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THE QUALITY OF PORCINE MESENCHYMAL STEM CELLS AND THEIR OSTEO- AND ADIPOGENIC CELL DERIVATIVES – THE LEVEL OF PROAPOPTOTIC BAD PROTEIN EXPRESSION*

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Abstract

The aim of the research was to evaluate the quality of porcine mesenchymal stem cells (MSCs) and MSC-derived osteoblasts/osteocytes (bone cells) and adipocytes (fat cells). This evaluation was performed on the basis of molecular analysis for proapoptotic BAD protein expression. MSCs isolated from the pig bone marrow were cultured *in vitro* for five weeks in three types of culture media: differentiating towards the osteoblasts/osteocytes (O) and adipocytes (A) and non-differentiating, control medium (C). In all groups of cells, the relative extent of BAD protein expression was estimated by western blotting. Significant differences in the posttranslational abundance of BAD proteins were noted between MSCs differentiated into the osteogenic and the adipogenic cell lineages ($P<0.05$). Summarizing the results, we conclude that posttranslational level of BAD protein expression can be used as a reliable marker for assessing the quality of both MSCs and their cell derivatives. Interestingly, the semi-quantitative profile of BAD protein expression in differentiated cells turned out to be lower than that observed in undifferentiated cells, demonstrating that the culture conditions used for pro-osteogenic or pro-adipogenic cellular transformation did not affect negatively the quality of MSCs.

Key words: BAD, protein, expression, MSCs, adipocytes, osteoblasts/osteocytes

Mesenchymal stem cells isolated from adult tissue, and cultured under appropriate conditions with the specific chemical compounds have the ability to differentiate in the bone, cartilage, adipose tissue and skeletal muscle (Caplan, 1991; Dominici et al., 2006). The mesenchymal stem cells brought high hopes for regenerative medicine due to participation in the process of damaged tissues or organs repair. This can

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facilitate and accelerate both the reduction of scarring after myocardial infarction and the restoration of normal cardiac contractile function (Quevedo et al., 2009). Moreover, adult bone marrow derived MSCs have been extensively investigated in the setting of arthritic disease and focal cartilage defects (Coleman et al., 2013). In addition, MSCs have also been successfully used to overcome the effects of osteoporosis and broken bone repair (Ratajczak and Kucia, 2005; Urbaniak-Kujda et al., 2005). In some of these treatments the non-differentiated MSCs are used while in others before administering the cells into the patient they must be differentiated towards the required cell type. Unfortunately, the *in vitro* culture conditions as well as differentiation can adversely affect the quality of cells. Therefore, an important aspect of the study is to assess the quality of a given cell after differentiation. Also, we should keep in mind that the process of differentiation is not as efficient as we might expect. The efficacy of cells subjected to differentiation to undergo desired direction of transformation is low (Cai et al., 2011; Alfaro et al., 2010). This means that there is still much to discover to elucidate the mechanisms underlying this process. The situation is complicated by the fact that there are differences in the expression of typical MSC markers between the species as well as within the same species between the lines (Bieback et al., 2012). These differences are most probably caused by different protocols for MSCs isolation and expansion as well as *in vitro* culture (Bieback et al., 2012). Taking into consideration all the above-mentioned reasons, it is justified to monitor MSCs quality in varied points of their *in vitro* culture. Assessment of the level of apoptosis as the quality marker of cells, oocytes and embryos is the generally accepted methodological approach (Opiela et al., 2008 a, b). Apoptosis is a genetically programmed, active process of cell death associated with the activation of many genes which requires energy input. It allows maintaining a balance between the processes of cell proliferation and death. It also ensures proper tissue homeostasis and proper development and growth of organs, embryogenesis, differentiation, and removal of cells (damaged, bearing mutations, infected and abnormal) (Ashkenazi and Dixit, 1998). Apoptosis is composed of three phases: excitation, execution and destruction (Kidd et al., 2000). Programmed cell death is mostly initiated in response to ligand binding to receptors on the surface of dying cells, which causes the so-called apoptosis activation by initiator caspases (Huppertz et al., 1999). Execution phase is controlled by proteins that can activate, block or delay apoptosis by modulating caspase activity. These proteins belong to the Bcl-2 protein family and have pro- and anti-apoptotic activity. The overabundance of proapoptotic proteins in relation to the quantity, concentration and activity of antiapoptotic proteins results in the induction of the irreversible step of programmed cell death – destruction (Vaux et al., 1988). BAD protein is a member of the proapoptotic protein family that interacts with antiapoptotic Bcl-XL protein. This latter process induces cell death probably by inhibiting the ability of Bcl-XL to block the release of cytochrome c from the mitochondria into the cytosol (Datta et al., 1997; Hanada et al., 2004). Afterwards, the dissociated form of BAD protein binds to adaptor protein 14-3-3 so it is retained in the cytoplasm (Krześlak, 2010; Słupianek et al., 2007).

The purpose of the study was to evaluate the quality of undifferentiated pig MSCs and differentiated MSC derivatives including either progenitor (immature)

and terminally specialized (mature) bone cells, i.e., osteoblasts and osteocytes, or adipose/fat cells (adipocytes). This evaluation was based on the determination of semi-quantitative profile of proapoptotic BAD protein expression, which was aimed at demonstrating the possible harmful effect of applied conditions of *in vitro* MSCs culture and inducible differentiation.

Material and methods

The *in vitro* culture of MSCs, adipocytes and osteoblasts/osteocytes

Isolation and expansion of MSCs

Bone marrow was collected under anaesthesia from gilts and piglets of the Landrace breed. All animal procedures were approved by the Local Animal Care Ethics Committee No. II in Kraków at the Institute of Pharmacology of the Polish Academy of Sciences.

MSCs were isolated from bone marrow according to Bosch et al. (2006) protocol with own modifications. Cells were suspended in DMEM supplemented with 10% FCS. Since the separation of single MSCs colonies, the culture was carried out in DMEM supplemented with 5% FCS to reach 80% confluence.

Differentiation of MSCs towards adipocytes and osteoblasts/osteocytes

After isolation of single MSCs colonies, part of the culture was subjected to differentiation towards osteoblasts/osteocytes and adipocytes in DMEM supplemented with 15% FCS, Glutamax 1x (Invitrogen, USA), 1 ml/100 ml medium of AA (Sigma) and the appropriate supplements according to Mao and Marion (2009). Adipogenic differentiation medium supplements were as follows: 0.5 μM dexamethazon (D2915, Sigma), 0.5 μM isobutylmethylxantine (I5879, Sigma) and 50 μl indomethacin (I7378, Sigma). Osteogenic differentiation medium supplements were as follows: 10 nM dexamethazon (D2915, Sigma), 20 mM β-glycerolphosphate (G9891, Sigma) and 50 μM L-ascorbic acid 2-phosphate (A8960, Sigma).

After 5 weeks, the differentiated cells were trypsinized, and resuspended in 50 μl PBS with protease inhibitors (Merck4Biosciences, USA) with a final concentration of 10 μl. The prepared samples were stored in liquid nitrogen until further analysis.

The determination of BAD protein expression

Determination of protein concentration

Thawed samples were evaluated spectrophotometrically using Nanodrop2000c to assess the protein concentration in each sample: MSCs not subjected to differentiation or untreated control (K), MSCs undergoing adipocyte differentiation (A) and MSCs undergoing osteocyte differentiation (O).

Determination of the proteins in the samples was performed by Bradford, using the Coomassie Plus reagent Bradford Assay Reagent (Thermo Scientific, USA) according to the procedure described by the manufacturer. Extinction values were read at 450 nm. A standard curve was prepared using solutions of 0.1%, 0.01% and 0.001% BSA.

Separation of proteins

Separation of proteins was performed by SDS-PAGE. The denatured sample was applied to the wells in 12% polyacrylamide gel. Electrophoretic separation was performed in Laemmli's system (25 mM Tris, 192 mM glycine, 0.1% SDS) (Laemmli, 1970) using polyacrylamide gel electrophoresis apparatus Mini Protean III BioRad (USA).

Two types of markers were used: Prestained SDS-PAGE Standards Low Range (Bio-Rad, USA) – visible on the membrane, and Biotinylated Protein Ladder (Cell Signaling, USA) – visible on X-ray film. After electrophoresis, the gel was carefully removed and washed in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol) (Opiela et al., 2008 a, b).

Western blotting

Transfer of proteins from the gel to the membrane

In order to carry out the transfer, the "sandwiches" were prepared. The first layer was paper (Extra Thick Blot Paper, Bio-Rad, Poland), then PVDF membrane (0.22 micron, BioRad, USA) which was applied on the polyacrylamide gel, and another layer of tissue paper. The sandwich was sealed on both sides of the tape by sponges. Proteins were transferred onto a PVDF membrane (Bio-Rad, USA) in transfer buffer (10 mM CAPS, 10% methanol), lasting 1 hour. A 50 V current was applied.

Protein labelling by antibodies

The free spaces on the membrane, not saturated with protein were blocked with blocking buffer TBS-T (25 mM Tris, 0.5 M NaCl, pH 7.5 + 0.1% Tween 20) supplemented with 0.25% (w/v) skimmed milk powder. Blocking, washing and all following incubations were performed at room temperature in the Snap id system (Millipore, USA).

Protein detection on the membrane

The blocked membrane was incubated for 10 min. at room temperature with primary antibody BAD (sc-7869, Santa Cruz Biotechnology, Inc, USA) at a concentration of 14 µg/ml, diluted in blocking buffer. After washing the membrane four times in TBS-T buffer, 0.1%, the membrane was re-incubated for 10 min. at room temperature with secondary antibody (Santa Cruz Biotechnology, Inc, USA) conjugated to horseradish peroxidase (HRP) at a concentration of 0.1 µg/ml.

Protein detection on the X-ray film

The membrane was developed using the West Femto (Thermo Scientific, USA) and Femto (Millipore, USA), as recommended by the manufacturer's procedure. After 5–10 min. incubation at room temperature the membrane was exposed to X-ray film (Thermo Scientific, USA). Films were developed manually using developer and fixer (Kodak, Poland) as recommended by the manufacturer's protocol.

The semi-quantitative analysis of protein expression

Films were scanned using Fluor-S MultiImager system (Bio-Rad, USA). The densitometric signal for each band was measured using Quantity One software (Bio-Rad, USA).

Stripping of antibody and membrane re-labelling

The membrane was stripped of antibodies by incubating 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 (Opiela et al., 2008 a, b). After 3 extensive washings, the membrane was blocked as written previously. Then, the membrane was incubated again with anti-actin antibody (Santa Cruz Biotechnology, Inc, USA) at a concentration of 0.4 mg/ml diluted in blocking buffer. The secondary antibody (Santa Cruz Biotechnology, Inc, USA) conjugated to HRP was used at a concentration of 0.15 μg/ml. In order to visualize the bands the protocol outlined above was performed.

Statistical analyses

Student's t-test was used to compare the expression level of BAD between analysed groups. The effect of MSC culture conditions was regarded as: significant at the level of random error probability value $0.01<P\leq0.05$, highly significant at the level of $0.001<P\leq0.01$, respectively.

Results

Differentiation of MSCs towards adipocytes and osteoblasts/osteocytes

After 4 weeks of MSCs (Figure 1) *in vitro* differentiation we obtained adipocytes (Figure 2) and osteocytes (Figures 3 and 3.1) which were confirmed by the expression of relevant markers typical for these cells at the transcript level (Opiela, unpublished data) as well as by morphological features. The established clonal lines (cell strains) of differentiated osteogenic MSC derivatives were comprised of both progenitor (immature) and terminally specialized (mature) bone cells, i.e. osteoblasts and osteocytes, respectively. After 6 weeks of *in vitro* culture in osteoblasts/osteocytes differentiation medium we obtained the characteristic mineralization in the culture flask (Figure 3.1). The ability of MSCs to differentiate towards adipocytes and osteoblasts/osteocytes supports their mesenchymal and stem origin.

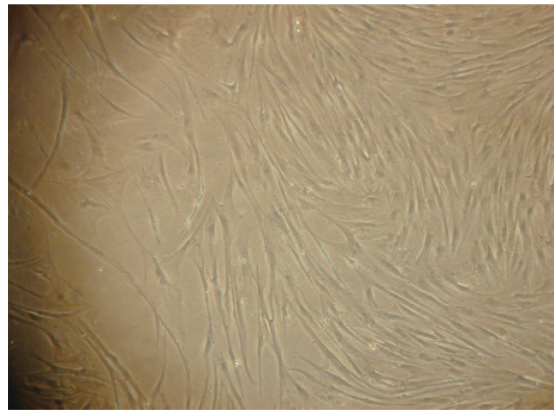


Figure 1. The colony of MSCs (x200)

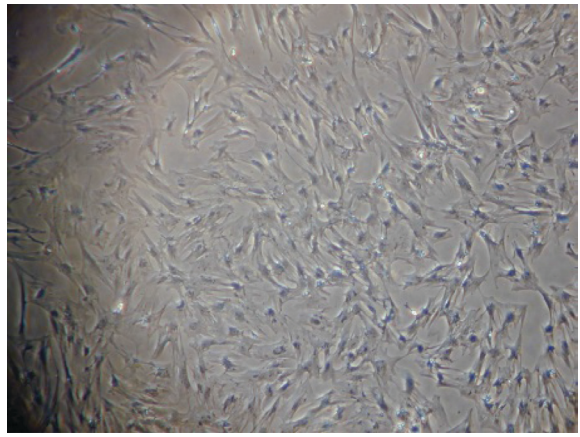


Figure 2. The colony of adipocytes after 4 weeks of differentiation (x200)

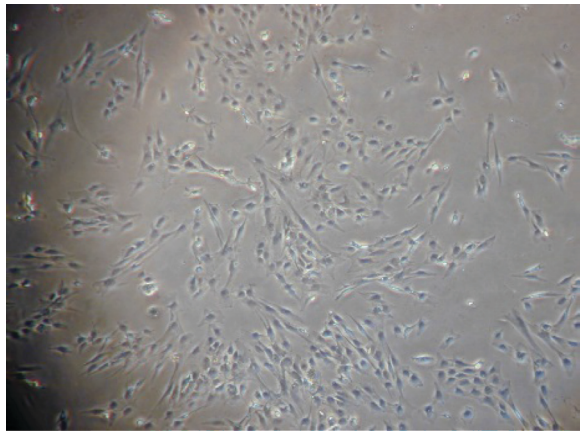


Figure 3. The colony of osteoblasts/osteocytes after 4 weeks of differentiation (x200)

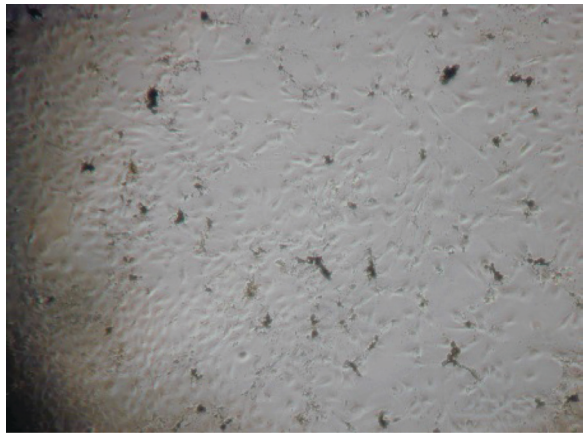


Figure 3.1. The colony of osteoblasts/osteocytes after 6 weeks of differentiation; visible dark spots represent mineralization of culture (x200)

Determination of BAD expression in MSCs, osteoblasts/osteocytes and adipocytes

BAD protein was present in all analysed groups of cells: not differentiated and MSCs subjected to differentiation towards adipocytes and osteoblasts/osteocytes. The product of BAD gene expression was a band at 24 kDa (Figure 4). The product of actin gene expression was a band at 46 kDa. Specific protein bands for BAD and actin were determined by applying a marker visible on the X-ray film by using the appropriate secondary antibody.

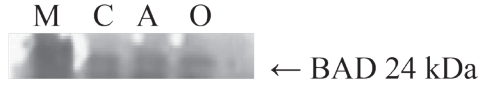
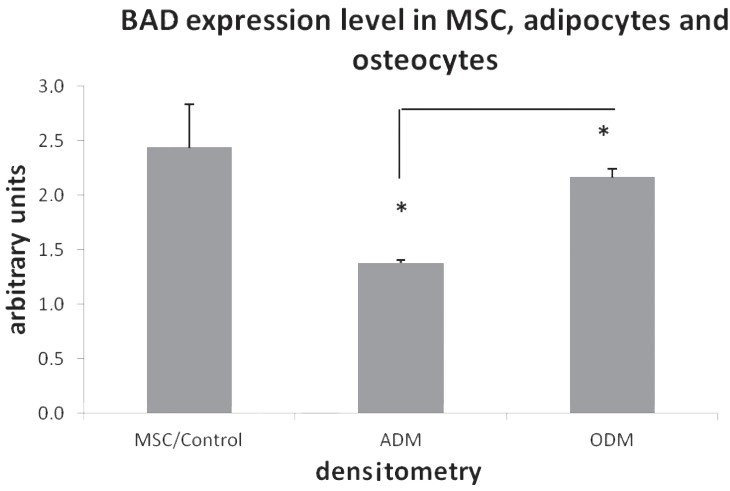


Figure 4. The picture of BAD protein bands on X-ray film after western blotting. M – marker; C – control, cells not subjected to differentiation; A – adipocytes; O – osteoblasts/osteocytes



*(P <0.05) the osteoblasts/osteocytes vs. adipocytes (Student's t-test).

Figure 5. Analysis of BAD protein expression in MSCs, adipocytes and osteoblasts/osteocytes by western blotting. The bars in the graph are the mean value of the calculated relative densitometric signals of the control signal from two independent experiments

For evaluation of BAD protein expression two repetitions were performed. To control the amount of applied protein in each experimental group, the expression of a house-keeping protein, actin was assessed as a control. The actin re-labelling was performed after BAD stripping procedure. The mean BAD protein expression was calculated on the basis of densitometric measurements of bands as shown in Figure 5. There were significant differences ($P<0.05$) in the level of BAD protein expression between adipocytes and osteoblasts/osteocytes (Figure 5). There were no significant

differences between cells undergoing differentiation (adipocytes and osteoblasts/osteocytes) and MSCs cultured in standard medium which served as control (Figure 5).

Discussion

We succeeded in differentiating the porcine MSCs into osteoblasts/osteocytes and adipocytes using human differentiation protocol with our modifications. As the *in vitro* culture and differentiation can adversely affect the quality of the cells, one of the important aspects is to assess the quality of differentiated cells.

In the present experiment the MSCs were differentiated into osteoblasts/osteocytes and adipocytes in the culture media used for human MSCs differentiation (Mao et al., 2009). It is known that there are differences in the expression of MSC markers between the human MSCs, mouse and pig (Bieback et al., 2012). For this reason, one cannot assume *a priori* that the medium composition that is optimal and effective for the human MSCs differentiation will also be one for the pig MSCs. Although there are examples of culture media composition used for the differentiation of porcine MSCs (Ringe et al., 2002; Juhásová et al., 2011), this does not change the fact that the phenotypes of MSCs lines differ within the same species, and as it is believed now, these differences are the result of varied protocols being in use to yield MSCs from bone marrow as well as varied *in vitro* culture conditions (Dominici et al., 2006; Izadpanah et al., 2008). Therefore, this situation justifies our research. Moreover, we suggest that it would be appropriate to check the quality of MSCs lines derived in each laboratory dealing with this issue. In our experiment, we proposed the estimation of the proapoptotic protein, BAD. Significant is the fact that the assessment of quality is based on the protein expression as protein is the most authoritative and reliable marker reflecting real gene expression because proteins are the final product of the information stored in gene DNA, contrary to RNA. Furthermore, by estimating the proapoptotic protein level, we examine the progress of apoptosis indirectly. The study of apoptosis advancement as a quality marker of the cells, oocytes and embryos is the generally accepted methodological approach (Opiela et al., 2008 a, b).

The significant differences ($P<0.05$) in BAD expression were found between osteoblasts/osteocytes and adipocytes. This result might suggest that the medium used for the MSCs differentiation into osteoblasts/osteocytes was not properly balanced, resulting in sub-optimal *in vitro* culture conditions. However, having in mind that the obtained arbitrary units for osteoblasts/osteocytes BAD expression were still lower than arbitrary units for control MSCs, it can be stated that the medium composition optimized for human MSCs differentiation (both adipocytes and osteoblasts/osteocytes) can be successfully applied in pigs. Moreover, there were no significant differences in the level of BAD protein expression between cells undergoing differentiation (A and O) and not subjected to differentiation (MSC-Control). This result again confirms the above formulated conclusion as well as that the medium used for the differentiation of adipocytes and osteoblasts/osteocytes does not affect the quality of the cultured cells. Surprisingly, as already mentioned, the highest expression

of BAD protein was detected in control MSCs but without significant differences. Whether it is a sign of not optimal medium applied when the *in vitro* culture lasts over 1 month should be verified by more complex analysis and elevated number of replicates. Another explanation may be increased responsiveness of these cells to suboptimal environmental conditions similarly as was the case with the so-called competent bovine oocytes BCB + (Opiela et al., 2008 a, b).

In conclusion, the differentiated cells expressed lower level of BAD protein than control MSCs, not subjected to differentiation. Moreover, there were no significant differences between these two types of cells and thus it can be concluded that the applied human culture differentiation media did not adversely affect the quality of cells. The present results show that out of three media used for *in vitro* culture and differentiation, the adipocyte differentiation medium turned out to be the most optimal. BAD protein can be used to assess the quality of porcine mesenchymal stem cells.

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Jakość mezenchymalnych komórek macierzystych świni oraz ich pochodnych zróżnicowanych w kierunku komórek szeregu osteo- i adipogennego – poziom ekspresji proapoptotycznego białka BAD

STRESZCZENIE

Celem przeprowadzonych badań była ocena jakości mezenchymalnych komórek macierzystych (MSCs; ang. *mesenchymal stem cells*) świni oraz komórek MSCs poddanych różnicowaniu *in vitro* w kierunku osteoblastów/osteocytów i adipocytów. Ocena ta polegała na oszacowaniu poziomu ekspresji proapoptotycznego białka BAD na podstawie analizy molekularnej. Wyizolowane ze szpiku kostnego komórki MSCs były hodowane *in vitro* przez 5 tygodni w trzech rodzajach pożywek: różnicującej w kierunku osteoblastów/osteocytów (O) i adipocytów (A) oraz nieróżnicującej, kontrolnej (C). Względny poziom ekspresji białka BAD był określany przy zastosowaniu metody *western-blotting*. Statystycznie istotne różnice zaobserwowano w potranslacyjnym, półilościowym profilu ekspresji białka BAD, w komórkach podlegających różnicowaniu w kierunku osteoblastów/osteocytów, w stosunku do komórek, w których proces różnicowania przebiegał w sposób adipozależny ($P<0,05$). Ponadto, odnotowano brak statystycznie istotnych różnic między niezróżnicowanymi komórkami macierzystymi z grupy kontrolnej a komórkami poddanymi indukowalnemu różnicowaniu w kierunku powstawania osteoblastów/osteocytów lub adipocytów. Podsumowując uzyskane wyniki badań, wnioskujemy, że potranslacyjny poziom ekspresji białka BAD, o aktywności proapoptotycznej, może być użyty jako miarodajny wskaźnik oceny jakościowej, zarówno mezenchymalnych komórek macierzystych, jak i ich zróżnicowanych pochodnych. Półilościowy profil ekspresji białka BAD, będącego agonistą śmierci apoptotycznej, okazał się być niższy w odniesieniu do zróżnicowanych komórek macierzystych, w porównaniu z subpopulacjami niezróżnicowanych komórek MSCs, co dowodzi, że procedura indukowalnego różnicowania (tj. transformacji proosteogennej lub proadipogennej) nie wpływa negatywnie na jakość hodowanych komórek macierzystych.