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Isolation and Characterization of Porcine Adult Muscle-Derived Progenitor Cells

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ABSTRACT

Here, we report the isolation of progenitor cells from pig skeletal muscle tissue fragments. Muscle progenitor cells were stimulated to migrate from protease-digested tissue fragments and cultured in the presence of 5 ng/ml basic fibroblast growth factor. The cells showed a sustained long-term expansion capacity (>120 population doublings) while maintaining a normal karyotype. The proliferating progenitor cells expressed *PAX3*, *DESMIN*, *SMOOTH MUSCLE ACTIN*, *VIMENTIN*, *CD31*, *NANOG* and *THY-1*, while *MYF5* and *OCT3/4* were only expressed in the lower or higher cell passages. Myogenic differentiation of porcine progenitor cells was induced in a coculture system with murine C2C12 myoblasts resulting in the formation of myotubes. Further, the cells showed adipogenic and osteogenic lineage commitment when exposed to specific differentiation conditions. These observations were determined by Von Kossa and Oil-Red-O staining and confirmed by quantitative RT-PCR analysis. In conclusion, the porcine muscle-derived progenitor cells possess long-term expansion capacity and a multilineage differentiation capacity. J. Cell. Biochem. 105: 1228–1239, 2008.

KEY WORDS: ADULT STEM CELLS; PIG; MUSCLE

Adult stem cells can function as multilineage contributors for development and maintenance of tissue, or regeneration after damage. For stem cell research, they are an accessible but heterogeneous source of cells, which can be used for medical applications like tissue engineering, stem cell transplantation or, in combination with gene therapy, to attenuate genetic disorders. Adult stem cells are quiescent undifferentiated cells that possess self-renewal ability and most likely reside in all body tissues. When exposed to stimuli caused by trauma or disease, they start to transiently amplificate and differentiate to become specialized cells of the regenerating tissue.

In skeletal muscle, several distinct stem cell populations have been identified which function in muscle growth and regeneration. These stem cells or progenitor cells can be used for studies regarding muscle diseases and tissue repair such as for restoration of muscular dystrophies including Duchenne muscular dystrophy [Shi and Garry, 2006; Peault et al., 2007]. Unfortunately, success in clinical trials has to date been limited.

In muscle development, the initiation of myogenesis starts with the expression of the muscle regulatory factors (MRFs) MyoD and Myf5. These skeletal muscle-specific basic helix–loop–helix transcription factors drive myogenesis, but terminal muscle development is subsequently coordinated by the MRFs Myogenin and MRF4 [Asakura et al., 2001; Pownall et al., 2002; Kassar-Duchossoy et al., 2004; Suelves et al., 2004; Shi and Garry, 2006]. The cellular state can be morphologically identified by the fusion of

Abbreviations: ALP, alkaline phosphatase; APC, allophycocyanine; aP2, adipocyte fatty acid binding protein; BACT, beta-actin; EGF, epithelial growth factor; FACS, fluorescence-activated cell sorting; FITC, fluorescein; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; h, human; HGF, hepatocyte growth factor; HP, high passage; HSC, haematopoietic stem cell; IGF, insulin growth factor; LP, low passage; MSC, mesenchymal stem cell; NCAM, neural cell adhesion molecule; PDGF, platelet-derived growth factor; PE, R-Phycoerythrin, PECAM, platelet endothelial cell adhesion molecule; pg, pig; PGK1, phosphoglycerate kinase 1; PM, proliferation medium; PPARγ2, peroxisome proliferator-activated receptor γ2; rb, rabbit; rt, rat; SMA, smooth muscle actin; TGF-β, transforming growth factor beta; UBQ, ubiquitin; VCAM, vascular endothelial adhesion molecule; VEGFR2, vascular endothelial growth factor receptor 2.

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cells thereby forming multinucleated myotubes; these cells express Myogenin, MRF4 and Myosin Heavy Chain (MYHC) [Feldman and Stockdale, 1991; Pownall et al., 2002; Jacquemin et al., 2004].

The first reported and best-characterized muscle progenitor cells are satellite cells [Mauro, 1961]. Quiescent satellite cells are identified by their position beneath the basal lamina in adult muscle fibers and biochemically by their expression of the paired-box transcription factor Pax7 [Seale et al., 2000; Asakura et al., 2001; Dhawan and Rando, 2005; Relaix et al., 2005]. Pax7 functions as a myogenic regulator upstream of the MRFs for the initiation of myogenesis [Zammit et al., 2006; Buckingham, 2007; Grefte et al., 2007; McKinnell et al., 2007]. Satellite cells are the contributors of post-natal skeletal muscle regeneration [Collins et al., 2005; Holterman and Rudnicki, 2005; Montarras et al., 2005], but also exhibit multilineage differentiation potential towards adipogenic and osteogenic lineages in both mouse and human [Asakura et al., 2001; Hashimoto et al., 2008].

Besides satellite cells, distinct populations of progenitor cells have been observed in muscle that possess multilineage differentiation capacities. These populations have been derived with different isolation techniques. For instance, muscle-derived stem cells (MDSC) were isolated using a preplating technique [Qu-Petersen et al., 2002]. After transplantation, these cells showed multilineage contribution towards myogenic [Lee et al., 2000; Qu-Petersen et al., 2002; Tamaki et al., 2007], osteogenic [Lee et al., 2000], neural cell lineages [Qu-Petersen et al., 2002] and endothelium of blood-vessels [Qu-Petersen et al., 2002; Tamaki et al., 2007]. In muscle, blood vessel-associated pericytes and a myoendothelial stem cell population have shown an efficient myogenic potential and a multilineage differentiation capacity towards osteogenic and chondrogenic lineages [Dellavalle et al., 2007; Peault et al., 2007; Zheng et al., 2007].

Mesoangioblasts associated with the muscle vascular system, are thought to be common progenitor cells for endothelial and mesodermal cells. In vivo, their regenerative capacities have been demonstrated in mouse and dog muscular dystrophy models [Zheng et al., 2007]. These cells are able to differentiate to adipocytes and osteoblasts [Peault et al., 2007]. Multipotent adult progenitor cells (MAPC) have been isolated from bone marrow, brain and muscle tissue, with differentiation capacities towards mesoderm, ectoderm and endoderm lineages [Jiang et al., 2002]. The muscle side population (SP) is a group of cells distinct from satellite cells consisting of a small percentage (~2–3%) of muscle progenitor cells. The cells have been shown to exhibit a haematopoietic lineage preference, but can also differentiate towards skeletal muscle cells [Asakura et al., 2002; Meeson et al., 2004; Montanaro et al., 2004; Schienda et al., 2006].

It is clear that the variety of muscle progenitor cells is wide. The diversity of tissue types (adipose tissue, nerves, vascular structures and connective tissue) within muscle requires the existence of numerous progenitor cells exhibiting several lineage differentiation plasticities [Zammit and Beauchamp, 2001]. The characterization of the progenitor cells is therefore of great importance to distinguish between the different populations. Expression profiles of biomolecular markers and cell differentiation capacities have resulted in a variety of defined classifications of several muscle progenitor cells.

Most muscle stem cells have been described in human and mouse. Stem cell lines derived from small animals such as the mouse encounter limitations due to the size and lifespan of this animal. Meanwhile, since the organ size and physiology of the pig show functional and anatomical similarity to those of the human, porcine stem cells may contribute in studies regarding regenerative therapies. However, not much is known about porcine muscle stem cells.

In this study, the isolation and characterization of porcine muscle-derived progenitor cells with maintenance of long-term expansion in vitro is described. Furthermore, plasticity of these cells to differentiate into myogenic, adipogenic and osteogenic lineages is demonstrated.

MATERIALS AND METHODS

PROGENITOR CELL ISOLATION AND CULTURING

To isolate progenitor cells, semitendinosus muscle tissue from the hind limb was dissected from a euthanized (pentobarbital) pig (hybrid York boar; 8 weeks of age). Around 50 mg of minced muscle tissue was digested with 0.8 mg/ml protease (Sigma, ST Louis, MO) in phosphate-buffered saline (PBS, Braun, Melsungen, Germany) for 1 h at 37°C as described previously [Doumit and Merkel, 1992]. Tissue fragments were washed in Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG, Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (FBS, Invitrogen) and plated in 0.1% gelatin-coated 6-well plates (Sigma). Cell liberation and proliferation was obtained in the presence of 5 ng/ml basic fibroblast growth factor (bFGF, Invitrogen) in proliferation medium (PM) consisting of DMEM-HG, 20% FBS, 50 μg/ml gentamycin with 1% antibiotic-antimycotic and 250 ng/ml fungizone (all from Invitrogen).

After 3 days, tissue debris was removed and adhered cells expanded by replating trypsinized cells (0.25% Trypsine/0.5 mM EDTA; Sigma) at a density of 2,000 cells/cm^2. The muscle cells were pre-plated repeatedly for removal of fibroblasts as described previously [Qu et al., 1998]. The isolated cells were expanded in PM containing 5 ng/ml bFGF and passaged every third day.

FLOW CYTOMETRY

Antibodies listed in Table I were used to characterize the cells by flow cytometry. Antibody incubations were performed in 0.5% Bovine Serum Albumin (BSA; Roth, Karlsruhe, Germany) in PBS for 1 h on ice and washed three times (0.5% BSA in PBS). Non-labeled antibodies were visualized using a PE-conjugated goat-anti-mouse antibody for the detection of pg-CD29, rt-CD31 and an APC-conjugated goat-anti-mouse antibody for pg-CD45, pg-MHC-class I and pg-MHC-class II detection. FITC-conjugated goat-anti-rabbit antibody was used to detect antibody ms-Flk-1 and pg-CD44. Appropriate nonspecific mouse IgG isotype control antibodies were used in the same concentration as the primary antibodies and analyzed parallel to the run. Specie cross reactivity of the antibodies were tested on freshly isolated porcine bone marrow cells (data not shown). Flow cytometric analysis was performed using FACS Calibur and Cell Quest software (both from Becton Dickinson, San Jose, CA).

TABLE I. Listing of Antibodies Used for Flow Cytometry

Antibody	Dilution	Clone	Specificity	Company
CD29	1:10	NaM160-1A3	Beta 1 integrin, MSC	BD
CD31	1:10	TLD-3A12	Platelet endothelial cell adhesion molecule (PECAM-1)	BD
CD44	1:20	MAC329	Hyaluronate receptor, cell-adhesion molecule, MSC	F
CD49d-FITC	1:20	BU49	VLA-alpha 4 integrin, receptor for VCAM	I
CD45	1:20	K252.1E4	Leucocyte common antigen, HSC	F
CD56-PE	1:200	MEM188	Neural cell adhesion molecule (NCAM)	I
CD56-FITC	1:10	NCAM16.2	Neural cell adhesion molecule (NCAM), myoblasts	BD
CD90-FITC	1:500	5E10	THY-1, primitive progenitor cells, MSC	BD
CD106-FITC	1:50	1.G11B1	Vascular cell adhesion molecule, MSC	F
CD146-PE	1:25	P1H12	Adhesion molecule, pericyte marker	BD
Flk-1	1:1,000		Vascular endothelial growth factor receptor 2 (VEGFR2)	UP
MHC-class I	1:200	PT85A	Class I major histocompatibility antigen, MSC	VMDR
MHC-class II	1:50	TH81A	Class II major histocompatibility complex (HLA-DQ)	VMDR

BD, Becton Dickinson, SJ; F, Fitzgerald, MA; I, ImmunoTools, Friesoythe, Germany; UP, Upstate, NY; VMDR, WA.

IMMUNOSTAINING

Cells were cultured in 8-well glass chamber slides, fixed in 4% paraformaldehyde (PFA; Electron Microscopy Science, Hatfield, PA; 15 min) and washed with 0.05% Tween-20 in PBS (PBST; 3 times 5 min). Permeabilization was performed in 0.5% Triton-X-100 (30 min). Additionally, the cells were blocked for endogenous biotin (avidin/biotin blocking kit; Vector Laboratories, Burlingame, CA) and peroxidases (0.3% H_2O_2 in 100% methanol; 30 min) and washed (PBST). Pre-incubation in blocking solution (30 min; 10% normal goat serum and 10% normal swine serum in 0.4% fish gelatin (Sigma) in PBST) was performed to avoid a-specific binding. Subsequently, the primary antibody (anti-NANOG: ab21603: 2 μg/ml, Abcam, Cambridge, UK; anti-DESMIN: D33: 5 μg/ml; anti-Smooth Muscle Actin (SMA): 1A4: 1 μg/ml, both from DakoCytomation, Glostrup, Denmark) was incubated for 1 h in 0.2× blocking solution. After washing (PBST), secondary biotin-labeled goat anti-rabbit antibody incubation (30 min) was performed. Detection of this secondary antibody was completed by the streptavidin/avidin-peroxidase conjugated complex (DakoCytomation) visualization of 3-amino-9-ethyl-carbazole (Sigma). Nuclei were counterstained with Mayer's haematoxylin (2 min; Fluka, Buchs, Switzerland) and rinsed in tap water (1 min). Slides were overlayed with glycergel (DAKO) and covered with glass. All steps were performed at RT.

For immunofluorescence cells staining cells were fixed in 4% PFA (15 min) and permeabilized in 0.1% Triton/PBS (15 min). Pre-incubated in blocking solution (10% NGS, 0.1% fish gelatin, 0.1% BSA in PBST) and subsequently incubated with primary mouse antibodies (anti-Vimentin: V9: 1 μg/ml, DAKO; anti-Desmin: D33: 10 μg/ml, DAKO; mIgG1: 10 μg/ml: DAKO). Detection of primary antibody was performed with a 488Alexa-dye-conjugated goat-anti mouse secondary antibody for 30 min. Nuclei were stained with DAPI and dehydration in alcohol series (70%, 90%, 100%; 5 min). Slides were overlayed with FluorSave (Calbiochem, San Diego, CA), covered with glass and encircled with nailpolish. Detection was visualized by fluorescence microscopy (Leica DMRE). All steps were performed at RT.

RNA ISOLATION AND REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

RNA isolation was performed with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The cells were washed twice in PBS before being lysed with RLT buffer. DNase treatment was performed with an RNase-Free DNase set (Qiagen). The cDNA was generated using Superscript III First strand Synthesis System for RT-PCR (Invitrogen) according to the protocol advised by the manufacturer. Quantitative real-time RT-PCR (qRT-PCR) on cDNA (1 μl) was performed on an iCycler with iQ™ SYBR® Green supermix (both from BIO-RAD, Hercules, CA) and a 12.5 pmol primer concentration per 25 μl reaction (Table II). Amplification of cDNA was performed in a three-cycle protocol, initiated by a denaturation step of 3 min at 95°C, followed by 40 repeats of 30 s at 95°C, 20 s at 51–63°C and 30 s at 72°C and subsequently 77 repeats of 15 s with a 0.5°C increase in temperature every repeat starting from 60°C. All samples were normalized against the three best reference genes (*GAPDH*, *PGK1* and *UBQ*) determined by geNorm software (Primer Design Ltd., Southampton, UK) [Kuijk et al., 2007]. Normalized qRT-PCR gene expression values were defined relative to the expression level of normalized proliferating cells using the delta Ct method. For conventional RT-PCR to detect mRNA levels, cDNA (1 μl) was amplified with HotStarTaq DNA Polymerase (Qiagen) with 12.5 pmol primer concentration in a 25 μl reaction (Table II). This was performed using a MyCycler Thermal Cycler (BIO-RAD) with initial denaturation at 95°C for 15 min, followed by 40 repeats of 95°C for 15 s, 55–58°C for 30 s, 72°C for 45 s and finally an elongation step of 72°C for 7 min. PCR products were visualized after agarose gel (1.25%) electrophoresis with 0.5 μg/ml ethidium bromide (Merck, Whitehouse station, NJ). Primer specificity was confirmed by product sequencing.

KARYOTYPING

Cells were incubated with colchicin (0.3 μg/ml; Sandoz, Holzkirchen, Germany) for 1.5 h in a humidified incubator (5% CO_2, 37°C) and detached using 0.25% Trypsin/0.5 mM EDTA for 1 min. The pelleted cells (5 min at 250 × *g*) were incubated in 1.5 ml hypotonic buffer (0.075 M KCl) for 20 min at 37°C and centrifuged (5 min at 250 × *g*). Cell pellets were fixed for 1 min in cold methanol/acetic acid (3:1; Merck), centrifuged and subsequently taken up in 500 μl fixative and stored at −20°C until further use. Cell suspensions were centrifuged (5 min at 250 × *g*) and diluted in 100 μl cold fixative. From 20 cm cell suspensions were drop-wisely applied with a Pasteur pipette onto absolute alcohol and diethyl ether wiped pre-chilled glass slides and air-dried. Giemsa staining (Sigma) was performed by putting slides in Sörensen's buffer, pH 6.8 (10 min), washing them in water and transferring in xylene (3 min).

TABLE II. Oligonucleotide Primer Sequences

Gene	Primer sequences (5′ → 3′)	Amplicon size (bp)	T_a (°C)	GenBank accession number (references)
PAX3	F: GCCAATCAACTGATGGCTTT R: GTACAGTGCTCGGAGGAAGC	181	55.0	NM008781
PAX7	F: GGTGGGGTTTTCATCAATGG R: GTCTCTTGGTAGCGGCAGAG	155	56.0	AY653213
MYF5	F: ACGACTAACCCCAACCAGAG R: GGCAACTGGAGAGAGAGAGGC	371	58.0	Y17154
MYOD	F: TCCGCGACGTAGATTTG R: ACGGGAAGTGCGAGTGTT	177	60.1	NM_001002824 [Blanton et al., 1999]
MYOGENIN	F: ATCCAGTACATCGAGTGCCTG R: GCATGGTTTCATCTGGGAAG	290	55.0	AY902465
DESMIN	F: CCGAGATCTACGAGGAGGAG R: GCTGAAAGAAGAAGCCGAGA	161	58.0	AF363284
MYHC	F: CCGTCTGGATGAGGCTGAG R: CCGTCTGGTAGGTGAGTTCC	179	60.1	NM214136
NANOG	F: CCTCCATGGATCTGCTTATTC R: GCATCTGCTGGAGGCTGAGGTA	210	55.0	AY596464 [Kuijk et al., 2008]
OCT3/4 (POU5F1)	F: GGTTCTCTTTGGGAAGGTGTT R: ACACGCGGACCACATCCTTC	313	55.4	AJ251914 [Kuijk et al., 2008]
THY-1 (CD90)	F: TCTCTTGCTAACAGTCTTG R: GGTAGTGAAGCCTGATAAG	265	54.5	BP156451
PPARy2	F: AGGAGCAGAGCAAAGAGG R: AGAGTTACTTGGTCATTCAGG	139	53.7	AF059245
αP2	F: AACCCAACCTGATCATCACTG R: TCTTTCCATCCCACTTCTGC	192	61.3	AF102872 [Iohara et al., 2006]
ALKALINE PHOSPHATASE (ALP)	F: CCAAAGGCTTCTTCTTGCTG R: TGTACCCGCCAAAGGTAAAG	195	60.0	AY145131 [Iohara et al., 2006]
COLLAGEN TYPE 1α1	F: CCCGTGCCCTGCCAGATC R: CCTCGACTCCCGTGGTTTCC	135	63.0	AF201723
OSTEOCALCIN	F: CAGGAGGGAGGTGTGTGAG R: TGCGAGGTCTAGGCTATGC	105	62.3	AY150038.1
CD31	F: CATTTCCAAAGTCAGCAGCA R: CATCATCATGCCTCCCTTCT	172	60.1	X98505 [Iohara et al., 2006]
BACT	F: GACTTCGAGCAGGAGATGG R: GCACCGTGTTGGCGTAGAG	233	56.0	AJ312193
UBQ	F: TTCGTGAAGACCTTGACTG R: GGACTCCTTCTGGATGTTG	186	51.1	M18159 [Kuijk et al., 2007]
PGK1	F: AGATAACGAACAACCAGAGG R: TGTCAGGCATAGGGATACC	126	56.4	AY677198 [Kuijk et al., 2007]
GAPDH	F: TCGGAGTGAACGGATTTG R: CCTGGAAGATGGTGATGG	219	51.1	AF017079 [Kuijk et al., 2007]

Bp, base pairs; T_a, annealing temperature.

Slides were overlaid with DEPEX (Serva, Heidelberg, Germany) and covered with glass. The cells were analyzed using a Leica DMRA fluorescence microscope and CytoVision Genus software (Applied Imaging, Hampshire, UK). All steps were performed at RT.

MYOGENIC DIFFERENTIATION

Murine C2C12 myoblasts were seeded onto 12-well plates (Greiner, Frickenhausen, Germany) and myotube formation was induced by the incubation in 2% FBS in DMEM-HG for 3 days. Muscle cells were seeded at a density of 50×10^3 cells onto 0.4 μm pore sized transwell insert membranes (Greiner) and allowed to attach. The inserts containing the muscle progenitor cells were cocultured with the C2C12 myotubes in 2% FBS in DMEM-HG. Half the medium volume was refreshed every 2–3 days for 3 weeks.

ADIPOGENIC DIFFERENTIATION

Cells were seeded onto 12-well plates (Corning, NY, USA) at a density of 2,000 cells/cm^2 and were kept in PM with 5 ng/ml bFGF for 3 days. Differentiation was induced with DMEM-HG containing 10% FBS with 0.5 mM isobutyl methylxanthine (IBMX; Sigma), 0.2 mM indomethacin (Sigma), 10 μg/ml insulin (from hog pancreas; Sigma) and 10^{-6} M dexamethasone (Serva) [Pittenger et al., 1999; Bosch et al., 2006]. Differentiation medium was changed every 2–3 days for 24 days. Afterwards, cells cultures were washed in PBS and fixed in 4% PFA (15 min) and subsequently incubated with Oil-Red-O (0.3% in 60% *iso*-propanol; 10 min), followed by washing in 60% *iso*-propanol (5 min) and subsequently in PBS. All steps were performed at RT.

OSTEOGENIC DIFFERENTIATION

Cells were seeded onto 12-well plates (2,000 cells/cm^2) and were kept in PM with 5 ng/ml bFGF for 3 days until they reached 80% confluency. Osteogenic differentiation was induced in DMEM-HG with 10% FBS containing 10 mM β-glycerol phosphate (Sigma), 0.5 mM ascorbic acid (Merck) and 10^{-8} M dexamethasone (Serva) [Pittenger et al., 1999; Li et al., 2005]. Differentiation medium was changed every 2–3 days for 24 days. The cells were washed in PBS, fixed in 4% PFA (15 min) and placed in a 5% silver nitrate solution (Sigma) for 1hr in front of a 60-W lamp, then rinsed in distilled water three times and incubated for 5 min in 5% sodium thiosulphate (Sigma) and washed in water [Vacanti et al., 2005]. All steps were performed at RT.

RESULTS

PROGENITOR CELL ISOLATION AND CULTURING

Semitendinosus muscle from 8-week-old pigs was dissected and cultured in vitro. After 24 h, cells emerged from fiber explants and

started to proliferate. These cells were passaged at a low density (2,000 cells/cm^2) during which the cells sustained a population doubling time between 30 and 48 h. The cells, termed muscle-derived progenitor cells (MDPCs), were cultured for more than 4 months with maintenance of morphology. The MDPCs were classified as low passage (LP); 1–9 passages and high passage (HP); 10 to >50 passages (Fig. 1A–C). The chromosomal content of the cells was analyzed by karyotyping the cells at various passages. High passage MDPCs, after approximately 30 passages equivalent to 120 population doublings (PDs) still showed a normal karyotype of 38 chromosomes (Fig. 1D). Expanded cell cultures were preplated (PP) several times for the removal of fast adherend fibroblasts. There population doubling time started from 24 h and reached 48 h around the 130 PDs (Fig. 1E). Here single MDPCs underwent aberrant divisions resulting in a multi nuclei phenotype and a decrease in doubling time.

CHARACTERIZATION OF MDPCs

To phenotype the MDPCs the expression profile of cell surface markers was determined. The MDPCs uniformly expressed CD29/CD44/CD56/CD90 and MHC-class I, markers of the mesenchymal lineage, but did not express CD31/CD45/CD106/CD146/Flk-1 and MHC-class II. Staining against CD49d, a receptor for vascular endothelial cell-adhesion molecule VCAM-1, resulted in a weakly positive and negative population (Fig. 2).

Immunofluorescence cell staining for VIMENTIN revealed to be positive for all cells (Fig. 3A), while DESMIN expression was absent (data not shown). Immunocytochemistry on MDPC-HP revealed nuclear expression of NANOG in about half of the cell population (Fig. 3B). Further analysis of non-stimulated cells showed that a subset of the cells expressed SMA (Fig. 3C).

Gene expression patterns of the MDPCs-LP and -HP were further determined with conventional RT-PCR. Expressions of *NANOG*,

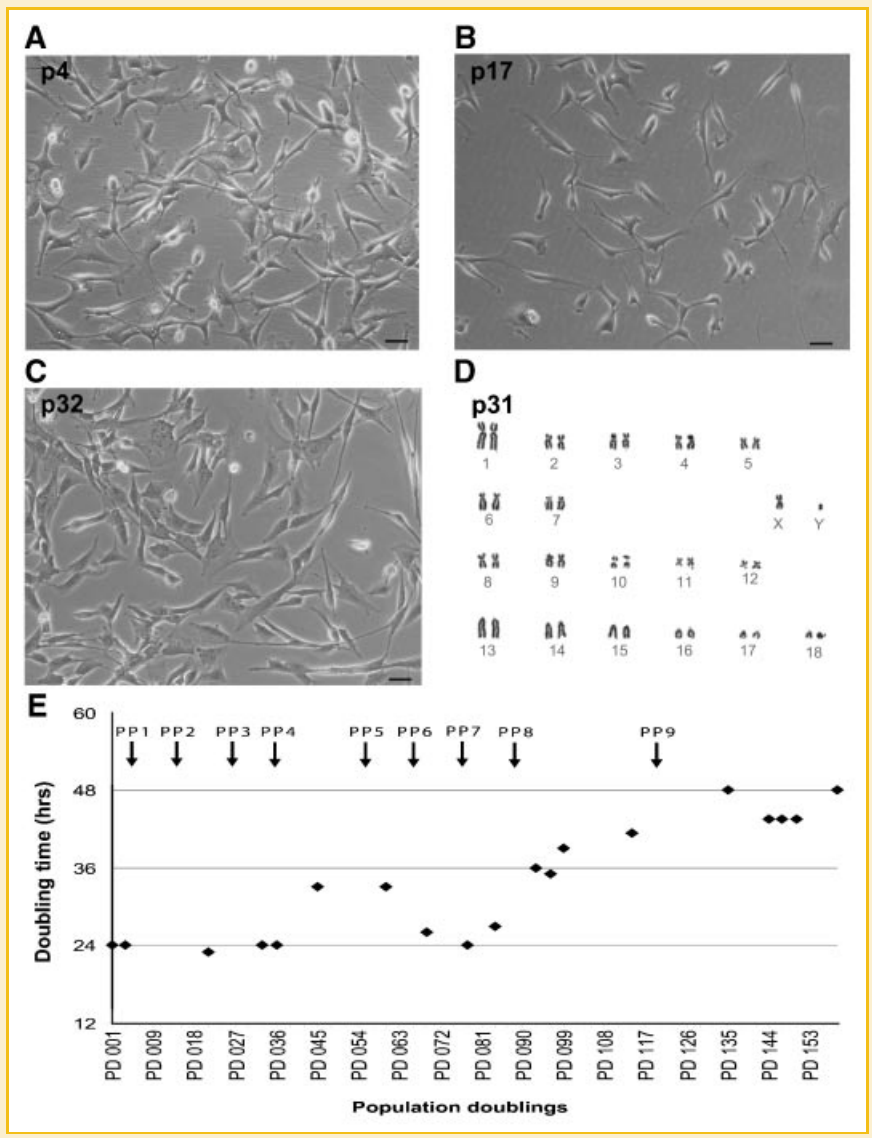

Fig. 1. In vitro MDPC expansion. A: Cell morphology of MDPCs-LP p4. B: MDPCs-HP p17 and C: MDPCs-HP p32. Scale bar = 10 μm. D: Karyotype of MDPCs-HP p31 showing a normal numerical porcine phenotype of 38 chromosomes. E: Cell doubling over prolonged culture-expansion with repeated preplating (PP).

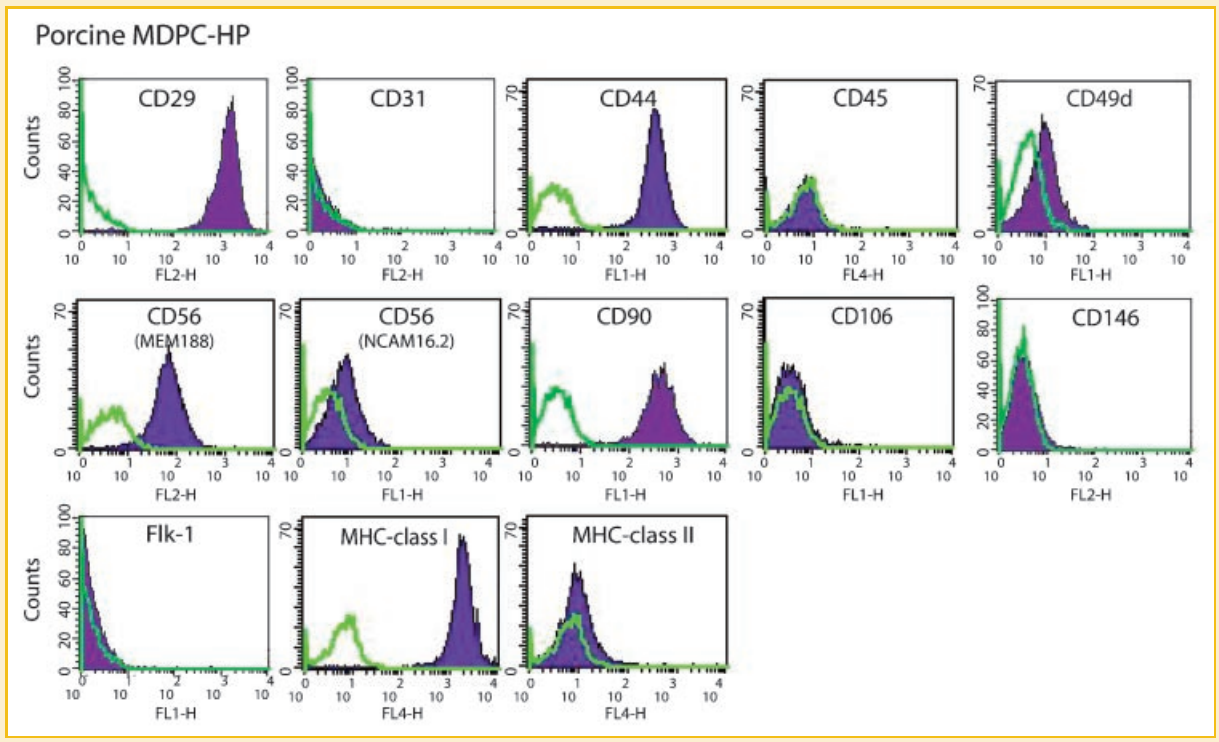

Fig. 2. Phenotyping of MDPCs-HP. Flow cytometric analysis with cell surface markers CD29, CD31, CD44, CD45, CD49d, CD56, CD90, CD106, CD146, Flk-1, MHC-class I and MHC-class II (purple). Corresponding fluorescent detection of nonspecific IgG isotypes represents negative control (green line).

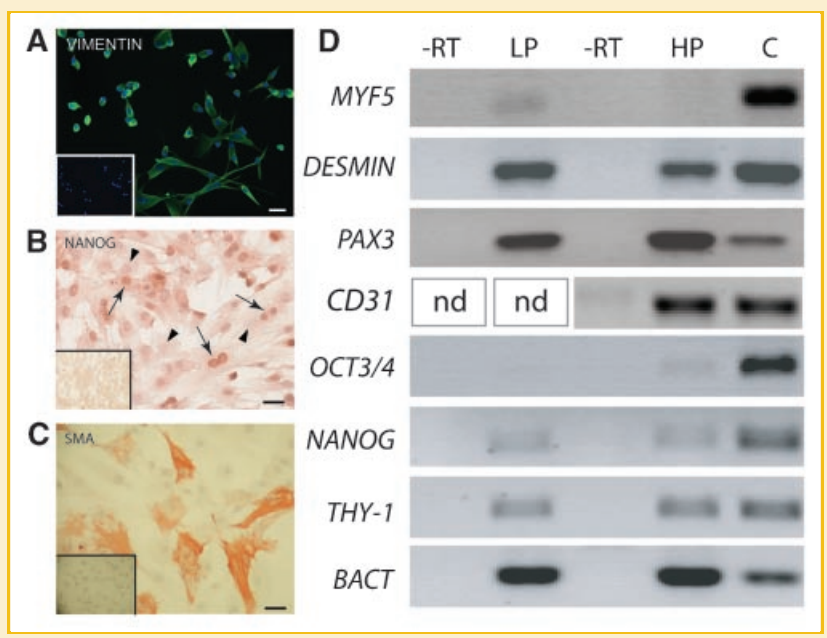

Fig. 3. Characterization of MDPCs-HP. A: Immunofluorescent detection of Vimentin (green). Corner frame shows negative control (mIgG isotype). Cells were co-stained with DAPI (blue). B: Detection of nuclear NANOG by immunocytochemistry (arrows; red). Cells were co-stained with haematoxylin (light blue). Arrowheads indicate nuclei lacking NANOG expression. Corner frame shows negative control (no primary antibody). C: Smooth muscle actin detection by immunocytochemistry (red). Cells were co-stained with haematoxylin (light blue). Corner frame shows negative control (mIgG1 isotype). Scale bar = 25 μm (A–C). D: PCR gene expression analysis of low (LP) and high passaged (HP) MDPCs. *BACT* was used as internal control. –RT represents RNA samples without reverse transcriptase during cDNA generation. C: control cDNA. nd = not determined.

THY-1, *CD31*, *DESMIN* and *PAX3* were observed in both MDPCs-LP and -HP. Low expression of *OCT3/4* was only observed in MDPCs-HP and *MYF5* mRNA expression only in MDPCs-LP (Fig. 3D). Expression of the muscle-specific genes *PAX7*, *MYOD* and *MYOGENIN* were not detected (data not shown). Surprisingly, expression of DESMIN could not be detected by immunocytochemistry (data not shown).

MULTILINEAGE DIFFERENTIATION OF MDPCs

To investigate the differentiation potential of the MDPCs, cells were cultured under various conditions. MDPCs-HP were cocultured with differentiated murine C2C12 myotubes to analyze the myogenic differentiation potential. MDPCs and C2C12 cells were separated by 0.4 μm membranes allowing secreted factors to diffuse, while preventing cell mixture. No myotubes were observed in the absence of C2C12 cells (non-stimulated cells, Fig. 4A). Multinucleated myotube formation was observed from day 19 of induction (Fig. 4B and D). In these differentiated cells DESMIN protein was detected by immunofluorescence (Fig. 4C). *MYHC* gene expression was detected after 3 weeks of co-culture, but not in non-stimulated cells (Fig. 4E).

To analyze the adipogenic differentiation capacity, cells were cultured for 24 days under conditions designed to stimulate adipogenic differentiation. This medium contained dexamethasone as adipogenic stimulator and IBMX and insulin to synergize the process. This resulted in the formation of adipocytes showing intracellular lipid droplets detected by Oil-Red-O staining (Fig. 5A–C). Genes involved in fat metabolism regulation are peroxisome proliferator-activated receptor γ2 (PPARγ2) and adipocyte specific gene (aP2) for the terminal stage of adipogenesis. The stimulated progenitor cells showed a markedly increased induction in *PPARγ2* expression and an increase in *aP2* expression relative to proliferating MDPCs-HP as determined by qRT-PCR. Interestingly, MDPCs showed a repression in both *COLLAGEN TYPE 1 α1* and *OSTEOCALCIN* gene expression, while *ALP* remained relatively constant (Fig. 5D).

Osteogenic differentiation of MDPCs was induced during 24 days. Here, ascorbic acid was used to activate collagen production, β-glycerol phosphate to induce the formation of alkaline

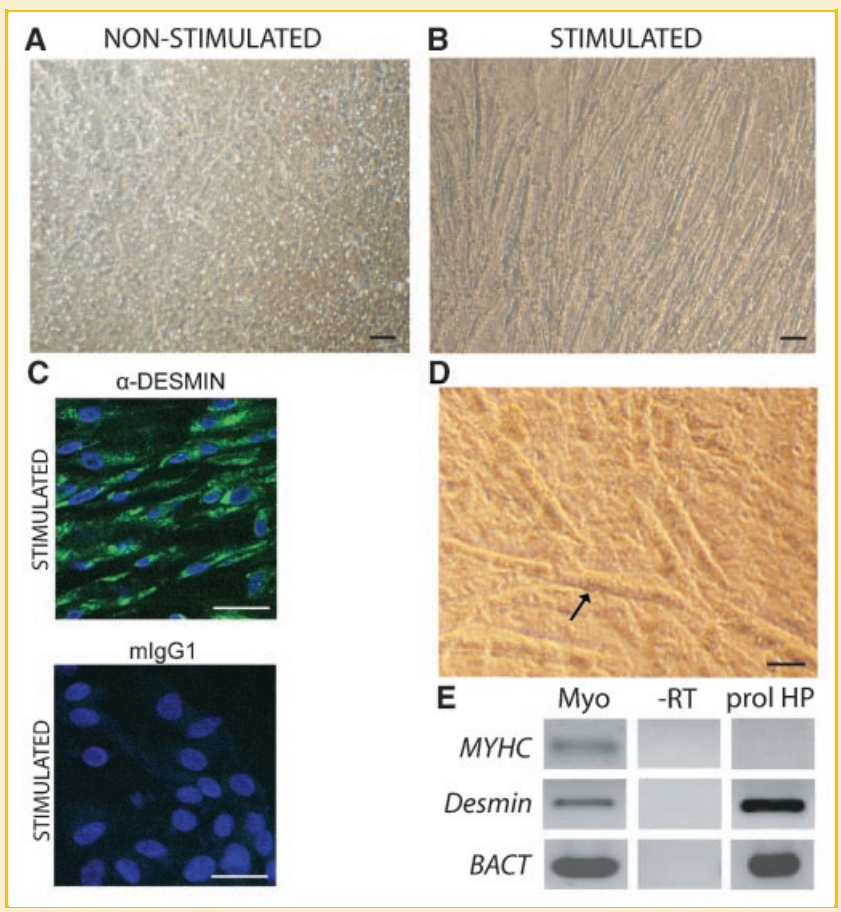

Fig. 4. Activation of myogenesis. A: Monolayer of non-stimulated MDPCs-HP in transwell inserts. B: Myotube formation in stimulated MDPCs-HP. C: Desmin staining of myotubes after stimulation (upper; green). Control staining using mIgG isotype (lower). Cells were co-stained with DAPI (blue). D: Multinucleated myotube in stimulated MDPCs-HP culture (arrow). Scale bar = 25 μm (A–D). E: RT-PCR analysis of stimulated MDPC-HP. Myo: myogenesis; Prol HP: proliferating MDPCs-HP. −RT represents RNA samples without reverse transcriptase during cDNA generation.

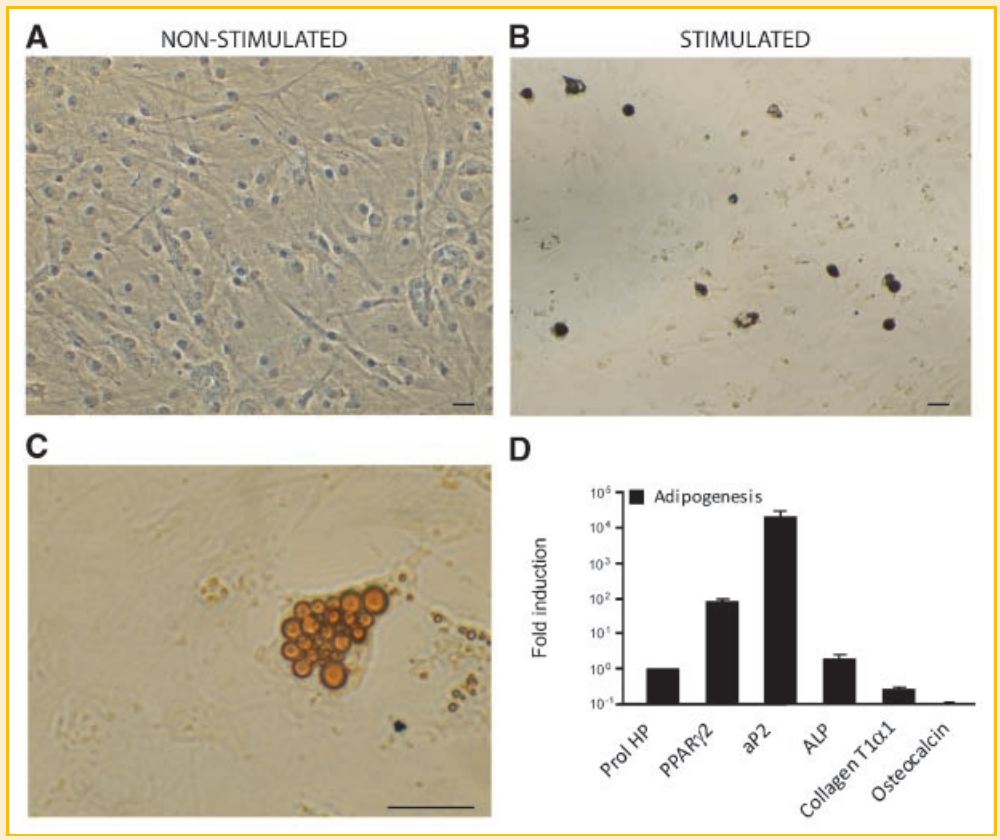

Fig. 5. Activation of adipogenesis. A: Oil-Red-O staining on non-stimulated MDPCs-HP. B: Red staining of intracellular lipid droplets in stimulated MDPCs-HP with Oil-Red-O. C: Higher magnification of intracellular lipid accumulation stained with Oil-Red-O. Scale bar = 30 μm (A–C). D: Normalized expression of adipogenic regulatory genes *PPARγ2*, *aP2* and osteogenic regulatory genes *ALP*, *Collagen Type 1α1* and *Osteocalcin* genes relative to proliferating MDPCs-HP as determined by quantitative RT-PCR (black bars). Prol HP: proliferating MDPCs-HP. Bars are mean + SD.

phosphates and dexamethasone to act as an osteogenic stimulator. This resulted in the formation of calcium depositions demonstrated by Von Kossa staining compared to unstimulated cells (Fig. 6A–C). Expression of genes involved in regulating bone formation was detected by qRT-PCR. The expression of *ALKALINE PHOSPHATASE* (*ALP*), coding for an enzyme released by osteogenic cells functioning in the process of bone mineralization, was increased relative to the expression level of proliferating MDPCs. The expression of *COLLAGEN TYPE 1α1*, involved in early phase of bone differentiation, showed also an increased induction [Zou et al., 2008]. No induction of *OSTEOCALCIN* expression, involved in the late stage of osteoblast development into matrix mineral, was observed. Notably, MDPCs revealed an upregulation in *PPARγ2* and in *aP2* expression (Fig. 6D).

All gene expression levels were quantified by normalization against reference gene expression values (*UBQ*, *PGK1* and *GAPDH*).

DISCUSSION

Here, we describe the isolation and characterization of a porcine progenitor cell population derived from muscle tissue. The MDPCs demonstrated a long-term expansion and multilineage differentiation capacity. The long-term expansion was indicated by a sustained morphology with a numeric karyotype over 120 population doublings. The occurrence of single large flat cells started to arise in culture after 5 months, resulting in proliferative senescence of the MDPC culture. The cells were cultured with presence of bFGF to maintain proliferation. bFGF is used to control proliferation of embryonic stem cells and sustain their undifferentiated state [Ludwig et al., 2006; Xiao et al., 2006]. Porcine muscle stem cells cultured without bFGF already exhibit proliferation senescence after the fifth passage [Fligger et al., 1998; Blanton et al., 1999]. This suggests that also in porcine MDPCs, bFGF signaling is necessary for long-term expansion.

The MDPCs were further analyzed by immunophenotyping revealing a CD29+/CD31−/CD44+/CD45−/CD49d+/−/CD56+/CD90+/CD106−/CD146−/Flk-1−/MHC-class I+ and MHC-class II− population. CD56 or NCAM is a protein expressed on myoblasts [Hawke and Garry, 2001; Mesires and Doumit, 2002]. Two antibodies were used to detect CD56. Clone MEM-188 reacted with MDPCs recognizes the 180 kDa NCAM protein, while clone NCAM16.2 recognizes a common domain on three isoforms including the 180 kDa isoform. This latter isoform has been used to characterize myoblasts [Holzer et al., 2005; Vilquin et al., 2005].

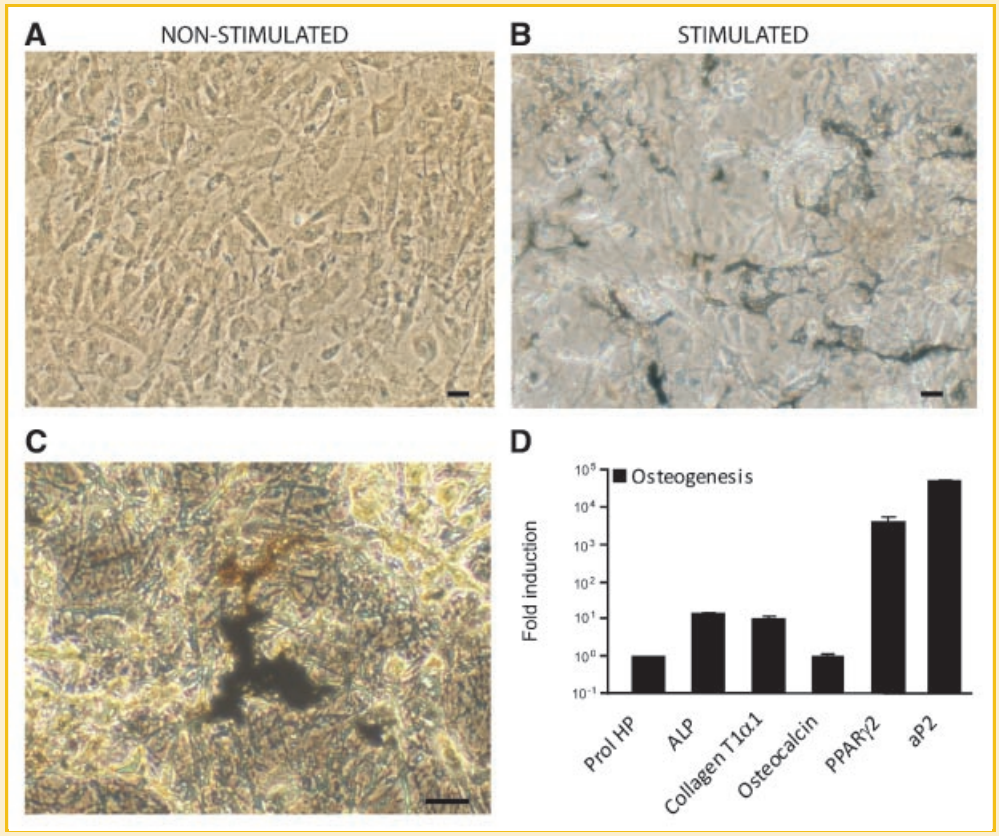

Fig. 6. Activation of osteogenesis. A: Von Kossa staining of non-stimulated MDPCs-HP. B: Detection of black calcium deposits in stimulated MDPCs-HP after Von Kossa staining. C: Higher magnification of calcium deposits visualized by Von Kossa staining. Scale bar = 20 μm (A–C). D: Normalized expression of osteogenic regulatory genes *ALP*, *Collagen Type 1α1*, *Osteocalcin* genes and adipogenic regulatory genes *PPARγ2* and *aP2* relative to proliferating MDPCs-HP as determined by quantitative RT-PCR (black bars). Prol HP: proliferating MDPCs-HP. Bars are mean + SD.

This NCAM16.2 antibody showed only a weak binding to MDPCs suggesting that the MDPC population did not consist of myoblasts. The absence of MHC-II, Flk-1 and CD45 (a pan-haematopoietic marker) confirms the non-haematopoietic origin of the cells [Zhang et al., 2003]. Absence of CD106 expression differs however from that of other previously described mesenchymal stem cell phenotypes [Cao et al., 2003; Kolf et al., 2007; Shetty et al., 2007]. The absence of CD146 expression suggests a lack of pericytes in the MDPC population [Dellavalle et al., 2007]. Similarly, expression of CD44 and MHC-class I suggests that MDPCs are other than MAPC [Jiang et al., 2002; Zeng et al., 2006].

In addition to the absence of *PAX7* gene expression, *MYOD* nor *MYOGENIN* expression was detected (data not shown). *MYF5* mRNA was detected in MDPCs-LP. Although mRNA of muscle intermediate filament protein DESMIN was detected, no protein expression was observed (data not shown). This suggests a non-myogenic orientated muscle cell population. Both *NANOG* and *OCT3/4* expression was detected in the MDPCs. *NANOG* and *OCT3/4* are genes important for the self-renewal capacity and the maintenance of pluripotency [Chambers and Smith, 2004; Kues et al., 2005; Carlin et al., 2006]. NANOG protein was visualized by immunocytochemistry. Its function in adult cells is unknown, but expression has been reported in other adult cells [Kim et al., 2008; Zuba-Surma et al., 2008]. Further, gene transcripts of *OCT3/4* were only detected in later passages (HP). This implies selection for *OCT3/4*-positive cells during culturing, accompanied with a decline in *MYF5*-positive cells. *THY-1* or CD90, which is expressed in mesenchymal stem cells and on myo(fibro)blasts [Fiegel et al., 2004; Rajkumar et al., 2005; Bosch et al., 2006], was additionally detected by RT-PCR in MDPCs-LP and -HP. The transcription factor PAX3 acts downstream of PAX7 [Kassar-Duchossoy et al., 2005; Relaix et al., 2005] and can initiate the myogenic program in early paraxial mesoderm [Darabi et al., 2008]. *PAX3* mRNA expression was observed in MDPCs-LP/HP indicating the mesodermal origin of the cells. Mesoangioblasts have been shown to undergo myogenic commitment in vitro when cocultured with C2C12 myoblasts [Grassi et al., 2004; Sampaolesi et al., 2006]. These cells exhibit a low osteogenic and adipogenic differentiation capacity [Peault et al., 2007]. Furthermore, mesoangioblasts have been suggested to be a source of progenitor cells in muscle [Cossu and Bianco, 2003]. The MDPCs described here seems similar to mesoangioblasts, but lack the expression of Flk-1.

Under appropriate conditions in coculture with C2C12 cells, MDPCs were able to differentiate towards the myogenic lineage. The MDPCs were also cultured in the presence of a variety of growth

factors and cytokines (including of IGF-I/II, EGF, HGF, PDGFs, insulin) to stimulate myogenic differentiation [Dodson et al., 1996; Blanton et al., 2000; Hawke and Garry, 2001; Shi and Garry, 2006]. None of the factors was successful in the induction of myogenic differentiation in a variety of concentrations and combinations (data not shown). However, coculture of the MDPCs with C2C12 myotubes did result in DESMIN positive multinucleated myotubes expressing *MYHC*, albeit not very robust.

Exposure of MDPCs to adipogenic differentiation factors resulted in a selection of differentiating adipogenic progenitor cells showing a significant increase in *PPARγ2* and *aP2* gene expression (normalized against the corresponding reference genes values), the transcriptional regulators of adipogenesis [Iohara et al., 2006; Zou et al., 2008]. Adipogenesis of the MDPCs was confirmed by detection of intracellular lipid droplets in a subset of the cell population. Surprisingly, during osteogenic differentiation of MDPCs, an upregulation of *PPARγ2* and *aP2* was also observed. Osteoblasts and adipocytes are derived from a common mesenchymal progenitor. Therefore, in a pool of osteo–adipo progenitor cells it is possible to make the switch from osteoblast to adipocyte commitment or both [Hasegawa et al., 2008]. *PPARγ2* expression observed during osteogenic differentiation could be caused by the presence of dexamethasone in the osteogenic differentiation medium stimulation *PPARγ2* expression and fat cell formation. This gene has been shown to repress bone regulatory genes like EGR2/Krox20, which regulates osteoblast differentiation and osteocalcin expression [Cui et al., 1997; Akune et al., 2004; Leclerc et al., 2005; Shockley et al., 2007; Zou et al., 2008]. However, after adipogenic stimulation, the expression of both *OSTEOCALCIN* and *COLLAGEN TYPE 1α1* mRNA was remarkably reduced possibly by downregulation of bone regulatory genes, while selectively directing adipogenesis. *ALKALINE PHOSPHATASE* and *COLLAGEN TYPE 1α1* showed an increase in gene transcripts relative to the proliferating state of the MDPCs, after osteogenic stimulation [Hwang et al., 2008; Zou et al., 2008]. The onset of bone differentiation was demonstrated by detection of local calcium deposit in the culture albeit weak indicated by the low level of *OSTEOCALCIN* expression.

The MDPCs exhibited a stable long-term high expansion characteristic. The fates of MDPCs could be triggered, by directing the cells towards a specific mesodermal lineage (myogenic, adipogenic and osteogenic) in culture. In most studies porcine muscle-derived progenitor cells exhibit more robust in vitro myogenesis regulated by MRF expression. These cells are freshly isolated cells, comprising a rich source of muscle progenitor cells with high myogenic potential. Flow cytometric sorting of specific population of muscle progenitor cells also showed the enrichment of myoblasts in the population. Here we show the characterization of long-term expanded culture of muscle-derived progenitor cells. The multipotency of the MDPCs can be due to the heterogeneous nature of the population induced by cell culture expansion, but may also be the result of a multilineage potential of single cells [Tamaki et al., 2007]. Derivation of clonal cell lines from the MDPCs will therefore be of our next interest. Overall, these findings emphasize the function of adult tissue as a source of progenitor cells able to contribute to the repair and regeneration of several tissue types. Porcine stem cells can be useful for studying tissue regeneration and can serve as an excellent model for human medical applications.

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