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Review Article

Experimental Limitations Using Reprogrammed Cells for Hematopoietic Differentiation

Katharina Seiler, Motokazu Tsuneto, and Fritz Melchers

Senior Research Group Lymphocyte Development, Max Planck Institute for Infection Biology, 10117 Berlin, Germany

Correspondence should be addressed to Fritz Melchers, melchers@mpiib-berlin.mpg.de

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We review here our experiences with the *in vitro* reprogramming of somatic cells to induced pluripotent stem cells (iPSC) and subsequent *in vitro* development of hematopoietic cells from these iPSC and from embryonic stem cells (ESC). While, in principle, the *in vitro* reprogramming and subsequent differentiation can generate hematopoietic cell from any somatic cells, it is evident that many of the steps in this process need to be significantly improved before it can be applied to human cells and used in clinical settings of hematopoietic stem cell (HSC) transplantations.

1. Introduction

The *in vitro* generation of hematopoietic stem cells (HSC) and mature hematopoietic cells from embryonic stem cells (ESC) promises to provide an alternative source of cells that could replace total bone marrow cells or HSC-enriched fractions of them. This is especially necessary in the case of human cells in clinical settings for HSC transplantations. In addition, studying hematopoiesis *in vitro* bypasses the need of donor cells, in particular to study hematopoietic disorders in human. ESC lines can be cultured long term and allow, in contrast to HSC, homologous recombination of DNA, that is, the insertion of exogenously modified genes into the appropriate sites in the genome. Thus, genetically altered, ESC-derived HSC might allow the proper genetic repair of defective cells of the hematopoietic system, including those of the innate and the adaptive immune system. However, for transplantations of human cells histoincompatibilities between the ESC-derived HSC and the transplanted host might be the cause of transplant rejections.

Since it has now become possible to generate ESC-like induced pluripotent stem cells (iPSC) from differentiated peripheral cells [1, 2], HSC as well as mature hematopoietic cells might in the future be generated from differentiated cells of a patient via iPSC. Somatic cells that are either mature, fully differentiated cells or are restricted in their ability to develop into a limited collection of cell types can be induced to become pluripotent, so that they exhibit higher differentiation capacity. This process is called reprogramming. It is not yet clear whether reprogramming will always equal dedifferentiation. The original, and most widely employed method to induce iPSC from somatic cells uses ectopic expression of the transcription factors Oct-4, Sox-2, and Klf-4, either with or without c-myc [1, 3–8]. However, concerns limiting clinical applications of patient-derived, that is, directly converted iPSC, include potential epigenetic differences between iPSC and ESC [9–18], and possible modifications of the genome by insertions and continued expression of the transcription factors that could affect the capacities of reprogrammed iPSC to properly differentiate. In our case of interest, we discuss some limitations to develop them into HSC and their differentiated hematopoietic cell lineages.

Several studies have improved the procedure of the generation of iPSC from a variety of different types of differentiated cells to find the most efficient method. In general, attempts to optimize both cell-intrinsic and exogenous factors to achieve optimal growth, survival, and differentiation requirements, first for the transfection phase and, thereafter, for the conversion from the differentiated cells to the iPSC have been made [1, 3–8]. Many studies exist showing that iPSC share the characteristic of ESC, that is, they can give rise to all cell types of a proper body, proven by the development

of chimeric animals and teratoma formation [1]. However, these qualitative analyses do not provide information about the quantitative efficiency of development. Thus, to investigate whether iPSC can replace ESC to study development and for clinical applications, efficiencies of development are needed.

Here, we summarize our experience with Oct-4/Sox-2/Klf-4-transduced mouse embryonic fibroblasts (MEF), mouse bone marrow-derived (MBM) hematopoietic progenitors, and mouse fetal liver-derived preB lymphocytes in the *in vitro* generation of iPSC that show varying levels of continued expression of the transduced transcription factors in iPSC and in differentiating hematopoietic cells. These levels of transgenic expression relate to the potency of the iPSC to differentiate subsequently *in vitro* to hematopoietic cells.

Hematopoietic development from ESC and iPSC is one of the best-studied differentiation programs. Culture systems have been developed that allow the differentiation of hematopoietic lineages *in vitro* from ESC and iPSC [19–27] which we have attempted to optimize for myeloid, T, NK, and B cells [28]. However, the efficient development and maintenance of *in vivo* reconstituting HSC from ESC and iPSC remains challenging. For a clinically relevant procedure of generating transplantable HSC, first, the best type of differentiated cell for conversion to iPSC with the best cell-intrinsic and extrinsic factors have to be found. Thereafter, improved methods need to be developed to generate and stabilize the pluripotent, long-term reconstituting potentials of transplantable HSC.

2. Reprogrammed Somatic Cells as New Sources for the Generation of Hematopoietic Cells

2.1. Step 1: From Differentiated Cells to iPSC. Somatic cells were first reprogrammed by somatic cell nuclear transfer [29–31]. Later, lineage-associated transcription factors were identified within a pool of 24 pluripotency-associated factors that had the potential to reprogram adult cells into pluripotent cells upon retroviral transduction [1]. Thus, transduction of mouse fibroblasts with Oct-4, Sox-2, Klf-4, and c-myc-generated iPSC by selection for *Fbxo15* activation that expressed pluripotency markers, generated teratomas upon subcutaneous injection, and contributed to different tissues upon blastocyst injection [1]. Transcription factor-based reprogramming has been optimized, so that c-myc was omitted and cells were selected with reactivation of *Nanog* and Oct-4 as well as by checking the ESC-like morphology [4, 6, 8, 32]. Facts, hypotheses, and unresolved issues of cellular reprogramming [33] and the maintenance and change of epigenetic memory in iPSC [34] have recently been discussed extensively. As summarized by Hanna et al. [33], gene expressions and biological characteristics of iPSC may be influenced by genetic backgrounds (different strains of mice, healthy donor-derived versus patient-derived iPSC), incomplete or heterogeneous iPSC formation, additional or alternate reprogramming factors, and transgene-expressing iPSC.

In our experiments, we have used the method of retroviral transduction with three vectors that constitutively express Sox-2, Oct-4, and Klf-4, respectively, and in which the transcription factor genes are not excisable, for example, by cre/lox-mediated deletion. We have generated iPSC lines from MEFs, and MBM. All of our iPSC lines express ESC-characteristic markers and form teratomas *in vivo* [28].

Continued transgene expression in our iPSC lines at different levels, even throughout differentiation to hematopoiesis *in vitro,* appeared possible. When this was measured, a remarkable difference became apparent. All MEF-iPSC lines showed expression patterns of the three transgenic transcription factors that were hardly above those of the corresponding endogenous genes, while all MBM-iPSC lines showed a markedly higher expression of Oct-4,Klf-4 and Sox-2. It appears that a higher threshold expression of the three factors is needed to reprogram MBM-iPSC than MEF-iPSC.

Distinct differentiated cells need different culture conditions, for example, different stromal cells or other cytokines (Figure 1). While MBM-derived cells do not grow well in the iPSC condition without IL-6 and SCF, MEF do. This may contribute to our observations that the efficiencies of establishing MEF-derived iPSC are higher than that of MBM-derived iPSC in our experiments. This indicates that the establishment of iPSC is more difficult if the original somatic cells from which the iPSC are intended to be induced do not fit iPSC media conditions on MEF and LIF.

The tissue culture conditions for the transduction and subsequent *in vitro* conversion to iPSC appear markedly different. Thus, when we consider the changes that MEF proliferating in medium alone, compared with MBM proliferating in medium substituted with SCF and IL-6 have to undergo after viral transduction to become iPSC MEF should find it easier to continue proliferation and survival in LIF-substituted media. Maybe the higher expression of the three transduced transcription factors is favourable for the more difficult conversion of MBM to iPSC. Thus, we suggest that the ability of cells to grow in "iPSC selection media" might influence their efficiency to reprogram.

2.2. Step 2: From ESC and iPSC to HSCs and Mature Hematopoietic Lineage Cells. For the differentiation of ESC towards several types of mature hematopoietic cells, two protocols have been developed—the formation of embryoid bodies (EB) that form in suspension culture and the cocultivation of ESC with stromal cells. In the first protocol, ESC are allowed to grow in suspension in the absence of feeder cells and LIF, differentiate spontaneously, and form spheroidal aggregates mimicking embryonic tissues, so called embryoid bodies [35–38]. Cells within developing EB can differentiate to mature cells, including hematopoietic lineage cells [39, 40]. Hematopoietic progenitor cells, which have the tendency to exist as mobile, nonaggregated single cells, must be freed by dissociating procedures from these EB aggregates.

In the second protocol, cocultivation of ESC with preadipocytic stromal cells allows a two-dimensional differentiation into hematopoietic cells without the formation of those complex aggregated structures and, thus, an easier, gentle isolation of progenitors of hematopoietic development [21, 27]. Furthermore, the use of the M-CSF-deficient stromal cell line OP9 avoids premature differentiation to myeloid

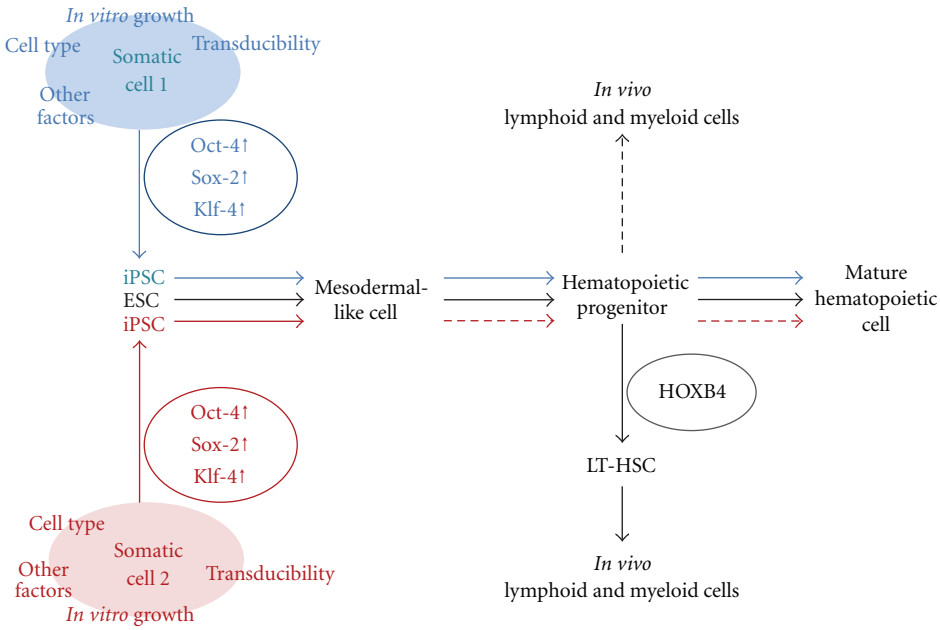

Figure 1: Overview of the current understanding of the efficiency to induce iPSC from different types of somatic cells, and subsequent development of iPSC into hematopoietic cells. Dashed lines implicate lower numbers of cells developing from the former cell type compared to full lines.

lineage cells and allows the development of T, NK, and B lymphoid cells [21]. In our *in vitro* differentiation experiments comparing ESC and iPSC [28]—the latter generated by retroviral transduction with Sox-2, Oct-4, and Klf-4—we did observe a reduced ability of iPSC-derived mesodermal-like cells to differentiate into hematopoietic progenitors in vitro. When Oct-4, Sox2, and Klf-4 were still highly expressed in the differentiating cells. The overexpression of Sox-2 appeared to be inversely related to hematogenic potency (data are summarized in Table 1).

In conclusion, our experiments suggest—as those of others [41]—that expression of virally transduced genes must be terminated before the induction of differentiation. The three different transcription factors appear to impede hematopoietic development to different extents. While Oct-4 and Klf-4 appear to be tolerated at continuously elevated levels to generate at least progenitors and precursors of T, NK, B, and myeloid cell development, levels of Sox-2 need to be downregulable for hematopoietic development. From these results, it appears that overexpression of the transgenic transcription factors inhibits development of Flk-1$^+$ mesodermal to CD45$^+$ hematopoietic progenitors. Constitutive expression has been shown by others not to affect the development of iPS cells into cells of the hematopoietic system [42, 43]. We would expect from our results that the transgenic expression of the three transcription factors in their iPSC lines should be as low as that of our MEF-iPSC lines.

If normal mouse or human somatic cells are used for the generation of iPSC the viral vectors should be deletable [44] without mutagenic consequences or should be introduced as proteins [45] or as synthetic modified mRNA [46].

3. Generation of HSC from ESC and iPSC Still Needs to Be Improved

Even if the procedures for the generation of iPSC will eventually be faithful and efficient enough to yield cells with the same differentiation potencies as those of ESC the subsequent efficient generation of transplantable, reconstituting HSC derived from ESC and iPSC cells still has been difficult until today. Murine iPSC can be used to generate new mouse strains in which bone marrow should, in most cases, become the source of normal numbers of long-term reconstituting HSC. In contrast, human iPSC, obviously, can not be used for such an *in vivo* development of HSC. Hence, the development of human HSC from ESC and iPSC must be attempted by differentiation in tissue cultures. The most successful method to obtain HSC *in vitro* from ESC is to transduce the cells with HOXB4 [23, 25, 47–54].

However, such retroviral modifications generate cells in which the "per cell" hematopoietic potency is still inferior to the same number of unseparated total bone marrow cells. Furthermore, retrovirally transduced cells carry the risk of mutations which might lead to malignant transformations, for example, leukaemia in the case of HOXB4 [55]. A few studies have reported transplantations of non-HOXB4-transduced cells resulting in long-term engraftment of both

Table 1: Differentiation of MBM- and MEF-derived iPSC lines in comparison to ESC lines. Numbers of cells indicate those developed from 4×10^3 undifferentiated cells (day 0). Expression levels represent amounts of mRNA determined by quantitative RT-PCR, normalized to GAPDH expression, and calculated as expression values of the respective genes in undifferentiated Bruce4 ES cells (day 0).

Cell line	Number of $Flk1^+$ cells on day 5	Number of $CD45^+$ cells on day 10	Sox-2 expression on day 5 relative to Bruce 4 on day 0	Sox-2 expression on day 10 relative to Bruce 4 on day 0	Oct-4 expression on day 5 relative to Bruce 4 on day 0	Oct-4 expression on day 10 relative to Bruce 4 on day 0	Klf-4 expression on day 5 relative to Bruce 4 on day 0	Klf-4 expression on day 10 relative to Bruce 4 on day 0
	$\times 10^5$	$\times 10^5$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$
J1 ES	2.7	5.5	1.3	0.2	31	0.2	480	1.7
Bruce 4 ES	3.2	23	0.5	0.04	41	0.08	32	0.04
MBM-iPS A	3.1	10	1.0	6.3	2600	120	3600	83
MBM-iPS C	2.2	0.1	18	1050	11000	2500	3700	700
MEF-iPS 1	2.6	7.3	16	21	12	56	3.2	5.8
MEF-iPS 5	4.0	18	45	71	0.65	70	22	5.6

the lymphoid and myeloid compartments, but none of them could reconstitute hematopoiesis in secondary transfers, [56–59]. The question remains which kind of progenitor is developed under these conditions.

It has been shown that yolk sac progenitors display minimal HSC potential [60–62]. In contrast, para-aortic splanchnopleura-derived cells can give rise to bone marrow reconstituting HSC which are capable of definitive hematopoiesis [60, 61, 63]. It might be that ESC differentiation *in vitro* generates only HSC capable of primitive, but not of definitive hematopoietic potency. That would explain the inability of ESC-derived hematopoietic progenitors to generate HSC with the capacity to develop into lymphoid cells upon transplantation. This possibility ignores the fact that ESC and iPSC can be differentiated into primitive, that is, erythrocytes expressing fetal-type haemoglobin, and definitive cells, that is, lymphocytes, *in vitro*. HOXB4 overexpression in hematopoietic cells derived from ESC and from yolk sac enables the detection of transplantable HSC [47] (Figure 1). Hence, HOXB4 works in two ways. One is to increase the number of transplantable HSC. The second is to make HSC transplantable by modifying the homing receptors. Therefore, the injection of hematopoietic cells from human ESC directly into the bone marrow results in the detection of repopulatable HSC [49]. In conclusion, we need to understand the molecular program that induces this switch in greater detail to induce the formation of long-term reconstituting HSC with definitive hematopoietic potential, as HOXB4 does, but without retroviral insertion.

Finally, nonhematopoietic cells provide niches in bone marrow for the proper hematopoietic differentiation that are yet to be defined, and that are missing in the culture conditions of differentiating ESC. Furthermore, long-term repopulating HSC that reside in the bone marrow are in a deeply quiescent (G_0) state and lose engraftment potential during their $S/G_2/M$ transit [64–67]. The present tissue culture conditions favor proliferation of HSC candidate cells. The development of conditions allowing cells to enter into and survive in the G_0/G_1 phase would be another important step towards establishing HSC *in vitro*.

4. Conclusions

Both stages of the *in vitro* development, first, from somatic, differentiated cells into iPSC and second, from iPSC into HSC are still so inefficient, even with murine cells, that the clinical use of human HSC derived from a patient's somatic cells are far from reality. It will need many more improvements at the various stages of reprogramming and differentiations of cells (Figure 1). Different somatic cell types represent different differentiation states, which have different growth abilities *in vitro*, different susceptibilities to be transduced by retroviral vectors and other yet unidentified factors, that make differently capable to become reprogrammed with different efficiencies. To allow effective reprogramming to iPSC, reversibly inducible or nonintegrative methods for reprogramming need to be used, since constitutive overexpression of reprogramming factors has been shown to interfere with differentiation. ES cells, and, to a lesser degree also, iPSC can be developed into all types of hematopoietic lineages in vitro. However, the reproducible generation of transplantable, engraftable HSC in vitro from pluripotent cells without overexpression of HOXB4 is still challenging (Figure 1).

Conflict of Interests

The authors have no conflicting financial interests.

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