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Pushing the Reset Button: Chemical-Induced Conversion of Amniotic Fluid Stem Cells Into a Pluripotent State

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doi:10.1038/mt.2012.192

In an article by Moschidou *et al.* appearing in this issue of *Molecular Therapy*, a major step toward factor-free derivation of a pluripotent cell type was made using a c-Kit$^+$ subpopulation of human first-trimester amniotic fluid cells, which could be converted into bona fide induced pluripotent stem (iPS) cells without the ectopic expression of the pluripotency factors.[1] After an extended cell culture period and exposure to the histone deacetylase (HDAC) inhibitor valproic acid (VPA), these so-called amniotic fluid stem cells (AFSCs) could be reset into a pluripotent state.

The potential of human embryonic stem cells (hESCs) to differentiate into virtually any cell type of the human body raises the hope that they can be used to treat a variety of human diseases.[2] Yamanaka and colleagues found that terminally differentiated cells from non-embryonic sources could be converted back into a pluripotent state, a discovery that has revolutionized stem cell research and regenerative medicine.[2] These so-called iPS cells have developmental potential similar to that of hESCs and may therefore be an optimal source for regenerative therapy, while sidestepping the traditional ethical concerns involving embryos.[3] iPS cells were initially derived using integrating viruses delivering the reprogramming factor combination Oct4, Sox2, KLf4, c-Myc or Oct4, Sox2, Nanog, Lin28 into the genome of the host cell.[4,5] Further refinement of reprogramming techniques using nonintegrating viruses,[6] messenger RNAs,[7] and minicircle plasmids[8] has reduced the risk of mutagenicity caused by integrated reprogramming factors. Future clinical applications of iPS cells will depend on the ability to improve the integrity of the genome of these cells in the absence of exogenous genetic manipulations. Therefore, the gold standard for induction of pluripotency would be a transgene-free technique using a fully chemically defined reprogramming approach of easily accessible cell types.

Terminally differentiated fibroblasts have traditionally been used as a starting cell population for reprogramming experiments. More recently, other cell types that already express some of the pluripotency factors were successfully reprogrammed using fewer transgenes or in combination with different chemical compounds.[9,10] In general, multipotent cell types that retain some differentiation plasticity such as adipose stromal stem cells[11] or neuronal stem cells[12] are more efficiently reprogrammed. Multipotent cells can also be found in the amniotic fluid that surrounds the developing fetus. It is well established that this heterogeneous cell population contains around 1% multipotent AFSCs.[13] Interestingly, these naive c-Kit$^+$ AFSCs share 82% transcriptome identity with hESCs—as well as the expression of the pluripotency markers Oct4, Sox2, Klf4, SSEA3, TRA-1-60, and TRA-1-81 (ref. 1). Although expression of the pluripotency markers was significantly lower than in hESCs, Moschidou *et al.* showed that the AFSCs could form embryoid bodies and differentiate into cells representing the three embryonic germ layers. However, upon injection into immunodeficient NOD/SCID mice, these cells did not form teratomas, one of the more important criteria for pluripotency. After modifying the epigenetic status using the HDAC inhibitor VPA, the investigators were able to establish functional pluripotency in the AFSCs.

Acetylation of histones leads to an open chromatin structure, which is generally associated with active transcription.[14] Therefore, it is reasonable to assume that the more accessible open chromatin structure is responsible for the increased expression of the pluripotency factors Oct4, Nanog, and Sox2. The induction of cell plasticity through manipulation of the cell signaling machinery has been examined previously. For instance, it has already been shown that the more mature, primed (epigenetically marked for differentiation) epiblast stem cells could be converted into a more naive state by stimulating different signaling pathways with small molecules.[15] Furthermore, treatment of human fetal fibroblasts with inhibitors of DNA methyltransferase and HDAC increases the expression of pluripotency-related genes.[16] It would be interesting to confirm whether other HDAC inhibitors, such as sodium butyrate, trichostatin A, or suberoylanilide could induce transformation effects similar to those described by Moschidou *et al.*[1] Interestingly, another small molecule, 5-aza-2′-deoxycytidine, which affects the overall DNA methylation status, has been shown to induce human AFSC differentiation along the cardiac lineage.[17]

It is also possible that the multipotent c-Kit$^+$ AFSC subpopulation underlies the enhanced reprogramming efficiency of amniotic fluid–derived cells.[18] However, it is not clear if the pluripotent AFSCs resulted from the reprogramming of an independent precursor cell, or from a chemically induced resetting of primordial stem cells. Moschidou *et al.*[1] tried to tackle this question by comparing the transcriptome of naive AFSCs, VPA-stimulated AFSCs, and the seminoma cell line TCam-2. However, seminomas are similar to embryonal carcinomas and germ cell tumors and therefore not

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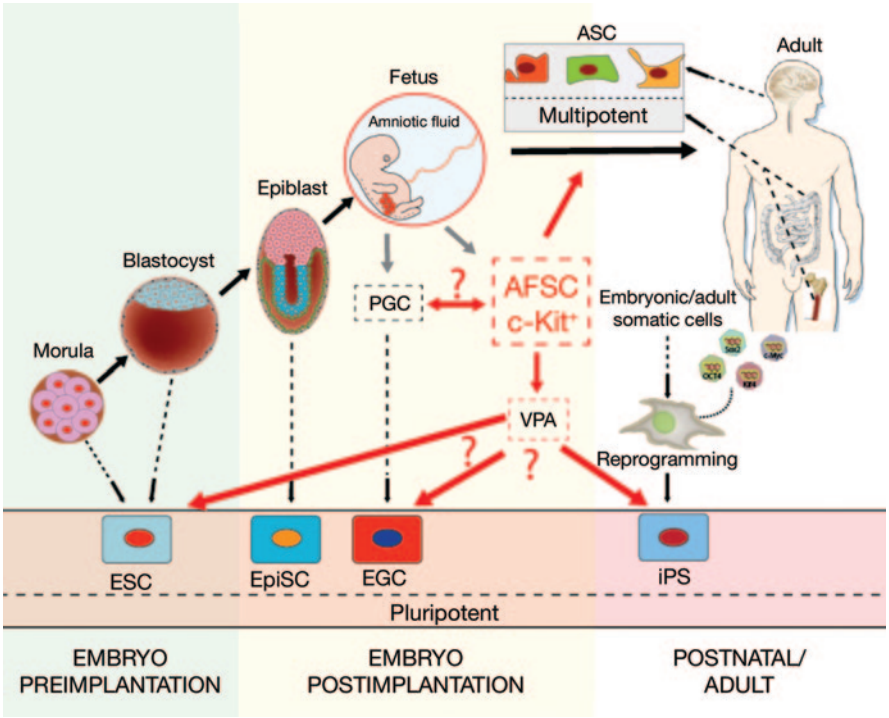

Figure 1 Developmental fates of embryonic and adult stem cells. During development it is possible to isolate pluripotent cells at different stages. Moreover, several reprogramming techniques now allow the induction of pluripotency to terminal differentiated cell types (iPS, induced pluripotent stem cell). Amniotic fluid stem cells (AFSCs) can easily be derived within the first trimester of pregnancy. AFSCs most likely originate from primordial germ cells (PGCs), and treatment with the histone deacetylase inhibitor valproic acid (VPA) converted these multipotent cells into pluripotent cell type. Therefore, the transcriptome of AFSCs is presumably comparable to the transcriptome of embryonic germ cells (EGCs). ASC, adult stem cell; ESC, embryonic stem cell; EpiSC, epiblast stem cell.

the most reliable control cell.[19] To shed light into the exact origin of AFSCs and the VPA-stimulated pluripotent AFSCs, it would be of interest to compare the transcriptome of these cell lines with iPS cells, hESCs, and additionally with the primordial germ cell (PGC)–derived pluripotent embryonic germ cells (EGCs) as described by Shamblott *et al.*[20] **(Figure 1)**Although VPA induced the expression of 273 hESC-specific genes, including various pluripotency genes, the overall transcriptome of VPA-stimulated AFSCs differs afterward more significantly from hESCs than before the drug treatment (82% vs. 78% genes in common). In addition, AFSCs share the expression of different genes mainly found in PGCs, and VPA stimulation of AFSCs also induces the transcription of genes involved in spermatogenesis. Therefore, it is likely that the multipotent c-Kit$^+$ AFSC subpopulation is derived from the PGCs persisting in amniotic fluid after their migration to the genital ridge. It is possible that lack of the tissue-specific microenvironment (niche) and signaling causes loss of PGC pluripotency and leads to establishment of a multipotent AFSC subpopulation. Finally, besides the great potential of amniotic fluid–derived iPS cells, caution must be exercised to examine whether the extended cell culture time of AFSCs (90 days) might lead to mutations and a higher risk of karyotype abnormalities similar to what has been observed for hESCs.[21] Besides the risk of mutation, the extended time required for establishing a pluripotent cell type may be a concern.

In summary, the identification of the c-Kit$^+$ AFSCs and the ability to convert them into a pluripotent cell type is an important advancement for regenerative medicine applications. In particular, the transgene- and virus-free induction of pluripotency could make AFSC-derived iPS cell lines useful for establishing a human leukocyte antigen–matching stem cell bank[22] and therefore for future clinical therapies.

ACKNOWLEDGMENTS

We gratefully acknowledge grants NIH DP2OD004437, NIH R01 HL113006, and CIRM RB3-05129 (J.C.W.) and support from the German Research Foundation (S.D.).

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Conditional Negative Selection of Gene-Modified Hematopoietic Stem Cells

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doi:10.1038/mt.2012.195

In this issue of *Molecular Therapy*, Barese and colleagues from the National Institutes of Health present a landmark study conducted in a clinically relevant large-animal model to evaluate the utility of the herpes simplex virus thymidine kinase (HSVtk) gene for conditional elimination of transplanted long-term repopulating hematopoietic cells, potentially representing true hematopoietic stem cells (HSCs).[1] This paper provides proof of concept for this approach, is rich in highly relevant experimental findings, and presents a stimulating discussion that will certainly motivate the field to pay even more attention to the important principle of conditional negative selection of gene-modified cells.

Indeed, despite the remarkable success achieved with the negative selection of gene-modified T lymphocytes in clinical trials,[2–4] the application of this principle to gene-modified stem cells has been far less explored. Although some studies have addressed suicide gene transfer as a safety mechanism to eliminate potential tumors occurring following the transplantation of pluripotent stem cell progeny (see references in Barese *et al.*[1]), studies addressing the utility of this principle for the more realistic and increasingly successful genetic modification of HSCs have been lacking. This is remarkable given that suicide gene transfer has been discussed over many years as a potential solution for the control of serious adverse events originating from the transformation of gene-modified hematopoietic cells since the first discovery of leukemias and premalignant clonal expansion induced by insertional mutagenesis.[5–7]

One explanation for the lack of experimental studies testing the utility of the suicide principle for HSC modification may be related to negative expectations. The HSVtk-mediated mechanism of suicide was known to be largely restricted to proliferating cells; human HSCs, however, typically spend weeks in quiescence before undergoing cell division, at least in homeostatic conditions. In addition, ganciclovir (GCV), the prodrug used for suicide induction of HSVtk-expressing cells, is myelotoxic when administered for prolonged periods of time, thus potentially also eliminating untransduced hematopoietic cells. Moreover, studies with HSVtk-modified tumor cells had suggested that epigenetic and genetic escape mechanisms, such as vector silencing, rearrangement of the transgene or the flanking chromosomal sequences, or point mutations, may constitute major limitations for the complete elimination of the target population.[8,9]

The new article from the Dunbar lab addressed all these limitations, with truly encouraging results.[1] First, this work revealed that the restriction of GCV-mediated cell killing to proliferating cells does not limit the potential to eliminate long-term repopulating hematopoietic cells, not even in the clinically relevant rhesus model that is far closer than any small-animal model to recapitulating the complex dynamics of human hematopoiesis, with coexistence of cycling and quiescent cells. The remarkable complete elimination of vector-transduced cell populations was assessed by very sensitive assays and persisted for at least 18 months after GCV application. These results imply the elimination of the pool of quiescent, long-lived lymphocytes, which must have originated from the transplanted HSCs and progenitor cells in the long time before administration of GCV. Whether GCV/HSVtk-mediated killing of quiescent hematopoietic cells is caused by mitochondrial damage, as speculated by Barese *et al.*, remains to be addressed.

Second, this study identified at least two drug regimens that allow killing of long-term repopulating, HSVtk-transduced hematopoietic cells while avoiding major unspecific myelotoxicity. These drug regimens are another important component of this study, with direct translational implications. The good news is that a single course of GCV over a period of five days was sufficient for cell elimination. Another, more continued course lasting for three weeks was also well tolerated and highly efficient. Thus, even if elimination would be more difficult, as in the case of a larger population of transduced cells and/or malignant transformation, the therapeutic index of GCV might be sufficient to be clinically useful in patients receiving gene-modified HSCs.

Third, the authors of this study found no evidence for escape mechanisms by genetic instability, but did report evidence suggesting that the specific activity of the HSVtk has an impact on the completeness of cell killing in their model. Although not directly addressed, this implies that a given threshold of HSVtk expression and activity has to be overcome to exploit this suicide principle in HSCs and their progeny cells. Engineering the HSVtk expression cassette and coding sequences provides room for further improvements (e.g., Preuss *et al.*[10]). Furthermore, Barese and colleagues make the interesting and somewhat challenging suggestion that promoters be designed that work only in transformed cells, which would be of special interest when a malignant gene-modified clone is to be eliminated while sparing the untransformed yet gene-modified cell population.

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