Reprogramming to iPS cells and their subsequent hematopoietic differentiation is more efficient from MEFs than from preB cells

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Efficiencies of the generation of induced pluripotent stem (iPS) cells from either mouse embryonic fibroblasts (MEF) or from mouse fetal liver (FL) derived preB cells and their hematogenic potencies were compared. In 10 days approximately 2% of the MEFs transduced with Sox-2, Oct-4 and Klf-4 developed to iPS cells, while only 0.01% of transduced FL-preB cells yielded iPS cells, and only after around 3 weeks. Subsequently, the generated iPS cells were induced to differentiate into hematopoietic cells in vitro. On day 5 of differentiation MEF-iPS yielded numbers and percentages of Flk-1+ mesodermal-like cells comparable to those developed from embryonic stem (ES) cells. Compared to ES cells further differentiation to hematopoietic and lymphopoietic cells was reduced, possibly because of persistent expression of the reprogramming factors. By contrast, FL-iPS cells developed lower numbers and percentages of Flk-1+ cells, and no significant further development to hematopoietic or lymphopoietic cells could be induced. These results indicate that the efficiencies of iPS generation and subsequent hematopoietic development depends on the type of differentiated cell from which iPS cells are generated.

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1. Introduction

One of the most promising future applications of embryonic stem (ES) cell research is the production of transplantable cells via directed in vitro differentiation. A major restriction for its applicability is incompatibility of the transplanted cells with the host’s immune system. Using induced pluripotent stem (iPS) cells for the production of patient-specific cells might prove to be an effective tool to provide immunotolerant cells for transplantation.

Cells from all three germ layers, including the liver, stomach, pancreas and neural progenitors have been used as sources for the generation of human and murine iPS cells [1–5]. The efficiency and duration of somatic cell reprogramming has been shown to be dependent on the target cell [6,7]. Cells that are at a later stage of differentiation such as terminally differentiated B lymphocytes showed different reprogramming kinetics compared to cells from an earlier developmental stage. Small molecules such as the TGFβ antagonist Alk-5, MEK inhibitors and the DNA methyltransferase inhibitor AKA were shown to accelerate reprogramming [8,9]. Despite standardized methods to confirm the ES cell-like state, such as alkaline phosphatase activity or the ability to form teratoma in immunocompromized mice [7,10] significant genomic and epigenomic differences have been found between iPS and ES cells [9,12–15]. Furthermore, it has been shown that iPS cells, which were derived from different tissues, possess variable potency during in vitro differentiation [13–15] and might retain an epigenetic memory of their tissue of origin [11].

In this study, mouse fetal liver (FL)-derived preB cells and mouse embryonic fibroblasts (MEFs) are used as sources of somatic cells for the generation of iPS cells with the intention to investigate whether cells from different origins are reprogrammed with different kinetics and different efficiencies. The iPS cells generated from FL-preB cells and from MEFs are then subjected to the same in vitro differentiation protocol [16] to compare their capacities to differentiate into different hematopoietic progenitors.

2. Methods

2.1. Preparation and maintenance of fetal liver-derived preB cells and embryonic fibroblasts

Embryonic fibroblasts were isolated on day 14.5 of gestation as previously described [17]. Feeder cells were produced by passaging MEFs three times, always after reaching confluency. For passaging, cells were washed twice with PBS and trypsinized with 0.05% Trypsin-EDTA for 5 min at 37 °C. To stop trypsinization, 1 volume culture medium [DMEM (GlutaMAX; Gibco-Invitrogen)/10% FCS...
2.4. MEFS of (Sigma–Aldrich) was added, and the cells were resuspended thereafter as single cell suspensions. The cells were then centrifuged for 5 min at 290 × g and 4 °C, resuspended in culture medium and reseeded at a 1:3 dilution. When cells reached confluence for the third time, MEFS were trypsinized as before and γ-irradiated to inhibit proliferation. Fetal liver preB cell lines, proliferating on stromal cells in the presence of IL-7, were generated from embryos at day 17.5 of gestation as described [18] and cultured in preB cell culture medium [IMDM (Gibco-Invitrogen)/2% FCS (Sigma–Aldrich) and 1% IL-7 ([J558/IL-7]) on λ-irradiated OP9 stromal cells [19]. FL-derived preB cells lines were passaged at subconfluency (max 70%) every 3–4 days onto fresh irradiated OP9 stromal cells and IL-7 and kept at 37 °C and 10% CO2.

2.5. Differentiation of pluripotent stem cells into hematopoietic progenitors

iPS cells and controls were differentiated as previously described [16], except that 1 × 105 cells/ml were used initially. Also, if passed on day 7 half the medium was changed on day 5 and 10. Cells were also trypsinized on day 10–12 to investigate all cells including strongly adherent cells.

2.6. Quantitative realtime RT-PCR

RNA was isolated using the SV Total RNA Isolation System (Promega). For quantitative analysis of mRNA expression, the QuantiTec SYBR Green PCR Kit (Qiagen) protocol was followed. In duplicates, 10 μg RNA (50 μg/ml) was added to each well of a 96-well plate that was kept on ice. For each reaction, 15 μl master mix (1.75 μl RNase-free H2O, 0.25 μl RT-mix, 0.5 μl primer pair (each 20 μM) and 12.5 μl SYBR-green mix) was added to each well. Reverse transcription with subsequent DNA amplification was performed using the following reaction conditions: Initial hold for 30 min at 50 °C and 15 min at 95 °C. 45 PCR cycles were run consisting of 20 s at 95 °C for melting, 40 s at 60 °C for annealing and 40 s at 72 °C for elongation. The change in fluorescence during DNA amplification was detected by 7900HT RT-PCR system (ABI Prism). Standard curves for each gene was made using purified RNA of undifferentiated Bruce 4 ESC to enable quantitative measurements of RNA samples. GAPDH expression was used for normalization of probes (primer sequences for GAPDH: 5′-TCTTCAATGGGTAGAACAGACA-3′ and 5′-CAGTGGCAATGAGATT′). Primers designed to bind the UTR sequence which is not present in the ectopic gene were used to determine endogenous gene expression (Sox-2: 5′-CAGACACCTGATGCCGCAGC-3′ and 5′-AGACTTTTGGGAGCTCTTC-3′; Oct-4: 5′-CCTCAACACTGAGG-3′ and 5′-GTTTCACTGAGGACCAAC-3′; Klf-4: 5′-AGGAAAGAGCCATTGGATGAC-3′ and 5′-CGACTCAGAGC-3′; Pdx-1: 5′-CCCTCTTCACTGAGGACCAAC-3′ and 5′-TGCAATTTGTGCGGACAC-3′).

3. Results

3.1. MEFs are reprogrammed with faster kinetics and higher efficiencies than FL-derived preB cells

MEFs and FL-preB cells were retrovirally transduced to constitutively express Sox-2, Oct-4 and Klf-4, with the aim to generate iPSCs. The change in cell morphology was recorded until colonies were picked, and established as iPSC cell lines (Fig. 1A and B). ES cell-like colonies appeared 20 days after preB cell transduction while MEFS required only 11 days before they showed a similar cell transformation to iPS-like cells. During this period of iPS establishment in vitro it became evident that the number of retrovirally transduced FL-preB cells steadily declined during the first week after transduction (Fig. 1C), while the number of transduced MEFs increased during that time. This indicates that the ES cell culture conditions, i.e. on irradiated MEFS and LIF (see Section 2 for details) support much better the survival and proliferation of retrovirally transduced MEFS, but not of transduced preB cells.

The different reprogramming efficiencies of the two different target cell sources were quantitatively compared by the numbers of iPS cells generated by retroviral transduction. From a total of
2 × 10⁶ transduced preB cells, 11 individual ES cell-like colonies sustained growth after picking between day 20 and 25 after transduction. Taking the transduction rate of preB cells into account, which was detected by Sox-2 expression 24 h after transduction (Fig. 1D), this accounted for a reprogramming efficiency of 0.01%. To obtain an accurate measure for retroviral MEF transduction, which formed at much higher frequencies in densely populated cell cultures during transformation, different transduced cell concentrations were tested for alkaline phosphatase (AP) activity 12 days post transduction and only positive colonies were counted (Fig. 1E and F). Considering transduced cells only, the reprogramming efficiency of MEFs was 2%.

After establishment of the FL-preB cell- and the MEF-derived iPS-like cell lines, the cells were analyzed for SSEA-1 expression and stained for alkaline phosphatase activity to ascertain their ES cell-like phenotype (Fig. 2A and B).

We conclude from these experiments that under identical conditions of transduction and subsequent cell culture, approximately 200-fold lower numbers of transduced FL-preB cells reached an iPS-like state, compared to MEFs. Furthermore, the transduced FL-preB cells required more time (20–25 days, compared with 12 days for MEF) to become fully reprogrammed.

3.2. Hematopoietic differentiation of FL-derived preB cells and MEFs

Previously BM-derived iPS cells were generated with the same retroviral vectors for Sox-2, Oct-4 and Klf-4 (17). They had been found to express increased levels of the transducing transcription not only at the undifferentiated iPS cell stage, but retain this elevated expression even after 5 and 10 days of in vitro induction first to Flk-1⁺, thereafter to CD45⁺ hematopoietic progenitor cells. While the differentiation to Flk-1⁺ progenitors had appeared unaffected, the subsequent differentiation between day 5 and 10 to CD45⁺ progenitors appeared severely reduced. The FL-preB cell- and MEF-derived iPS cell lines produced in this study were applied to the same in vitro differentiation protocol. As controls that would compare these differentiation capacities to the cell lines used in the previous study one BM-iPS cell line (BM-iPS A), one MEF-iPS cell line (MEF-iPS S) and the wild-type Bruce4 ES cells were also induced to the in vitro hematopoietic differentiation protocol.

Five days after the start of differentiation, all cell lines had proliferated on confluent OP9 stromal cells to a comparable degree, i.e. had expanded 50–100 fold (Fig. 3A). However, the development of Flk-1⁺ mesodermal cells differed. All MEF-iPS cell clones, but only one of all tested FL-iPS cell clones (FL-iPS 4) showed similar Flk-1 surface antigen expression compared to the Bruce 4 ES cell control (Fig. 3B). This indicates that the FL-iPS cell lines have a reduced capacity to differentiate to Flk-1⁺ mesodermal-like cells.

CD41 was expressed at normal levels on differentiated MEF-iPS cells, when compared with differentiated ES cells, i.e. 20–25% of the differentiated cells were CD41⁺ (Fig. 3C). By contrast, less than 3% became CD41⁺ in all the differentiated FL-iPS cell clones (Fig. 3C).

To investigate whether this reduced FL-iPS cell differentiation is delayed in time, differentiating iPS cells were cultured for two additional days before passaging at day 7. Even though Flk-1 and CD41 expression on FL-iPS cells increased during these two days while it decreased on differentiating MEF-iPS cells, further differentiation to CD45⁺ hematopoietic progenitors 5 days after the first passage and while cultured in the presence of SCF and FLT3L, was
**Fig. 2.** Pluripotency markers AP (A) detected by histochemical staining in one representative iPS clone and SSEA-1 and (B) detected by FACS on representative iPS clones (FL-iPS 1-10 and MEF-iPS 1-9).

**Fig. 3.** Proliferation (A) and differentiation (B) to Flk-1+ cells and to CD41+ cells (C) at day 5 (black) and 7 (gray), and to CD45+ cells at day 10 (black) and 12 (gray) of differentiation culture of FL- and MEF-iPS clones. Differentiation was initiated by seeding FL-iPS cells and MEF-iPS cells in parallel cultures with control ES cells (Bruce 4 ES, J1 ES) and control iPS cells (BM-iPS A) onto irradiated OP9 stromal cells in the absence of LIF. (B,C,D) Flk-1, CD41 and CD45 surface antigen expression detected by FACS. One point represents one cell line. Dead cells were excluded by DAPI staining.
not improved (Fig. 3C and D). In fact, the change of the differentiating ES- and iPS cells at day 5 to the SCF/FLT3L-containing medium appears to be the optimal time for this change, as a change at day 7 lead to a reduction in the number of CD45⁺ hematopoietic progenitors developing 5 days later (Fig. 3D).

Previously all BM-derived iPS cell clones had shown a reduced capacity to differentiate to CD45⁺ progenitors, and subsequently to T- and B-lineage cells [16]. This might have been the result of a continued overexpression of Sox-2, Oct-4 and Klf-4 even 10 days after the induction of differentiation. It was also found that the quantity of Sox-2 overexpression appeared to relate to the inhibition of hematopoietic potential. Therefore, we tested Sox-2, Oct-4 and Klf-4 overexpression in the MEF- and FL-iPS cell lines before and after induction of differentiation. The mRNA expression levels for these three transcription factors were tested by quantitative realtime RT-PCR (Fig. 4). Indeed, transduced Sox-2, Oct-4 and Klf-4 were overexpressed at least 1000-fold in all FL-iPS cell lines compared to ES cells before induction of differentiation. This appears to be even higher (around 1000-fold) than in BM-iPS A (Fig. 4A). After 7 days of differentiation, total mRNA levels were reduced in all MEF-iPS and FL-iPS cell clones, as well as in the BM-iPS clone A but remained at higher levels compared to differentiated ES cells. In contrast to our recent study, in which each BM-iPS cell clone had a different Sox-2, Oct-4 and Klf-4 expression level in the undifferentiated state, undifferentiated FL-iPS cell clones showed similar mRNA expression levels but differed in their ability to reduce total and endogenous transcription factor overexpression during differentiation.

3.3. Capacities of MEF-iPS and FL-iPS cell clones to differentiate to T- and B-lineage cells

iPS cultures differentiated for 5 days on confluent OP9 stromal cells, and thereafter to CD45⁺ hematopoietic progenitors were then cultured either on OP9 stromal cells, FLT3L and IL7 to induce B cell differentiation, or on DLL1-OP9-stromal cells, FLT-3L and IL-7 to induce T cell differentiation for 7–10 days (Fig. 5A). In B lineage-directed differentiation CD19⁺ B220⁺ were detectable in differentiating cell cultures of control ES cells as well as of MEF-iPS clones, but not of FL-iPS clones (Fig. 5B and C). The expression of CD19, and the lack of expression of IgM indicates, that these B-lineage cells differentiated to a preB-like state of development, expected to expand by proliferation on stroma and IL-7.

In the T-lineage-directed differentiation cultures DN2 (CD25⁺CD44⁻) cells, as well as a few DP (CD4⁺CD8⁺) cells became detectable (Fig. 5B and C), again in differentiating ES- and MEF-iPS cultures, but only few in FL-iPS cultures.

We conclude that MEF-iPS have, lower than ES-cells, detectable potencies to develop B- and T-lineage cells in vitro, while FL-iPS cells, that essentially already lack capacities to develop CD45⁺ hematopoietic progenitors, also lack, as expected, capacities to develop detectable numbers of B- or T-lineage cells.

4. Discussion

One major reason for the observed differences in the efficiencies to develop to iPS cells is likely to be found in the different tissue
culture conditions that allow the proliferation of MEFs, respectively FL-preB cells before and after transduction with the three transcription factors Sox-2, Oct-4 and Klf4. FL-preB cells proliferate on irradiated OP9 preadipocytic mesenchymal stromal cells [19], but are transferred to a distinctly different culture condition, namely to irradiated MEFs and LIF. In contrast, transduced MEFs are transferred after transduction to the much more familiar environment of irradiated MEFs and LIF. Hence, it is understandable that MEFs continue to proliferate after transfection, while in the more drastically changed in vitro conditions transfected FL-preB cells do not (Fig. 1C).

Therefore the efficiency of iPS generation from transfected FL-preB cells is lower, as increased proliferation has been correlated with more efficient reprogramming [6]. Transduced preB cells grown on OP9 stroma and LIF [7] are likely to encounter the same inability to proliferate, as we have not been able to replace IL-7 in our OP9-dependent preB cell cultures by other cytokines, notably by LIF, to keep these preB cells proliferating.

Although the iPS clones generated from MEFs and from FL-preB cells both have the morphological appearance, and the capacities to express the ES cell-related markers, alkaline phosphatase and SSEA-1, it is likely that at least the FL-iPS are not really ES cell-like. Future experiments probing the pluriptivity of the FL-iPS should investigate whether mice can be developed from these FL-iPS. It is also possible that internal gene regulatory mechanisms of preB cells need to undergo more dramatic changes than MEFs need, maybe with the help of other transcription factors, to become reprogrammed to fully potent iPS. In this context it is notable that Hanna et al. [7] have transduced preB cells with either C/EBPa, or have knocked down the expression of PAX5 to increase the capacities of mature B cells to become reprogrammed to iPS cells.

This might suggest that earlier stages of B lymphocytic development, such as pro/preB- or even Common Lymphoid Progenitors may be nearer to an iPS cell than the preB cells which we used in the present study.

In addition these iPS clones have severely reduced hematopoietic and lymphopoietic capacities, that may, at least in parts, be a consequence of the continued expression of the transducing transcription factors even in differentiating cells at day 10 of differentiation, i.e. at a time when hematopoietic progenitors have developed in vitro from ES cells. Previously, highly elevated expression levels of the Yamanaka factors in undifferentiated and differentiated BM-derived iPS cells have been suspected to be the reason for a reduced hematopoietic potential [16].

5. Conclusion

Our results do not offer any evidence that epigenetic programming toward B lineage in preB cells could increase the capacities of iPS cells to differentiate to hematopoietic progenitors, T-lineage or B-lineage cells in vitro.
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