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Epigenetic Rejuvenation of Mesenchymal Stromal Cells Derived from Induced Pluripotent Stem Cells

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SUMMARY

Standardization of mesenchymal stromal cells (MSCs) remains a major obstacle in regenerative medicine. Starting material and culture expansion affect cell preparations and render comparison between studies difficult. In contrast, induced pluripotent stem cells (iPSCs) assimilate toward a ground state and may therefore give rise to more standardized cell preparations. We reprogrammed MSCs into iPSCs, which were subsequently redifferentiated toward MSCs. These iPS-MSCs revealed similar morphology, immunophenotype, in vitro differentiation potential, and gene expression profiles as primary MSCs. However, iPS-MSCs were impaired in suppressing T cell proliferation. DNA methylation (DNAm) profiles of iPSCs maintained donor-specific characteristics, whereas tissue-specific, senescence-associated, and age-related DNAm patterns were erased during reprogramming. iPS-MSCs reacquired senescence-associated DNAm during culture expansion, but they remained rejuvenated with regard to age-related DNAm. Overall, iPS-MSCs are similar to MSCs, but they reveal incomplete reacquisition of immunomodulatory function and MSC-specific DNAm patterns—particularly of DNAm patterns associated with tissue type and aging.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are heterogeneous cell preparations and only a small subpopulation often referred to as "mesenchymal stem cells" possesses multilineage differentiation potential (Dominici et al., 2006). MSC preparations are greatly affected by starting material, such as bone marrow (BM) or adipose tissue (AT), and cell-culture media. Furthermore, they acquire functional changes during culture expansion ending in replicative senescence (Wagner and Ho, 2007). So far, MSCs are scarcely defined by fibroblastoid plastic adherent growth, a panel of nonspecific surface markers, and their capacity to differentiate toward adipogenic, osteogenic, and chondrogenic lineages (Dominici et al., 2006).

In this regard, induced pluripotent stem cells (iPSCs) converge to a better-defined ground state of pluripotency (Hackett et al., 2013). They can be differentiated into all cell types of the organism and—while in pluripotent state—cultured virtually indefinitely without signs of replicative senescence. Epigenetic profiles, such as DNA methylation (DNAm) patterns, are reorganized during reprogramming of somatic cells into iPSCs and closely resemble those of embryonic stem cells (ESCs) (Huang et al., 2014). In particular, senescence-associated DNAm, which is acquired during in vitro expansion (Koch et al., 2013), and age-related DNAm, which accumulate during aging of the organism (Horvath, 2013), are reversed to ground state. In comparison to primary cells, iPSCs are therefore better defined and offer a good starting point for large-scale generation of standardized derivatives, such as iPSC-derived MSCs (iPS-MSCs).

Several groups described strategies to derive MSC-like cells from either ESCs (Barberi et al., 2005; Boyd et al., 2009) or iPSCs (Liu et al., 2012; Diederichs and Tuan, 2014; Zhang et al., 2011). These approaches were based on coculture with primary MSCs, growth factor combinations, or spontaneous differentiation in embryoid bodies (EBs). So far, it has not been analyzed whether DNAm patterns of iPS-MSCs resemble those of primary MSCs.

RESULTS

Redifferentiation of iPSCs toward iPS-MSCs

We have recently reprogrammed MSCs from human bone marrow into iPSCs (Shao et al., 2013). These iPSCs were now redifferentiated toward iPS-MSCs using two alternative protocols: (1) the culture medium was simply exchanged to initial MSC-culture medium that comprised 10% human platelet lysate (hPL) or (2) iPSCs were allowed initially to differentiate into EBs in ultralow attachment plates for 7 days in differentiation medium (Figure S1A available online). Thereafter, cells were cultured under

standard culture conditions for MSCs with 10% hPL. After 35 days (four passages), the cells revealed a typical fibroblastoid growth pattern; these cells are referred to as iPS-MSCs in this manuscript (Figure 1A). iPS-MSCs passaged on gelatin-coated (Figure 1B) or noncoated (Figure S1B) tissue culture plastic exhibited significantly higher proliferation rates than primary MSCs of the corresponding passage. The immunophenotype of iPS-MSCs was essentially identical to primary MSCs ($CD29^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD14^-$, $CD31^-$, $CD34^-$, and $CD45^-$), albeit CD105 was less expressed in iPS-MSCs (Figures 1C and S1C). Furthermore, differentiation of iPS-MSCs toward osteogenic and chondrogenic lineage was equivalent to MSCs. Adipogenic differentiation was also induced in iPS-MSCs, although accumulation of lipid droplets was less pronounced than in primary MSCs (Figures 1D and S1D). These results on in vitro differentiation potential were further validated by upregulation of lineage-specific marker genes (Figure 1E). Taken together, iPS-MSCs fulfilled the minimal criteria for definition of MSCs (Dominici et al., 2006) - even though less prone to adipogenic differentiation. Because both redifferentiation protocols (with or without EB formation) did not reveal significant differences, we used the one-step differentiation protocol without EB formation and with gelatin coating for subsequent experiments.

iPS-MSCs Reveal Similar Gene Expression as MSCs

Global gene expression was compared in MSCs, iPSCs, and iPS-MSCs. Hierarchical cluster analysis revealed close relationship between iPS-MSCs and MSCs (Figure 2A), which was confirmed by pairwise correlation coefficients (Figure 2B). Gradual changes in gene expression were already observed during the first week of differentiation (Table S1): MSC marker genes including ecto-5′-nucleotidase (*NT5E;* CD73), CD44 antigen (*CD44*), alanyl aminopeptidase (*ANPEP*; CD13), and neural cell adhesion molecule 1 (*NCAM1*; CD56) were already upregulated. On the other hand, pluripotency genes were rapidly downregulated upon differentiation toward iPS-MSCs (Figures 2C, S2A, and S2B). Mesodermal genes typically expressed in MSCs were expressed at a similar level in iPS-MSCs (Figure 2D). Pairwise comparison of gene expression in MSCs, iPSCs, and iPS-MSCs revealed relatively few significantly differentially expressed genes between MSCs and iPS-MSCs (2-fold differential expression and adjusted p value <0.01; Figure 2E; Table S2): 339 genes were higher expressed in iPS-MSCs, and these were particularly enriched in gene ontology (GO) categories of transcriptional regulation, cell adhesion, and development; 214 genes were higher expressed in MSCs that were particularly enriched in GO categories for T cell activation and immune response (Figure 2F). Therefore, we used a surrogate assay to determine suppression of T cell proliferation in coculture with iPS-MSCs or MSCs. Indeed, MSCs significantly suppressed T cell proliferation in a dose-dependent manner, whereas this was not observed in iPS-MSCs, indicating lower immunomodulatory function (Figure 2G). To further classify gene expression profiles of iPS-MSCs, we used PhysioSpace analysis, a bioinformatics tool to interpret gene expression differences between two distinct cell types in terms of physiologically relevant expression patterns (Lenz et al., 2013) that provided further evidence that iPS-MSCs converged toward MSCs (Figure S2C). Overall, gene expression profiles supported the notion that iPS-MSCs closely resemble MSCs, even though there are differences in their immune function.

DNA Methylation Profiles of iPS-MSCs

Subsequently, we have analyzed DNAm profiles of MSCs, iPSCs, and iPS-MSCs (each of corresponding donors). Hierarchical clustering demonstrated that iPS-MSCs and MSCs cluster together (Figure 3A). At day 7 of differentiation toward iPS-MSCs the methylome was between pluripotent and nonpluripotent cells (Table S1). However, even after 5 weeks of differentiation 39,753 CpGs remained significantly differentially methylated between iPS-MSCs and MSCs (>20% differential DNAm; adjusted p value <0.01; Table S2), whereas only 13,896 CpGs reached this level of significance in iPS-MSCs versus iPSCs (Figure 3B). Overall, DNAm levels were higher in iPSCs and iPS-MSCs as compared to primary MSCs. Nevertheless, redifferentiation was associated with gradual loss of highly methylated and gain of unmethylated CpG sites (Figure S3A). DNAm was further analyzed in relevant genes – for example hypermethylation of POU class 5 homeobox 1 (*POU5F1*; OCT3/4) and Nanog homeobox gene (*NANOG*), and hypomethylation of surface marker genes *NT5E* (CD73*)* and endoglin (*ENG*; CD105) (Figure 3C). Notably, these DNAm patterns revealed high similarity between primary and redifferentiated MSCs in many genes, particularly in *NT5E*. Comparison of DNAm changes with expression changes of corresponding genes revealed some association, but there was no universal linear correlation (Figures S3B and S3C). Furthermore, DNAm differences of iPS-MSCs and MSCs were enriched in intergenic regions and shore regions of CpG islands (Figures 3D and S3D).

Comprehensive Analysis of DNAm Changes in iPS-MSCs

We have recently demonstrated that iPSCs maintain donor-specific characteristics in their DNAm pattern: 1,091 CpGs with the highest variation in different MSC preparations remained methylated at similar level in corresponding iPSCs (Shao et al., 2013). Here, we demonstrate that this donor-specific pattern was also maintained upon redifferentiation into iPS-MSCs (Figures 4A and S4A).