Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow[1]

LIN SONG AND ROCKY S. TUAN[2]

Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland, USA

SPECIFIC AIMS

Transdifferentiation is a process whereby a cell type committed to and progressing along a specific developmental lineage switches into another cell type of a different lineage through genetic reprogramming. Although this process has been well studied and established in amphibian systems, it is unclear whether mammalian cells possess the same potential. Recent in vivo transplantation studies showed that adult mesenchymal stem cells (MSCs) were able to differentiate into mesoderm-derived cell types as well as cells with neuroectodermal and endodermal characteristics, suggesting that transdifferentiation occurs in mammalian systems. However, there are concerns over these findings because of the possibility of progenitor cell contamination and cell fusion. In this study, we developed an in vitro differentiation strategy to assess whether human mesenchymal stem cells (hMSCs) precommitted to a given mesenchyme cell lineage can transdifferentiate in response to inductive extracellular cues.

PRINCIPAL FINDINGS

1. Fully differentiated osteoblasts from hMSCs were capable of transdifferentiation into adipocytes and chondrocytes

We tested whether hMSCs exposed to osteogenesis-inducing factors can maintain their potential of differentiation into adipocytes and chondrocytes. Osteogenic differentiation of MSCs is characterized by three distinct phases—cell proliferation, cessation of proliferation and secretion of extracellular matrix, and mineralization of extracellular matrix—resulting in mature osteoblasts. It has been widely accepted that as osteogenic differentiation progresses, the multiple differentiation potentials of mesenchymal cells gradually become more restricted, such that terminally committed osteoblasts are unable to differentiate into other cell types. However, our results showed that hMSCs cultured under osteogenic conditions for 10, 20, or 30 days maintained their ability to differentiate into adipocytes and chondrocytes. Even the fully differentiated osteoblasts (after 30 day induction), identified by expression of alkaline phosphatase, bone sialoprotein, and osteocalcin, histochemically detectable alkaline phosphatase activity and the elaboration of calcified extracellular matrix, were able to change their differentiation program and became lipid-producing adipocytes and chondrocytes that produced sulfated proteoglycan, collagen type II, and link protein.

2. Fully differentiated adipocytes and chondrocytes derived from hMSCs were capable of transdifferentiation into other mesenchymal lineages

We subjected hMSCs to an adipogenic or chondrogenic differentiation program and switched the culture environment once mature adipocytes or chondrocytes were formed. hMSCs that had differentiated into adipocytes after 20 days transdifferentiated into osteoblasts or chondrocytes upon exchange of the inducing extracellular factors. Similarly, chondrocytes derived from MSCs induced in serum-free, 3-dimensional alginate culture in the presence of TGF-β3 could be dissociated and subsequently induced to differentiate into osteoblastic cells and adipocytes. Further support for transdifferentiation came from the RT-PCR detection of the respective lineage-specific mRNA transcripts after the second round of differentiation, i.e., osteocalcin (OC) for osteogenesis, lipoprotein lipase (LPL) for adipogenesis, and collagen type II (COL II) for chondrogenesis. Irrespective of the lineage-specific program the hMSCs were subjected to, expression of a number of lineage-specific gene transcripts appeared to be upregulated. During induction of osteogenesis, for instance, expression of PPARγ2, an adipocyte marker, and collagen type X, a chondrocyte marker, were detected; during induction of adipogenesis, several genes characteristic of osteoblasts and chondrocytes were expressed. Although expression of these lineage-specific genes was detectable, histological and immunohistochemical analyses did not reveal multiple differentiation phenotypes.

---

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-1100fje; doi: 10.1096/fj.03-1100fje

[2] Correspondence: Cartilage and Orthopaedics Branch, National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, 50 South Dr., Room 1503, Bethesda, MD 20892-8022, USA. E-mail: tuanr@mail.nih.gov

0892-6638/04/0018-0980 © FASEB

3. pOC-EGFP transfected hMSCs differentiated into osteoblasts and then transdifferentiated into adipocytes and chondrocytes

Cell fusion has been suggested as a means for MSCs to undergo differentiation and acquire differentiation-specific characteristics as a function of their location. Although our in vitro differentiation approach avoided the possibility of MSC fusion with cells in a target tissue to acquire their characteristics, it could be argued that contaminating progenitor cells could be present in the starting cell population and that these cells could be unresponsive to one type of influence, such as osteogenic induction, but be responsive to other influence, such as adipogenic or chondrogenic inductions. Thus, once the culture environment was switched, these progenitor cells would appear to be "transdifferentiated" from another precommitted cell type. hMSCs isolated by plastic adhesion were a largely heterogeneous cell population, and recent studies have shown that even cells derived from a single clone can be heterogeneous in differentiation capacity and stages. To validate the phenomena observed here, analysis of the second differentiation step should ideally be carried out with a homogeneous population of committed cells. Expression of osteocalcin, an osteoblast-associated matrix gene, has been considered a valid marker for fully differentiated osteoblasts. For sorting osteogenically committed cells, we transfected hMSCs with a GFP expression vector driven by human osteocalcin promoter. During osteogenic differentiation of hMSCs, expression of endogenous osteocalcin was up-regulated >5-fold, reaching a maximum around day 15 (**Fig. 1*A***), and diminished beyond day 20. The temporal expression profile of the osteocalcin-GFP construct followed closely that of endogenous osteocalcin gene expression (Fig. 1*A*), reaching maximal expression by day 15. The dramatic decrease of OC-GFP expression after 15 days could be the result of plasmid instability in transfected cells and/or cell death caused by exogenous DNA since, in a positive control constitutively expressing GFP (transfected with pCMS-EGFP), the percentage of positive cells was decreased by 2-fold every 5 days until it lost its expression completely by day 25. Thus, for cell sorting, transfected cells were induced to undergo osteogenesis for 15 days, then green fluorescent cells were collected by fluorescence activated cell sorting (FACS) (Fig. 1*B*). The GFP-expressing cells accounted for ~5% of the whole cell population; upon culture, all exhibited alkaline phosphatase activity (Fig. 1*C*), a characteristic for osteoblasts. Once placed in adipogenic medium (Fig. 1*D*), these homogeneous, fully differentiated osteoblastic cells were capable of adipogenesis. By limiting dilution, we determined that 6 of 16 colonies derived from osteoblasts formed adipocytes vs. 8 of 12 colonies using mock transfected cells. When GFP-expressing osteoblasts were cultured in 3-dimensional alginate culture, they formed large, spherical cell clusters (Fig. 1*E*, a) surrounded by sulfated extracellular proteoglycan (Fig. 1*E*, b), resembling native chondrocytes formed in hyaline cartilage. More than 97% of these cell clusters stained histochemically positive for collagen type II (Fig. 1*E*, c) and proteoglycan link protein (Fig. 1E, d), indicating that fully differentiated, homogeneous osteoblasts were capable of giving rise to chondrocytes, i.e., undergoing chondrogenesis. These results confirmed the true transdifferentiation potentials of differentiated cells derived from hMSCs to a level comparable to that of the original hMSCs: terminally differentiated cells can reprogram their genome and switch their phenotype in response to inductive extracellular cues.

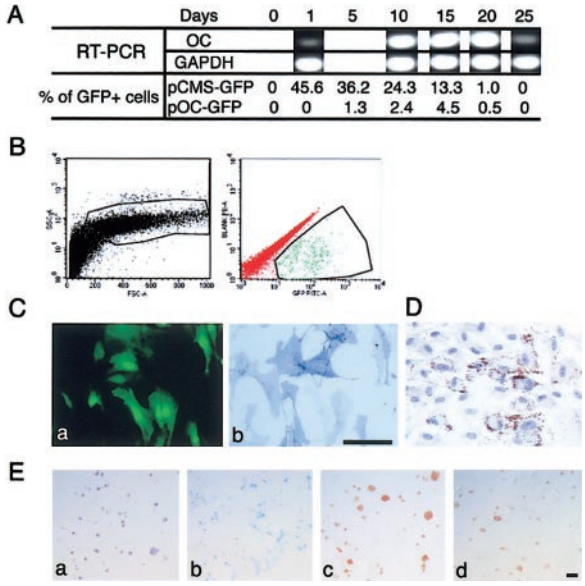

Figure 1. pOC-EGFP transfected hMSCs differentiated into osteoblasts, then transdifferentiated into adipocytes and chondrocytes. *A)* Time course of endogenous osteocalcin expression and GFP expression in pOC-EGFP transfected cells undergoing osteogenic differentiation. Endogenous osteocalcin expression was examined by RT-PCR. Cells transfected with pOC-EGFP construct or pCMS-EGFP control construct were cultured in osteogenic medium or control medium, respectively. pOC-EGFP expression paralleled that of endogenous osteocalcin. Aliquots of cells were collected at each time point and the % of GFP-positive cells was determined with FACS. Loss of constitutive GFP expression (pCMS-EGFP) was seen after day 25. *B)* Fully differentiated osteoblasts, expressing osteocalcin promoter-driven GFP, were sorted by FACS. Left: FACS profile of total cell population; right: only OC-expressing, GFP-positive cells were collected for further analyses. *C)* Fluorescence micrograph of sorted GFP-positive osteoblasts (a) showing homogeneous staining for alkaline phosphatase (b). *D)* Sorted GFP-positive osteoblasts derived from hMSCs transdifferentiated into lipid-producing adipocytes detected by Oil red O stain. *E)* Sorted GFP-positive osteoblasts transdifferentiated into chondrocytes in 3-dimensional alginate culture. H&E stain (a); Alcian blue stain (b); immunohistochemical staining of collagen type II (c) and link protein (d). Scale bar, 50 μm.

4. Fully differentiated osteoblasts from hMSCs were capable of dedifferentiation and retention of multidifferentiation potential

An interesting phenomenon during the transdifferentiation process was the extensive cell proliferation that preceded the phenotypic switch. When hMSCs were cultured in osteogenic medium for 30 days, adherent cells formed large osteoblastic nodules (**Fig. 2*A*, a**). When the osteogenesis-inducing medium was replaced with control medium, spindle-shaped fibroblast-like cells started to migrate out of the calcified matrix nodule (Fig. 2*A*, b) and were morphologically similar to the original hMSCs. Since cell division is required for demethylation, a critical step for genome reprogramming, perhaps without the pressure of inducing factors, fully differentiated MSC-derived cells could resume cell proliferation, modify their gene expression profile, and return to a more primitive stem cell-like stage.

Accompanying the phenotypic changes observed was a fluctuation in the expression of lineage-specific transcription factors: Cbfa 1 for osteogenesis, Sox 9 for chondrogenesis, and PPARγ2 for adipogenesis (Fig. 2*B*). As expected, expression of Cbfa 1 was up-regulated during osteogenesis, whereas both Sox 9 and PPARγ2 were down-regulated compared with undifferentiated hMSCs. On the other hand, expression levels of all three transcription factors decreased during osteoblast dedifferentiation, which suggested that cells might return to an uncommitted developmental stage from a fully determined cell type. The dedifferentiated cells

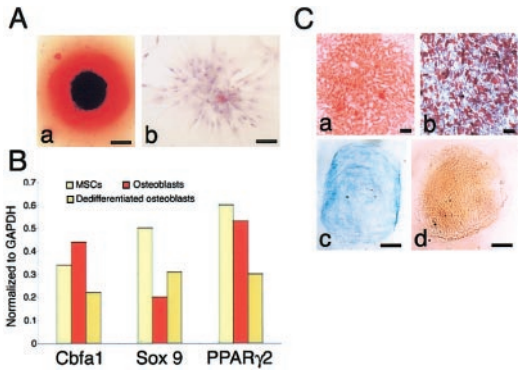

Figure 2. Osteoblasts derived from osteogenic differentiation of hMSCs were capable of dedifferentiation and retention of multidifferentiation potential. *A)* A fully differentiated osteoblastic nodule in osteogenic culture of hMSCs (30 days) stained with alizarin red (a). Fibroblastic cells derived from the osteoblastic nodule migrated out and proliferated (b, H&E). *B)* Expression levels of lineage-specific transcription factors (Cbfa1, Sox9, and PPARγ2) during osteogenesis and osteoblast dedifferentiation. *C)* Cells derived from the osteoblastic nodule differentiated into 3 mesenchymal lineages: osteoblasts (a, alizarin red stain), adipocytes (b, Oil red O stain), and chondrocytes (c, Alcian blue stain; d, immunohistochemical staining of collagen type II). Scale bar, 50 μm.

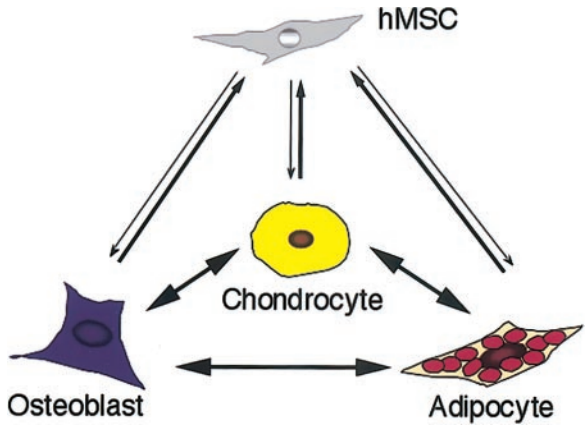

Figure 3. A transdifferentiation model of hMSCs. Osteoblasts, adipocytes, and chondrocytes differentiated from hMSCs were able to transdifferentiate into other mesenchymal cell types. Fully differentiated cells were also capable of dedifferentiation into a primitive stem-like cell type and retention of multiple differentiation potential.

derived from the osteoblasts not only exhibited similar morphology as MSCs, but also exhibited MSC-like multidifferentiation potentials. As shown in Fig. 2*C*, these fibroblast-like cells formed mature osteoblasts (a), adipocytes (b), and chondrocytes (c, d).

CONCLUSIONS AND SIGNIFICANCE

In this study we showed that hMSCs that have been precommitted to one mesenchyme cell lineage can transdifferentiate into other cell types in response to inductive extracellular cues (**Fig. 3**). These precommitted cells proliferate and are able to dedifferentiate into a primitive stem cell stage through genome reprogramming. These findings suggest the importance of cell dedifferentiation and transdifferentiation in the development and maintenance of mammalian tissues and regulation of lineage commitment. The simplicity of the in vitro differentiation system used here should allow us to identify molecular regulators, such as cell cycle proteins, transcription factors, or other signaling molecules, that control cross-lineage commitment among different cell types. Identification of these factors will not only shed light on fundamental mechanisms regulating development, but provide tools to manipulate adult stem cells for cell-based approaches in regenerative medicine. Our findings are also relevant to understanding the pathogenesis of skeletal diseases. For example, aged and osteoporotic patients have a higher fat-to-bone ratio than young and healthy counterparts, possibly due to the conversion of bone to fat cells. Understanding how pathways mediating the transdifferentiation between osteoblasts and adipocytes are regulated should be relevant to the development of therapeutic control of bone loss in osteoporosis. FJ