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Heps with Pep: Direct Reprogramming into Human Hepatocytes

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The limited supply and expansion capacity of primary human hepatocytes presents major challenges for pharmaceutical applications and development of cell-based therapies for liver diseases. Now in *Cell Stem Cell*, two papers demonstrate efficient direct reprogramming of human fibroblasts into induced hepatocytes, which exhibit metabolic properties similar to primary hepatocytes.

The liver is a unique organ that performs a broad spectrum of functions. It stores reserves of iron, vitamins, and minerals and detoxifies alcohol, drugs, and other chemicals that accumulate in the bloodstream. The liver also produces bile, albumin, and blood-clotting factors. Finally, the liver performs an essential metabolic activity by storing glycogen. These tasks are managed by one cell type, the hepatocyte, which constitutes the main cellular unit of the liver. Genetic disorders or injuries that prevent the liver from carrying out these essential activities result in life-threatening diagnosis and end-stage liver diseases that require organ transplantation. Thus, generating large quantities of hepatocytes as an alternative to liver transplants is a major objective for drug development and regenerative medicine. However, freshly isolated hepatocytes come in limited supply, often from donated organs that are of poor quality, and are impossible to expand in large quantities in vitro. Therefore, deriving hepatocytes from stem cell populations such as human pluripotent stem cells (hPSCs) presents an attractive alternative to primary cells. Now in *Cell Stem Cell*, two studies, from the groups of Lijian Hui and Hongkui Deng, demonstrate an additional approach by directly reprogramming fibroblasts into human induced hepatocytes (hiHeps) (Du et al., 2014; Huang et al., 2014).

hPSCs have been used advantageously to produce hepatocytes for disease modeling (Rashid et al., 2010) and for developmental studies. However, generation of cells displaying all the functional characteristics of mature hepatocytes has been proven difficult. Indeed, hPSC-derived hepatocytes uniformly express fetal markers such as AFP and lack key metabolic activity associated with adult cells such as cytochrome p450, especially CyP3A4. Importantly, recent improvements involving 3D cultures (Ogawa et al., 2013), small molecule screens (Shan et al., 2013), and also in vivo maturation with coculture of endothelial cells (Takebe et al., 2013) have resulted in important functional improvements, including the expression of inducible CyP3A4 and diminished AFP expression.

The development of robust pluripotent stem cell differentiation protocols has been impaired by the lack of knowledge concerning the mechanisms that regulate the functional maturation of the human liver after birth. Direct reprogramming approaches could bypass this last limitation by avoiding the need to mimic a complex path of development in vitro. Accordingly, previous reports have shown that overexpression of Gata4/HNF1alpha/Foxa3 or HNF4a/FoxA1/FoxA2/FoxA3 in mouse embryonic fibroblasts, following genetic ablation of p19, enables the production of induced hepatocyte-like cells (iHep) (Huang et al., 2011; Sekiya and Suzuki, 2011). These cells can be expanded in vitro while displaying a limited metabolic activity, and they retain the capacity to colonize the failing liver of mice lacking fumarylacetoacetate hydrolase (Fah−/−), a common animal model of liver failure (Azuma et al., 2007).

The two reports published in this issue of *Cell Stem Cell* have successfully extended this approach to human fetal cells. Huang et al. (2014) report that overexpression of

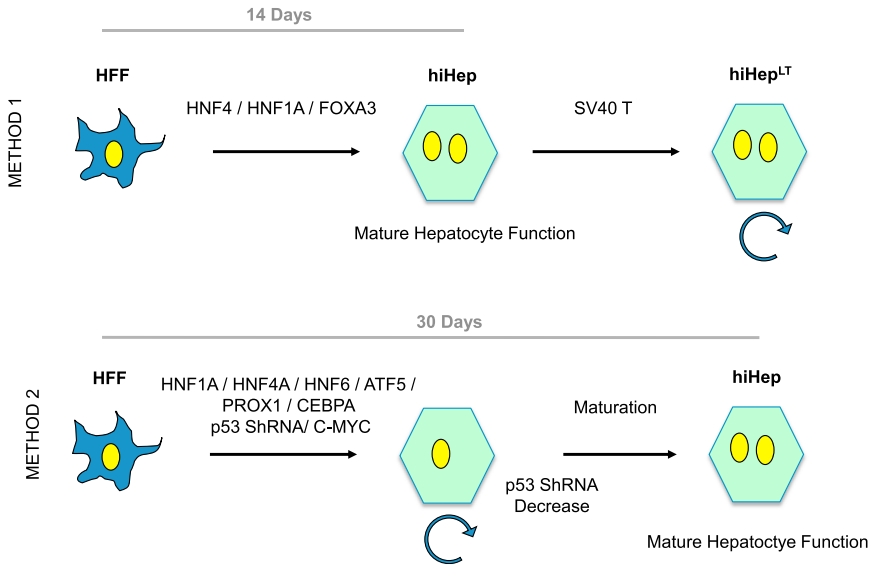

Figure 1. Direct Reprogramming of Human Fetal Fibroblast into Induced Hepatocytes
Human fetal fibroblasts (HFFs) are transduced with two different cocktails of transcription factors. The transduced cells are then grown in media supporting hepatocytes survival and function. With method 1, the proliferative capacity of hiHep cells is increased by overexpressing SV40 T antigen 14 days after transduction. In method 2, expression of c-*myc* and shRNAs targeting p53 allow proliferation of reprogrammed cells. The expression of shRNA directed against p53 decreases 25 days after transduction, and the cells are then transferred into a medium allowing functional maturation. The resulting hiHep display functional characteristics of primary hepatocytes including Albumin and Alpha-Antitrypsin secretion, phase I enzyme activity including Cyp3A4, phase II metabolic enzyme, and phase III drug transporter expression, cholesterol uptake, and glycogen storage.

HNF4 (instead of GATA4), HNF1A, and FOXA3 in fibroblasts allows the production of hiHeps with a conversion rate close to 20%. The second publication relies on a more complete set of factors (C-MYC, HNF1A, HNF4A, HNF6, ATF5, PROX1, CEBPA, and p53 ShRNA) that follow a developmental rationale, since the cocktail includes transcription factors not only involved in liver bud induction (HNF1A, HNF4A, HNF6) but also in hepatocyte specification/maturation (ATF5, PROX1, and CEBPA). Following this approach, nearly 80% of the resulting hiHep cells express Albumin after 30 days of maturation. The efficiency of both methods is remarkable and suggests that direct reprogramming could be a valuable substitute for large-scale production of hepatocytes. Interestingly both groups have developed novel strategies to bypass the lack of proliferation associated with direct reprogramming. Huang et al. (2014) used overexpression of SV40 large T antigen to establish hiHep^{LT} cells, which can be grown in vitro. However, the resulting cells display decreased metabolic activity when compared to nontransformed hiHep cells. Du et al. (2014) used simultaneous overexpression of C-MYC and knockdown of p53 during reprogramming (Figure 1). This provides the advantage of avoiding forced proliferation of mature cells, which would likely be detrimental to their functionality.

In both cases, hiHeps display an outstanding panel of metabolic activities including inducible expression and activity of CyP3A4, at levels comparable to those of primary hepatocytes. Impressively, Du et al. (2014) used freshly isolated hepatocytes from two donors, which represents the best possible control. Both publications also show that hiHeps and primary hepatocytes share a mutual gene expression profile and the authors rightly focus on the genes commonly expressed. Nevertheless, a broad number of genes are also differentially expressed, demonstrating differences between hiHep cells and their natural counterparts. This discrepancy simply confirms that engineering cells identical to primary hepatocytes in vitro is utopic. Artificially generated cells are likely to exhibit specific characteristics imposed by culture systems that only partially replicate the complexity of the natural hepatic environment.

Finally, both studies validate the functionality of hiHep cells using three different mice models for acute liver failure. Of particular interest, hiHep^{LT} can colonize the livers of FRG (Fah−/−/Rag2−/−/Il2rg−/−) mice and mice injected with concanavalin A, improving their survival. These results are impressive since only adult primary hepatocytes have been shown to function in the FRG model (Azuma et al., 2007). Similarly, Du et al. (2014) show that hiHep can colonize efficiently the liver of Tet-uPA/Rag2−/−/γc−/− mice and secrete high levels of human albumin in the plasma of the transplanted mice. Both studies also show that primary hepatocytes have a higher capacity for colonization with consistently higher levels of Albumin secretion. Thus, these in vivo data certainly confirm the unprecedented level of functionality acquired by hiHep cells but also suggest that they could be a limited substitute for primary hepatocytes in the context of cell-based therapy.

Together these publications establish that production of highly functional human hepatocyte-like cells using direct reprogramming approaches is feasible and thus represent an exciting step toward the production of an infinite supply of cells for drug development pipelines and therapies for liver diseases. However, several challenges remain to be addressed, including the transfer of this technology to adult somatic cells especially from patients with inherited metabolic disorders for the purpose of disease modeling. Additionally, the use of direct reprogramming for cell-based therapies also remains uncertain. Indeed, the technologies currently available to generate hiHeps and to increase their proliferative capacity are not compatible with in vivo use. Additionally, hiHeps are a mixed cell population, since each cell originates from a unique reprogramming event. This could introduce a large degree of variability and confounds quality controls essential for moving this methodology into the clinic. Thus, despite the significant progress achieved by the current publications, further basic studies are still required to uncover the mechanisms that orchestrate human liver development and that ultimately establish the functional diversity of hepatocytes in vivo.

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Reprogramming Can Be a Transforming Experience

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Since the inception of nuclear reprogramming, the parallels between this process and tumorigenesis have become increasingly apparent. Recent studies by Abad et al. and Ohnishi et al. have now formalized this connection by demonstrating that the same transcription factors used for reprogramming to pluripotency drive tumor initiation in vivo.

Although experimental conditions allowing nuclear reprogramming in vitro have been well established (Stadtfeld and Hochedlinger, 2010), whether an in vivo environment would also support induced pluripotent stem cell (iPSC) formation from adult somatic cells has remained essentially unexplored. This open question within the field is now addressed in two new studies using doxycycline-inducible reprogramming factors (Oct3/4, Klf4, Sox2, and c-Myc; OKSM) in transgenic mice (Abad et al., 2013; Ohnishi et al., 2014). They demonstrate that doxycycline-inducible expression of OKSM in adults can ultimately lead to formation of teratomas in multiple organs. Because the cell-of-origin of this tumor type is typically a pluripotent cell, these studies indicate that the in vivo milieu is perfectly amenable to nuclear reprogramming. Indeed, ex vivo culture of cells derived from these teratomas or circulating cells of induced mice yields iPSCs in the absence of further OKSM induction. Abad and colleagues additionally demonstrate that cells reprogrammed in this setting acquired a more primitive, totipotent state than in-vitro-derived iPSCs. However, it remains to be determined how iPSC potency can be so dramatically affected in these distinct experimental settings.

While these discoveries are highly significant for the iPSC field, of potentially greater interest are the unexpected insights gained into the association between nuclear reprogramming and cellular transformation (Figure 1). A prominent acute effect of OKSM induction in both studies was the formation of dysplastic lesions in multiple tissues, potentially representing early reprogramming steps en route to pluripotency. As might be expected given the initial dependence of in vitro reprogramming on continual expression of exogenous OKSM factors, Ohnishi et al. found that tissue dysplasia was most often reversed upon doxycycline withdrawal at early time points after OKSM induction. A more prolonged induction period resulted in formation of tumors in multiple tissues consisting of undifferentiated dysplastic cells, which were distinct from teratomas. Strikingly, these tumors were resistant to doxycycline withdrawal and were maintained independently of transgenic OKSM expression. The kidney seemed particularly disposed to developing these tumors and was a focus of study by the authors. Critically, several lines of evidence indicated that these tumors were derived from kidney tubule cells that had partially reprogrammed toward a pluripotent state (partially reprogrammed transformed cells; PRTCs). Transcriptional profiling confirmed that PRTCs had lost tubule-cell identity and adopted elements of an embryonic stem cell (ESC) gene-expression program. Indeed, iPSCs could be rapidly generated in vitro from tumor cells upon further OKSM induction. Further, modification of the reprogramming cassette to omit specific OKSM factors demonstrated that the presence of *Oct3/4*, considered the most critical reprogramming component (Takahashi and Yamanaka, 2006), was essential for tumor resistance to doxycycline removal. When comparing gene expression between PRTCs and ESCs, key differences were