration of epidermal stem cells in electric fields is time- and voltage-dependent. (**A** and **B**) Trajectories of cells with the starting points of each cell set at the center without EFs (A) or under an EF (B). EF = 200 mV/mm for six hours. (**C**) Directedness of epidermal stem cells at different electric field strengths and different time points. ** $p<0.01$ compared with no EF controls. ## $p<0.01$ compared with the directedness value at two hours.

Stem cells (SCs) play a pivotal role in tissue maintenance and repair throughout adulthood. ESCs residing in the epidermis and in hair follicles of the skin are thought to be involved in wound healing (*2*). However, little is known about how ESCs participate in wound healing. Based on previous studies, we hypothesized that endogenous EFs arising at wound sites might be an important cue for ESC-induced initiation of wound repair. In a rat wound model, we probed for known markers of ESCs, α6-integrin and keratin 19 (*3*), in the skin tissue surrounding wounds. Double-positive cells around the wounds' edges markedly increased when endogenous EFs were enhanced using local administration of prostaglandin E2. In contrast, ESCs significantly decreased when the endogenous EFs were attenuated using local administration of furosemide. These results suggest that endogenous EFs may act as an important factor that induces ESCs in the niches around wounds to migrate into the wound to aid in repair. In order to examine the effects of direct physiologically induced EFs on the migration of ESCs, we stimulated ESCs isolated from rat epidermis using physiological levels of EFs, which were the same strength as endogenous EFs detected at wounds. In the presence of EFs, ESCs displayed a directional migration towards the cathode, whereas in the absence of EFs, ESCs randomly migrated (Figures 1A and 1B).

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The directedness of ESCs was significantly dependent on both the field strength and the duration of an EF (Figure 1C). Furthermore, EFs of similar magnitude as those endogenously generated significantly promoted the migration of ESCs. Each of the measured parameters that reflect cell migration speed (*4*)—trajectory speed (Tt/T), displacement speed (Td/T), and *x*-axis displacement speed (Dx/T)—increased significantly in the presence of an EF and in a field strength-dependent fashion. Our results suggest that endogenous wound EFs might be an important cue for the initiation of ESC migration from their niches to a wound site, where they can participate in wound healing (*5*).

Because F-actin polymerization is a key step in cell migration (*6*), we investigated whether it could be involved in EF-induced ESC migration. F-actin was shown to be evenly distributed in ESCs in the absence of EFs, but instead displayed an asymmetric polymerization in the presence of EFs and accumulated toward the cathodal side of the migrating cells as shown by fluorescence staining of F-actin by phalloidin. This asymmetric distribution toward the cathodal side is in accordance with cell migration. Pretreatment with an F-actin polymerization inhibitor, latrunculin A, abolished EF-induced asymmetric distribution of F-actin and significantly inhibited EF-induced cathodal migration of ESCs (Figure 2A). These results further suggest that F-actin might be a major molecular mechanism responsible for EF-induced directional migration of ESCs.

Next, we investigated how ESCs might detect an EF signal. We hypothesized that epidermal growth factor receptors (EGFRs) might be an important molecule for sensing the electric signal because they are located on the cell surface of ESCs. Additionally, physiological DC EFs have been shown to up-regulate EGFR expression on the cell surface

of epithelial cells and induce a redistribution of these receptors towards the cathodal side of the cells (*7*). Our study showed that pretreating ESCs with the EGFR inhibitor AG1478 one hour before EF application significantly reduced cathodal directedness and migration speed of ESCs in an EF (Figure 2B). Further, this inhibition of EGFR signaling also abolished the EF-induced asymmetric distribution of F-actin in ESCs. These results indicate that EGFR might play an important role underlying ESCs' ability to detect a bioelectric signal.

Given that both extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidylinositol-3-kinase (PI3K) play important roles in EF-induced migration of epithelial cells (*8*), we further investigated the relationship between both of these signal transduction pathways and ESC cell migration. Western blot analysis revealed no significant changes in ERK1/2 or PI3K protein expression levels in ESCs following EF stimulation. However, the phosphorylation levels increased significantly for both proteins detected by their respective site-specific phospho-antibodies. Pretreating ESCs with an ERK1/2 inhibitor (U0126) or a PI3K inhibitor (LY294002) prior to EF stimulation significantly reduced the EF-induced directional migration of ESCs (Figure 2C). Further, these pretreatments also significantly inhibited the EF-induced asymmetric distribution of F-actin towards the cathodal side and the phosphorylation of ERK1/2 and PI3K. Taken together, these data indicate that both ERK1/2 and PI3K signal transduction pathways may be involved in the EF-induced directional migration of ESCs. Therefore, it's possible that EGFRs on the cell surface of ESCs both detect bio-electric cues and then activate ERK1/2 and PI3K signal transduction pathways to induce cell migration (*9*).

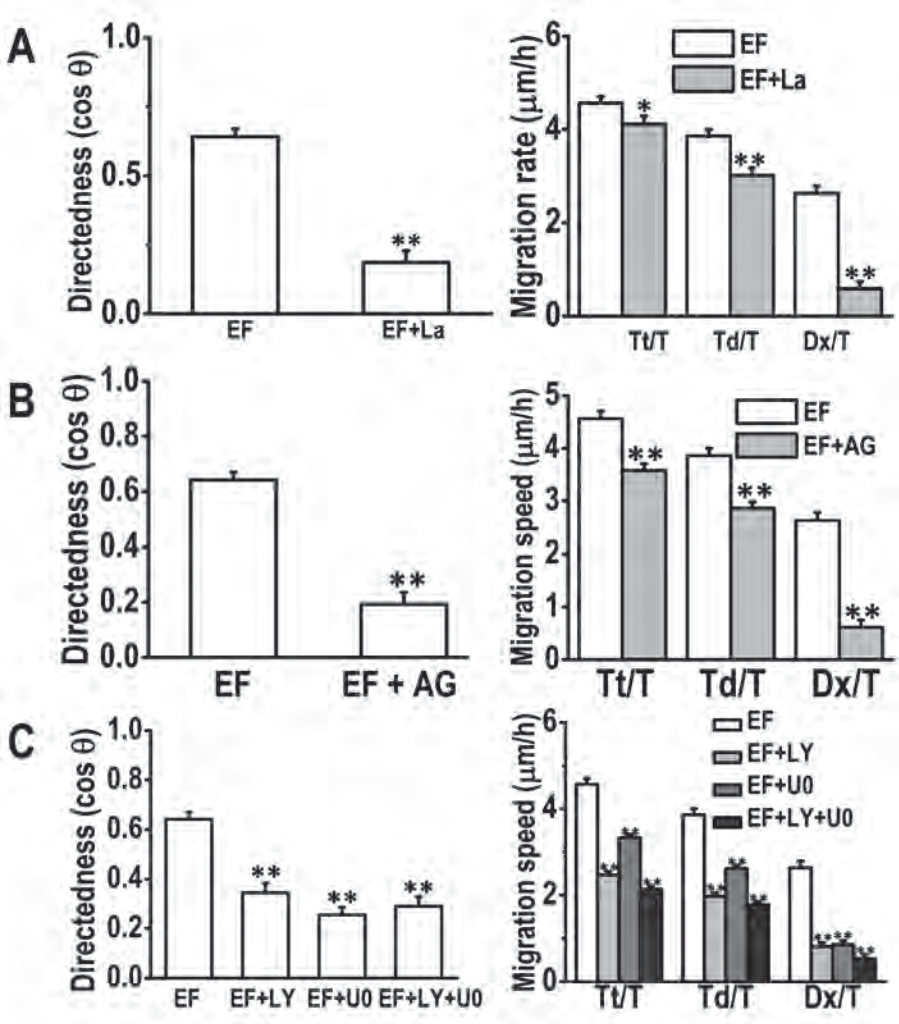

Figure 2. Mechanisms for electric field (EF)-guided migration of epidermal stem cells (ESCs). (**A**) Relationship of F-actin with EF-guided migration of ESCs. Latrunculin A (LA), an F-actin polymerization inhibitor, was added before EF stimulation. (**B**) Relationship of epidermal growth factor receptors (EGFRs) with EF-guided cell migration. AG1478 (AG), an EGFR inhibitor, was added before EF stimulation. (**C**) Relationship of extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidylinositol-3-kinase (PI3K) signal transduction pathways with EF-guided cell migration. U0126 (UO) and LY294002 (LY) are ERK1/2 and PI3K inhibitors, respectively. *p<0.05; **p<0.01, compared with the EF group. Tt/T, trajectory speed; Td/T, displacement speed; Dx/T, x-axis displacement speed.

In addition to inducing ESC migration, electric signaling seems to promote ESC differentiation. Stimulating ESCs with EFs of a physiologically relevant magnitude for one week reduced the number of cells expressing α6-integrin and keratin 19 (K 19), and increased the number of cells expressing K 1 and K 10, which suggests that physiological EFs might promote ESCs to differentiate into epidermal cells. Our previous work also revealed that applied EFs promoted the osteogenic differentiation of rat bone marrow-derived mesenchymal SCs in vitro. In addition, physiologically induced EFs have been demonstrated to promote the differentiation of embryonic SCs into cardiocytes and myocytes (*10*).

In summary, endogenous EFs at wound sites may play an important role in the initiation of reparative responses by ESCs. Our studies demonstrate that EFs not only initiate the migration of ESCs, but also promote their differentiation. We hope these findings will aid in the development of new approaches to promote wound healing.

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Initiating Scar Formation - The Dermal "Template Defect" Theory

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Previous studies on the mechanism of scar formation generally focused on cellular events and the role of growth factors. Clinical evidence indicated that loss of dermal tissue in deep cutaneous injuries led to scar formation, while dermal transplantation into the wound site reduced scarring (*1, 2*). These results demonstrated that scar formation was closely related to dermal defects. Repair, therefore, starts in the dermal tissue and the subsequent cellular events are simply responses to regeneration of the dermal defect. Here we review the evidence that certain characteristics of dermal tissue act as a template to modulate cell functions that are essential to the regeneration of skin structure and function. We also present data to support the theory of a dermal "template defect."

What are the Responses to Dermal Defects in Wound Healing?

In order to investigate the effect of dermal defects on the wound healing process, studies were carried out in Sprague Dawley rats with full-thickness wounds (i.e., both epidermal and dermal layers were damaged). Animals were randomized into the following groups: (1) natural healing without graft coverage, (2) split-skin graft (contains very little dermal tissue), (3) full-thickness graft (includes full thickness dermal tissue), and (4) acellular dermal matrix (ADM) + split-skin graft (mimics full thickness dermal tissue).

In all groups, histology revealed that, in neogranulation tissues, fibroblasts transformed from a spindle shape (young) to a stellate (mature) shape, which coincides with a change from parallel-orientated collagen fibers to a reticular arrangement. Stellate-shaped fibroblasts are typical in normal skin. This desirable process was most evident in the full-thickness grafting and ADM + split-skin groups compared to the split-skin graft group, suggesting that greater dermal tissue damage results in worse wound tissue remodeling (*3*). Meanwhile, the naturally healing wounds, without graft coverage, presented with poor mechanical compliance, more and prolonged differentiation from fibroblast into myofibroblast, and higher expression of extracellular matrix and scar-favorable factors such as TGF-β1, fibronectin, and integrin $\alpha_2\beta_1$. In the dermal tissue grafting groups, wound compliance was significantly improved, and extracellular matrix and pro-scar factor expression were much lower. The degree of improvement was closely associated with the thickness of the dermal graft (*4, 5*).

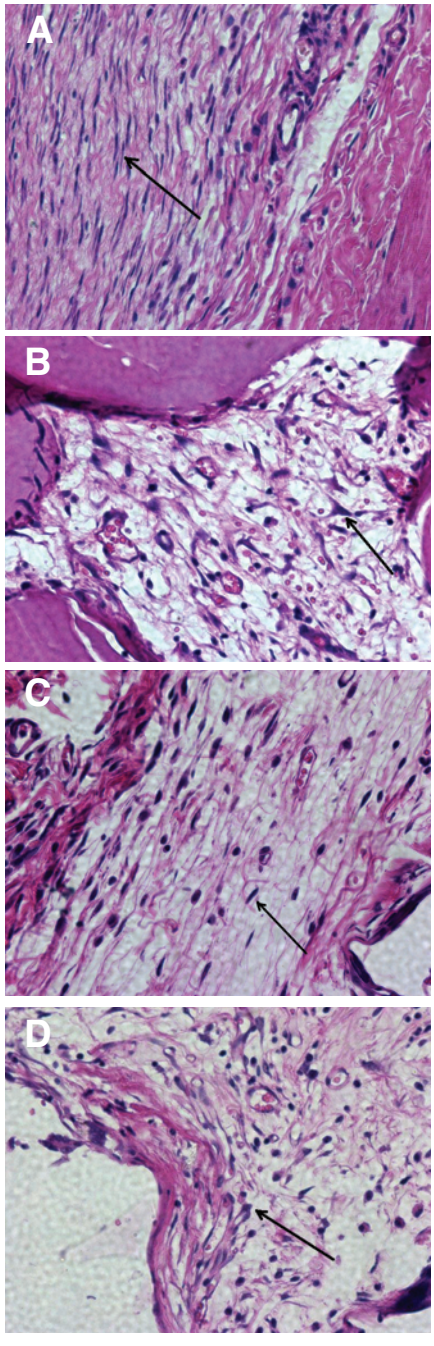

Figure 1. The influence of dermal 3-D structure and components on fibroblast and collagen arrangement (H&E stain, magnification: 400x). From reference *3*. Samples taken outside of decalcified bone matrix (DBM) (**A**) or PUA sponge (**C**), showing spindle-like fibroblasts (arrows), similar to granulation tissue, containing collagen fibers growing parallel to the long axis of the cell. In contrast, in samples from within the DBM (**B**) or sponge (**D**), the fibroblasts (arrows) grew in a polymorphous fashion, with collagen fibers aligned with the structure in a multidirectional way, similar to normal skin.

Clinical studies have also shown that when full-thickness skin donor sites are covered with ADM + split-skin graft, wounds have decreased expression of collagen, less differentiation from fibroblasts into myofibroblasts, less endothelium proliferation, lower expression of TGF-β1 and its receptors TGF-βR1&2 and Smad3, in addition to increased apoptosis (*6, 7*). This indicates that dermal defects may influence wound healing in multiple ways. Reducing dermal defects by dermal tissue grafting leads to less scar formation, which raises the question: why does dermal tissue have this influence, and what is the root cause: structure, components, or both?

The Role of 3-D Dermal Structures in Regulating Fibroblast Function

In order to study how the 3-D structure of different materials influences fibroblast cell function (*3*), we implanted decalcified bone matrix (DBM) or a polyurethane (PUA) sponge (with porous 3-D structures) subcutaneously in rats and analyzed tissue samples collected postoperatively one or two weeks later. There were two major histological findings. First, fibroblasts residing within and those residing outside of the DBM or sponge pores presented with very different morphologies, despite being from the same wound (Figure 1). Second, cells growing on the two different implantation materials were not significantly different in terms of morphology and collagen arrangement.

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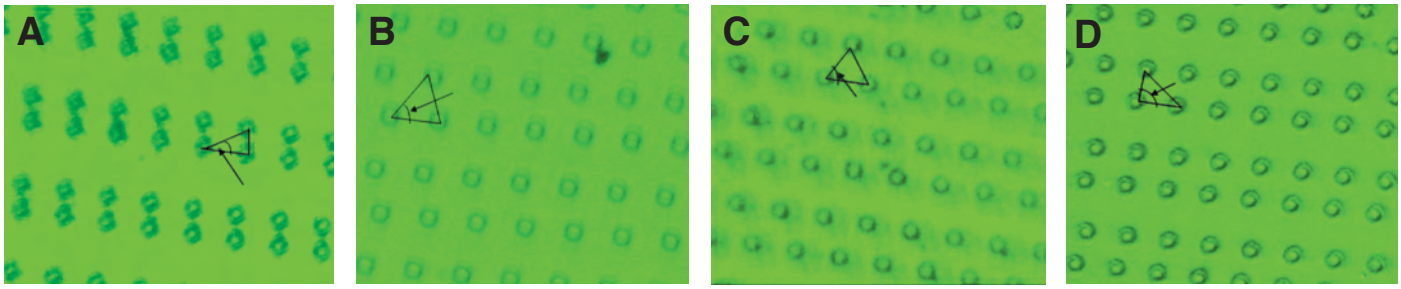

Figure 2. Triangular arrays of microdots of collagen I on polydimethylsiloxane microprinted with vertex angles of (**A**) 20º; (**B**) 40º; (**C**) 60º; (**D**) 80º. Magnification: 200x. Arrow indicates the vertex angle. From reference *8*.

The Influence of Tissue Structure On Cell Function

In order to understand the role of 3-D structure and its relation to other components, we conducted further studies. We found that myofibroblasts could be induced to differentiate from fibroblasts on 2-D culture systems coated with fibronectin, laminin, collagen, and dissolving 3-D matrix, but were not similarly induced using a 3-D culture system containing the same components (*3*). However, myofibroblasts were generated if the 3-D structure was compressed and damaged. In this study, we found that a 3-D cultural system could inhibit fibroblast to myofibroblast differentiation. Since myofibroblast differentiation from fibroblasts could be thought of as a characteristic of scar formation, these results indicate that the 3-D structure might be an important factor in determining cell fate.

Tissue Structure is a Template To Orient Cell Function

To clarify the influence of different 3-D structures on cell function, we subcutaneously implanted 3-D collagen membranes with pore sizes of 200 μm, 500 μm, and 1,000 μm into rats and took samples for dynamic observation at one week, two weeks, and three weeks postoperation. Analysis of the tissue samples revealed changes in cell proliferation and apoptosis corresponded with different pore sizes (*3*). These results suggest that different 3-D structures can influence cell biology and we thus propose that the 3-D structure of the supporting matrix can act as a template in modulating cell function.

The Integrity and Continuity of Tissue Structure is the Key To the Dermal "Template Defect"

Clinical studies found that during ADM + split-skin graft transplantation, the gaps of dermal matrix left by the skin graft mesher were more frequently filled by granulation tissue where increased differentiation from fibroblast to myofibroblast was observed, and it appeared that the integrity or continuity of the tissue had a significant influence on scar formation. In order to test this, we cut biomaterials with 3-D structure into fragments, so that the integrity and continuity of biomaterials structure was damaged**.** The fragments of biomaterials were then implanted subcutaneously into rats and, as controls, biomaterials with intact structure were implanted in the same way in a different group of animals. Samples were taken three weeks postoperatively. We found that the group implanted with fragmented biomaterials presented more granulation hyperplasty when compared to the group with intact structures (*3*). This confirmed our prior research (see above) that the 3-D tissue structure could act as a template in modulating cell biology, and clearly showed that once integrity and continuity of tissue was damaged, the 3-D tissue structure was also destroyed, disallowing it from acting as a template for tissue repair and regeneration.

Different 3-D Structures Produce Different Cell Adhesion Angles and Differentially Influence Cell Function

The 3-D structure of dermal tissue is irregular in morphology and therefore a mathematical algorithm—which uses an approximation of multiple curves—is needed. Cells form different adhesion angles when adhering to a matrix with varied curvatures. By examining the behavior of cells adhered at different angles, we can assess the impact of alternate 3-D structures on skin healing. To do this, we developed a cell culture system using molecular self-assembling and microprinting technology to come up with a matrix containing four different cell adhesion angles (20°, 40°, 60°, and 80°) (Figure 2). Our data demonstrated that cell proliferation, α-SMA expression, and cell-secreted hydroxyproline levels varied for each adhesion angles (*8, 9*), which indicates that cells may behave differently depending upon the angle at which they adhere to the matrix structure.

In summary, our studies indicate that dermal defects and their level of severity affect hypertrophic scar formation. The various components within the tissue can influence wound healing, which depends upon the structural composition of the derma. Further, tissue structure appears to serve as a template that guides cell function, while the tissue's integrity and continuity is a prerequisite for repair to take place. Loss of dermal tissue integrity and continuity due to trauma leads to a lack of the template defect, which may be one important mechanism that hinders the recovery of cell function, resulting in scar formation. Taken together, these observations lead us to hypothesize that the dermal "template defect" explains why scars form while damaged tissue heals rather than the skin being repaired.

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Dedifferentiation: A New Approach for Skin Regeneration

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Dedifferentiation is an important biological phenomenon whereby cells regress from a specialized function to a simpler state reminiscent of stem cells (*1*). Dedifferentiation occurs during wound repair and regeneration, and determines a species or organ's capacity for regeneration. For the skin, epidermal stem cells (EpiSCs) are a regenerative cell type that plays an important role in wound repair as well as in tissue engineering of replacement skin (*2*). After a skin injury, dedifferentiation may be a required for the epidermis to regenerate by cell proliferation and redifferentiation. Here, we discuss our studies investigating the occurrence of dedifferentiation of mature epidermal cells into stem cells or stem cell-like cells, exploring their possible signaling pathways, and evaluating the tumorigenic risk of dedifferentiation-derived EpiSC-like cells (Figure 1).

In 2001, we found groups of cells that were positive for the EpiSC markers β1 integrin and keratin 19 in the spinous and granular layers (between the basal layer and the stratum corneum) of biopsy samples from patients with traumatic leg ulcers treated with topical recombinant human epidermal growth factor (rhEGF) (*3*). Further, we found that these stem-cell islands were specific to those samples treated with rhEGF and were not found in the control samples (did not receive rhEGF) and normal skin. In the follow-up observation, the patients treated with rhEGF showed better regenerated epidermal structure than the controls, whose ulcers remained almost unchanged. Our results therefore showed epidermal stem-cell islands only in wounds treated with rhEGF, which suggests that rhEGF might induce cell reversion and formation of stem-cell islands, and might be involved in the wound healing process.

We then tried to demonstrate this epidermal cell dedifferentiation by establishing dedifferentiation-inducing models in vivo and in vitro. First, we transplanted human epidermal sheets lacking basal EpiSCs and transit amplifying (TA) cells onto full-thickness skin wounds in nude mice (*4, 5*). These transplanted skin grafts showed a significant number of cells positive for the EpiSC and TA cell markers cytokeratin 14 (CK14), CK19, and β1 integrin in the suprabasal layer compared with those before transplantation and normal epidermal sheets, indicating that some of the differentiating cells in the grafted epidermal sheets may have dedifferentiated into stem cells or stem cell-like cells. Next, we isolated these dedifferentiation-derived cells from the grafted epidermal sheets and cultured them in vitro. These cells showed characteristics of EpiSCs, as evidenced by their morphology, a small round shape with large nuclear-to-cytoplasm ratio; the characteristic expression of CK19, β1-integrin, Oct4, and Nanog; their rapid adhesion to type IV collagen; their high colony-forming efficiency; their long-term proliferative potential; and their capacity to regenerate a skin-equivalent tissue. Based on these studies, we concluded that epidermal cell dedifferentiation could be influenced by the wound microenvironment. Second, we established in vitro dedifferentiation induction models using several different treatments, including injury-inducing stimulations such as ultraviolet (UV) radiation, heat, and oxidant injury. Further, we treated cells with various growth factors, including basic fibroblast growth factor (bFGF) and EGF, since these molecules have been shown to play an important role in wound healing (*6*). We found that treating epidermal cells with UV radiation or bFGF resulted in relatively stable dedifferentiation and the induction procedure was easily controllable (*7, 8*). Dedifferentiation was confirmed by assessing five different cell characteristics: (1) morphology (showing smaller cell size, fewer organelles, and higher nuclear-cytoplasmic ratio), (2) phenotype (reexpression of EpiSC and TA cell markers and redistribution of α6 integrin and CD71), (3) proliferation ability (regaining the high colony-forming efficiency, marked cell-cycle progression, and enhanced telomerase activity), (4) redifferentiation capacity (reconstructing a well-formed epidermis with regular stratification), and (5) gene-expression profile (genes controlling cell adhesion and mitotic cell cycle were up-regulated, but those controlling epidermal cell development, differentiation, and keratinization were down-regulated).

We then used both the in vivo and in vitro models to investigate the signaling pathways involved in the epidermal reversion process (*9*). In the grafted epidermal sheets of the dedifferentiation-inducing animal model, we observed a significantly increased expression of Wnt family members (Wnt-1, Wnt-4 and Wnt-7a) and an elevated nuclear accumulation of β-catenin and its target genes, cyclin D1, and c-myc. When an inhibitor of the Wnt/β-catenin pathway was applied to the wounds post-transplantation until the sheets were removed for detection, epidermal cell dedifferentiation was not detectable as no significant differences were shown in the number of cells positive for the EpiSC markers in the sheets before and after grafting. This demonstrated that the Wnt/β-catenin signaling pathway is responsible for epidermal cell dedifferentiation in vivo. Further, we investigated whether dedifferentiation could be induced by activating the β-catenin pathway. We cultured aged human epidermal cells in vitro and treated them with a highly specific GSK-3β inhibitor which significantly increased the amount of β-catenin in cell nuclei. The results showed that epidermal cell dedifferentiation could be induced by GSK-3β inhibitor, as they exhibited morphologic, phenotypic, and functional characteristics of EpiSCs. In addition, we found the involvement of extracellular signal-regulated kinase (ERK) in UV-induced epidermal cell dedifferentiation in vitro (*8*). Therefore, dedifferentiation and regeneration are more complicated than previously thought and the multiple induction factors and signaling pathways work together to produce these processes.

Finally, a better understanding of the mechanisms underlying epidermal cell dedifferentiation may uncover safety concerns since some of these molecular factors are also associated with carcinogenesis. Therefore, before dedifferentiation-derived EpiSCs can be applied clinically,

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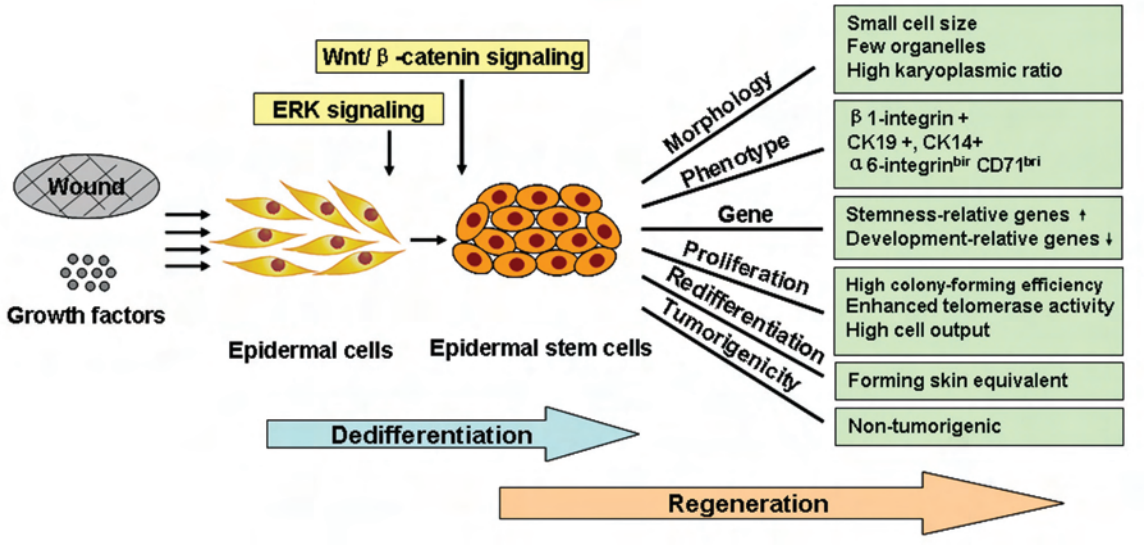

Figure 1. Schematic illustration of epidermal cell dedifferentiation. Differentiated or differentiating epidermal cells will undergo dedifferentiation in response to internal and external signals. Producing a wound injury or introducing growth factors can induce cells to re-enter the cell cycle, proliferate, dedifferentiate, and redifferentiate. We postulate that the Wnt/β-catenin pathway and ERK pathway underlie epithemal cell dedifferentiation. Dedifferentiation may be a crucial, perhaps even necessary, step for tissue regeneration when endogenous stem cells are lost or exhausted.

a full evaluation of the carcinogenic risk surrounding the process is critical. In this regard, we injected EpiSCs that were harvested from our in vivo and in vitro dedifferentiation models subcutaneously into the right flank of nude mice and assessed their tumorigenic potential. We did not observe any palpable tumor nodules, regarded as xenografts, during 30 days of observation. In contrast, human melanoma cell Bowes (HMCBs), used as a positive control, formed obvious xenografts at the injection sites within a short time. These results suggest that dedifferentiation-derived EpiSCs are unlikely to be cancer-causing in vivo. Additionally, genetic instability can predispose cells to become cancerous by increasing the rate of DNA mutations and chromosomal alterations, therefore it is crucial for cells to stay genetically intact during dedifferentiation. Thus, we also evaluated the genetic stability of all dedifferentiation-derived EpiSCs. The EpiSCs that were induced by UV exposure showed a small amount of double stranded DNA damage, appearance of gH2AX foci, activation of cell cycle checkpoint factors including p53 and p21, and signs of apoptosis. Therefore, one should be cautious when using UV exposure as an external factor to induce dedifferentiation, even though the UV-induced EpiSCs did not form tumors in vivo. By comparison, EpiSCs induced by the application of bFGF showed no obvious signs of DNA damage or activation of related response factors, which make this method a more promising candidate for cell dedifferentiation.

When it comes to regeneration, however, the situation may not be as simple. To induce endogenous regeneration in a wound's microenvironment, different genes and molecules that control dedifferentiation, redifferentiation, and patterning may need to be expressed at the appropriate levels and in the correct temporal and spatial context. This complexity is why mammals are unable to completely regenerate tissues the way that their lower counterparts can. Thus, stimulating and regulating regeneration-competent cells in situ is the most desirable way to restore damaged and diseased tissues (*10*). Only in this way, may humans one day acquire the ability to replace lost, diseased, and worn down structures.

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Intermittent Hypoxia Stimulates Neurogenesis in the Brain

Lingling Zhu, Ph.D., Kuan Zhang, M.D., Ming Fan, Ph.D.*

Adult neurogenesis is the process of generating functional neurons from adult neural precursor cells, and it occurs in restricted regions of the mammalian brain (*1*). Neurons in the adult central nervous system (CNS) have little ability to regenerate once injured. As a result, the main regenerative medicine-based treatment for CNS diseases is cell-replacement therapy. Previous experiments have been performed in which embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), and induced pluripotent stem cells (iPSCs) were differentiated into nerve cells in vitro and transplanted into injured brains of animal models and patients with accidental damage or Parkinson's disease. However, brain cell transplantation is an invasive procedure and the injected cells do not survive for long following transplantation. More importantly, the allografted stem cells could form teratomas. These issues currently limit the utility of cell-replacement therapy for neurological diseases. Therefore, novel and more efficient ways to enhance endogenous neurogenesis for nerve cell replacement are urgently needed.

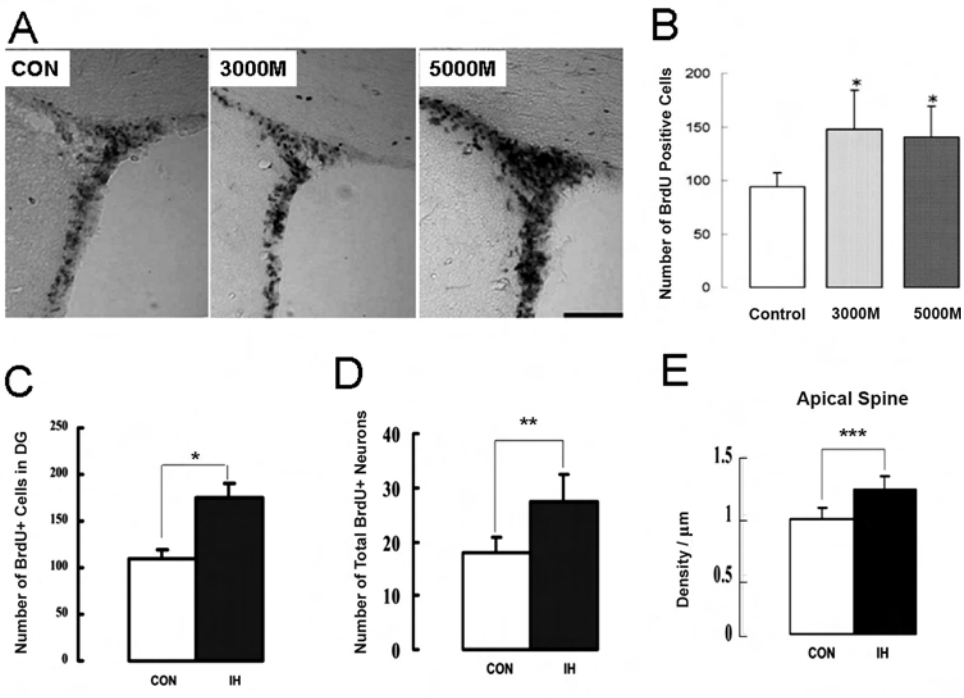

Figure 1. Effects of intermittent hypoxia on the multiple stages of neurogenesis in the subventricular zone (SVZ) and dentate gyrus (DG) of the adult rat brain. (**A**) Representative photographs of BrdU-labeled cells in the lateral ventricular zone after intermittent hypoxia. Black granules are BrdU-positive cells. Scale bar = 100 µm. (**B**) The number of BrdU-positive cells in the SVZ after intermittent hypoxia (IH) treatments mimicking 3,000 and 5,000 m altitudes. The number of BrdU positive cells increased by up to 42%, N=6 (*p<0.05 relative to control). (**C**) The number of BrdU-positive cells increased significantly in the DG of the hippocampus after IH (3,000 m group), by 64%, N=4 (*p<0.001 relative to control). (**D**) IH increased the total number of the newborn neurons by 43.5%, N=4 in the DG subregion of hippocampus in the 3,000 m group (**p<0.05 relative to control). (**E**) IH markedly increases the density of apical oblique (AO) dendritic spines (***p<0.001 relative to control). Each bar represents the mean ± SEM.

Neurogenesis occurs continuously throughout adulthood in the subgranular zone (SGZ) of the mammalian dentate gyrus and subventricular zone (SVZ), but generally requires initiation by some type of stimulus, including intrinsic and extrinsic factors, such as an enriched environment, physical exercise, and growth factors (*2*). Neurogenesis is a dynamic process with multiple stages. First precursor cells begin proliferating, then these cells differentiate into lineage-restricted immature neurons, and finally the newborn cells mature into fully functional and integrated neurons (*3*). The activation of endogenous neurogenesis in the adult brain raises the possibility of repairing damage in the CNS of an adult after an injury or degenerative neurological disease (*4*). Here, we discuss a new method, intermittent hypoxia (IH), to stimulate neurogenesis in situ.

IH has been found to prevent permanent damage and to have a protective role in the CNS following brain injury (*5*). However, little is known about the effect of intermittent hypoxia on neurogenesis in a normal, uninjured brain or its role in treating neurodegenerative diseases. We sought to investigate this by testing the effects of two IH conditions on Sprague Dawley (SD) rats by exposing them to hypobaric hypoxia in a special chamber that mimics high altitude conditions at 3,000 and 5,000 m above sea level, four hours per day for a two week period (*6, 7*). We observed that both of the IH conditions promoted the proliferation of NSCs in the SVZ of the lateral ventricles (Figure 1A and 1B). In the IH treated groups, the total number of BrdU-labeled cells in the dentate gyrus (DG) subregion of the hippocampus was 64% (3,000 m group) (Figure 1C) and 72% (5,000 m group; data not shown) higher compared to the normoxia control group two weeks after treatment. Moreover, we determined that IH (3,000 m group) increased the total number of newborn neurons in the DG using a double staining with BrdU (a marker of dividing cells) and NeuN (a marker of mature neurons) by 43.5% (Figure 1D), and most of newly generated neurons migrated into the granule cell layer (GCL) at 14 days after IH treatment compared to the normoxia group (data not shown). We also measured the density and length of the dendritic spines of pyramidal neurons

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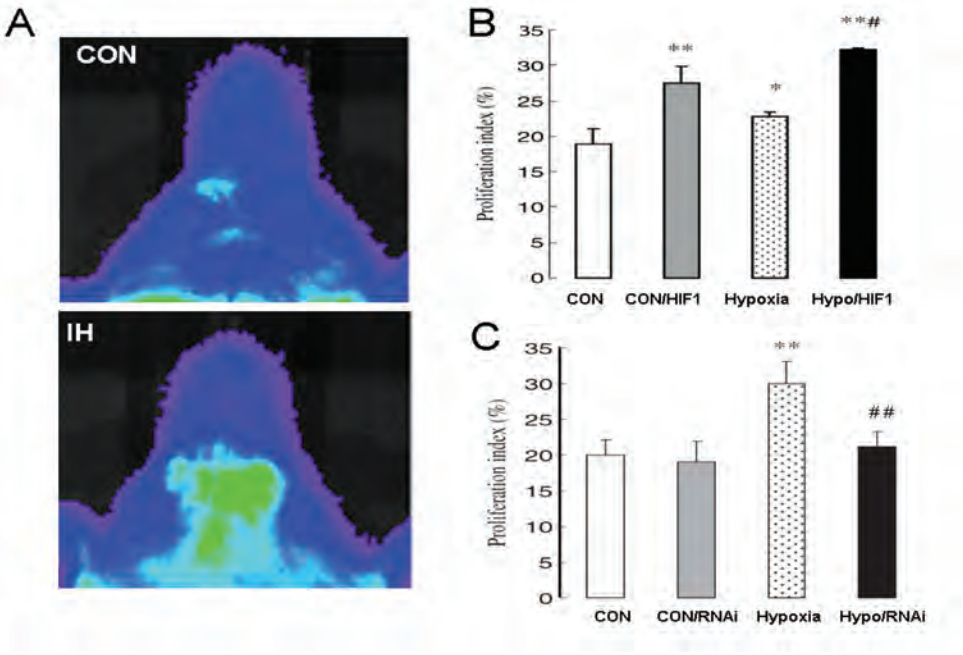

Figure 2. Intermittent hypoxia (IH) activates HIF-1 alpha and its role in neurogenesis. (**A**) Transgenic mice that ubiquitously express a bioluminescent luciferase-HIF prolyl hydroxylase reporter gene showed enhanced HIF-1 alpha activity in the brain after IH. (**B**) Overexpression of HIF-1 alpha in the brains of normoxia control mice (CON/HIF1 group) caused a similar proliferative effect on neurogenesis to IH in nontransgenic mice (Hypoxia). IH in HIF-1 alpha transgenics resulted in an additive effect on neurogenesis (Hypo/HIF1). *p<0.05 and **p<0.01 compared with the control group (CON); $^{\#}p$<0.05 compared with the Hypoxia group. (**C**) Knocking down HIF-1 alpha (Hypo/RNAi) inhibits the neuronal proliferation induced by hypoxia (Hypoxia). The data are the means ± SD. **p < 0.01, as compared with the control group. $^{\#}$ $^{\#}p$ < 0.01, as compared with hypoxia group.

in the hippocampus of Thy1 transgenic mice, which express yellow florescent protein (YFP) in their neurons. IH increased the length of both apical oblique (AO) and basal shaft (BS) dendritic spines (data not shown), and enhanced the density of AO dendritic spines but not the BS dendritic spines (Figure 1E). Taken together, these data indicate that IH can enhance multiple stages of the neurogenesis process within the adult rat brain, including NSC proliferation, the survival and migration of newborn neurons, and spine morphogenesis of mature neurons.

To further understand the molecular mechanism underlying this process, we investigated the role of a key transcription factor for hypoxia, hypoxia-inducible factor 1 (HIF-1). Using transgenic mice that ubiquitously express the bioluminescent reporter gene consisting of firefly luciferase fused to an activator region of the gene encoding HIF prolyl hydroxylase, we detected an activation of HIF-1 alpha in the brain after intermittent hypoxia (Figure 2A). Next, to elucidate the potential role of HIF-1 alpha in neurogenesis after hypoxia, we overexpressed HIF-1 alpha using an adenovirus construct. We found that overexpressing HIF-1 alpha mimics the proliferation effect of hypoxia (Figure 2B). In contrast, knockdown of HIF-1 alpha with RNAi using the pSilencer 1.0-U6 plasmid as the vector targeting HIF-1 alpha mRNA inhibited hypoxia-induced proliferation (Figure 2C). These results demonstrate that intermittent hypoxia increases neurogenesis in the adult brain through the HIF-1 alpha signal pathway (*8, 9*).

These intermittent hypoxia treatments seem to induce a previously unknown regenerative capacity of mature neurons, which raises the possibility of repairing the adult brain after damage from an injury or degenerative neurological disease (*9*). Thus, we next attempted to treat ischemic brain injury and the effects of Parkinson's Disease (PD) in rat models using IH. Preliminary results indicate that IH promotes the recovery of cerebral ischemic injury rats as measured by neurological behavioral tests (data not shown) and enhances neurogenesis in the striatum in the PD model (*7*). Moreover, recent studies have also reported that intermittent hypoxia alleviates the symptoms of depression (*10*).

In conclusion, our data indicate that intermittent hypoxia is a new and non-invasive method that can be used to stimulate neurogenesis in the adult brain. Further, we hope this technique may be applied as a new strategy to treat brain injury and neurodegenerative diseases.

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Autologous Stem Cell Therapy for Chronic Lower Extremity Ischemia

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Critical limb ischemia (CLI) is an advanced stage of peripheral arterial disease (PAD). Only about two-thirds of patients with CLI can be revascularized with conventional bypass surgery or endovascular therapy, while the remaining one-third have no chance for the treatment, resulting in major amputation and potential morbidity and mortality. As a result, exploring new, minimally invasive strategies for revascularization of severely ischemic limbs is of major importance.

The role of endothelial progenitor cells (EPCs) in angiogenesis was first observed by Asahara *et al.* in 1997 (*1*), who demonstrated that vasculogenesis (de novo vessel formation via hemangioblasts/EPCs) plays an important role in the natural compensatory revascularization process for PAD, which was previously believed to include only angiogenesis (a sprouting of small endothelial tubes from preexisting capillary beds) and arteriogenesis (enlargement of preexisting collateral arterioles). The bone marrow provides a large reservoir of stem cells including EPCs and mesenchymal stem cells (MSCs), and these stem cells can be mobilized into peripheral blood. Thus it was hypothesized that therapeutic angiogenesis with EPCs harvested from both bone marrow and mobilized peripheral blood might improve limb ischemia.

The first clinical trial using bone marrow mononuclear cell (BM-MNC) transplantation to treat CLI patients was reported in 2002 (*2*). Following harvesting and enrichment of 500 mL of bone marrow, autologous BM-MNCs were injected intramuscularly into patient's ischemic limbs. The results showed remarkable improvement of limb ischemia without notable adverse events. To avoid the pulmonary complications of general anesthesia, we improved the protocol of harvesting bone marrow under local anesthesia and intramuscularly transplanting BM-MNCs under intra-spinal anesthesia. We successfully started the first clinical trial in China in early 2003 after obtaining approval from the hospital ethics committee (*3*). Improved clinical parameters were observed in the transplanted patients, even diabetics (*4*), including elevated transcutaneous oxygen pressure, decreased need for major amputation, reduced pain, improved ankle-brachial index, and richer collaterals on angiography (see Figure 1).

To investigate whether the therapeutic efficacy is due to the transplanted bone marrow cells, we treated 22 patients suffering from bilateral lower extremity ischemia with two different doses for each extremity by intramuscular injection transplantation (*5*). We found the extremities transplanted with less than 10^5 BM-MNCs had no improvement, while the extremities transplanted with more than 10^8 BM-MNCs had marked clinical improvements, indicating that neither low cell dose ($<10^5$ cells) nor the action of injecting the cells by needle puncture could produce therapeutic angiogenesis and collateralization.

Intramuscular transplantation under intra-spinal anesthesia is still risky and not tolerable for some patients in poor general condition. Encouraged by the results from an animal study (*6*), we performed a clinical trial in which BM-MNC delivery was via intra-arterial administration under local anesthesia. We advanced a balloon catheter to get as close as possible to the occluded lesion, then injected the BM-MNCs within five minutes of inflating the balloon and blocking blood flow. We observed a similar clinical improvement using this delivery route when compared with intramuscular delivery (*7*).

Successful BM-MNC transplantation requires acquisition of 400–500 mL bone marrow, which can yield 1–3x10^9 mononuclear cells. However, acute loss of such a large amount of blood can be dangerous and even intolerable for elderly patients, especially those in poor general health. Granulocyte colony-stimulating factor (G-CSF) can mobilize bone marrow stem cells, including EPCs, into peripheral circulation. We used this treatment, isolating mobilized peripheral blood mononuclear cells (PB-MNCs) and using them to treat PAD patients. Relatively good results were achieved in PB-MNC transplant patients (*8*), but there was a concern based on previous results (*8*) that some patients might suffer from cardiovascular or cerebral events after mobilization, presumably due to increased blood viscosity caused by white blood cell enrichment, even with anti-thrombosis prevention therapy.

As a shortened mobilization period should decrease the cardiovascular or cerebral events and BM-MNCs contain a greater number of stem cells than PB-MNCs, we modified the method by harvesting a total of 200 mL bone marrow two to three days after mobilization with G-CSF. In a clinical trial we were able to obtain more than ten times the yield of BM-MNC following G-CSF mobilization, and observed fewer complications and better clinical improvement in ischemic status (*9*). This is therefore a preferable method since it requires less bone marrow suction, provides more stem cells, has good therapeutic results, and is safer.

The percentage of implanted $CD34^+$ stem cells used in this study was generally between 0.5% and 2.3% of total implanted MNCs, but there is no proven correlation found between $CD34^+$ count and therapeutic response. Given that $CD34^+$ stem cells play the main angiogenic role, we hypothesized that transplanting more of these cells might improve patient outcome. Enriching for $CD34^+$ stem cells to a concentration of close to 80% before injection achieved a better outcome in a small number of patients (unpublished).

More than 1,000 patients with PAD have undergone autologous stem cells transplantation in the past eight years in China. Short-term efficacy and safety of autologous stem cell transplantation has been confirmed, but medium-term results have not been as satisfactory (*10, 11*). In a clinical trial from March 2003 to June 2005, patients were treated by autologous BM-MNC transplantation and 65 were followed up (mean follow-up time was 21.5 months, range 8–56 months). Despite eight patients dying of heart or brain diseases, the total success rate was 70.8% with a recurrence rate of 10.7%. Long-term efficacy of over a year was found in 42 patients (64.6%) and of over two years in 24 patients (36.9%). Twelve patients who had repeated treatments (two to four times) had continued clinical improvement for over 37 months. This suggests that repeated stem cells transplantation may be a promising path to achieve better and more sustainable outcomes.

Safety regarding stem cell use remains a concern. In our clinical trial, pathological examination of 23 amputated limbs from patients who had received stem cell transplantations showed no signs of neoplasm formation and neo-bone formation, and no other unwanted

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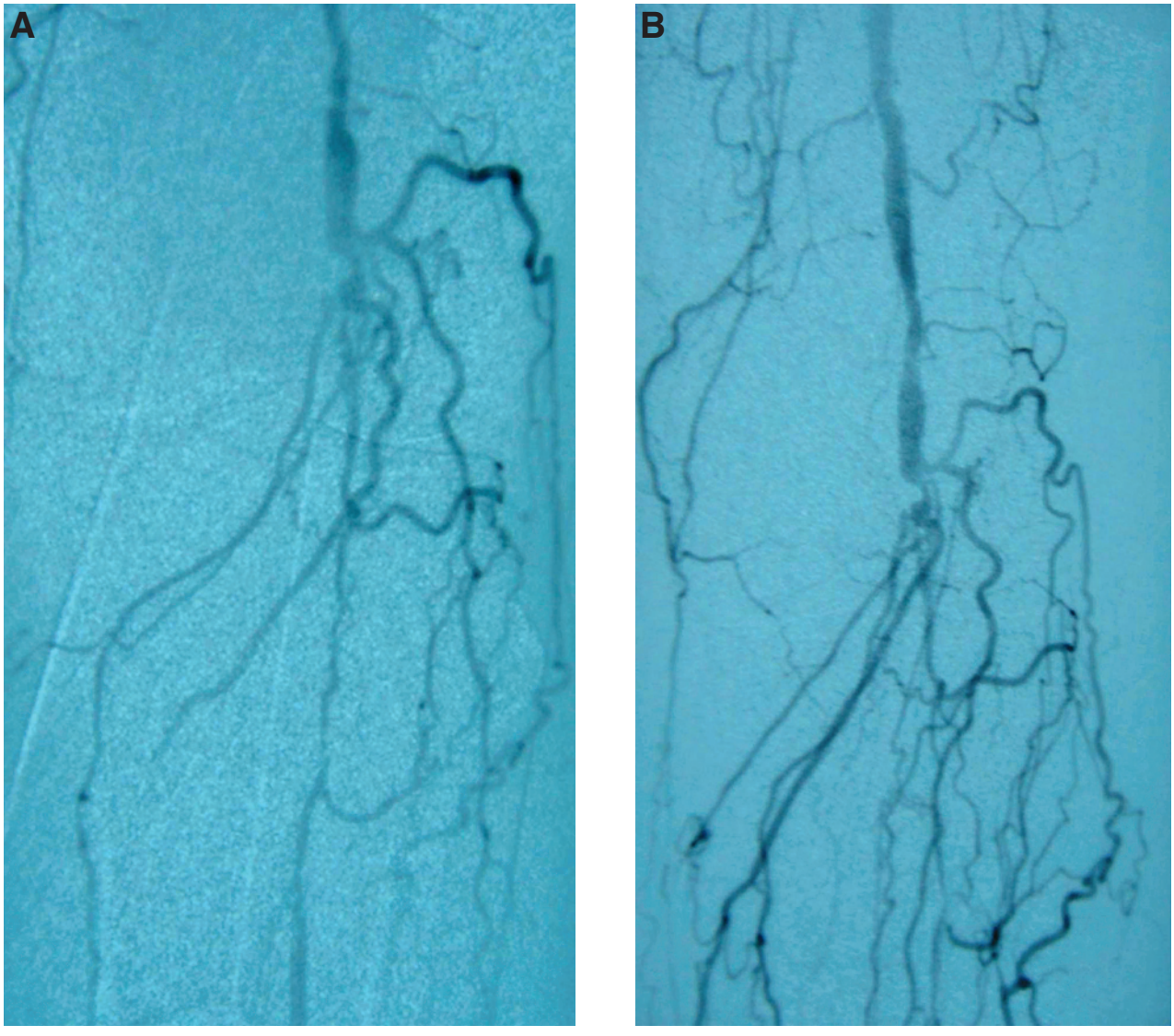

Figure 1. Representative angiograms showing the vasculature of an ischemic leg. (**A**) preimplantation and (**B**) two months postimplantation, showing richer collaterals.

complications or adverse effects were observed in the more than 300 subjects who had undergone autologous stem cell transplantation for lower extremity ischemia.

In summary, although autologous stem cells transplantation proved effective and safe for PAD, the long-term efficacy and safety still requires additional, multicenter randomized clinical trials, as well as further studies on how to improve efficacy.

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Maintaining Learning Ability During the Aging Process?

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Age-associated memory impairment (AAMI) occurs in many mammalian species, including humans. Unlike Alzheimer's disease, in which degeneration of select neurons leads to a near complete loss of cognitive abilities, AAMI is a functional decline likely involving alterations of neuronal spines and synapses without significant neuron loss and thus may be more amendable to treatment or prevention (*1, 2*). Spines are small protrusions found on the neuronal dendritic arbor and form the fundamental structure for learning and memory synapses together with presynaptic elements. With respect to shape, spines can be classified as thin, mushroom, or other. Thin spines are highly motile, plastic, and distinguished by their capacity for expansion, such as in the formation of new connections related with learning. Mushroom spines are stable and form stronger connections with presynaptic elements, which make them a good candidate for the storage of long-term memories. Recent in vivo time-lapse imaging studies have also suggested that thin spines are "write-enabled" and linked to learning, while the larger, mushroom spines are "write-protected" and represent memory traces (*3–5*).

Nonhuman primates (NHPs) are a particularly attractive model for studies on age-related cognitive decline for two main reasons. First, rhesus monkeys (*macaca multatta*)—the NHP model used in our studies—share a number of significant physiological traits with humans. These primates have 28-day menstrual cycle with similar ovarian hormone fluctuations and also experience menopause characterized by low estrogen levels (*6*). Second, NHPs are an excellent model to study cognitive impairment linked to the dorsolateral prefrontal cortex (dlPFC), which plays a dominant role in goal-directed behavior and is highly vulnerable to aging (*7*). For the past decade, we used NHPs (both male and female, with age ranges of 9–14 years old for "young" animals and 22–35 years old for "aged" animals) as a model to investigate the cognitive and neuronal changes in the aging cerebral cortex and to explore potential interventions to slow down age-related cognitive decline.

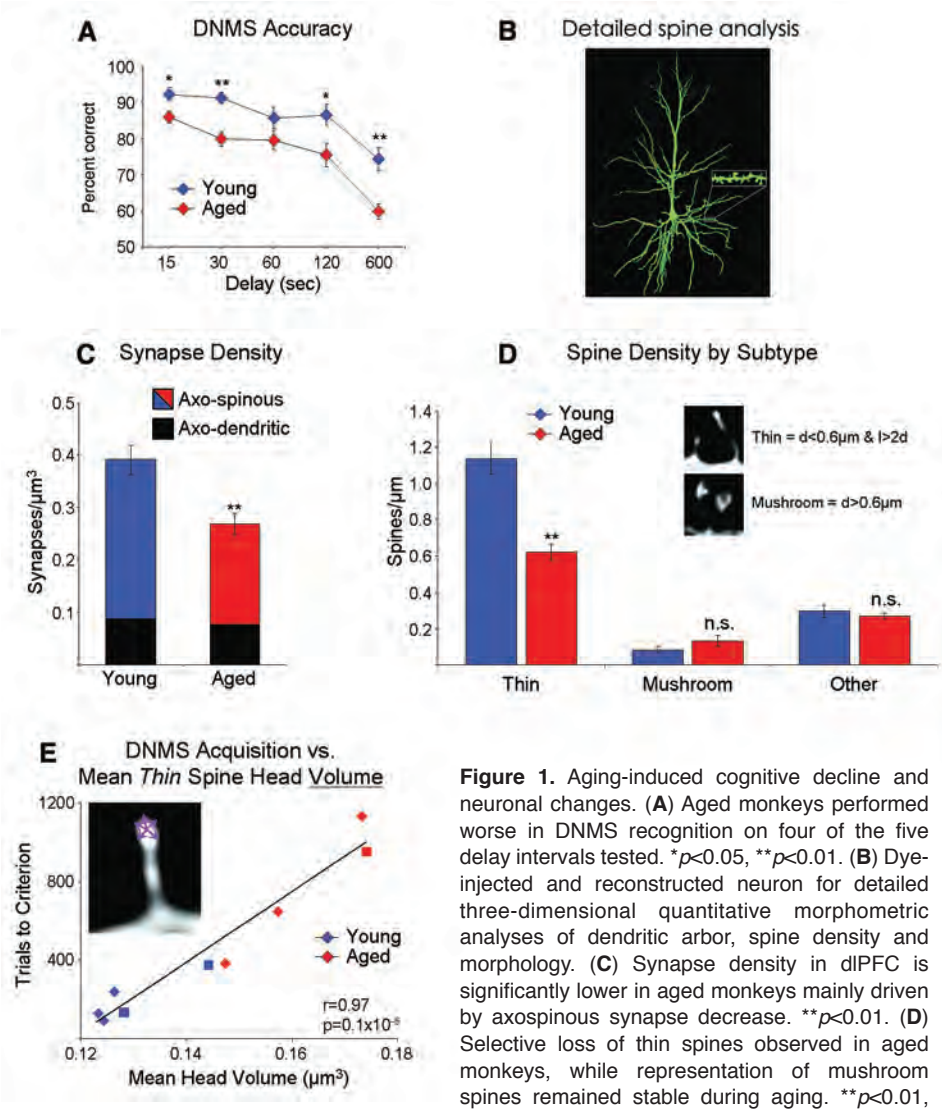

Figure 1. Aging-induced cognitive decline and neuronal changes. (**A**) Aged monkeys performed worse in DNMS recognition on four of the five delay intervals tested. *p<0.05, **p<0.01. (**B**) Dye-injected and reconstructed neuron for detailed three-dimensional quantitative morphometric analyses of dendritic arbor, spine density and morphology. (**C**) Synapse density in dlPFC is significantly lower in aged monkeys mainly driven by axospinous synapse decrease. **p<0.01. (**D**) Selective loss of thin spines observed in aged monkeys, while representation of mushroom spines remained stable during aging. **p<0.01, d, spine head diameter; l, spine length; n.s., not significant. (**E**) Head volume analysis shows that DNMS acquisition correlates most strongly with the mean volume of thin spines.

Behavioral Characterization

Our cognitive tests showed an age-related decrease in both acquisition and performance on the delayed non-matching-to-sample (DNMS), a task dependent on the dlPFC (DNMS acquisition) and medial temporal lobe (DNMS performance) (Figure 1A).

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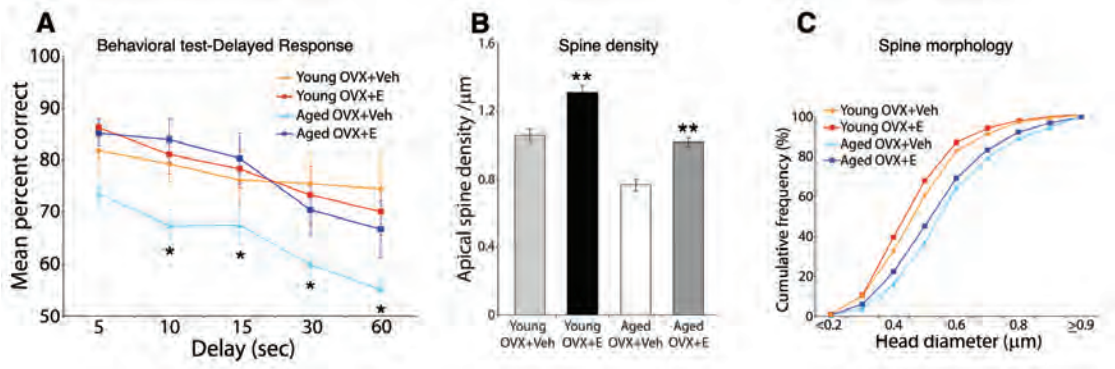

Figure 2. Effects of cyclic estrogen treatment on age-related cognition decline and neuronal changes. (**A**) Estrogen replacement therapy significantly restored performance of aged monkeys on delayed response (DR) tasks to the level of both young treatment groups. Young OVX+Veh and Young OVX+E monkeys performed equally well on DR tasks. (**B**) Significant effect of estrogen was seen in both young and aged spine densities. (**C**) Cumulative frequency analysis of aging and estrogen effects on spine size. Aging-induced selective loss of small thin spines was partially reversed by estrogen treatment in both young and aged monkeys. *p<0.05, **p<0.01.

Age-Related Neuronal Changes and Its Correlation with Behavior

Detailed, quantitative morphometric analyses were performed by microinjection of individual neurons with fluorescent dye followed by three-dimensional tracing and high resolution confocal imaging of dendritic spines and electromicroscopic analysis of synapse density (Figure 1B). A substantial aging-induced loss of neuronal spines (33%, p<0.01) and synapses (32%, p<0.01) in pyramidal neurons across all branch orders of both apical and basal dendrites from monkey dlPFC area 46 was observed (Figure 1C, D). The age-related loss of spines was specific to spines previously referred as "thin" spines (46%, p<0.01). In contrast, no age-related change in either density or morphology of mushroom spines was observed. Both synapse density and the overall average spine size of an animal were correlated with acquisition of DNMS (i.e., learning a task). Most interestingly, the strongest correlations were found between morphometric indices of thin spines and DNMS acquisition. As showed in Figure 1D, head volume of thin spines was 97% correlated with learning (Pearson correlation, p<0.001, Figure 1E), which indicates that more thin spines allow for better DNMS acquisition. This data supports our hypothesis that in the dlPFC, the small, highly plastic class of thin spines could be particularly sensitive to cognitive aging. In contrast, mushroom (memory) spines do not seem to be vulnerable to aging, which, together with the above evidence, might explain how the aging brain maintains expertise in tasks such as playing the piano despite a pronounced decline in the ability to learn new things.

This study provided neurobiological support for the old saying that we can't teach an old dog new tricks, but we can age gracefully by emphasizing learning in our youth (*8*).

Estrogen and the Aging Brain

Menopause is an important physiological and psychological milestone for many women. While some clinical studies have shown that hormone therapy (HT) following menopause might protect against cognitive decline, other studies have shown an increased risk for neurologic complications associated with the drugs (*9*). These inconsistencies spurred us to investigate the interaction between estrogen and aging brain in NHPs (*10*). Unlike rats, which experience a chronic high estrogen state at reproductive senescence, the NHP menopause is very similar to humans, characterized by loss of circulating estrogen. A cohort of young (10 years old) and aged (22 years old) female rhesus monkeys were surgically ovariectomized (OVX) and then given a regularly repeated injection of 100 µg of either estrodiol cypionate (E) or vehicle (Veh) every 21 days. At various stages pre- and postoperatively, all monkeys underperformed in cognitive assessment tests for multiple tasks, including the delayed response (DR) test, a task dependent on the dlPFC and DNMS. The first important finding in our study was that the estrogen treatment initiated shortly after OVX could restore wild-type performance on the DR task in aged monkeys experiencing cognitive decline, but had no effect in the young monkeys, where resilience in cognitive function was observed even in the absence of estrogen (Figure 2A). Further morphological analysis in the dlPFC revealed that the age-related neuronal spine density decrease was driven mainly by selective loss of thin spines, and this decrease was partially reversed by the cyclic estrogen treatment (Figures 2B and 2C). The data clearly indicated that cyclic estrogen treatment—which mimics physiological estrogen fluctuations—and its early initiation during peri-menopause in aged primates can maintain or significantly restore the structural and functional integrity of the neocortex.

In conclusion, our decade-long NHP studies reveal that selective loss of neuronal thin spines is responsible for age-related decline of cognitive performance, especially in learning capability. Selective synaptic preservation of mushroom spines is the key to the aging-related maintenance of long-term prefrontal-dependent expertise. Our studies highlight the importance of thin spines in healthy cognitive aging. Initiation of cyclic estrogen therapy during peri-menopause is an effective way to restore the age-related cognitive impairment through spinogenesis, partially by restoring or preserving the number of the thin spines.

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Establishing A Comprehensive Wound Repair and Tissue Regeneration Center: A De Novo Model

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The technological and economic successes that followed the recent political reforms in China have led to lifestyle changes resulting in an altered disease pattern for diabetes and chronic wounds. For example, the prevalence of diabetes mellitus in China is high, at 9.7% (*1*); 5%–15% of those with the disease may be expected to suffer from complications such as foot disease and other chronic wounds. The percentage of the population in China that is over 60 years of age is high, complicating disease treatment due to slower healing in aged individuals (*2*). These factors have negative consequences on the nation's health care burden, requiring a rethinking of health and disease management strategies.

The spectrum of chronic wounds found in China is broad, from burn injuries to trauma damage to diabetic foot disease. Recent epidemiology studies show that the prevalence of chronic wounds among hospitalized patients has increased significantly, with diabetic wounds, post-trauma chronic wounds, and pressure ulcers listed as the top three (*3*). To achieve our vision of improved wound management across the country, we outline a strategy to offer concerted patient care, while continuing to pursue research excellence into understanding disease pathology and exploring the role of technologies in improving treatments. One novel strategy is to use information technology to connect rural community health clinics with hospital-based specialists in order to provide better management of patients with chronic wounds. An example is in Shanghai, where since April 2011, specialists at the 9th People's Hospital link with physicians in the community using rapid 4G wireless technology that permits fast data transfer—particularly of images—and quick communication (*4, 5*). This telemedicine system (Figure 1) reduces the need for patients to travel to specialist departments found only in large cities, saving time and money, while at the same time securing rapid diagnoses for patients who do need urgent specialist intervention so that they can more quickly be transported to a hospital such as the 9th People's Hospital in Shanghai.

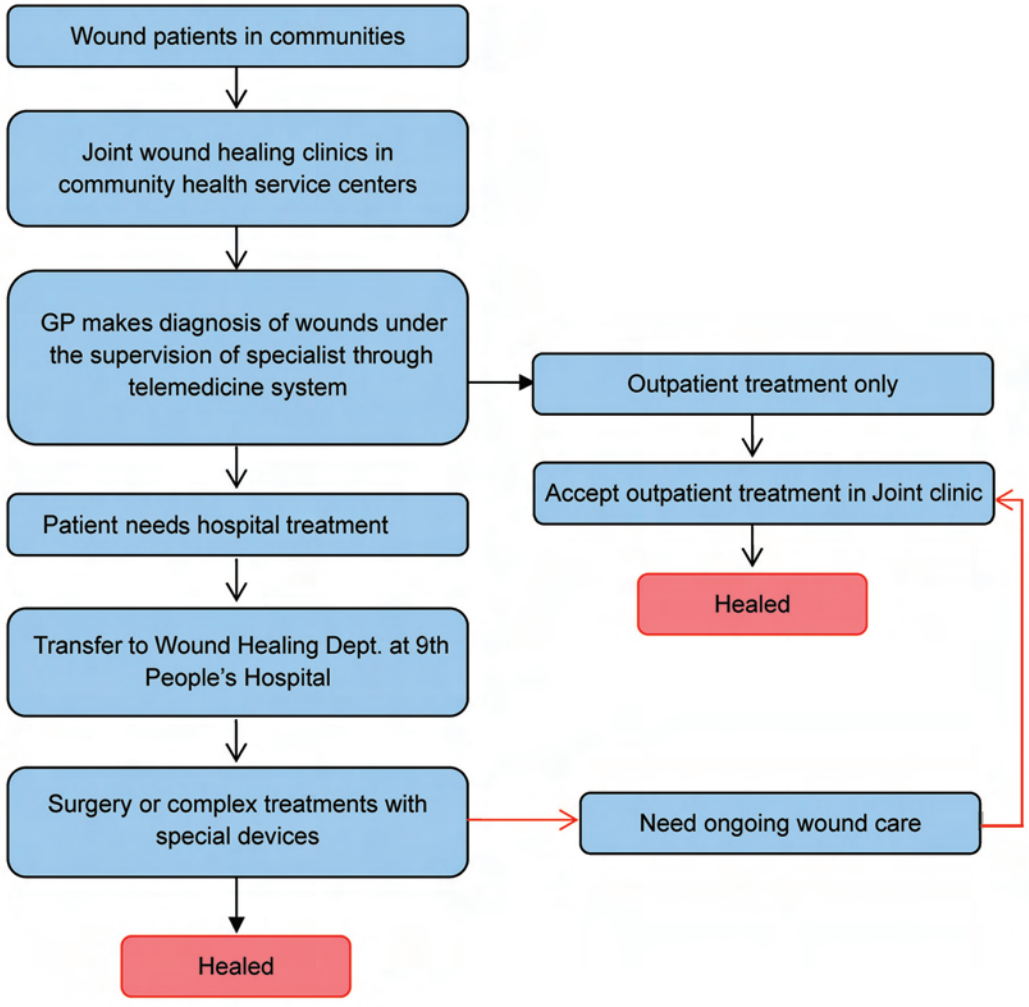

Figure 1. Two-way referral pathway between the wound healing department and the community health service centers.

Thus far, 125 patients (54 males and 71 females) with wounds of differing etiologies have been treated in this way since the telemedicine

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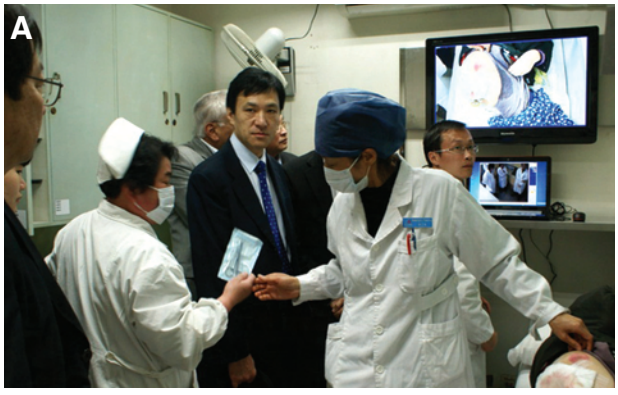

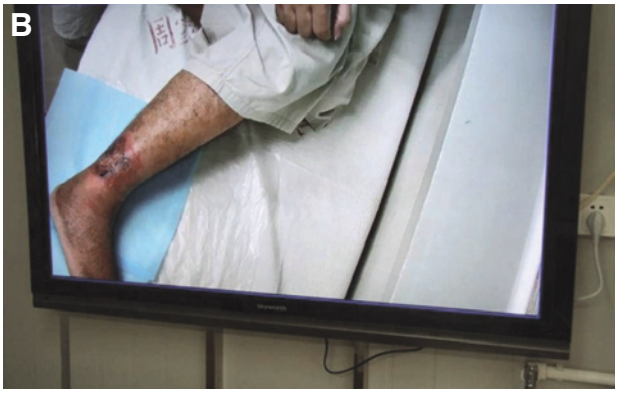

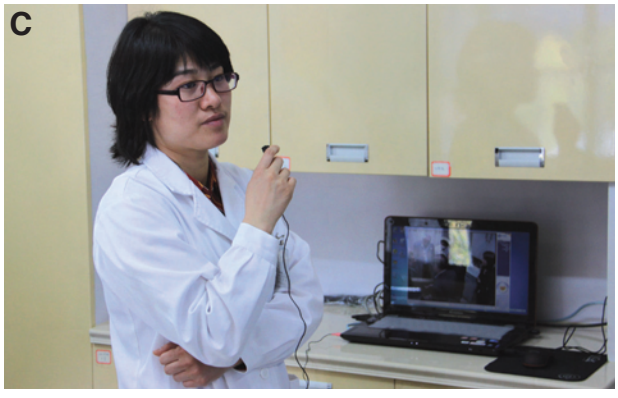

Figure 2. (**A**) During the First Sino-European Joint Meeting of Wound Healing, experts from Europe visited the telemedicine system at Zhou-jia-qiao Community Health Service Center of Shanghai Changning District. (**B**) A community doctor treating a wound under the supervision of a specialist from the Wound Healing Department of 9th People's Hospital. (**C**) A specialist from the Wound Healing Department using the telemedicine system to make an online diagnosis.

system was first put in place. Of these, seven attended the specialist clinic and wounds were reported to be healing in 121 of the 125 patients. From this early experience it would appear that only a small number of patients require hospitalization, while the remainder may be managed in the community setting with specialist support. This concept of "small ward, big clinic" is new in China (Figure 2) and our aim is to further audit and examine all aspects of the system following the initial success.

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Repairing Peripheral Nerve Gaps with Nerve Extracellular Matrix-Derived Scaffolds and Mesenchymal Stem Cells

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A nerve autograft for bridging nerve gaps remains the gold standard for peripheral nerve repair because it contains structural and biological components ideally matched to the requirements of the peripheral nerve. However, the use of nerve autografts has some disadvantages, including donor site morbidity and the limited amount of expendable autogenous nerve graft tissue available. As an alternative, allotransplantation of a nerve guide/conduit can be used to treat nerve defects up to a few centimeters long (*1*).

In addition to serving as a conduit, the neural extracellular matrix (ECM) of a nerve allograft plays an important role in peripheral nerve gap repair. In a previous study, we extracted ECM from a peripheral nerve and found that the complex benefited nerve regeneration in vitro (Figure 1) (*2*). As a result, we believe that the best alternative for peripheral nerve gap repair is an acellular nerve allograft, which recreates in vivo structures and contains essential components needed for peripheral nerve regeneration (*3*).

Different techniques have been used to obtain acellular nerves, including thermal, radiation, and chemical treatments. After removing the cellular component, chemically extracted acellular nerve grafts have intact cylindrical collagen tubes with a basal lamina component and can be reoccupied by Schwann cells following grafting (*4*). Our previous work using chemically extracted nerve bundles (*5*) was carried out using small rodents. There are marked differences in peripheral nerve structure between humans and rats that make the acellular technique more difficult. We refined the extraction method (*6*) and were able to obtain chemically extracted acellular nerve allografts (CEANA) from canine and human sciatic nerves, which have more interfascicular epineurium, and thicker perineurium and epineurium than the rat sciatic nerves. The non-cell components, including laminin and fibronectin, and the structure of the neural basilar membrane, which is very important for Schwann cell migration and axon growth, were preserved after the nerve allograft was extracted, while the cellular components, such as the axon and myelin sheath, which are immunogenic, were removed successfully. After verifying its low immunogenicity, we successfully repaired 5 cm defects in the sciatic nerve in a canine model (*7*).

To examine the length of the defect that could be repaired with our nerve allografts, we treated 8 and 10 cm defects in the sciatic nerve with CEANA in a canine model. The results suggested that an 8 cm segment acellular nerve allograft is the longest distance that allows nerve regeneration with good function. There were many more regenerated axons across the nerve allograft and motor end-plates in the reinnervated muscles in the 8 cm group than in the 10 cm group (*8*). After approval from the hospital ethics committee, 21 patients with nerve defects were treated with CEANA. All patients regained sensation and motion. Electromyogram studies performed during the average follow-up period of 39.2 months found that the average length of the CEANA was 5.8±2.4 cm. Seventy-one percent of the outcomes were excellent or good based on the recovery of sensation and motor function (*9*).

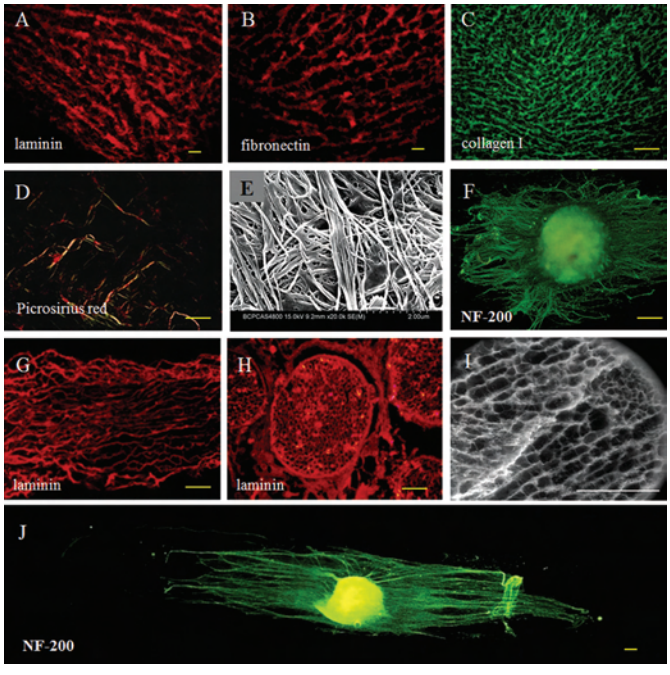

Figure 1. The composition and morphology of nerve-derived ECM (A-F) and acellular nerve allografts (G–J). Immunofluorescence staining shows that nerve-derived ECM contains laminin (**A**), fibronectin (**B**), and collagen I (**C**). Picrosirius red staining (**D**) demonstrates that nerve-derived ECM is rich in collagens I and IV. Scanning electron microscopy (SEM) reveals (**E**) that the nerve-derived ECM has a nanofibrous structure, with diameters of 30–130 nm. Rat dorsal root ganglion (DRG) explants were grown on ECM films for seven days (**F**), then fixed and stained with neurofilament 200 kDa (NF-200) to visualize neurite outgrowth. Most of the neurite outgrowth from the DRG extended along the ECM films. Laminin immunofluorescence staining (**G** and **H**) demonstrates the preservation of the basal lamina composition after chemical decellularization, while scanning electron microscopy (**I**) shows that the acellular nerve grafts preserve their three-dimensional scaffolding structure. NF-200 immunofluorescence staining (**J**) demonstrates that the neurite outgrowth from the DRG extends unidirectionally, parallel to the aligned films from the acellular nerve grafts. Scale bars represent 100 μm.

Although nerve gaps can be repaired with CEANA, the repair length was shorter, growth speed of the axons slower, and number of axons lower than that using nerve orthotopic transplantation. We found that essential neurotrophic factors in the allografts were removed during the chemical extraction of the cellular components. We were able to

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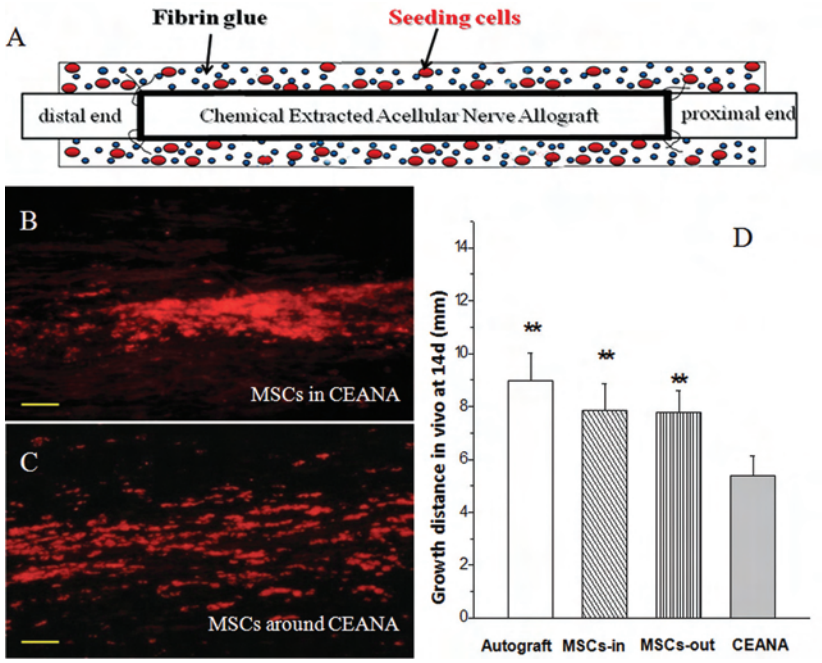

Figure 2. (**A**) Diagram showing repair of a sciatic nerve defect with an acellular nerve graft supplemented with the injection of fibrin glue containing MSCs around the graft. The distribution of PKH26-labeled MSCs in acellular allografts 14 days postoperatively with the MSCs injected (**B**) inside and (**C**) around the graft is shown. (**D**) Quantitative analysis of the distance of axonal regeneration by immunostaining. Data are the mean ± standard deviation. ** $p<0.01$, compared to CEANA alone. Scale bars represent 100 μm.

enhance the regenerative effects of CEANA using adenoviral transfection with hepatocyte growth factor (HGF) (*10*) or the sustained local release of nerve growth factor (NGF) (*11*) around the acellular nerve graft. However, there are many growth factors involved in nerve regeneration and current efforts to use cytokines in a clinical setting are limited by the methods of administration.

Numerous factors promoting nerve growth, including NGF, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor, and matrix metalloprotease 2, are secreted by mesenchymal stem cells (MSCs), which may be exploited as a "drug factory" to obtain the desired healing response in injured nerve tissue. We constructed a nerve graft using an acellular nerve graft, containing MSCs inside or around the graft, to repair a 10 mm defect in a murine sciatic nerve, after verifying that the MSCs embedded in the fibrin glue could survive and were able to secrete growth factors such as NGF and BDNF in vitro. Twenty-four mice were divided into four groups of six each and attempted repair of a 10 mm sciatic nerve defect was carried out using nerve autografts, acellular nerve grafts, acellular nerve grafts supplemented with MSCs (5×10^5 cells) in fibrin glue around the graft, or acellular nerve grafts in which MSCs (5×10^5 cells) were injected into the graft. The mouse sciatic nerve functional index was evaluated using walking-track testing every two weeks. The weight of the triceps surae muscle and the histomorphometry of the triceps surae and repaired nerves were examined 8 weeks postoperatively. At this time, the repair with the nerve autograft gave the best functional recovery, while the nerve repaired with the acellular nerve grafts with MSCs (inside or outside the graft) achieved better functional recovery and a higher axon number than with the acellular nerve graft alone (Figure 2). Supplying MSCs in fibrin glue around acellular nerves is easy, maintains the nerve structure, and can support nerve regeneration in a similar way to the direct injection of MSCs into an acellular nerve for long nerve defects, but may avoid destroying the nerve graft (Figure 2). The technique is simple and is another option for stem cell transplantation (*3*).

Although peripheral nerve gaps can be repaired with CEANA and MSCs, many problems need to be resolved before using the technique on large animals or humans. One is the size of the nerve gap that can be repaired using a CEANA loaded with MSCs. Another is the fate of MSCs seeded around the CEANA. We plan to examine these in future studies.

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Enhancing Diabetic Foot Care and Reducing Amputation Using Multidisciplinary Care Teams

Zhangrong Xu, M.D.*, Yufeng Jiang, M.D., Hongbin Gu, M.D.

There are currently 92 million diabetic patients and 145 million people with prediabetes in China (*1*). Among those with diabetes, diabetic foot disorder is becoming a serious burden, which significantly affects a patient's quality of life and China's health care resources. A recent survey determined that one-third of the amputations in the central urban hospitals are due to diabetic foot disease. The average medical costs for these patients are 36,700 RMB (about 5,478 USD) per person (*2*). Further, a current epidemiologic survey showed that diabetes is now the leading cause of chronic wounds for hospitalized patients, up from 4.91 % in 1996 to 35.3 % in 2008 (*3*). Nearly 40% of Chinese diabetic patients who have had an amputation died within a five-year follow-up period (*4*). Unfortunately, there are no college-level training programs for podiatry in China, or in other Asian countries. To add to these difficulties, most hospitals in China do not have specialized diabetic foot-related clinics, which contributes to the inaccessibility of appropriate health care and specialists, such as orthopedic surgeons, vascular surgeons, wound surgeons, dermatologists, diabetologists, and other clinical staff. However, we have to face the dramatically increasing prevalence of diabetes and its more severe consequence, diabetic foot disease. Considering these circumstances, multidisciplinary medical teams are needed to provide the professional care and preventive measures that decrease the need for amputations in patients with diabetic foot disorder as well as treat the various other complications arising from diabetes (*5, 6*). To meet this objective, in 2003, we sent one doctor and one nurse to the Royal Prince Alfred Hospital of Sydney University for intensive practice and training sessions in which they learned how to treat diabetic foot disease. This has allowed us to gradually establish a diabetic foot clinic, which consists of a group of clinical staff including diabetologists, a vascular surgeon, an orthopedic surgeon, a wound surgeon, and a radiologist who treat patients with refractory diabetic foot ulcers, and other complicated foot diseases, at least once a week. This multidisciplinary team approach has proven effective and efficient for providing immediate and adequate care to the limb-threatening diabetic foot disorder. Our extensive experience over the years has allowed us to incorporate new technologies and treatment strategies in our diabetic foot clinic, including vascular intervention (stents and deep balloon angioplasty), transplantation of autologous peripheral blood stem cells to treat peripheral vascular disease, autologous platelet-rich gels, and negative-pressure therapy (Figure 1). Further, we have also integrated hyperbaric oxygen therapy and skin-graft or skin-flap reconstruction for treating recalcitrant ulcers, which are frequently large and slow to heal. Our multidisciplinary care model combined with patient-centered care and the recruitment of new technologies has allowed us to increase our serious diabetic foot patient intake from 12 in 2001 to 214 in 2010. We now treat patients who are referred to us from all over China and have received a few cases from other countries (Mongolia and Russia). We have successfully decreased the amputation rate in our clinic from 11.5% in 2003 to 7.2% in 2010, with only 4% of this total considered a major amputation.

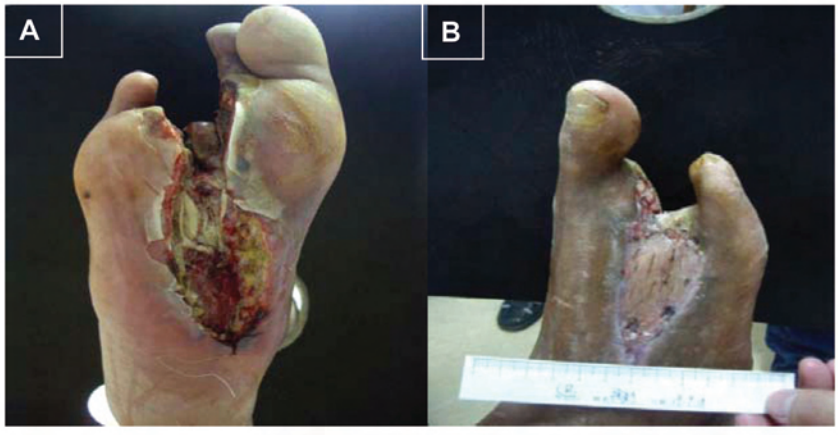

Figure 1. A patient with a deeply infected, ischemic, necrotic diabetic foot ulcer. (**A**) Deeply infected and ischaemic foot ulcer after radical debridement and intravenous antibiotics. (**B**) The wound healed after vascular intervention, wound debridement, negative pressure treatment, hyperbaric oxygen therapy, and skin-graft application.

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DNA Oxidation in Epigenetic Reprogramming

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The discovery of hydroxylated methylcytosine (5hmC) in mammalian DNA (*1, 2*) in 2009 immediately sparked intense investigation of its molecular impact and biological relevance. The enzymes responsible for the generation of 5hmC have since been better characterized, and this new modification's involvement in epigenetic regulation of embryonic development and disease has started to be elucidated. Our current model links ten-eleven translocation (Tet)-mediated oxidation of 5-methylcytosine (5mC) with active DNA demethylation via the recognition and excision of the final oxidation product, 5-carboxylcytosine (5caC), by a DNA glycosylase known as thymine-DNA glycosylase (TDG). Although DNA oxidation mediated by the Tet family dioxygenase, Tet3, plays a role in DNA demethylation and gene activation in the paternal DNA of the male pronucleus, events downstream of 5hmC and 5caC formation still need to be investigated to fully understand the mechanisms underlying epigenome reprogramming in the early embryo.

DNA Oxidation is Required for Activation of Pluripotency Genes in Early Embryos

DNA methylation is important in epigenetic developmental control. Active genes generally have low levels of methylation in their promoter and enhancer regions, and demethylation is required for transcriptional activation of epigenetically silenced genes. The best-known case of demethylation is the genome-wide removal of 5mC from the paternal pronuclear DNA in zygotes, originally reported based on the observation of decreased genome-wide 5mC signals in the male pronucleus (*3*) and a partial loss of methylation at some single copy genes and repeats (*4*). However, both the mechanism of DNA demethylation and its biological relevance are largely unclear. Our lab recently found that the Tet3 enzyme is specifically enriched in the male pronucleus in zygotes and is responsible for the removal of 5mC in the paternal genome (*5*). The 5hmC signal increases drastically in the paternal pronucleus around the pronuclear stage PN3, a time point when the 5mC signal starts to decrease (Figure 1). In Tet3-deficient zygotes derived from conditional knockout mice, paternal-genome conversion of 5mC into 5hmC fails to occur and the level of 5mC remains constant. The loss of 5mC in the paternal genome is thus caused by its conversion to 5hmC. Failure in paternal DNA oxidation impedes demethylation at the promoter and enhancer regions of pluripotency genes such as *Oct4* and *Nanog*, and delays the subsequent reactivation of the paternally derived transgene *Oct4*-EGFP in early embryos. As a consequence, female mice depleted of Tet3 in the germ line display severely reduced fecundity and their heterozygous mutant offspring lacking maternal Tet3 suffer an increased incidence of developmental failure.

Somatic cell nuclei injected into eggs undergo profound epigenetic reprogramming including DNA demethylation. The cytoplasm of oocytes from the germinal vesicle (GV) stage onwards contains substantial reprogramming activity. Our nuclear transfer (NT) experiment using Tet3-deficient oocytes suggested that Tet3 might be one of the cytoplasmic factors contributing to the reprogramming activity. Strikingly, the Tet3 protein in oocytes is translocated specifically to the pseudo-pronucleus formed from the transferred somatic nucleus following the injection. The somatic DNA in the pseudo-pronuclei undergoes oxidation, but this does not occur in NT embryos derived from Tet3-null oocytes. Activation of silenced pluripotency genes in somatic cells, such as *Oct4*, is known to be crucial for reprogramming cells and successful cloning. We found that demethylation of the *Oct4* promoter is impaired in the absence of Tet3 because the *Oct4* promoter region retains a higher level of methylation in the pseudo-pronucleus formed in the Tet3-null oocytes. Impaired demethylation of the *Oct4* promoter, in turn, postpones gene activation in NT embryos, which was demonstrated using a somatic *Oct4*-EGFP transgene. These observations support the idea that Tet3-mediated 5mC oxidation is involved in the epigenetic reprogramming of donor nucleus in somatic cell nuclear transfers.

Our findings suggest that reprogramming in somatic cell nuclear transfer (SCNT) shares a common mechanism with paternal genome remodeling in fertilized eggs. The incomplete DNA demethylation and embryonic gene reactivation observed in SCNT using Tet3-deficient

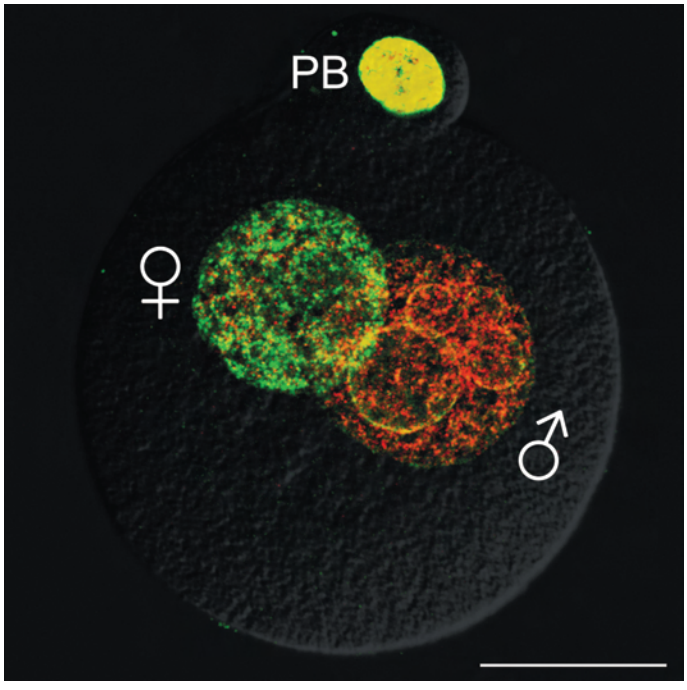

Figure 1. DNA oxidation in the male pronucleus in mouse zygotes. The 5hmC signal is shown in red (male pronucleus) and the 5mC signal in green (female pronucleus) in this image of immunofluorescence staining. PB, polar body. Scale bar represents 25 μm.

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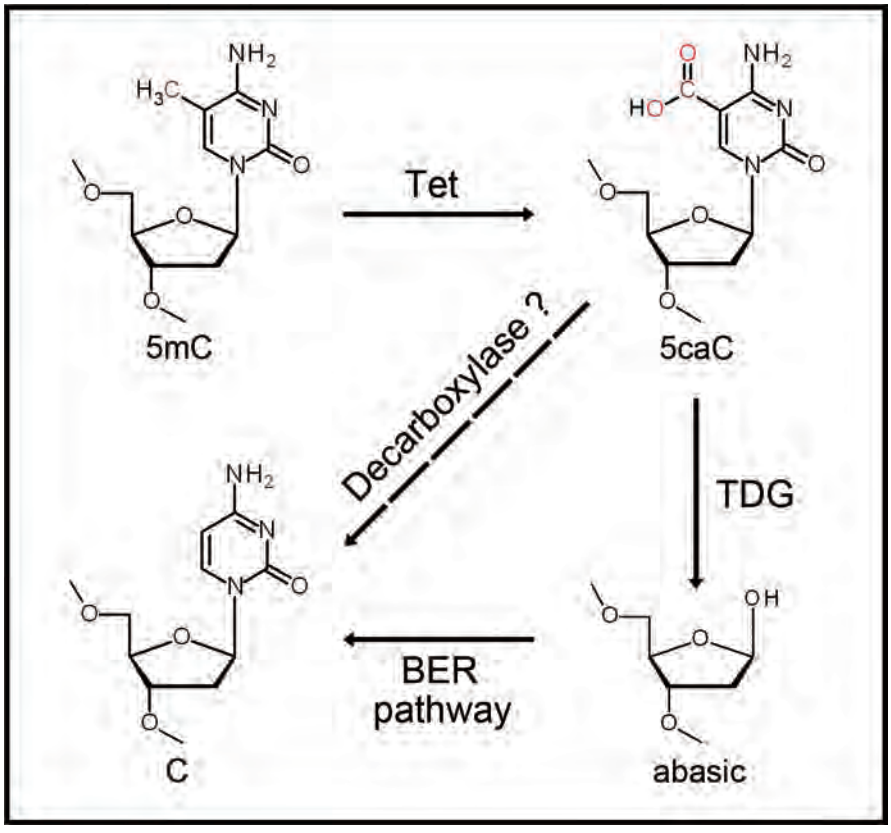

Figure 2. Pathways of active DNA demethylation. Decarboxylation of 5-carboxylcytosine (5caC) towards demethylation has been proposed but not experimentally substantiated. 5mC, 5-methylcytosine. TDG, thymine-DNA glycosylase, BER, base-excision repair.

oocytes seems to parallel the defective paternal reprogramming in Tet3-null zygotes upon natural fertilization. Further investigation is needed to determine the exact extent of 5mC oxidation in both fertilized and cloned embryos. Although a recent study indicates a DNA replication-dependent loss of 5hmC in the two-cell embryo, the possibility remains that active demethylation of paternal DNA involves further oxidation (*6, 7*) of 5hmC and subsequent decarboxylation, since this idea has not been formally excluded.

Tet-Mediated Oxidation of 5mC and Subsequent Excision of 5caC by TDG Constitutes a Demethylation Pathway

Multiple mechanisms can contribute to DNA demethylation, including the direct removal of the exocyclic methyl group from cytosines via a C-C bond cleavage, enzymatic removal of 5-hydroxylated methyl groups such as formaldehyde, or replacement of the methylated cytosine base and nucleotide through DNA base-excision repair (BER) and nucleotide excision repair pathways, respectively (*8, 9*). In theory, all of these processes can be triggered by Tet-mediated hydroxylation of 5mC. However, the events following 5mC hydroxylation have remained unclear. Our recent work demonstrates that 5hmC or 5mC in DNA can be oxidized to 5-carboxylcytosine (5caC) by Tet dioxygenases (*6*). 5caC is in turn specifically recognized and excised by TDG (Figure 2). This is confirmed in vivo, as depletion of TDG in mouse ES and iPS cells leads to the accumulation of 5caC (*6*).

The finding that TDG is involved in 5caC excision is surprising but provides a mechanism underlying its role in transcriptional regulation and embryonic development. TDG has been previously implicated in DNA demethylation, an assertion supported by its ability to excise the deamination product of 5mC or 5mC itself from DNA (*9*). However, no enzyme has been identified that deaminates 5mC to yield a mispaired thymine for TDG to act on. Moreover, TDG itself lacks glycosylase activity to excise 5mC directly. While the hypothesized demethylation mechanism involving deamination and/or base excision still awaits experimental proof, our work provides clear evidence that TDG mediates the demethylation process by recognizing and removing the oxidation products of 5mC (Figure 2).

The proposed demethylation pathway fits well with the puzzling distribution patterns of Tet, 5hmC, and 5mC. Tet1 and 5hmC are relatively enriched in CpG-rich active promoters that are unmethylated, while 5hmC is underrepresented in the majority of Tet1binding sites in ES cells (*9*). These apparently conflicting genomic distribution patterns of Tet1 (enzyme), 5mC (substrate) and 5hmC (one of the products) can be accounted for by speculating that active promoters are kept free of 5mC because the bound Tet1 converts stochastically occurring 5mC to 5hmC. At some of the Tet1 binding sites, 5hmC is further oxidized into 5caC, which is then removed quickly by TDG-mediated BER repair. In such a case, 5mC becomes undetectable in active promoters due to their transient existence in a small proportion of cells. Likewise, in many of the Tet1 binding sites, 5hmC is underrepresented because it is quickly converted to 5caC which is rapidly removed in cells. In this context, Tet-TDG dependent demethylation acts as an anti-silencing mechanism of gene regulation.

The fact that a relative higher steady-state level of 5hmC but not 5caC exists in ES cells suggests that oxidation to 5caC might be a rate-limiting step of DNA demethylation. It warrants further investigation to address what proportion of 5hmC is subjected to further oxidation by Tet enzymes and what cellular factors and genomic contexts induce consecutive DNA oxidation and even decarboxylation during cell differentiation and lineage reprogramming.

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