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MMP-2, *TIMP-2*, *TAZ* AND *MEF2a* TRANSCRIPT EXPRESSION IN OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION OF PORCINE MESENCHYMAL STEM CELLS*

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Abstract

Mesenchymal stem cell (MSC) differentiation is regulated intrinsically by transcription factors and extrinsically by the extracellular matrix. We tested whether matrix metalloproteinase-2 (*MMP-2*) and its inhibitor *TIMP-2*, *MEF2a* and *TAZ* transcription factors are involved in porcine MSC differentiation towards adipocytes and osteocytes. Flow cytometry and immunofluorescence were used to investigate the expression levels of multipotent cell surface markers CD73 and CD105. Real-time PCR was performed to detect the osteogenic- and adipogenic-specific markers, osteocalcin and aP2, respectively, and to estimate the *MMP-2*, *TIMP-2*, *MEF2a* and *TAZ* transcript expression levels in three groups of cell, i.e., undifferentiated MSCs, adipocytes (A) and osteocytes (O). We showed that at the transcript level, the differentiation of MSCs towards adipocyte fate may involve *MMP-2*, *TIMP-2* and *TAZ*. We also show that the differentiation of MSCs toward osteocyte fate may involve *TIMP-2*, *MEF2a* and *TAZ*. Our research provides preliminary data on the possible role of the *MMP-2*, *TIMP-2* and *TAZ* transcripts in adipogenic differentiation and of the *TIMP-2, TAZ* and *MEF2a* transcripts in the osteogenic differentiation of porcine MSCs. We report for the first time the possible involvement of *MEF2a* in the osteogenesis of porcine MSCs. Our work may provide additional evidence for the MMP-independent function of *TIMP-2* during osteogenesis.

Key words: mesenchymal stem cells, *TIMP-2*, *TAZ*, *MEF2a*, transcripts, osteogenic, adipogenic, differentiation

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Clinical trials using human mesenchymal stromal stem cells as therapy in several diseases are ongoing and include their use in the treatment of myocardial injury (Jackson et al., 2001), sepsis (Gonzalez-Rey et al., 2009), renal ischemia (Lin et al., 2003) and diabetes (Itakura et al., 2007; Li et al., 2012). Mesenchymal stem cell (MSCs) may also be used as a vehicle for gene therapy and drug delivery due to their anti-inflammatory, anti-proliferative, angiogenic and immunomodulatory properties (Li et al., 2012; Rho et al., 2009). MSC-derived osteoblasts/osteocytes have also been used in cell-based skeletal therapies (Prockop, 1997; Chang et al., 2004 a). Establishing the clinical application of MSC-derived therapeutic cells necessitates that MSCs can be effectively and safely differentiated towards a desired cell type. Due to the low efficiency of MSC differentiation into specific cell types (e.g., osteocytes, adipocytes, myoblasts or endothelial cells), it would be beneficial to identify the genes responsible for controlling their cell fate choice to enhance the differentiation protocols (Rosland et al., 2009).

Mesenchymal stem cell (MSC) differentiation is regulated intrinsically by transcription factors and extrinsically by the extracellular matrix (Philip et al., 2005; D'Amour et al., 2006; Daley et al., 2008; Page-McCaw et al., 2007). This interaction facilitates the stepwise stages of stem cell maturation into the desired somatic cell. *MMP-2*, also called gelatinase-A, is one member of a family of multidomain zinc endopeptidases called matrix metalloproteinase proteins (MMPs), which are digestive proteolytic enzymes of extracellular matrix components and several other cell surface molecules (Vu and Werb, 2000; Visse and Nagase, 2003). The main role of MMPs is to degrade the extracellular matrix proteins collagen, lamin, proteoglycan and fibronectin to facilitate cell migration (Vu and Werb, 2000; Visse and Nagase, 2003). MMPs control angiogenesis, embryogenesis, tissue remodelling and apoptosis and are involved in the pathogenesis of many diseases, including cancer, cardiovascular disease and Alzheimer's disease (Łukaszewicz et al., 2008). In mice, the loss of *MMP-2* function leads to bone development abnormalities which indicates the importance of this protein in osteogenesis. *MMP-2* is also responsible for the degradation of type IV collagen, which is the main component of the vascular basement membrane (Lipka and Boratyński, 2008).

MMP function is regulated by a family of inhibitors called tissue inhibitors of metalloproteinases (TIMPs), of which there are four: *TIMP-1, TIMP-2, TIMP-3* and *TIMP-4* (Brew et al., 2000). Exciting reports on a high transcript expression level of several MMPs and their inhibitors in MSCs (i.e., *MMP-1, -2* and *-19* and *TIMP-1, -2* and *-3*) (Panepucci et al., 2004; Cronwright et al., 2005) have formed the basis for the hypothesis that these factors have key functions in the differentiation of MSCs (Mannello, 2006; Mannello et al., 2006). However, it is not clear how these factors work: structural proteins of the extracellular matrix, cytokines and growth factors have the potential to interact with MMPs and may thus induce the differentiation of MSCs under the influence of the microenvironment. Conversely, active growth factors and chemokines can be activated through the proteolytic action of MMPs and through differentiation, which promotes the colonisation of the specified region (Lisignoli et al., 2006). Son et al. (2006) showed that MSCs secrete *MMP-2*, and De Becker et al. (2007) discovered that *MMP-2* is functionally involved in MSC migration (Son et

al., 2006; De Becker et al., 2007). Kasper et al. (2007) showed that MSC behaviour is controlled by MMP activity, which in turn, is regulated by the mechanical stimulation of cells; therefore, the MMP/TIMP balance seems to play an essential role in transferring mechanical signals to MSCs (Kasper et al., 2007). The involvement of both MMPs and TIMPs in controlling MSC mobilisation, homing properties and differentiation into various cell types is presented elsewhere (Cronwright et al., 2005; Mannello, 2006; Kasper et al., 2007).

The latest research of Hoshiba et al. (2012) showed that the ECM plays an important role in MSC differentiation by regulating the expression of various transcription factors that control the balance of osteogenesis and adipogenesis (Hoshiba et al., 2012). The transcription factor *Runx2* drives MSC differentiation into osteoblasts (Komori et al., 1997). PPARγ (peroxisome proliferator-activated receptor gamma), which is a ligand-activated transcription factor, is critical for adipocyte differentiation (Rosen et al., 1999; Hu et al., 1995; Lecka-Czernik et al., 2002). The differentiation of each lineage is mutually exclusive and transcriptionally controlled by *TAZ* (transcriptional coactivator with a PDZ-binding motif) (Hong et al., 2005). As proven in murine (Hong et al., 2005, 2009) and human MSCs (Hoshiba et al., 2012), *TAZ* is a *Runx2* transcriptional coactivator for osteocalcin expression and directly inhibits the ability of *PPARγ* to stimulate transcription of the endogenous fatty-acid-binding protein aP2 (Hoshiba et al., 2012; Hong et al., 2005, 2009). Therefore, *TAZ* is a transcriptional modifier of MSC differentiation that promotes osteoblast differentiation (Li et al., 2007; Cho et al., 2010) while simultaneously inhibiting adipocyte differentiation. Consistent with this finding, bone marrow-derived MSCs depleted of *TAZ* show decreased osteogenesis and increased adipogenesis. *TAZ* tunes the balance between osteoblast and adipocyte development (Hong et al., 2009). In a study performed using zebrafish, *TAZ*-depleted embryos survived for up to 8 days after fertilisation and had developmental abnormalities (Hong et al., 2009).

In vertebrates, the *MEF2* family of transcription factors are encoded by four genes: *MEF2a*, *MEF2b*, *MEF2c* and *MEF2d* (Shore and Sharrocks, 1995). The expression of *MEF2* proteins in many cell types (e.g., neurons, chondrocytes, muscle) occurs concomitantly with the activation of their differentiation programs, and the balance between the transcription-activating function of *MEF2* and the repressive function of histone deacetylases dictates the development of these cells (Arnold et al., 2007; Chang et al., 2004 b, Potthoff and Olson, 2007). It is also thought that *MEF2* will be found to regulate the differentiation of additional cell types, in which its functions have yet to be investigated (Potthoff and Olson, 2007). Therefore, we decided to analyse the expression of *MEF2a* to determine whether it plays a role in the differentiation of porcine MSCs into adipocytes and osteocytes. The pig as an animal model for the study of stem cell therapy or regenerative medicine has several advantages in comparison with conventional rodent models which are presented in our earlier review paper (Opiela and Samiec, 2013).

In this study, we verified the hypothesis that matrix metalloproteinase-2 (*MMP-2*), its inhibitor *TIMP-2* and the transcription factors *TAZ* and *MEF2a* are involved in the differentiation of porcine MSCs into adipocytes and osteocytes.

Material and methods

Animal care and permission to conduct research on animals

Three outbred Polish Large White (PLW) pigs of either sex, weighing 15–20 kg each, were maintained under conventional conditions in the pigsty of the Department of Biotechnology of Animal Reproduction in Balice, Poland. The veterinary care was provided. All animal procedures were approved by the Local Animal Care Ethics Committee No. II in Kraków.

Unless otherwise indicated, all chemicals and media were purchased from Sigma (Poznań, Poland).

Isolation and *in vitro* culture of porcine mesenchymal stem cells

Porcine MSCs (pMSCs) were obtained from the bone marrow of PLW pigs. The bone marrow was aspirated under general anaesthesia. Bone marrow samples were resuspended in phosphate buffered saline (PBS; Biomed, Lublin, Poland) (1:1), and 6 ml of this solution was layered over 3 ml of Ficoll-Paque (StemCell Technologies, USA) and centrifuged. The mononuclear cell fraction was collected and resuspended in PBS and centrifuged. This procedure was repeated twice. The pelleted cells were resuspended in pMSC medium (low-glucose-DMEM) supplemented with 10% foetal bovine serum (FBS)/1% antibiotics/1% Glutamax (Invitrogen, USA) in 75 cm^2 tissue culture flasks (T75) in 17 ml of medium. The next day, the medium was changed, and the adherent cells were allowed to form colonies until reaching 80% confluence. Colonies were harvested using 0,05% trypsin/0.25% EDTA treatment. For the pMSC passages, 0.25 to 0.5×10^6 cells were seeded into one T75 flask (Ezzelarab et al., 2011; Opiela et al., 2013 b).

Expression of MSC surface markers

The expression of surface markers in MSC cultures for phenotypic characterisation was performed by flow cytometry analysis and whole mount immunofluorescence.

Flow cytometry

Cell cultures were trypsinised, washed in PBS and fixed with 2% formaldehyde solution for 3 min. Nonspecific binding was prevented by incubating the cells in 5% BSA for 15–30 min. Approximately 10^5–10^6 cells were incubated with 10 µg/ml of the primary antibodies or isotype controls for at least 30 min at room temperature. The following antibodies were used: PE MouseAnti-Human CD73 (Cat# 550257) and PerCP-Cy™5.5 Mouse anti-Human CD105 (Endoglin) (Cat# 560819) both obtained from BD Pharmingen™ (USA). After washing in PBS with 0.2% Tween 20, cells were analysed using a DAKO Galaxy flow cytometer (orange and red fluorescence). The WinList 7.0 software (Verity SH) was used for further calculations.

Whole mount immunofluorescence

The expression of surface markers in MSC cultures for phenotypic characterisation was performed by immunofluorescence staining. Cells were cultured until

80% confluence was reached, washed in PBS and fixed with 1% formaldehyde solution for at least 24 h in 4ºC. Fixed cells were washed three times in 0.1% TBS-T (Tris-Buffered Saline with 0.1% Tween 20) buffer. Then MSC were incubated with 0.2% Triton X-100 solution for 15 minutes. Nonspecific binding was prevented by incubating the cells in 3% BSA for 30 min. MSC were incubated with primary antibodies against CD73 (dilution 1:22 goat polyclonal IgG, sc-14682, Santa Cruz Biotechnology, Dallas, USA) and CD105 (dilution 1:50 goat polyclonal IgG, sc-19793, Santa Cruz Biotechnology) at 4°C overnight. After three washes in 0.1% TBS-T, MSC were incubated with donkey anti-goat IgG-FITC antibody (sc-2024, Santa Cruz Biotechnology) at dilution of 1:100 at room temperature for 60 minutes in the dark. MSC were then washed three times in TBS-T and counterstained with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, USA) at room temperature for five minutes in the dark. Subsequently, cells were observed using a fluorescence microscope (Nikon Eclipse Ti-U) and photographed using a digital camera (Nikon Digital Sight ds-fi1c).

***In vitro* differentiation of pMSCs into adipocytes and osteocytes**

Adipogenic differentiation medium (ADM) comprised of the pMSC medium described above was supplemented with 0.5 µM dexamethazon (D2915, Sigma), 0.5 µM isobutylmethylxanthine (I5879, Sigma) and 50 µl indomethacin (I7378, Sigma), according to Mao and Marion (2009). For osteogenic differentiation, osteogenic medium (ODM) was used (pMSC medium with 10 nM dexamethasone (D2915, Sigma), 20 mM β-glycerophosphate (G9891, Sigma) and 50 µM L-ascorbic acid 2-phosphate (A8960, Sigma) (Mao and Marion, 2009; Opiela et al., 2013 a, b). Both media were changed every 3 days. After 4 weeks, differentiation was confirmed using qPCR for specific markers.

Real-time PCR quantification

Total RNA was isolated as described previously (Chomczynski, 1993). The relative expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalise the marker gene expression levels in each sample. The KAPA SYBR FAST One-Step qRT-PCR Master Mix Kit Universal (Kapa Biosystems, USA) was used to perform a relative quantification of gene expression. Each PCR reaction (total volume of 20 µl) consisted of 1 µl of total RNA (100 ng) and 19 µl of reaction mixture, which consisted of 10 µl KAPA SYBR FAST qPCR Master Mix (2X), 0.5 µl of each 10 µM forward and reverse primer (Table 1), 0.4 µl of KAPA RT Mix (50X) and 7.6 µl RNase-free water. The qRT-PCR reaction was conducted under the following conditions: 5 min at 42°C (for the first-strand synthesis); 5 min at 95°C; 40 cycles of 10 s at 95°C for denaturing, 20 s at 58°C for annealing and 20 s at 72°C for extension. Experiments were performed using a Mastercycler Realplex (Eppendorf, UK Limited, Cambridge). GAPDH was used as an endogenous standard. The results for the individual target genes were normalised according to the relative concentration of the endogenous standard. To minimise the error associated with the differences in the quantity of applied template, the analyses were run in triplicates, and the results were averaged.

Quantitative PCR (qPCR) of adipocytes and osteocytes markers

The results for adipocytes and osteocytes were normalized with those obtained from undifferentiated MSCs. The primers used were pig adipocyte Protein 2 (aP2)5'-GGCCAAACCCAACCTGA-3' (forward) and 5'-GGGCGCCTCCATCTAAG-3' (reverse); pig osteocalcin 5'-TCAACCCCGACTGCGACGAG-3' (forward); and 5'-TTGGAGCAGCTGGGATGATGG-3' (reverse). GAPDH was used as an endogenous standard 5'-CTGCCCCTTCTGCTGATGC-3' (forward) and 5'-GACAACTTCGGCATCGTGGA-3'(reverse).

Quantitative PCR (qPCR) of *MMP-2*, *MEF2a*, *TAZ*, TIMP transcripts expression in MSCs, adipocytes and osteocytes

The results for *MMP-2*, *MEF2a*, *TAZ* and *TIMP-2* in adipocytes, osteocytes and undifferentiated pMSCs were normalised with control, undifferentiated MSCs collected at day 6. The primers are presented in Table 1.

Table 1. Quantitative RT-PCR primer sequence

Gene	Sequence of primers (5'→3')	Product size (bp)
TIMP-2	F1 - TCAAGGGACCTGACAAGGAC	150
	R1 - AGTCACAGAGCGTGATGTGC	
MEF2A	F1 - AGATCTTCGCGTTGTCATCC	142
	R1 - GGTGGTCACAGACACCACTG	
MMP-2	F1 - GTGGTGCGTGTGAAGTATGG	150
	R1 - GCCATCCTTGTCGAAGTTGT	
TAZ	F1 - ACGGTCTCCAATCACCAGTC	140
	R1 - TGGGAGTGTAGCTCCTTGGT	
GAPDH	F1 - CTGCCCCTTCTGCTGATGC	151
	R1 - GACAACTTCGGCATCGTGGA	

Determination of *MMP2* protein expression

Protein separation was performed by SDS-PAGE. The denatured samples of undifferentiated MSCs, adipocytes and osteocytes were applied to the wells in 12% polyacrylamide gel. Electrophoretic separation was carried out using the Laemmli system (25 mM Tris, 192 mM glycine, 0.1% SDS) (Laemmli, 1970). Western-blotting was performed as previously described (Opiela et al., 2013 a). The blocked membrane was incubated for 10 min at room temperature with the primary antibody *MMP-2* (mouse, monoclonal ab80737, Abcam) at a concentration of 1 µl/ml, diluted in blocking buffer. After washing the membrane four times in 0.1% Tween 20 in Tris-buffered saline (TBS-T 0.1%) the membrane was re-incubated for 10 min at room temperature with the secondary antibody (goat anti-mouse IgG-HRP, sc-2031 Santa Cruz Biotechnology, Inc, USA) conjugated to horseradish peroxidase

(HRP) at a concentration of 0.07 µl/ml along with antibiotin antibody at a concentration of 0.06 µl/ml (Biotinylated Protein Ladder Detection Pack, Cell Signaling Technology). The membrane was developed using West Femto (Thermo Scientific, USA), according to the manufacturer's protocol. After a 5-min incubation at room temperature, the membrane was exposed to X-ray film (Thermo Scientific, USA). The films were developed manually using developer and fixer (Kodak, Poland), according to the manufacturer's protocol. Two types of markers were used: Prestained SDS-PAGE Standard Low Range (Bio-Rad, Warszawa, Poland) visible on the gel and membrane and Biotinylated Protein Ladder Detection Pack (Cell Signaling Technology) visible on an X-ray plate. The films were scanned using the x-ray equipment from Fluor-S-Multiimager (Bio-Rad). The densitometric signal for each of bands was measured using the Quantity One software (Bio-Rad). The membrane was stripped of antibodies by incubating it in a stripping buffer (100 mM 2-mercaptoethanol, 20% (w /v) SDS, 62.5 mM Tris-HCl; pH 6.7) at 60°C for 60 min. After 3 extensive washings and further blocking, the membrane was incubated again with the anti-actin antibody (Santa Cruz Biotechnology, Inc., USA) at a concentration of 0.67 µl/ml diluted in blocking buffer. The secondary antibody (Santa Cruz Biotechnology, Inc, USA) conjugated to HRP was used at a concentration of 0.13 µl/ml. To visualise the bands, the protocol outlined above was performed.

Experimental design

We performed *in vitro* culture in 3 groups of cells: undifferentiated MSCs that were cultured in standard medium without supplements for 1 week and MSCs subjected to either adipocyte differentiation or osteocyte differentiation protocols. The RNA for real-time PCR was isolated for undifferentiated MSC controls after 1 week in culture and after 4 weeks of differentiation for adipocytes and osteocytes. When cells reached 80% confluence, the culture was terminated. Flow cytometry (FC) and immunofluorescence (IF) were used to investigate the pluripotent characteristics of pMSCs by estimating the CD73 and CD105 cell marker expression levels. Real-time PCR was run to detect osteogenic- and adipogenic-specific markers, osteocalcin and aP2, respectively, and to estimate the *MMP-2*, *MEF2a*, *TIMP-2* and *TAZ* transcripts expression levels in all three groups of cells. We also analysed the MMP-2 protein expression level using Western blot.

Statistical analysis

Each experiment was performed in triplicate to confirm the trend in our results. pMSCs were obtained from three different sources on each occasion. Statistical significance was obtained from the data obtained in 6 experiments that were performed in triplicate for MSCs and Os and from 4 experiments that were performed in triplicate for As. Differences in the *MMP-2*, *MEF2a*, *TIMP-2* and *TAZ* transcript levels and *MMP-2* protein expression was assessed using ANOVA followed by Tukey's post hoc test. Differences with a probability value less than 0.05 were considered significant.

Results

Surface marker expression of MSCs

Immunophenotyping showed that 64% of the analysed MSCs expressed CD105 and that nearly 26.7% expressed CD 73 (Figure 1). Immunofluorescence of CD105 and CD73 showed that nearly all cells expressed CD105 and 96–98% of cells expressed CD73 (Table 2 and Figures 2 and 3).

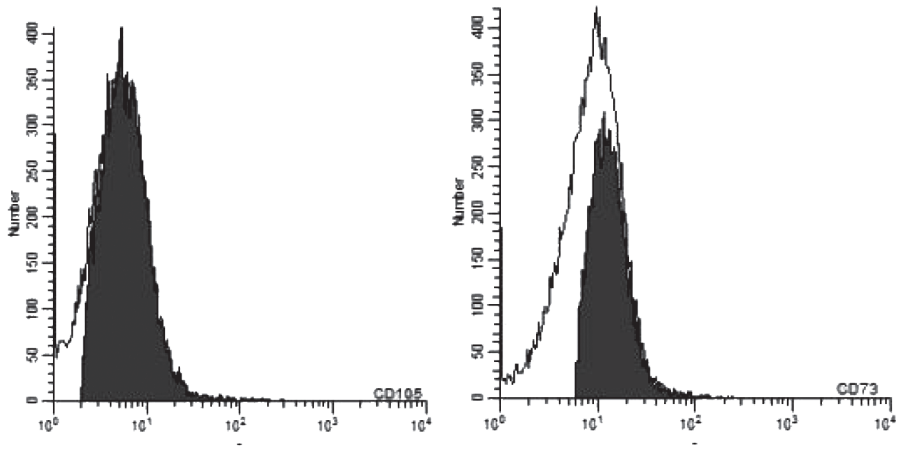

Figure 1. Study of typical surface marker expression in MSCs using flow cytometry. Subtracted histograms (CD73 – control sample), (CD105 – control sample)

Table 2. The expression of CD105 and CD73 estimated by immunofluorescence staining of porcine MSCs

MSC			
replication	DAPI	CD105	% positive cells
1	100	99	99
2	147	144	97.9
3	123	123	100
	DAPI	CD73	
1	160	155	96.8
2	109	107	98.1
3	133	129	96.9

Ability of MSCs to differentiate towards adipocytes and osteocytes

Real-time PCR of both the osteocalcin transcripts of osteocytes and the *aP2* transcripts in adipocytes was used to confirm their successful differentiation. The relative expression level of *aP2* in adipocytes was 52.2, and no expression was detected in osteocytes. The relative expression level of osteocalcin in osteocytes was 77.1, and in adipocytes, it was 2.5. Undifferentiated MSCs served as controls (reference group).

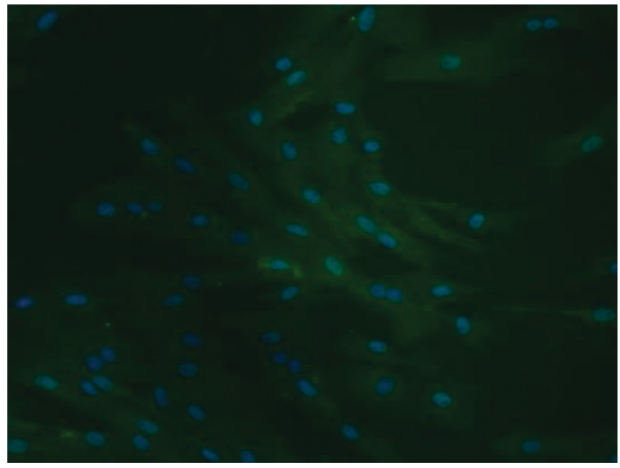

Figure 2. The cell surface marker CD105 of porcine BMSCs was examined by immunofluorescence staining. Blue fluorescence represented nucleus, and green fluorescence represented the positive expression of CD105

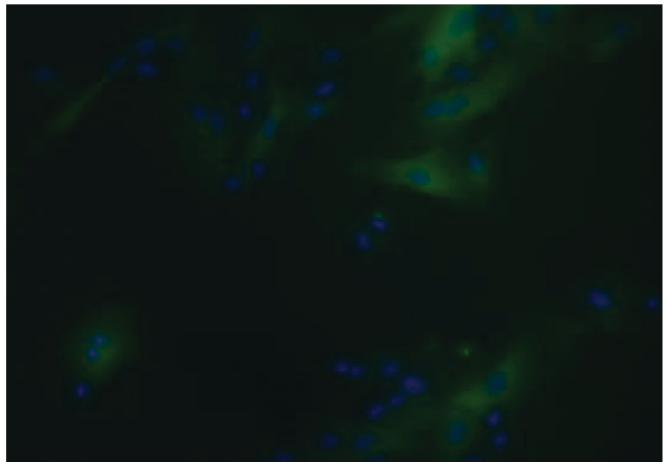

Figure 3. The cell surface marker CD73 of porcine BMSCs was examined by immunofluorescence staining. Blue fluorescence represented nucleus, and green fluorescence represented the positive expression of CD73

Transcript expression levels of *MMP-2*, *TAZ*, *TIMP* and *MEF2a* in MSCs, adipocytes and osteocytes

Differences in the transcript expression levels were analysed for MSCs, adipocytes (A) and osteocytes (O) as presented in Table 3. We showed that at the transcript level, the differentiation of MSCs towards adipocytes involves *TIMP-2* and *TAZ* because a significant upregulation of expression was observed in adipocytes compared with MSCs ($P<0.05$). The differentiation of MSCs towards osteocytes may involve *TIMP-2* and *MEF2a* because a highly significant upregulation of these genes was observed ($P<0.001$ and $P<0.01$, respectively); this differentiation also may involve

TAZ because a significant upregulation (P<0.05) was observed between osteocytes and MSCs. We report for the first time the possible involvement of *MEF2a* in osteogenesis of porcine MSC because a significant increase in its transcript level was observed in differentiated cells.

Table 3. Transcript expression levels of *MEF2A*, *MMP-2*, *TAZ* and *TIMP* in adipocytes, osteocytes and MSCs (mean ± SD)

Sample	*MEF2a*	*MMP-2*	*TAZ*	*TIMP-2*
Reference sample	1	1	1	1
MSC	1.22±0.42 A	1.33±0.43 a	0.94±0.29 a	0.93±0.2 aC
O	1.66±0.46 B	1.25±0.49	1.25±0.26 b	2.2±0.7 AD
A	1.51±0.28	0.82±0.49 b	1.29±0.43 b	1.48±0.42 bB

(a, b – P<0.05; A, B – P<0.01; C, D – P<0.001), MSC and O – 6 experiments in triplicate, A – 4 experiments in triplicate.

Protein expression of *MMP-2*

The differential semi-quantitative *MMP-2* protein expression level in three cell types was analysed: undifferentiated MSCs, adipocytes and osteocytes. We showed that there is no significant difference in the *MMP-2* protein expression level among the three analysed cell types (Figures 4, 5).

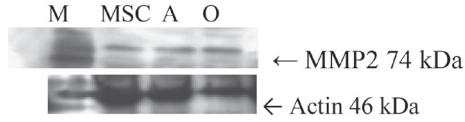

Figure 4. Image of *MMP2* and actin protein bands on X-ray film after Western blot. M – marker; MSC – control; A – adipocytes; O – osteocytes

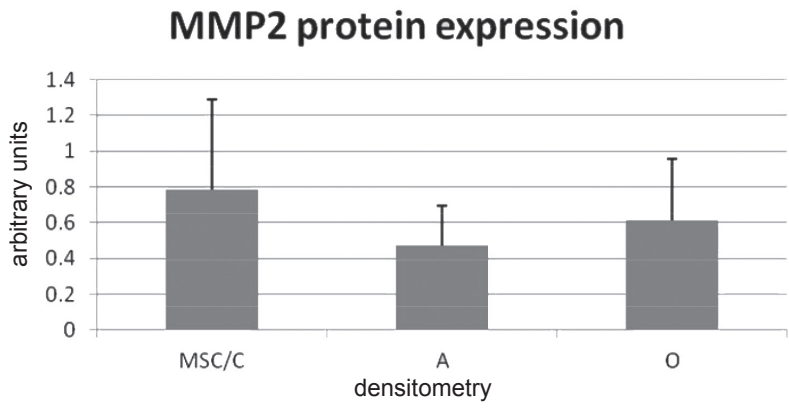

Figure 5. Analysis of *MMP2* protein expression in MSCs, adipocytes and osteocytes by Western blot. The bars in the graph are the mean value ± SEM of the calculated relative densitometric signals of the control signal from three independent experiments

Discussion

Because of ethical and logistical barriers, human stem cells cannot be used in clinical trials. Therefore, pigs are an attractive model for preclinical studies and thus offer an alternative path for improving clinical medicine. The goal of this study was to provide a transcriptomic estimation of the genes that are hypothesised to be involved in porcine MSC differentiation into osteocytes and adipocytes to increase the efficacy of successful MSC differentiation into these desired cell types.

In our experiment, the MSCs were isolated from porcine bone marrow and selected based on plastic adherence. MSCs from animal origin have been defined as cells that adhere to a plastic surface and are able to undergo trilineage differentiation (Li et al., 2012; Dominici et al., 2006). The condition regarding cell surface antigen expression is not obligatory in case of animal MSCs. Moreover, unfortunately, the low availability of specific antibodies for large animals, including pigs limits their characterisation (Kolf et al., 2007). In the cases of CD73 and CD105, there is a limited cross-reactivity of anti-human antibodies with animal cells, an issue discussed by Boxall and Jones (2012).

Presently, it is accepted that pMSCs do express the markers CD29, CD44, CD49, CD90, CD73, CD105 and SLA-I and do not express the markers CD11b, CD14, CD31, CD34, CD45, CD117, CD133 and SLA-II (Li et al., 2012). Regarding the expression of CD105 our results are clear and obvious as both analysis FC and IF showed positive expression of this marker in obtained MSC. However, the results obtained by FC showed that expression of CD73 can be estimated as negative. This result was not confirmed by immunofluorescence as we showed positive expression of this marker in analysed MSC cells. The antibody against CD73 was different in both analyses and in our opinion this is responsible for contradictory results.

We began our studies to test the hypothesis that differences in the expression levels of *MMP-2* and *TIMP-2* exist between differentiated and undifferentiated MSCs. The *MMP-2* transcript level was even significantly lower ($P<0.05$) in adipocytes compared to control, undifferentiated MSCs. We did not observe any differences in *MMP-2* expression between MSCs and osteogenic cells but noted significantly high differences ($P<0.001$) in the *TIMP-2* transcript expression levels. Nevertheless, our results are in agreement with the newest understanding of TIMP function. Based on our understanding of their role in apoptosis regulation, growth, migration, angiogenesis and remodelling, apart from being endogenous inhibitors of MMPs, TIMPs also display MMP-independent function, which appears to also be true for differentiation. Because we observed differences in *TIMP-2* but not *MMP-2* expression during osteogenic differentiation, our results may support this new model of TIMP function. Moreover, Lovelock et al. (2005) observed that TIMPs induce fibroblast proliferation, migration and differentiation into myofibroblast and that this effect was MMP-independent, as shown through the use of MMP inhibitors (Lovelock et al., 2005). Therefore, it is believed that TIMPs act by stimulating cardiac fibroblast proliferation and phenotypic differentiation into myofibroblasts and that they contribute to the remodelling of the cardiac ECM and wound healing (Vanhoutte and Heymans, 2010). It is possible that TIMPs' MMP-independent differentiation function may be

a result of their 'cytokine-like' properties. A comprehensive review of the MMP-independent biological properties of the four TIMP family members is presented elsewhere (Vanhoutte and Heymans, 2010).

Nevertheless, we are aware that the lack of differences in the transcript expression levels of *MMP-2* between osteogenic and undifferentiated cells does not clearly mean that *MMP-2* has no role in the differentiation process of these cells. Although we obtained the same results at the protein level through a semi-quantitative analysis of MMP-2 protein expression based on a Western blot analysis of densitometric band measurements, a more rigorous quantification of *MMP-2* and *TIMP-2* activity using zymography would provide more definitive proof for the involvement of these factors in the differentiation process.

As previously stated, *TAZ* coactivates Runx2 and represses PPARγ; *TAZ* is thus a coactivator of bone development and a repressor of adipocyte differentiation (Hong et al., 2005). MSCs that were depleted of *TAZ* showed increased adipogenesis, and cells containing shRNA against *TAZ* demonstrated enhanced Oil Red O staining (Hong et al., 2005). We showed a significant increase in the *TAZ* transcript levels in osteocytes and adipocytes compared with control MSCs, and these results are in agreement with previous reports (Hong et al., 2005). Scheideler et al. (2008) studied the transcriptional regulation of the adipocyte/osteoblast balance in human multipotent adipose-derived stem (hMADS) cell-derived cell line to determine whether the global expression profiles were different between the stages before and after lineage commitment. They extracted differentially expressed transcripts from time points 5 to 6 specifically in one differentiation pathway and found that *TAZ* derepressed at the time of commitment in osteoblastogenesis but had a delay in adipogenesis after the commitment. This repression is mediated by miRNAs and is a predominant early mechanism before final cell commitment (Scheideler et al., 2008). Moreover, according to recent reports, PPAR*γ,* which is a master adipogenic factor, is regulated by factors other than *TAZ,* including myocyte enhancer factor-2 interacting transcriptional repressor (MITR) (Chen et al., 2011), BMP2 (Takada et al., 2010), cytokines and noncanonical Wnts (Takada et al., 2012).

MEF2 plays a central role in the activation of the genetic programs that control cell differentiation, proliferation, morphogenesis, survival and apoptosis (Potthoff and Olson, 2007). *MEF2*, in combination with additional transcription factors, drives and amplifies the myogenic differentiation program (Wang et al., 2001) and is required for proper muscle development (Shen et al., 2006). *MEF2* is also a regulator of the other core cardiac transcription factors that are required for cardiomyocyte differentiation, as *Mef2a*-null mice exhibit perinatal lethality from cardiovascular defects (Naya et al., 2002). *MEF2* plays a crucial role in neural crest development (Verzi et al., 2007; Miler et al., 2007). During development, *MEF2* may also be involved in angiogenesis by promoting cell survival (Hayashi et al., 2004) and vascular remodelling (Chang et al., 2006). The *MEF2* protein regulates dendrite morphogenesis and the differentiation of post-synaptic structures (Shalizi et al., 2006). Interestingly, *MEF2* appears to play a role in synaptic plasticity, suggesting an important role for it in learning and memory (Potthoff and Olson, 2007). Our results would extend this long list of *MEF2* roles because we observed significant differences between

MSCs and osteocytes (P<0.01). We report for the first time the possible involvement of *MEF2a* in the osteogenesis of porcine MSCs because a significant increase in its transcript level was observed in differentiated cells. However, considering the broad range of *MEF2a* roles, we also assume that the elevated *MEF2a* expression in osteocytes may be, in part, a result of the proliferation or survival or apoptosis transcriptional programs. Our previous research (Opiela et al., 2013) analysed the expression of proapoptotic protein Bad in MSC, A and O and found a lower expression of Bad in differentiated cells compared with control MSCs. Therefore, we exclude the possible role of *MEF2a* in apoptosis in these cells.

Conclusions

In conclusion, the therapeutic use of MSCs could benefit from a better understanding of MSC differentiation mechanisms that allow for the optimal transformation of these cells. Our research provides preliminary data on the role of *MMP-2*, *TIMP-2* and *TAZ* in adipogenic differentiation and *TIMP-2, TAZ* and *MEF2A* in osteogenic differentiation of porcine MSCs. We report for the first time the possible involvement of *MEF2a* in osteogenesis in porcine MSCs because a significant increase in its transcript level was observed in differentiated cells. Our work may also provide further evidence for an MMP-independent function of *TIMP-2* because no difference in *MMP-2* expression was detected between osteogenic and undifferentiated cells. Our result warrants further research to elucidate the differentiation molecular pathway and the involvement of *MEF2a*, *TAZ*, *MMP-2* and *TIMP-2*.

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Conflict of interest statement

The authors declare that they have no competing interests.

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