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# Adipogenic differentiation of culture-expanded bone marrow derived porcine mesenchymal stem cells

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ABSTRACT

In order to use Mesenchymal stem cell populations for obesity and related metabolic syndrome studies in cell culture system, as a prerequisite, we evaluated the potency of these stem cells to undergo adipogenic differentiation. Porcine stem cells were chosen to study adipogenesis in due to the fact that pig has a natural tendency to get obese and the species is considered to be the most desired biomedical model for human applications. Porcine MSCs have been exposed to adipogenic induction media following a 21day protocol and observed under microscope for detecting stages of differentiation. At the terminal differentiation stage; morphologically, the cells appeared rounded with numerous large cytosolic lipid spheres. Upon staining with Oil Red O, the lipid spheres stained bright red. Based on this, proprietary medium was found to differentiate MSCs more efficiently than medium formulated on previous reports. Both, the differential morphologic feature corresponding to the adipocyte and positive Oil Red O staining confirmed about successful adipogenic differentiation. We envision that stem cell based culture system from porcine species would aid for studying molecular adipogenesis and subsequent identification of therapeutic targets for obesity and other metabolic diseases.

**Key words**: Adipogenic differentiation, Bone marrow, Porcine MSCs, Obesity model

Obesity, insulin resistance and diabetes are the major metabolic syndromes manifested as the result of dysregulated lipogenesis and steroidogenesis and these pose great challenge to the healthcare community throughout the world. These threats call for intensive research in search of therapeutic targets. Several attempts are being made worldwide to alter the lipid biosynthesis. This includes targeting of various adipogenic enzymes (Matsuzaka and Shimano 2009), enzymes regulating fatty acid metabolism like acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1) (Chakravarthy *et al.* 2005, Mao *et al.* 2006, Miyazaki *et al.* 2007, Ntambi *et al.* 2002). Alternatively, inducing non shivering thermogenesis to bypass the caloric contribution is recently considered worth promising (Harms and Seale 2013).

It is established that nuclear sterol regulatory element binding protein-1c (SREBP-1c) is a master regulatory factor of *de novo* lipogenesis and steroidogenesis (Jeon and Osborne 2012) and therefore, plays a pivotal regulatory role in lipid metabolism (Knebel *et al.* 2012). Recently, the elongase of very long chain fatty acids family 6 (ELOVL6) is anticipated to be a potential therapeutic target. This is a rate-limiting enzyme catalyzing the chain elongation and therefore controls tissue fatty acid composition (Green *et al.* 2010, Matsuzaka *et al.* 2007). Furthermore, it is demonstrated that SREBP-1 regulates ELOVL6 expression (Kumadaki *et al.* 2008, Matsuzaka *et al.* 2002) and ELOVL6-deficient mice to become obese and develop hepatosteatosis (Shimano 2012). These data reinforce the idea that ELOVL6 might be a potential therapeutic target for the metabolic diseases.

Cell culture based system has remained popular to study from the very stage of early development of fat tissue *in vitro*. For this purpose, 3T3 L1 rodent cell line (sub-clone of 3T3 fibroblast from Swiss albino strain) is most frequently used (Green and Kehinde 1975). Since mesenchymal stem cells (MSCs) have the inherent potency to differentiate into adipocyte (Jiao *et al.* 2012, Krampera *et al.* 2007), we consider these cells of porcine origin as an alternative resource.

Increasing evidences are in support of the fact that pig is more close species to human than any other laboratory animal. Because of their similar genetic makeup and their close resemblance in anatomy and physiology to that of human, they are often employed in biomedical research for human disease (Samstein and Platt 2001). That is why swine has been used in biomedical research in a variety of areas like cardiovascular, gastrointestinal, metabolic, liver, reproductive and infectious disease. The extensive use of swine species for research studies in arteriosclerosis, heart transplants, bioprosthesis heart valves, intestinal transplants,

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diabetes and pancreatic transplants, liver transplants, uterine cell physiology, bronchial and tracheal stent, renal transplants, immune and antibiotic and vaccine trials imply its inlaid potentials (Crick *et al.* 1998, Esteban *et al.* 2010, Swindle *et al.* 2012, Ye *et al.* 2014). Development of human organ by producing human-pig chimera using human pluripotent stem cells is currently under consideration (http://www.bbc.com/news/health-36437428). Therefore, swine species and cells from porcine origin have great potential for human therapy.

However, literature on use of porcine stem cells for adipogenesis is very limited. Present study was conceived to establish a porcine mesenchymal stem cell line for adipogenesis in culture. Earlier, we had demonstrated the harvest of bone marrow derived MSCs following either invasive method from live animal or from long bones of dead/slaughtered animal (Santra *et al.* 2015, 2017). In the current study, we demonstrated these isolated stromal MSCs are suitable for induced adipogenic differentiation. We believe that this seminal work would lay the basic framework to enhance the use of these adult stem cells as a model for further functional studies related to metabolic disorders, also establish these cells as a valuable resource for other developmental study as well as for therapy in regenerative medicine.

## MATERIALS AND METHODS

*Cells:* For the present study, porcine mesenchymal stem cells previously isolated and characterized earlier (Santra *et al.* 2017, 2015) were used.

### *Culture medium*

*Maintenance medium:* For culturing cells, the MSC medium was prepared with 1:1 mixture of DMEM (Gibco) and TCM199 (Himedia). The basal medium is additionally supplemented with 10× Antibiotic/Antimycotic solution (Himedia), 10× MEM Non essential amino acid (MEM NEAA, Invitrogen), 1× 2-Mercaptoethanol (Gibco) and 10% Fetal Bovine Serum (South American type; Gibco). Following mixing the complete medium is filtered by 0.2 µm syringe filter (MDI).

*Differentiation medium:* We have used two types of adipogenic differentiation media, one formulated based on previously published reports (Boyd *et al.* 2009) and the other one (HiAdipoXL™ Adipocyte Differentiation Medium) was procured from commercial source (Himedia). This commercial medium consists of Part A (HiAdipoXL™ basal medium) and Part B (HiAdipoXL™ adipogenic differentiation supplement). The complete adipogenesis differentiation medium was prepared by thawing Part B at 2–8°C overnight and transferring the entire content to Part A under aseptic conditions. This medium was further supplemented with 10× antibiotic-antimycotic solution (Himedia) and stored at 2–8°C until use.

*Maintenance and sub-culturing of cells:* MSCs were propagated in the maintenance medium at 37°C in a 5% $CO_2$ humidified incubator (Galaxy 170R, New Brunswick, Eppendorf). Cells were observed every day under the inverted microscope (Nikon) for checking morphology and confluency. When the cells reached about 70% confluency, they were sub-cultured. Briefly, the spent medium was aseptically removed and the monolayer was washed gently using appropriate volume of DPBS (Gibco). Following the washing step, cells were treated with 0.25% Trypsin-EDTA (Himedia) at 37°C until all the adherent cells start detaching off the flask. Once the cells were found detached from the surface, the trypsin activity was neutralized by adding 1 ml of fresh complete medium. The cell suspension was centrifuged at 1,200 rpm (Eppendorf) at room temp for 5 min to remove the traces of trypsin. After discarding the supernatant, cells were resuspended in complete culture medium and seeded in the ratio of 1:3. To ensure even distribution of cells, plates were gently rocked back and forth and side to side and cells were incubated at 37°C in a 5% $CO_2$ humidified incubator until they grow to about 70% confluency.

*Induction for adipogenic differentiation:* At 70–80% confluency, the cell culture plate was taken out from incubator and the spent medium was aseptically removed. Then appropriate volume of complete differentiation medium was added. The cells were observed microscopically and the medium was replaced with fresh complete differentiation medium after every 48 h. The adipogenic differentiation procedure was continued for 18 to 21 days.

### *Oil Red O staining of adipocytes*

*Preparing oil-red-o stain:* The stock solution was prepared by dissolving 300 mg of Oil Red O powder (Sigma) in 100 ml of 99% isopropanol (Sigma). Next the working solution is prepared by mixing 30 ml of Oil Red O stock solution with 20 ml deionized water. After allowing at room temperature for 10 min, the solution was filtered by Whatman No. 1 filter paper.

*Staining of adipocytes:* For detection of *in vitro* adipogenesis, cells from incubator were removed and placed in the fume hood. The media was aspirated off the plate and the plate was gently rinsed with sterile 2 ml DPBS along the sides of each well without disturbing the monolayer. Then, 2 ml of 10% formalin was added along the sides of each well and incubated for at least 30 min at room temperature. After fixation, the cells were washed gently with 2 ml of sterile water. Then 2 ml of 60% isopropanol was added to cover the bottom of each well and kept for 2–5 minutes. After decanting off the isopropanol, 2 ml of the Oil Red O working solution was added along the side of each well, to fully cover the cultured cells and incubated for 5 min at room temperature. After a careful rinsing with tap water, 2 ml of the hematoxylin counterstain (Sigma) was added into each well and kept for 1 min. Finally, cells were washed with warm tap water and observed under phase contrast microscope.

*Image capturing and visual examination:* For routine visual assessment of growth dynamics as well as

identification and characterization of adipocytes based on Oil Red O staining, Nikon Eclipse T*i*-S inverted fluorescence microscope (Nikon, Japan) was used. The images were captured using Nikon Elements imaging software.

## RESULTS AND DISCUSSION

Primary focus of this study was to establish a cell type of porcine origin with potential to undergo adipogenic differentiation upon induction. Mature adipocytes are difficult to maintain in culture and the structural integrity of cell organelles during fixation and subsequent processing for microscopy are affected. Moreover, stage specific phenomena cannot be followed in such culture. Therefore, detailed in-depth study warrants a cell culture system that would facilitate easier observation over a time course as well as minimal manipulation during the course of differentiation. Cultured 3T3–L 1 cells of mouse origin possess many of the biochemical characteristics of mature adipocytes and hence, are greatly suited in researches related to metabolism of lipid (Joost and Schurmann 2001, Porras and Santos 1996). However, mouse cell line may not be reliable for understanding human metabolism and diseases (Mestas and Hughes 2004). Since pig is considered to be more close to human (Swindle *et al.* 2012) and used in gene and cell therapy, xenograft and allograft procedures and other types of regenerative medicine, it would be rational to establish a porcine cell line to serve as study model for the said purpose.

Therefore, we have isolated mesenchymal stem cells from bone marrow (Fig. 1A), as per protocols published previously (Santra *et al.* 2015, 2017). These cells were propagated in MSC culture medium. The cells undergo a phase of clonal expansion as appeared by the appearance of focal adhered colony of clustered cells (Fig. 1B). These colonies gradually expand and coalesce with the neighbouring colonies and form monolayer (Fig. 1C). These culture-expanded cells could be maintained for several passages (over 50) by means of standard subculture procedure.

These cells were positive for several MSC markers as well as multiple pluripotency factors demonstrated earlier (Santra *et al.* 2017). They can also be cryopreserved for indefinite time for future use upon requirement. MSCs have inherent potential to differentiate into three mesodermal lineages namely, osteocytes, adipocytes and chondrocytes (Jiao *et al.* 2012, Krampera *et al.* 2007). These culture expanded cells have been demonstrated earlier for their potential to undergo osteogenic differentiation (Agarwal 2013, Santra 2016) indicating about their nature as stem cells.

In the current study, we checked their adipogenic differentiation potency. Therefore, these cells were subjected to adipogenic induction media and could differentiate into adipocytes (Fig 1D). During adipogenic differentiation, the cells appear rounded and possess numerous large cytosolic lipid spheres, revealed by Oil red

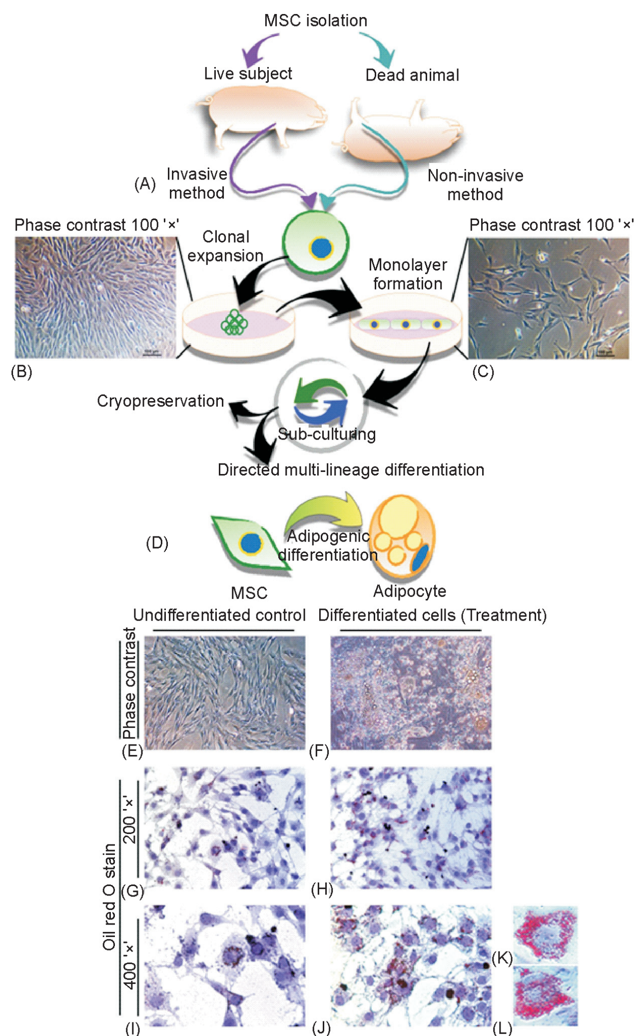


Fig.1. Isolation of porcine mesenchymal stem cells (MSCs) and induction of adipogenic differentiation *in vitro.* (A) Schematic representation of MSC isolation from pig by either invasive or from long bones of freshly slaughtered animals, *in vitro* expansion, sub-culturing and utility. Harvested MSCs were first adapted to artificial media and clonally expanded cells form monolayer. These cells were maintained for several passages, amenable to cryopreservation, and had the potency to undergo directed multi-lineage differentiation. Therefore, these cells are fit for subsequent uses in regenerative medicine.(B) Phase contrast image (100×) of clonally expanded grown MSCs in artificial cell culture media.(C) Phase contrast image (100×) of MSCs on monolayer over plastic culture dish.(D) Schematic diagram of adipogenic differentiation of porcine MSCs. (E & F) Phase contrast images of undifferentiated MSCs and adipogenic differentiated cells, respectively. The characteristic morphological features indicate differentiated cells have fat globules.(G & H) Photomicrograph (200×) of Oil Red O staining. The treatment panel shows more Oil Red O staining compared to control, indicating enhancement of adipogenic differentiation upon induction. (I & J) Photomicrograph (400×) of Oil Red O staining. The treatment panel shows more Oil Red O staining (appeared as red dots) compared to control, indicating successful induction to adipogenic lineages.(K & L) The photographs show typical signet rings in differentiated adipocytes, possessing numerous large cytosolic lipid spheres as revealed by bright red granules by Oil red O staining.

O staining (Deutsch *et al.* 2014). On microscopical examination, the terminally differentiated adipocytes appear as more rounded as compared to undifferentiated MSCs (Figs 1E, 1F). Additionally, lipid vesicles formed in the adipocytes could be observed microscopically as intracellular oil droplets and therefore the differentiated cells appeared more granular as opposed to MSCs (Figs 1E, 1F).

Oil red O is a popular fat-soluble dye, used for staining of neutral triglycerides (Mehlem *et al.* 2013, Deutsch *et al.* 2014, Andres-Manzano *et al.* 2015). When stained, Oil red O can make fat better visible and thus helps in differential identification of adipocytes. When the differentiated cells were stained with Oil red O, the lipid droplets stained bright red (Figs 1G-1L). Presence of more number of red cells in treatment group (Figs 1 H, 1J, 1K, 1L) compared to control group (Figs 1G, 1I) confirms higher efficiency of adipogenic differentiation of these bone marrow derived porcine MSCs. Like 3T3–L1 cells, adipocytes generated from porcine MSCs possess typical signet ring (Figs 1K, 1L) (Green and Kehinde 1975). Additionally, it is found that the proprietary adipogenic induction medium differentiates MSCs more efficiently than a medium formulated on previous reports (Boyd *et al.* 2009). Our earlier studies demonstrated that the bone marrow derived MSCs were capable of differentiating into osteoblast (Agarwal 2013, Santra 2016). The present study establishes their adipogenic potential. Therefore, these multipotent stem cells may be used as a model for investigations on mammalian adipogenesis.

The observations presented in this article report the cytological features of cultured porcine MSCs and provide a basis from which one can begin to study the biochemical changes at different stages during adipogenic differentiation. Their persistent stability on plastic cell culture dish during sub-culturing, consistence revival from suspended life in cryopreserved form and their multi-lineage differentiation potential make these cells a valuable cell source for biomedical application. We anticipate that these cells would be another popular model like 3T3– L1 for obesity and other related studies.

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