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A liver divided

By Benjamin Boettner, Associate Editor

Three separate teams have directly converted human fibroblasts to proliferative liver cells and thus eliminated a key drawback of using induced pluripotent stem cells to treat damaged livers—the inability of the resulting differentiated cells to repopulate damaged liver.[1–3]

Each team had a different twist on getting the cells to divide, but the common thread was that fibroblasts were directly programmed to cell states that are part of the hepatocyte differentiation pathway. Next steps could include taking the protocols' lentiviral manipulations out of the equation and further improving the maturation of the resulting hepatocytes.

Patients with chronic liver disease live for an average of 12 years with compensated cirrhosis before they enter a state of rapid decline marked by ascites, encephalopathy and other complications.[4] Because the demand for livers for transplantation exceeds the supply, a logical alternative is to use induced pluripotent stem (iPS) cell technology.

The idea is straightforward: take a patient's own cells, usually fibroblasts, revert them to an iPS cell state and then differentiate them *in vitro* into an autologous supply of hepatocytes. The problem comes at the last step, with different research groups reporting that iPS cell differentiation to mature hepatocytes is impeded and the resulting hepatocytes have dramatically diminished ability to proliferate.

Three teams have now developed independent protocols that overcome these limitations.

A team led by Sheng Ding and Holger Willenbring developed a method involving the retroviral introduction of three pluripotency genes—*OCT4*, *SOX2* and *KLF4*—into human newborn fibroblasts and brief exposure of the cells to medium containing endoderm-promoting factors together with the small molecule CHIR99021, which stimulates wingless-type MMTV integration site (WNT) pathway signaling.

Ding is a senior investigator at the **Gladstone Institute of Cardiovascular Disease** and a professor of pharmaceutical chemistry at the **University of California, San Francisco**. Willenbring is an associate professor in the **UCSF School of Medicine**'s division of transplant surgery.

Lijian Hui at the **Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences** took a different approach, converting human fetal fibroblasts directly into hepatocytes by transducing them with lentiviruses carrying three genes: *HNF1 homeobox A* (*HNF1A*), *hepatocyte nuclear factor 4α* (*HNF4A*; *TCF*) and *forkhead box A3* (*FOXA3*). Together, those genes promote hepatocyte lineage commitment and maturation.

The team removed the proliferation barrier by overexpressing the viral SV40 large T antigen in the resulting hepatocytes. SV40 is an inhibitor of cell cycle–regulating proteins including p53.

Hui is a professor at the Shanghai Institutes' Institute of Biochemistry and Cell Biology.

Finally, Hongkui Deng, Yan Shi and Shichun Lu introduced lentiviruses expressing a set of three hepatocyte fate conversion factors—*HNF1A*, *HNF4* and *one cut homeobox 1* (*ONECUT1*; *HNF6*)—into primary human fetal limb fibroblasts. The group then used a second set of maturation factors including *activating transcription factor 6* (*ATF6*), *prospero-related homeobox 1* (*PROX1*) and *CCAAT enhancer binding protein-a* (*CEBPA*) and siRNAs to inhibit p53 and *c-Myc* (*MYC*), thus releasing the cells from cell cycle inhibition.

Deng is a professor at **Peking University**'s college of life sciences and a principal investigator at the Key Laboratory of Chemical Genomics. Shi is an associate professor at the **Peking University Shenzhen Graduate School** and an investigator at the **Key Laboratory of Chemical Genomics**. Lu is a chief physician at the **Chinese PLA General Hospital**.

Hepatocyte differences

The UCSF team's procedure converted fibroblasts into what the group calls induced multipotent progenitor cell–derived endodermal progenitor cells (iMPC-EPCs). By growing the fibroblasts in medium that favors endoderm differentiation, the group prevented the cells from reverting to a pluripotent iPS cell–like state and instead directly converted them to the endoderm lineage from which hepatocytes normally emerge.

In culture, iMPC-EPCs spontaneously gave rise to hepatocyte-like cells, dubbed iMPC-Heps, that kept dividing. The results thus provided proof of principle that direct lineage conversion of fibroblasts to hepatocytes via iMPC-EPCs maintained proliferation.

The team then screened and identified a set of small molecules that increased iMPC-EPCs' conversion and an additional set of small molecules that, together with hepatocyte-promoting factors, improved differentiation into iMPC-Heps.

The resulting cells closely resembled proliferative fetal primary hepatocytes as judged by their expression of hepatocyte markers *HNF4A*, *albumin* genes, *α_1-antitrypsin* (*AAT*; *A_1AT*; *SERPINA1*) and *cytokeratin 8* (*CK8*; *KRT8*).

In an immune-deficient mouse model of liver failure, transplanted iMPC-Heps kept proliferating for at least 6 months and repopulated 2% of liver parenchyma. Animals receiving transplants survived significantly longer than nontransplanted controls.

Previous iPS cell–based studies only achieved about 0.05% liver repopulation.

iMPC-Heps continued to mature and produced functional, drug-metabolizing cytochrome P450 (p450) enzymes and albumin levels that were tenfold higher than those in controls.

Data were published in *Nature*.

Gladstone has filed for a patent covering the reprogramming conditions to generate hepatocytes from fibroblasts, and the IP is available for licensing.

"The authors cut short the amount of time that fibroblasts were exposed to regulatory proteins that induce pluripotency so that the

cells did not revert to a pluripotent state but rather only reverted to a multipotent state. By starting from the multipotent state, they could generate liver-like cells that appear better than if they had started from the pluripotent state," said Kenneth Zaret.

Zaret is a professor of cell and developmental biology at the **Perelman School of Medicine at the University of Pennsylvania** and associate director of the **University of Pennsylvania**'s Institute for Regenerative Medicine.

The two Chinese teams did not include passage through a multipotent iMPC-EPC-like state in their protocols and generated cells that were already further downstream in the hepatocyte lineage. To tackle the proliferation problem, the teams interfered with the expression of central cell cycle regulators.

Both teams termed the resulting cells HiHeps (human induced hepatocytes) and reported that the cells had some of the hallmarks of adult hepatocytes. The HiHeps also had higher hepatocyte activity than the iMPC-Heps produced by the UCSF team.

HiHeps expressed the genes relevant for hepatocyte identity, metabolic activity and detoxification mediated through p450 enzyme systems. Indeed, both groups showed that the HiHeps were able to metabolize model drugs.

Transplanted HiHeps also colonized liver parenchyma in mouse models of liver injury with repopopulation efficiencies of 0.3%–4.2% (Shanghai Institutes for Biological Sciences) and up to 30% (Peking University), producing substantial quantities of albumin and improving survival.

The two approaches were published in *Cell Stem Cell*.

The Shanghai Institutes for Biological Sciences has filed for a patent covering HiHep derivation from human fibroblasts. The IP is not available for licensing. The researchers at Peking University did not disclose the IP status of their method.

Growing up hepatocytes

It is early days for all three approaches, making head-to-head comparisons for potential in human regenerative approaches difficult. Regardless, each approach needs to do away with viral integration and improve final hepatocyte maturation before it can be further translated.

Salman Khetani, an assistant professor of mechanical and biomedical engineering at **Colorado State University** and cofounder and member of the scientific board of **Hepregen Corp.**, said that there were some notable differences between the UCSF findings and those from the Chinese teams.

The former, he said, showed "unsurpassed longevity of transplanted cells that lasted for more than six months." The two groups from China, however, had "more impressive *in vitro* functional characterization data relative to freshly isolated primary human hepatocytes."

> **"For clinical applications, it seems highly likely that a nonviral method of delivery of the regulatory proteins will be key. The viral method could disrupt genes in the cell, with long-term, unanticipated consequences."**
>
> ***—Kenneth Zaret, Perelman School of Medicine at the University of Pennsylvania***

Wolfram Goessling, an assistant professor in the Department of Medicine at **Harvard Medical School** and the **Harvard Stem Cell Institute**, said, "A crucial difference between the protocols is the pervasive use of chemicals in the study by the Gladstone Institute and UCSF, which circumvented direct inhibition of cell cycle regulators like the tumor suppressor p53 and present an advantage."

In all cases, Zaret said, viral delivery of the reprogramming factors needs to be replaced.

"For clinical applications, it seems highly likely that a nonviral method of delivery of the regulatory proteins will be key. The viral method could disrupt genes in the cell, with long-term, unanticipated consequences," he said.

Both Zaret and Khetani wanted to see more long-term data on the efficacy of transdifferentiated hepatocytes.

"Protocols to better mature the iMPC-Heps/HiHeps into functional hepatocytes *in vitro* so they can demonstrate better functions after transplantation *in vivo* should be a prime goal," Khetani said.

He added that various microscale and 3D engineering techniques that simulate a tissue environment *in vitro* have been previously applied to primary hepatocytes and could potentially be used with the induced hepatocyte-like cells to achieve better maturation.

Ruslan Semechkin said that the analysis of the final hepatocyte products needs to be more comprehensive before they are deemed a histocompatible source for modeling and treating liver diseases. Semechkin is CSO at **International Stem Cell Corp.**

"Most inherited liver disease phenotypes are observed only in fully differentiated cells," he said. Thus, "the degree to which human fibroblasts can be differentiated into hepatocytes will affect the extent to which the disease can be modeled *in vitro* and in follow-up studies *in vivo*."

"More activities and markers that closely resemble those of primary hepatocytes should be assessed," Semechkin continued. "Hepatic characteristics should be demonstrated using drug metabolism experiments on the gene expression and functional level. In-depth analysis of a number of parameters including hepatic transport proteins, mature hepatic transcription factors, albumin secretion, production of bile acids and bilirubin as well as mitochondrial functions would be informative."

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> ***—Salman Khetani, Colorado State University***

He said that International Stem Cell "would be interested in rapid testing of the protocol presented in the *Nature* paper." He also wanted to see a head-to-head comparison of the transdifferentiated hepatocytes versus pluripotent stem cell–derived hepatocytes.

Better characterization and *in vitro* maturation will also provide a basis for the cells to be used in disease modeling and drug testing.

Ding told *SciBX*, "We are planning to further optimize the process, especially focusing on hepatocyte *in vitro* maturation, before developing clinical-stage GMP conditions."

Ding's group also has identified small molecules to differentiate several other lineages including cardiomyocytes and pancreatic cells.

The team from the Shanghai Institutes for Biological Sciences is planning to test techniques that could eliminate viral integration events from the procedure, including the use of mRNAs and small molecules.

The Peking University group did not comment on its future plans.

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   Contact: Holger Willenbring, University of California, San Francisco, Calif.
   e-mail: willenbringh@stemcell.ucsf.edu
   Contact: Sheng Ding, Gladstone Institute of Cardiovascular Disease, San Francisco, Calif.
   e-mail: sheng.ding@gladstone.ucsf.edu
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   Contact: Lijian Hui, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
   e-mail: ljhui@sibcb.ac.cn
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   Contact: Hongkui Deng, Peking University, Beijing, China
   e-mail: hongkui_deng@pku.edu.cn
   Contact: Yan Shi, Peking University Shenzhen Graduate School, Shenzhen, China
   e-mail: shiyan@pkusz.edu.cn
   Contact: Shichun Lu, Chinese PLA General Hospital, Beijing, China
   e-mail: lsc620213@aliyun.com
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COMPANIES AND INSTITUTIONS MENTIONED

Chinese Academy of Sciences, Beijing, China
Chinese PLA General Hospital, Beijing, China
Colorado State University, Fort Collins, Colo.
Gladstone Institute of Cardiovascular Disease, San Francisco, Calif.
Harvard Medical School, Boston, Mass.
Harvard Stem Cell Institute, Cambridge, Mass.
Hepregen Corp., Medford, Mass.
International Stem Cell Corp. (OTCBB:ISCO), Carlsbad, Calif.
Key Laboratory of Chemical Genomics, Beijing, China
Peking University, Beijing, China
Peking University Shenzhen Graduate School, Beijing, China
Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pa.
Shanghai Institutes for Biological Sciences, Shanghai, China
UCSF School of Medicine, San Francisco, Calif.
University of California, San Francisco, Calif.
University of Pennsylvania, Philadelphia, Pa.