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REGULAR ARTICLE

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Porcine mesenchymal stem cells

Induction of distinct mesenchymal cell lineages

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Abstract The potential of mesenchymal stem and progenitor cells (MSC) to replicate undifferentiated and to mature into distinct mesenchymal tissues suggests these cells as an attractive source for tissue engineering. The objective was to establish a protocol for the isolation of porcine MSC from bone marrow and to demonstrate their ex vivo differentiation into various mesenchymal tissue cells. MSC from passage 2 were selected for differentiation analysis. Differentiation along the osteogenic lineage was documented by deposition of calcium, visualization of alkaline phosphatase activity, and by analysis of osteogenic marker genes. Adipocytes were identified morphologically and by gene-expression analysis. Deposition of type II collagen and histological staining of proteoglycan indicated chondrogenic differentiation. Therefore, porcine MSC may be introduced as a valuable model system with which to study the mesenchymal lineages for basic research and tissue engineering.

Keywords Porcine mesenchymal stem cells · Isolation · Osteogenesis · Chondrogenesis · Adipogenesis · Tissue engineering · Cell culture

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Introduction

Adult bone marrow contains mesenchymal stem and progenitor cells (MSC), which give rise to different mesenchymal tissues. MSC can be isolated and grown in vitro (Haynesworth et al. 1992). Important characteristics of human MSC are their ability to proliferate in culture with an attached fibroblastic morphology, the presence of specific marker proteins on their surface, and their in vitro multilineage potential (Pittenger et al. 1999).

Purified and expanded MSC from various species have been shown to differentiate along osteogenic (Jaiswal et al. 1997), chondrogenic (Johnstone et al. 1998; Mackay et al. 1998), adipogenic (Pittenger et al. 1999), tenogenic (Young et al. 1998), and marrow stromal lineages (Cheng et al. 2000).

In the present study, we established a protocol for the isolation of porcine MSC to compare their phenotype and their multilineage potential with human MSC. Here we report that the techniques used for purification, expansion, osteogenic, chondrogenic, and adipogenic differentiation of human MSC can be adopted for analysis of porcine MSC, which may serve the increasing demand for stem and progenitor cells in tissue engineering. As a result of the presented similarities between porcine and human MSC, porcine MSC are likely to be considered as a valuable model system for skeletal research.

Materials and methods

Isolation, culture, and differentiation of MSC

Femur and tibia bones from 6- to 8-month-old porcine donors (n=4, 100–130 kg) were sawn and gelatinous bone marrow was extracted under sterile conditions. Porcine MSC were isolated according to modifications of a method developed for human MSC (Haynesworth et al. 1992). Briefly, gelatinous bone marrow (3–4 g per sample) was resuspended in PBS and dispersed mechanically by passing through syringes fitted with a series of 16-, 18-, and 20-gauge needles. Cells were centrifuged, resuspended, and plated in complete DME medium (Biochrom), containing 10% fetal bo-

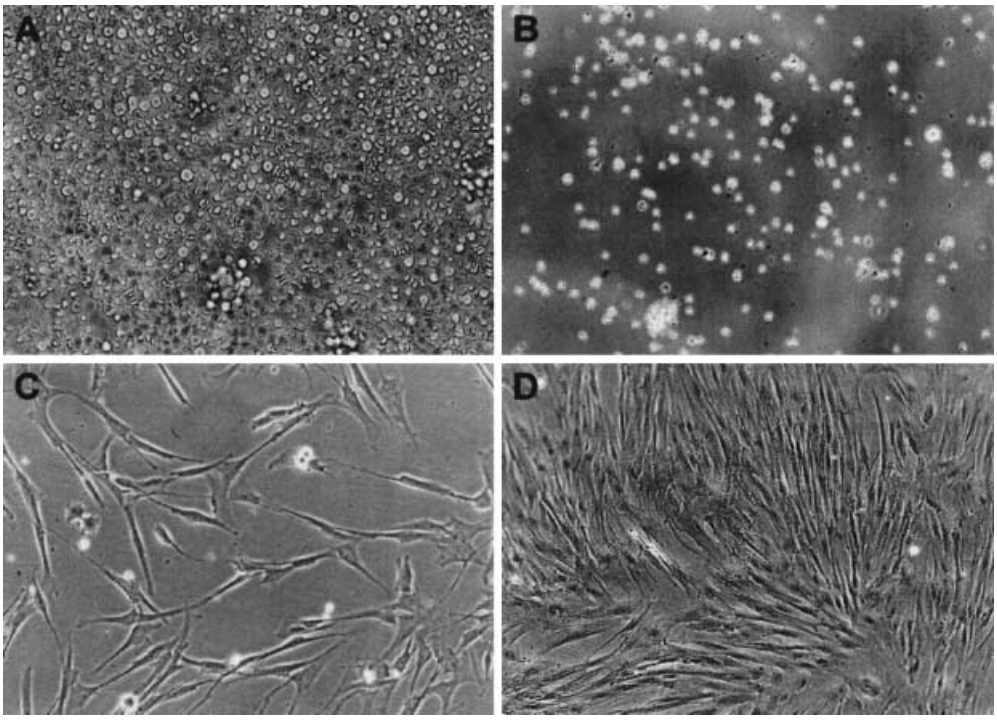

Fig. 1A–D Morphological appearance of porcine mesenchymal stem and progenitor cells (MSC). Porcine bone marrow-derived cells were purified according to protocols described for human MSC. Bone marrow cells were plated (**A**) and consisted of round-shaped erythrocytes and nonadherent cells at day 1. By day 3 (**B**), cells adhered to the culture plate and nonadherent cells were removed by medium exchange. By day 6 (**C**), cells exhibited a stretched fibroblast-like phenotype. Subcultivated cells, expanded up to at least passage 7 (**D**), presented a stable fibroblast-like morphology. **A** ×400; **B**, **C** ×200; **D** ×100

Table 1 Osteogenic and adipogenic marker genes

Gene	Accession number	Oligonucleotides (5→3′) (up/down)	Product size (bp)
GAPDH	AF017079	CTG CCC CTT CTG CTG ATG C GAC AAC TTC GGC ATC GTG GA	151
type Iα1 collagen	AF201723	CCA AGA GGA GGG CCA AGA AGA AGG GGG GCA GAC GGG GCA GCA CTC	232
osteocalcin	AW346755	TCA ACC CCG ACT GCG ACG AG TTG GAG CAG CTG GGA TGA TGG	204
osteonectin	AW436132	TCC GGA TCT TTC CIT TGC TTT CTA CCT TCA CAT CGT GGC AAG AGT TTG	187
aP2	AF102872	GGC CAA ACC CAA CCT GA GGG CGC CTC CAT CTA AG	167
PPARγ2	AF103946	GCG CCC TGG CAA AGC ACT TCC ACG GAG CGA AAC TGA CAC	238

vine serum (lot 228U; Biochrom) from selected lots (Lennon et al. 1996).

The differentiation potential was demonstrated by culture of porcine MSC (passage 2) under conditions that were favorable for osteogenic (Bellows et al. 1986; Maniatopoulos et al. 1988; Jaiswal et al. 1997), chondrogenic (Johnstone et al. 1998), or adipogenic (Gimble et al. 1992; Pittenger et al. 1999) development.

Polymerase chain reaction

Total RNA was isolated as described previously (Chomczynski 1993). Subsequently, total RNA (5 µg) was reverse transcribed after annealing with 500 ng oligo-$(dT)_{12–18}$ primers (Gibco) and 5 U Superscript reverse transcriptase (Gibco) in 70 µl (Gubler and Hoffmann 1983). The relative expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize marker gene expression in each sample in different concentrations. Real-time PCR using the i-Cycler PCR system (BioRad) was performed with 1 µl of the single-stranded cDNA sample, using the SYBR Green PCR Core Kit (Applied Biosystems). Relative quantitation of marker genes (Table 1) was performed according to the $\Delta\Delta C_t$ method as described (Winer et al. 1999).

Histological methods and immunohistochemistry

Osteoblasts exhibit high levels of alkaline phosphatase, which were visualized by staining with Sigma fast BCIP/NBT (Sigma). Von Kossa staining identified deposition of mineralized bone matrix. Proteoglycan secreting chondrocytes were stained with alcian blue 8GS (Roth) at pH 2.5. Presence of collagen was analyzed by azan staining. Adipocytes were identified morphologically and by staining with oil red O (Sigma). For immunohistochemistry of type II collagen, cryosections (6 µm) were incubated for 1 h with primary antibodies (rabbit anti-human type II collagen; DPC-Bier-

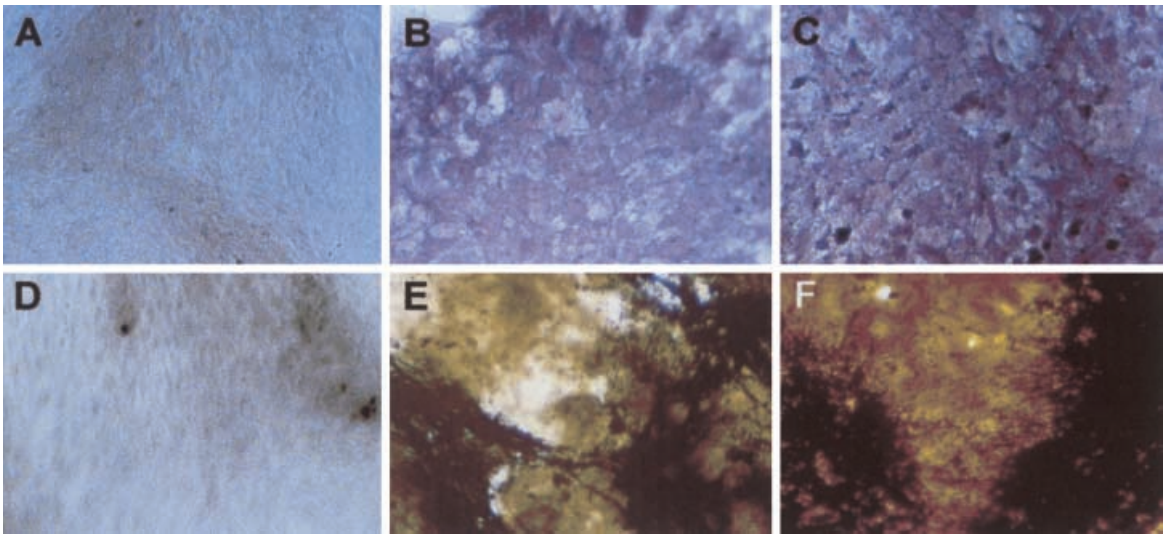

Fig. 2A–F Histochemical analysis of porcine MSC (passage 2) undergoing osteogenic differentiation. **A–C** Visualization of alkaline phosphatase activity (ALP) in osteogenic differentiation of porcine MSC. At day 6 (**A**), porcine MSC cultures displayed no ALP activity, which increased during prolonged cultivation under osteogenic conditions as shown for day 12 (**B**) and day 24 (**C**). **D–F** Von Kossa staining of mineralized bone tissue. At day 24 (**F**), the whole cell layer of osteogenic MSC was heavily covered with mineralized bone matrix. **A–F** ×40

mann). Subsequently, sections were incubated with biotinylated anti-rabbit antibody and peroxidase-conjugated streptavidine (Dako). The color reaction was developed by AEC substrate kit (Dako), followed by counterstaining with hematoxylin (Merck).

Results

Porcine bone marrow-derived cells consisted mostly of round-shaped erythrocytes and nonadherent hematopoietic cells (Fig. 1A). By day 3, cells adhered, and nonadherent cells were removed due to the exchange of culture medium (Fig. 1B). Morphologically, porcine MSC appeared as single, stretched cells leading to large clusters of stellate cells as they multiplied (Fig. 1C). Porcine MSC, which were cultivated and expanded over at least passage 7, still presented a stable fibroblast-like phenotype (Fig. 1D) and demonstrated no obvious reduction in mitogenic properties (data not shown).

Porcine MSC (passage 2) were cultured up to 30 days in monolayer or high-density cultures, respectively, and were stimulated to differentiate into distinct mesenchymal lineages.

During osteogenic stimulation, MSC continued to proliferate and readily formed multilayers showing differentiation along the osteogenic lineage (Fig. 2). At day 6, staining of alkaline phosphatase (ALP)-positive cells (Fig. 2A) or deposition of mineralized bone matrix, indicated by von Kossa staining, could not be detected (Fig. 2D). Expression of ALP was first detected by day 12 (Fig. 2B) leading to a steadily increasing number of ALP-positive cells at day 24 (Fig. 2C). Deposition

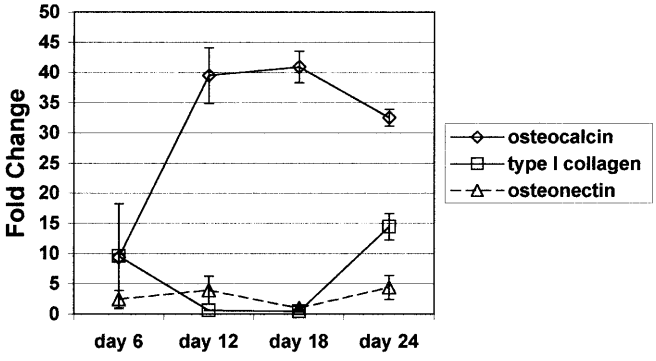

Fig. 3 Relative fold induction of osteogenic marker genes in porcine MSC cultures undergoing osteogenic differentiation. The relative fold induction of *osteocalcin*, *type Iα1 collagen*, and *osteonectin* gene expression in osteogenic cultures compared with nonosteogenic controls was calculated using the $\Delta\Delta C_t$ method. The mean of each triplicate well is plotted and the *error bars* represent SD

of mineralized matrix was first detected by day 12 (Fig. 2E). At day 24, almost the whole cell layer was heavily covered with mineralized matrix (Fig. 2F). Gene expression analysis of osteogenic cultures and nonosteogenic controls was performed for characteristic osteogenic marker genes (Fig. 3). Already by day 12, *osteocalcin* was induced up to 39-fold in osteogenic cultures compared with nonosteogenic controls. Instead, by day 24, *type Iα1 collagen* was upregulated in the late differentiation phase, whereas *osteonectin* was only marginally induced.

The chondrogenic potential of porcine MSC was characterized by histology and immunohistochemistry (Fig. 4). After 10 days of culture, MSC exposed to transforming growth factor-β_1 (TGF-β1) evolved a compact and homogeneous pellet structure, showing an increased cellular density. By day 30, alcian blue staining revealed a homogeneous deposition of proteoglycan within the whole section of the pellet culture (Fig. 4A). Azan staining demonstrated a nonhomogeneous distri-

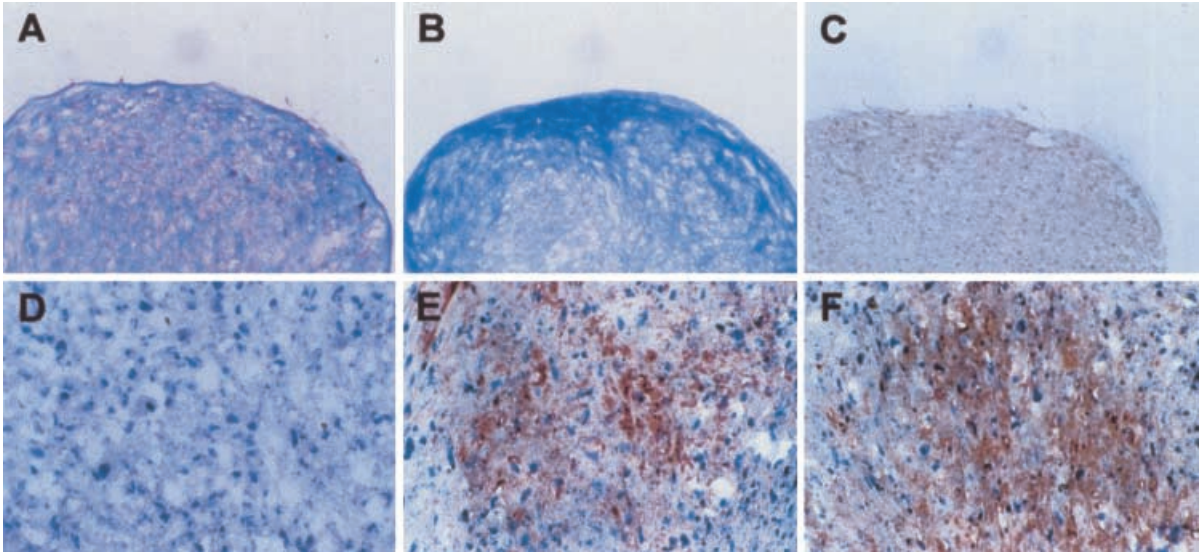

Fig. 4A–F Analysis of porcine MSC (passage 2) undergoing chondrogenic differentiation. **A–F** Analysis of porcine MSC micromass cultures in the presence of 10 ng/ml TGF-β_1. At day 30, porcine MSC showed a homogenous alcian blue staining of proteoglycan (**A**), whereas collageneous matrix was detected by azan staining (**B**) within the border zone of the pellets. Von Kossa staining revealed the absence of mineralized matrix components (**C**). **D–F** At day 10, cartilage-specific type II collagen could not be detected immunohistochemically (**D**). At day 20, staining of type II collagen was evident within the central part of the pellet structure (**E**) and was increased by day 30 (**F**). **A–C** ×200; **D–F** ×400

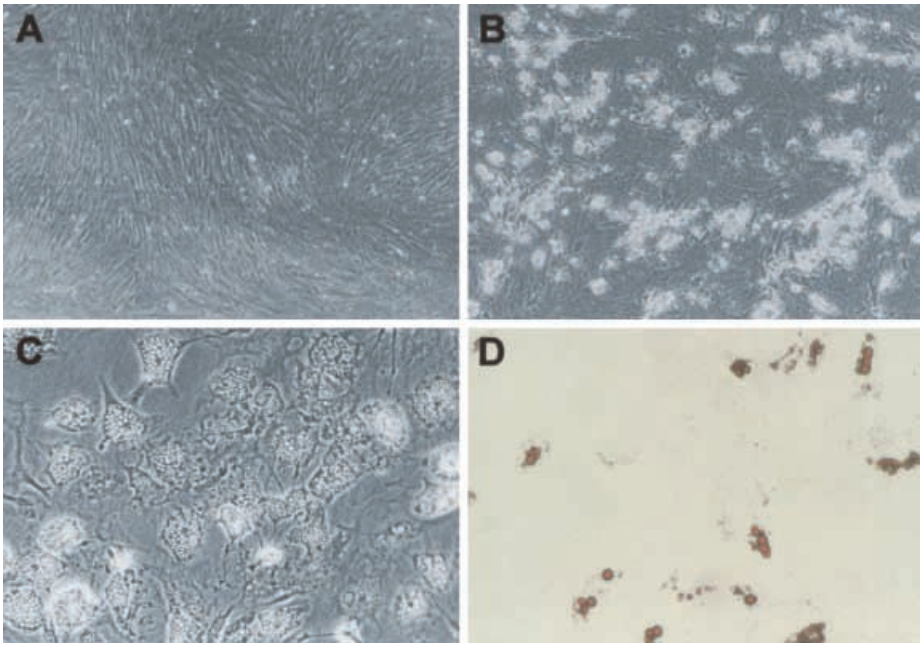

Fig. 5A–D Morphological appearance and histochemical analysis of porcine MSC (passage 2) undergoing adipogenic differentiation. Porcine MSC differentiating along the adipogenic lineage displayed a typical swirling growth pattern at day 1 after reaching confluence (**A**). At day 14, almost 30% of the cell layer was covered with adipocytic cells filled with lipid droplets (**B**). Magnification of individual cells demonstrated the characteristic phenotype of adipocytes by lipids filling the whole cytoplasm (**C**). Oil red O staining (**D**) documented the presence of neutral lipid droplets within cells differentiating along the adipocytic lineage. **A, B** ×100; **C, D** ×200

bution focused on the border zone of the pellet, whereas the central part showed less deposition of collagen matrix (Fig. 4B). Throughout the whole culture period, von Kossa staining (Fig. 4C) was negative and demonstrated the absence of mineralized matrix components in chondrogenic MSC cultures. Immunohistochemical staining of type II collagen displayed a steadily increasing amount of cartilage-specific collagen after prolonged cultivation of chondrogenic MSC. By day 10, type II collagen could not be detected (Fig. 4D), whereas the central part of the pellet cultures stained positive for type II collagen at day 20 (Fig. 4E). By day 30, staining of type II collagen was even more pronounced and was still localized only in the center of the pellets (Fig. 4F). Control MSC, not treated with TGF-β1, exhibited a more fibrous pellet structure, which appeared likely to be connective tissue-related by day 30 (data not shown).

Adipocytes were easily identified morphologically and by staining with oil red O (Fig. 5). During the early

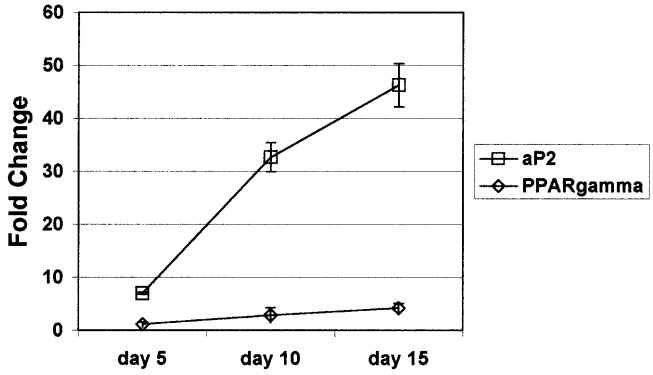

Fig. 6 Relative fold induction of adipogenic marker genes in porcine MSC cultures undergoing adipogenic differentiation. The relative fold induction of *aP2* and *PPARγ* gene expression in adipogenic cultures compared with nonadipogenic controls was calculated using the $\Delta\Delta C_t$ method. The mean of each triplicate well is plotted and the *error bars* represent SD

phases of adipogenesis, MSC grew in monolayers, showing a swirled growth pattern typical for MSC cultures (Fig. 5A). By day 2, the first cells filled with lipid droplets appeared (data not shown), which increased in number, covering almost 30% of the cell layer at day 14 (Fig. 5B). Magnification of individual cells demonstrated the typical phenotype of adipocytic cells displayed by characteristic lipid droplets filling the whole cytoplasm of single cells (Fig. 5C) and by oil red O staining (Fig. 5D). Gene expression analysis of distinct marker genes documented the potential of porcine MSC to undergo adipogenic differentiation (Fig. 6). At day 15, *adipocyte fatty acid-binding protein 2* (*aP2*) was induced up to 45-fold and *peroxisome proliferator-activated receptor γ* (*PPARγ*) was induced up to fourfold in adipogenic compared with nonadipogenic controls.

Discussion

Isolation and culture of porcine MSC

The isolation and expansion of porcine bone marrow-derived MSC was feasible, using selected culture medium and protocols, as described for the isolation of human MSC (Haynesworth et al. 1992; Lennon et al. 1996). One of the major problems in isolating MSC from distinct model organisms such as pigs remains the lack of appropriate species-specific MSC marker molecules. Here, porcine MSC presented morphological features known from mesenchymal stem cells derived from other species (Wakitani et al. 1995; Johnstone et al. 1998; Worster et al. 2000).

Differentiation potential of porcine MSC

Marrow-derived cells have been well documented for osteogenesis (Maniatopoulos et al. 1988; Jaiswal et al. 1997). Also, as described here, porcine MSC treated with dexamethasone (Dex) clearly demonstrated osteogenic differentiation in long-term cultures. As reported by others, uncommitted stem cells are recruited by Dex toward the osteogenic lineage, presumably by leading to bone cell differentiation at the expense of growth and proliferation (Bellows et al. 1990). In porcine MSC cultures, Dex is sufficient to induce the deposition of mineralized bone matrix and to upregulate bone-related marker genes such as *osteocalcin* (Carlson et al. 1993), *type Iα1 collagen* (Fisher et al. 1987), and *osteonectin* (Termine et al. 1981). Osteogenic potential has also been shown for neonatal pig bone marrow stromal cells (PBMSC). Upon incubation with medium containing Dex, PBMSC formed mineralized nodules, which demonstrated ALP-positive cells and a calcified type I collagen-rich matrix (Thomson et al. 1993). Therefore, Dex may stimulate both uncommitted stem and committed stromal cells. Although it has been suggested that osteoblasts and adipocytes share common precursors within the adult stromal system (Bennett et al. 1991), porcine MSC stimulated with Dex showed no obvious differentiation into adipocytic cells under cell culture conditions used here. Depending on the presence of Dex in primary or secondary cultures of marrow stromal cells, even an inverse relationship between the differentiation of adipocytic and osteogenic cells in marrow stromal cells has been postulated (Beresford et al. 1992).

As reported previously, the interactions between cells, as well as the lack of interaction between cells and substratum, are essential for in vitro chondrogenesis and maintenance of the chondrocyte phenotype (Solursh 1991). In this respect, high-density cultures of porcine MSC stimulated with TGF-β1 and Dex evolved a compact, cartilaginous structure on the basis of histochemical analysis and cartilage-specific type II collagen deposition. Here, TGF-β1 seems to play the pivotal role in the in vitro chondrogenic differentiation of porcine MSC cultures. The chondrogenic potential of TGF-β1 on MSC is not surprising, since in embryonic cartilage TGF-β1 is expressed abundantly and may be involved in the chondrogenic transformation of primitive mesenchymal condensations (Cancedda et al. 1995). Additionally, members of the TGF-β superfamily of growth factors have been shown to induce chondrogenesis in MSC from various species (Mackay et al. 1998; Yoo et al. 1998; Worster et al. 2000). In contrast, the role of Dex in chondrogenesis is quite unclear, as demonstrated by the impairment of chondrogenic development in murine condylar cartilage in the presence of Dex (Silbermann et al. 1987); whereas chondrogenesis is induced in organoid cultures of murine embryonic cells (Zimmermann and Cristea 1993). Although Dex induced weak chondrogenesis of rabbit bone marrow-derived mesenchymal progenitors (Johnstone et al. 1998), porcine control MSC, not treated with TGF-β1 but with Dex, failed to differentiate along the chondrogenic lineage. This is consistent with studies using

PBMSC to investigate the role of glucoconjugates on stromal cell differentiation. Dextran sulfate induced PBMSC monolayers to retract into circular cell aggregates, which were positive for type II collagen, whereas stimulation with Dex resulted in an unchanged phenotype (Noble et al. 1995).

To promote adipogenic differentiation, porcine MSC were treated with defined medium containing insulin. Adipogenic induction was apparent by the accumulation of vacuoles filled with neutral lipids and by the induction of distinct adipogenic marker genes. Development of adipocytes from MSC has been suggested to be a two-step process: (1) determination of preadipocytes, which have lost their developmental capacity while retaining their fibroblast-like phenotype; and (2) commitment and terminal differentiation, which is characterized by the formation of lipid droplets filling the whole adipocyte. Here, 5-azacytidine or insulin (Sager and Kovac 1982) promotes the determination of preadipocytes. One of the early preadipogenic marker genes is the transcription factor *PPARγ,* which converts murine fibroblasts into adipocytic cells, when overexpressed in 3T3 cells (Tontonoz et al. 1994). Later in adipogenesis, genes directly related to lipid metabolism are expressed, for example *aP2,* which is induced by *PPARγ* (Hollenberg et al. 1997; Gregoire et al. 1998).

As discussed here, porcine MSC from bone marrow have been isolated routinely, and the mesenchymal differentiation pattern exhibits a reproducible and stable phenotype. MSC may be a source of easily accessible mesenchymal progenitor cells for the evolving field of tissue engineering to regenerate bone, cartilage, tendon, and adipose tissue, autologously. Due to the similarity between porcine and human MSC, swine may provide a useful animal model system to study and evaluate tissue-engineered applications based on mesenchymal stem cells.

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