Adipogenic differentiation potential of porcine bone marrow mesenchymal stem cells grown in different basal media formulations

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ABSTRACT

Bone marrow-derived porcine mesenchymal stem cells (pMSC) are precursors of multiple lineage cells. However, the differentiation potential of pMSC might vary due to the culture media in which they are isolated and grown. In this study the effects of αMEM, aDMEM, M199, αMEM/M199, aDMEM/M199 and αMEM/aDMEM was determined on pMSC expression of adipogenic marker genes PPARγ, C/EBPα and ApN at passage 5 and 10; and differentiation potential of pMSC at passage 5. Relative expressions of the genes were determined by qPCR assay. Adipogenic differentiation progress was determined by microscopic evaluation and accumulation of lipid vesicles in cells after day 21 differentiation was confirmed by Oil Red-O staining. Results indicated varying expressions of adipogenic marker genes at passage 5 and 10 in pMSC grown in different culture media. Adipogenic differentiation characteristics also varied in different culture media, grown cells depending on the expression of adipogenic marker genes. Classification in order of differentiation potential revealed that the pMSC grown in M199 and αMEM/M199 was the best, followed by aDMEM/M199 and αMEM/aDMEM the intermediate and αMEM and aDMEM the least suitable media. Effect of media interaction analysis indicated that M199 media might have played a significant role in up-keeping of the desired marker gene expression and adipogenic differentiation potential of the pMSC. In conclusion, adipogenic differentiation potential of pMSC varied due to the differences in basal media composition and the M199 played a significant role in skewing pMSC towards adipogenic lineage.

INTRODUCTION

The porcine mesenchymal stem cells (pMSC) have immense potential for research and therapeutic applications as they are precursor of multi-lineage cells. They are capable of differentiation into their lineages, the osteogenic, adipogenic and chondrogenic cells (Pittenger et al., 1999), and the other lineages cells such as tendon (Gordon et al., 2005), endothelial cells (Portalska et al., 2012), nerve cells (Rooney et al., 2009), hepatic cells (Lee et al., 2004), myocytes (Wakitani et al., 1995), and cardiomyocytes (Guo et al., 2018).
Role of adipocytes in glucose uptake, angiogenesis, blood pressure and immunity has been well documented (Rosen and MacDougald, 2006). Adipocytes play a significant role in energy metabolism and maintenance of body temperature homeostasis. Thus differentiation of MSC into adipogenic cells are considered as an excellent model for research on obesity, diabetes and bone disorders (Wang et al., 2016). At the initiation of adipogenesis, the MSC proliferation stops, but the morphology does not change. This is followed by activation of the early initiators c-Fos, -Jun and CCAAT enhancer-binding proteins β and δ (C/EBPβ and C/EBPδ) that drive MSC towards adipogenic commitments (Patel and Lane, 2000). Activation of these proteins leads to the expression of key transcription factors Peroxisome proliferator-activated receptor (PPARγ) and CCAAT enhancer-binding proteins (C/EBPα) that determines the fate of the MSC. The committed cells gradually show the changes in morphology and the typical adipocyte phenotype with an accumulation of lipid vesicles in the cells. The entire process involves activation of several signal cascades and convergence of the activity of these transcription factors resulting in expression of Glucose transporter (GLUT4), Lipoprotein lipase (LPL), fatty acid-binding protein 4 (FABP4) and Adiponectin (ApN) (Moseti et al., 2016). Expression of ApN in bone marrow cells curtail mesenchymal progenitor cells towards adipogenesis and increased osteogenic differentiation ability (Lee et al., 2009). This adipocytokine (the ApN), is functionally active in the early and further downstream adipogenic differentiation process (Kadowaki et al., 2006) therefore is the most important gene in this line. The adipogenesis and osteogenesis maintain an inverse relationship, thus is relevant for bone formation. Increase in adipogenic capacity causes resorption and reduction of bone formation resulting in major disorders called osteoporosis (Wang et al., 2016). With the increase in bone age, a concomitant decrease in osteoblast numbers and the increase in the number of adipocytes observed in differentiating MSC (Duque, 2008). Factors such as cell density, adequate oxygen tension and age of the cells affect the skewness of adip/oosteogenic precursor cells in MSC (Muruganandan et al., 2009). The conditions such as age, pathological state of donor determine the stemness and differentiation potential of MSCs.

The micro-environment contributed by the media also plays a significant role to affect the oxygen tension and influence the number of colonies formed during pMSC differentiation (Bosch et al., 2006). So far the Minimum Essential Media alpha (αMEM) (Ayatollahi et al., 2012; Ferrari et al., 2014; Dulbecco’s Modified Eagle’s Medium (DMEM) (Ullah et al., 2015; Sanjurjo-Rodriguez et al., 2017); DMEM/Nutrient mixture F12 (DMEM/F12) mixed media (Ullah et al., 2015); Roswell Park Memorial Institute (RPMI) 1640 medium (Ullah et al., 2015) and Tissue culture Medium M199 (M199) (Hashmani et al., 2013; Sidney et al., 2015) are used for MSC culture in different species. It has been observed that equine MSC grown in DMEM/F12 and RPMI1640 were less amenable for osteogenic differentiation (Watchrarat et al., 2017). Porcine MSC grown in advanced Dulbecco’s Modified Earle’s Media (aDMEM) after 15 passages retain only the adipogenic differentiation with concomitant loss of osteo-chondrogenic differentiation potential (Vacanti et al., 2005). In previous studies, we have shown that pre-differentiation basal media for pMSC culture plays essential roles in the maintenance of cell proliferation and osteogenic differentiation potential (data submitted elsewhere).

Keeping in view the above background in mind we have evaluated the effects αMEM, advanced DMEM (aDMEM), M199 and their 1:1 mix αMEM/M199, aDMEM/M199, αMEM/aDMEM media on 1) the expression of key adipogenic regulator PPARγ, C/EBPα and ApN genes in pre-differentiation porcine bone marrow MSC and 2) adipogenic differentiation characteristics of the different media grown pMSC subpopulations.

**MATERIALS AND METHODS**

All the basal media αMEM, aDMEM, M199 used for the study and adipogenic differentiation media (#AL512, HiAdipoXL™) were purchased from HiMedia Laboratories, Mumbai, India. The mixed-media αMEM/M199, aDMEM/M199 and αMEM/aDMEM were prepared by mixing respective media at 1:1 ratio. Fetal bovine serum (FBS) and the supplements β-mercaptoethanol, Glutamax and Antibiotic-Antimycotic solution were purchased from Gibco Life Sciences, USA. All the other general chemicals if not mentioned otherwise were purchased from Sisco Research Laboratories (SRL), Mumbai, India.

**Porcine MSC**

The porcine MSC used for this study were derived from the femur and tibia bone marrows of three young pigs aged between one to one and half years utilising the plastic adherent properties of the cells in αMEM, aDMEM, M199, αMEM/M199, aDMEM/M199 and αMEM/aDMEM containing 10% FBS, 0.1% β-mercaptoethanol, 4mM Glutamax and 1% Antibiotic-Antimycotic solution as supplements. The derived cells were grown at 37°C temperature...
with 5% CO₂ and >85% relative humidity. The
grown cells were passaged at 70–80% confluence by
0.025% Trypsin-EDTA (#T4049, Sigma-Aldrich,
USA) treatment and continued to grow until passage
10. At passage 5, they were tested to be positive for
CD105 (all animal and all media), CD90 (all pigs
and all media) and CD73 (all pigs and all media) in
all the three pigs across the media. The expressions
of other genes varied between the pigs. The CD90
expression was high in pig #1: aDMEM/M199,
aDMEM; pig #2: αMEM, aDMEM, aDMEM/M199,
αMEM/aDMEM; pig #3: αMEM, αMEM/M199 and
low in pig #1: αMEM, M199, αMEM/M199 and
αMEM/aDMEM; pig #2: M199; pig #3: aDMEM,
M199, αDMEM/M199, αMEM/aDMEM grown
cells. The CD14 expression was high in pig #1:
M199, aDMEM/M199; pig #2: M199; pig #3:
aDMEM/M199 grown cells. The CD34 was low in pig #1,
#2 and #3: aDMEM, aDMEM, aDMEM/M199,
αMEM/aDMEM; pig #3: αMEM, αMEM/M199 and
αMEM/aDMEM grown cells. The CD14 expression was high in pig #1:
aDMEM/M199; pig #2: αMEM, aDMEM, M199
and αMEM/M199; pig #3: αMEM, αMEM/M199,
αMEM/M199, low in pig #1: αMEM, aDMEM, M199
and αMEM/M199; pig #2: αDMEM/M199; pig #3:
adMEM, M199, αMEM/aDMEM) and no expres-
sion observed in pig #1 and #2: αMEM/aDMEM
grown cells. The CD34 was low in pig #1, #2 and
#3: aDMEM/M199; pig #2 and #3: αMEM;
αDMEM/M199, αMEM/M199, αMEM/aDMEM;
pig #3: αDMEM and no expression observed pig
#1: αMEM, aDMEM, M199, αMEM/M199 and
αMEM/aDMEM; pig #2: aDMEM and M199 and
pig #3: M199. The CD45 expression was low in pig #1,
#2 and #3; αDMEM/M199 grown cells and no expres-
sion observed in pig #1: αMEM,
adDMEM, M199, αMEM/M199 and αMEM/aDMEM;
pig #2: aDMEM, M199 and pig #3: M199. The CD45 expression was high in pig #1:
aDMEM/M199; pig #2: aDMEM, M199 and pig
#3: M199. The CD73 was low in pig #1, #2 and
#3: αDMEM, aDMEM, M199, αMEM/aDMEM
grown cells. The CD45 expression was low in pig #1,
#2 and #3: aDMEM, aDMEM, M199 grown
cells. The CD105 was low in pig #1, #2 and
#3: aDMEM, aDMEM, M199 grown cells.

Cell culture and isolation of total RNA

The passage 4 and 9 porcine MSC of all the 3 pigs
grown in different media were seeded at a density of
2x10^5 cells/cm² in sterile 6-well culture plates in
triplicates and allowed to multiply until 70–80%
confluence. At this stage of growth spent media
were discarded, cell surface rinsed with 1X PBS, and
the cells lysed by treating with 0.4ml/well TRIZol®
LS reagent (#10296028, Ambion™ Life Technology,
USA). Before the addition of TRIZol® LS reagent, the
culture vessels were placed on (-20°C) cold gel pack.
The cell lysates from each well were collected and
stored at -80°C until further use. The total RNA
from each TRIZol treated samples were extracted
according to the manufacturer’s protocol. The RNA
yields were determined by taking the absorbance at
260nm and 280nm wavelength using NanoDrop ND-
2000 UV-Vis Spectrophotometer (Thermo Fischer
Scientific, USA). The purity of RNA was determined
by the ratio of absorbance at 260/280nm. Then
total RNA aliquots of each sample were denatured
and separated by 1% Agarose gel electrophoresis
under 0.2 mol/L 3-(N-morpholino) propane sulfonic
acid (MOPS) buffer (pH 7.0) and stained using ethidiu-
um bromide as per the published protocol (Kannan
et al., 2019). >1.5 ratios of 28S/18SrRNA bands con-
firmed Integrities of each isolated RNA samples by
densitometry analysis.

qPCR primer design and testing amplification efficiency

The primers of test genes PPARγ, C/EBPα, ApN
and endogenous control GAPDH gene were designed
(Table 1) using Primer3 web tool (http://bioinfo.ut
.ee/primer3-0.4.0/primer3/) and synthesised from
a local supplier (M/s Xcelris Labs limited, Ahmed-
abad, Gujarat, India). The efficiency of all the gene
primers was tested in duplicate 15μL reaction mix-
tures. Each reaction mixture contained 6.5μL of
cDNA template (50, 10, 2 and 0.4ng) for test genes
in duplicate, nuclease-free water for non-template
control (NTC) in triplicate and diluted total RNA
(5ng) for negative reverse transcriptase (RT) in
duplicate. Besides, each reaction mixture contained
0.5μL each of forward and reverse primer and 7.5μL SYBR
2X master mix. The efficiency of each pair of
gene-specific primers was calculated using the for-

mula: E = [10(-1/slope)-1]*100, where the slope is
obtained from the slope value between average Ct
and log quantity.

qPCR assay

The expression of adipogenic marker genes (PPARγ,
C/EBPα, ApN) and the endogenous control
(GAPDH) was estimated by SYBR green-based
qPCR assay. In brief, 10μL reaction mixtures were
prepared in triplicate. The PCR reaction was carried
out in 96 wells clear Light Cycler 480 Multi-well
plate (#05102413001, Roche, USA) using the Light
Cycler 480 instrument (Roche Diagnostics, USA).
Each reaction mixture contained 4μL template
[10ng cDNA for the gene of interest/ 10ng total
At the end of 21 days differentiation, the spent adipocytes by Oil-O-red staining confirmed the presence of lipid vesicles in differentiated and undifferentiated pMSC at the end of differentiation. The melt curve was generated by initial denaturation for 1 min at 95°C followed by annealing at 55°C and increasing temperature 1°C per 10s from 55 – 95°C. For melt curve analysis images were captured at each 10s intervals. The GAPDH expression was used to normalise variations in the amount of target genes mRNA in all samples. The relative mRNA abundance of target genes was calculated from comparative cycle threshold (Ct) using the formula \( \frac{\Delta Ct_{\text{target}}}{\Delta Ct_{\text{reference}}} \) as described by (Pfaffl, 2004). The \( \Delta Ct \) (target) was the difference between Ct of the target gene in calibrator (αMEM), and target gene in the test (other media) and \( \Delta Ct_{\text{reference}} \) is the difference between Ct of reference gene (GAPDH) in calibrator and reference gene in the test.

### Adipogenic differentiation

The passage 4 cells from different media were seeded in triplicate at a density of 5x10^3 cells/cm^2 per well in a sterile 24-well plate in 1mL of each media and grown until 90–95% of cell confluence. The spent medium in each well was replaced with an equal volume of adipocyte differentiation medium and incubated at 37°C with 5% CO₂ and >85% relative humidity. Until 21 days of differentiation period, at every 3 days of culture, spent media was discarded leaving a film of media on the top of the cells and 1mL of fresh induction medium was added gently from the well sides without disturbing the cell surface. The differentiating MSC were examined every day by the same individual under the Olympus IX51 inverted microscope (Olympus, Japan) at 100x magnification. Cell images were captured every three days using DP-73 camera and CellSens Entry 1.8 software (Olympus Soft Imaging Solutions, GmbH, Germany). The comparative differences in the progression of adipogenesis were recorded in terms of morphological changes, the formation of lipid vesicles, presence of contaminating osteocytes and undifferentiated pMSC at the end of differentiation.

### Confirmation of lipid vesicles in differentiated adipocytes by Oil-O-red staining

At the end of 21 days differentiation, the spent media was discarded cells were rinsed with PBS and fixed in 10% formalin for 10 min and directly subjected to staining using EZStain™ adipogenic staining Kit, (Cat # CCK013, HiMedia Laboratories, Mumbai, India). In brief, the cells after differentiation were exposed to Oil Red-O staining solution for 10 – 15 min. At the end of the incubations, the cells were washed three times gently with water. The deposition of lipid droplets stained with Oil Red-O was examined in several fields and images captured using DP-73 camera and CellSens Entry 1.8 software (Olympus Soft Imaging Solutions, GmbH, Germany).

### Statistical analysis

The log 2 fold mRNA expression data of passage 5, passage 10 independently and pooled passage 5 and 10 were analysed by full factorial design using General linear model and Multivariate analysis option. The calculated Log 2 Fold gene expression was considered as dependent variables and media and passages as fixed factors. The significance of differences among the means was determined by LSD post hoc test at p ≤ 0.05.

The effects of interaction between any two media on different gene expression were analysed considering the cells were cultured in the absence (0,0), presence of single (1,0/0,1) and combinations (1,1) of two different basal media formulations using full factorial design, gene expressions as dependent variables and media as fixed factors. Gene expression in the absence of a particular media signified that the cells were cultured in a basal media other than the two particular media used in single or in combinations. Thus two-way ANOVA with interaction was conducted using the following model: \( Y_{ijk} = \mu + M_1 + M_2 + (M_1 \times M_2)_{ij} + e_{ijk} \). Where, \( Y_{ijk} \) represented estimated gene expression (value of Log 2 Fold); \( M_1 \) is the \( i^{th} \) observation of M1 medium, \( M_2 \) is the \( j^{th} \) observation of M12 medium; \((M_1 \times M_2)_{ij}\) is the interaction between M1 and M2 at \( ij^{th} \) intersection; \( e_{ijk} \) is the unexplained error of the model.

The interactions were considered significant at \( p \leq 0.05 \).

### RESULTS AND DISCUSSION

#### Adipogenic marker gene expressions

The efficiency of PPARγ, C/EBPα, ApN and GAPDH set of primers was calculated to be 1.93, 1.97, 2.0 and 1.93, respectively. The expression of target genes in cells grown in different media was compared concerning the expression of the same gene in the cells grown in reference αMEM.
Table 1: List of different adipogenic marker and endogenous control (GAPDH) gene qPCR primers used for relative quantification of expression in pre-differentiation pMSC.

| Genes | Accession number | Primer | Sequence (5’ TO 3’) | Target (bp) | Size (bp) | Tm (℃) |
|-------|------------------|--------|---------------------|------------|-----------|--------|
| PPARγ | NM_214379.1      | F      | CACGAAGAGCCTCCAACCTC | 391-496    | 105       | 59.99  |
|       |                  | R      | ACCCTTGACCTCCTCACAAG |            |           | 60.11  |
| C/EBPα| XM_003127015.2   | F      | CGGTGGCTCTAAGATGAGG | 1373-1503  | 130       | 60.25  |
|       |                  | R      | GTAGGAATCGGGAGGCTGAG |            |           | 59.97  |
| ApN   | AY589691.1       | F      | ACACTGGGAATCTGGAGC  | 264-368    | 104       | 59.01  |
|       |                  | R      | TGAACG TGACATAGGGCC |            |           | 59.90  |
| GAPDH | NM_001206359.1   | F      | CCATCTTCCAGGAGGAGTC | 109-500    | 392       | 58.92  |
|       |                  | R      | AACGCAGGGATATTCTCGG |            |           | 59.12  |

Table 2: Effects of media interactions on the expression of adipogenic marker genes in pMSC. Probability values are indicated for the individual medium/their interactions (X).

| Gene | Media Interaction | αMEM*M199 | aDMEM*M199 | αMEM*aDMEM |
|------|-------------------|-----------|------------|-------------|
| PPARγ|                   | X: 0.003  | X: 0.111   | X: 0.016    |
|      | αMEM: 0.649       | aDMEM: 0.032 | aDMEM: 0.025 |
|      | M199: 0.005       | M199: 0.000   | M199: 0.000   |
| C/EBPα| X: 0.026         | X: 0.100   | X: 0.004   |
|      | αMEM: 0.029       | aDMEM: 0.090 | aDMEM: 0.056 |
|      | M199: 0.018       | M199: 0.000   | M199: 0.050   |
| ApN  | X: 0.000          | X: 0.029   | X: 0.089   |
|      | MEM: 0.079         | aDMEM: 0.000 | MEM: 0.995   |
|      | M199: 0.053       | M199: 0.019   | aDMEM: 0.000   |

Figure 1 described the relative mRNA expression of Peroxisome proliferator-activated receptor (PPARγ), CCAAT enhancer-binding proteins (C/EBPα) and Adiponectin (ApN) in pMSC derived and grown in αMEM (A), aDMEM (D), M199 (M), αMEM/M199 (A/M), aDMEM/ M199 (D/M), αMEM/aDMEM (A/D) media at passage 5 (P5), passage 10 (P10) and overall (P5 and P10). The GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as an endogenous control for normalisation of test gene expression. The fold changes of gene expression were calculated using the method of Pfaffl, 2004 considering expression in aDMEM cells as calibrator. Values of each bar are mean ± SEM of triplicate samples from 3 pigs. Different letters ‘a to f (P5), ‘i to n’ (P10) and ‘u to z’ (overall) among the media indicated on top of each bar and the “*” mark between the passages on top represented significant difference at p ≤ 0.05.

At passage 5, expression of PPARγ was up-regulated (p<0.05) in the cultures maintained in M199, aDMEM/M199 (D/M) and αMEM/aDMEM (A/D) media. At this passage, the expression of PPARγ in aDMEM (D) and αMEM/M199 (A/M) was not different from a culture maintained in αMEM (A) (reference medium). At passage 10, the up-regulation (p<0.05) of PPARγ continued in M199 but reversed to down-regulation (p<0.05) in aDMEM, aDMEM/M199 and αMEM/aDMEM grown cultures. The level of PPARγ in αMEM/M199 was same as αMEM. Overall PPARγ expression was up-regulated in M199 (M) and aDMEM/M199 (D/M) and down-regulated in aDMEM culture.

Within a single medium between passage 5 and passage 10, the PPARγ expression was found to be down-regulated (indicated by asterisk *) in aDMEM and aDMEM/M199 media. For the remaining cultures, the expression of this gene between two passages was not significantly different.
The highest level of up-regulation of PPARγ gene was observed in culture maintained in aDMEM/M199 (D/M) medium at passage 5.

**C/EBPα**

At passage 5, the expression of C/EBPα was up-regulated (p<0.05) in all cultures maintained with different media. At passage 10, C/EBPα was up-regulated only in culture maintained in M199, down-regulated (p < 0.05) in aDMEM and αMEM/M199 culture and same αaMEM in the remaining aDMEM/M199 and αMEM/aDMEM cultures. Overall, the C/EBPα up-regulation (p<0.05) was recorded in M199, aDMEM/M199 and αMEM/aDMEM culture.

Within a single medium between passage 5 and passage 10, the C/EBPα expression was significantly reduced (indicated by asterisk*) for cultures maintained in aDMEM, M199, αMEM/M199 and aDMEM/M199.

The highest level of up-regulation of C/EBPα gene was observed in culture maintained in M199 (M) medium at passage 5.

**ApN**

At passage 5, ApN expression was down-regulated (p<0.05) in all cultures propagated in different media except M199, where its expression was similar to the level of the reference culture (αMEM). At passage 10, expression of ApN was up-regulated (p<0.05) in M199 (M) whereas in all other cultures, it was down-regulated (p<0.05). Overall, the expression of ApN was same as reference culture in M199, whereas down-regulated expression (p<0.05) was recorded in cells grown in all other media.

With in a single medium between passage 5 and passage, 10ApN expressions was not different in all the cultures.

To understand the extent of each media influence the expression of adipogenic marker genes, we further analysed the data. The gene expression (PPARγ, C/EBPα and ApN ) data in the absence (0, 0), presence of single (1, 0 / 0, 1) and combinations (1, 1) of two different basal media formulations were arranged. The gene expression in the absence of a particular media signified that the cells were cultured in a basal media other than the two media and its combinations. Triplicate of each sample was analysed by qPCR, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as an endogenous control gene and αMEM expression as calibrator. The results are presented in Figure 2 and Table 2. The p values indicated in graphs and Table 2 were considered significant at p≤0.05. The log 2 Fold mRNA expression at passage 5 and passage 10 of three different pig samples were pooled to obtain the marginal means for plotting the graph. Expression of PPARγ was found to be affected by aDMEM, M199, and combined αMEM/M199 and αMEM/aDMEM media; C/EBPα was affected by the αMEM, M199 and combined αMEM/M199 and αMEM/aDMEM and the ApN expression was affected by a DMEM, M199 and the combined αMEM/M199 and aDMEM/M199 media (Figure 2 and Table 2).

**Adipogenic differentiation characteristics**

Figure 3 described at day 0 the cells in αMEM (a), aDMEM (b), M199 (c), αMEM/M199 (d), aDMEM/ M199 (e) and αMEM/aDMEM (f) were spindle-shaped. By day 12 the cells in αMEM (g), M199 (i) and αMEM/M199 (j) showed plenty of oval adipoblast-like cell morphology, few adipocyte-like and MSC like cells (indicated by arrow), the aDMEM, aDMEM/M199 and αMEM/aDMEM had spindle-shaped undifferentiated cells (rectangle); cells of aDMEM (h) and aDMEM/M199 (l) showed patches of nodular structures typical of osteogenic cells (inset) along with adipogenesis changes. By day 21, cells grown in all the media had more adipocytes; however, in M199 (o) and αMEM/M199 (p) had plenty of differentiated adipocyte-like cells showing dense small round vesicles; in aDMEM/M199 (q) and αMEM/aDMEM (r) had an intermediate number of differentiated adipocyte-like cells, and the αMEM (m) and aDMEM (n) had the least number of adipocyte-like cells. Magnifications of respective images are as denoted by the scale bar (50μm).

The undifferentiated pMSC in the different media were all spindle-shaped with no distinct changes in their morphologies at passage 4. However, the pMSC grown in different media responded differently to the induction of adipogenic differentiation. The differentiated cells differed in the initial morphological changes, accumulation of round vesicles in adipocyte like cells, sizes of round vesicles formed, presence or absence of undifferentiated cells and presence of contaminating osteoblast/osteocytes in the population by day 21 of differentiation. By day 3 of differentiation, the αMEM, aDMEM (Figures 3 and 4), and αMEM/aDMEM media grown cells maintained the typical spindle-shaped MSC morphology. However, many cells in M199, αMEM/M199 had shown oval-shaped adipoblast like morphology, and some cells showed accumulation of round vesicles.

By day 12, the pMSC grown in αMEM, M199 and αMEM/M199 showed predominant cells with adipoblast-like oval morphology, some adipocyte-like cells containing multiple round vesicles and
Figure 1: Effects of media and passages on adipogenic marker gene expressions in porcine bone marrow mesenchymal stem cells (pMSC).

Figure 2: Effects of media interactions on PPARgamma, C/EBPalpha and ApN gene expressions

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Figure 3: Comparative adipogenic differentiation progress of the different media grown porcine bone marrow mesenchymal stem cells (pMSC).

limited undifferentiated spindle-shaped cells; in aDMEM, aDMEM/M199 and αMEM/aDMEM few maturing adipocytes like cells packed with small round vesicles were observed amidst large spindle-shaped enlarged cells.

By the day 21, the pMSCs grown in αMEM showed a varying number of adipocyte-like cells containing small to large sizes round vesicles, some contaminated osteoblast- and osteocyte-like cells; in aDMEM, a limited number of differentiated adipocyte-like cells exhibited small-size round vesicles and also showed the presence of osteoblast- and osteocyte-like cells (Figure 3, inset). The M199, αMEM/M199 grown cells formed dense areas of mature adipocytes with small and large vesicles that covered the entire surface of the differentiated cells. In these two media grown cells, no undifferentiated MSC and osteocyte- or osteoblast-like cells were observed. In aDMEM/M199 and αMEM/aDMEM grown cells although dense areas of mature adipocytes were observed a few undifferentiated spindle-shaped and differentiated osteoblast- and osteocyte-like cells were also present.

The round vesicles observed in the differentiated cells stained positive for Oil Red-O stain thus confirmed that the accumulated small round vesicles were lipid vesicles of adipocytes.

Figure 4 described M199 grown pMSC by day 3 of differentiation induction (a) changed to spherical shape (line circles) an indication of pre-adipocytes (adipoblast). At the same time, very few cells from aDMEM (b.) were spherical, and most of the cells maintained undifferentiated morphology (dotted circle). A large number of adipocytes with a huge accumulation of lipid vesicles observed in M199 category differentiated cells (c) whereas undifferentiated cells and osteogenically differentiated cells were present along with multiple adipocytes in aDMEM (d) by day 21 of differentiation as evidenced by Oil-O-Red staining. Magnifications of respective images are as denoted by the scale bar (50 µm).

Basal media plays a significant role in the maintenance of cell proliferation and differentiation potential of the mesenchymal stem cells. Our earlier studies indicated the M199 when mixed with either of αMEM or aDMEM at 1:1 ratio supported the proliferation, and the αMEM/aDMEM supported the osteogenic differentiation of porcine MSC (data submitted elsewhere). In the current study, we reported that different basal media influence the adipogenic differentiation depending upon the expression of adipogenic marker genes in the pre-differentiation pMSC.

The αMEM, M199 and aDMEM are most commonly used basal media for MSC culture in different species. The αMEM and DMEM require compulsory serum supplementation; however; Hams F12 and M199 are developed as serum-free media (Arora, 2013). Therefore Hams F12 and M199 contain some extra components that are not available in αMEM and DMEM media. Mixing of two media helps to combine the advantages; however, do not replace the statistical design based concentrations optimisation method. Mixing of two media, DMEM and Ham’s F12 are used earlier for the culture of Chinese Hamster Ovary (CHO) cells, ATCC cancer cell line CCL-61 (Maun et al., 2019), rat adult neural stem/progenitor cells (Toda et al., 2003), rat brain microvascular endothelial cells (Simon et al., 2001) and upper-airway basal stem cells (Vaidyanathan et al., 2020).

Any basal medium that contains only a set of fixed
bio-chemicals with serum supplements may not fulfill the nutritional requirements of the dynamically changing demand of MSC for proliferation and cell differentiation. Such deficiency might be reflected as problems associated with the long term culture. The problems of proliferation (Ferrari et al., 2014) and differentiation potentials (Vacanti et al., 2005) are indeed observed for porcine MSC grown long term in the αMEM and DMEM. Improvisation of in vitro culture basal media composition, supplements, and growth factors is thus imperative for MSC culture. Constituents wise the aDMEM (http://himedialabs.com/TD/RSL019) contain a limited number of biochemical, αMEM (http://himedialabs.com/TD/AL081) has the maximum, and the M199 (http://himedialabs.com/TD/AL014) has many biochemical that is not available both in αMEM and aDMEM. Since M199 is used for embryo culture across the species, it is expected to contain many components that might help the proliferation and up keeping of the MSC properties. The results of this study provided additional proof of this concept.

Bone marrow stroma is the home for haematopoietic stem cells and non-hematopoietic MSC. The bone marrow-derived plastic adherent cells thus might naturally contain MSC, its precursors and derivative subpopulations, therefore, would show different marker expression and functional properties. The interesting fact that emerged from this study was that although the surface marker expression of the same media derived MSC of three pigs were different, the basal media in which they were grown affected the expression of adipogenic markers and the adipogenic differentiation characteristics.

Two master regulators control the adipogenic and osteogenic differentiation of MSC the Peroxisome proliferator-activated receptor γ (PPARγ) for adipogenic lineage cells and the Runx-related transcription factor 2 (RUNX2) for the osteogenic lineage cells (James, 2013). During adipogenesis, several signal cascades are activated by the increased expression of the key transcription factors Peroxisome proliferator-activated receptor-γ (PPARγ) and members of CCAAT/enhancer-binding proteins (C/EBPs) family (Muruganandan et al., 2009). The PPARγ are nuclear hormone receptor gene superfamily, expression of which is restricted to adipocytes (James, 2013). The PPARγ exists in two isoforms. The PPARγ1 and PPARγ2 and the expressions of both are highly inductive for adipogenesis (Muruganandan et al., 2009). It has been shown that heterozygous PPARγ mice had reduced ability of adipogenesis; however, show increased bone mass due to increased osteogenesis (Cao et al., 2015). Other proteins known to influence adipogenesis is the basic leucine-zipper proteins called C/EBPs. The C/EBPs expressed in adipocytes and existed in seven isoforms of which the C/EBPα, C/EBPβ and C/EBPδ are involved in promoting adipogenesis. The cyclic AMP (cAMP) response element-binding protein (CREB) a transcription factor regulates the expression of C/EBPα and PPARγ (Rosen and MacDougald, 2006). The C/EBPα and PPARγ synergistically stimulate each other’s expression, and the levels of both remain elevated in mature adipocytes (Muruganandan et al., 2009). Mouse embryonic fibroblast cells that lack C/EBPα expression show low PPARγ expression (Otto and Lane, 2005). A similar relationship between these two gene’s expression was observed in this study, as well. The passage 5 up-regulation of PPARγ and C/EBPα genes in all the media grown cells compared to the reference medium (αMEM) indicated their role in changing the expression of...
both the genes. This was further supported by the data on the effects of media interaction on these gene expressions. The up-regulation of PPARγ and C/EBPα observed in M199 media grown passage 5 cells indicated they were committed for adipogenic differentiations which was evident from the differentiation results. The maintained up-regulation of these two genes at passage 10 indicated such commitments might not have changed over the passages. In interaction analysis, the M199 was found to exert a significant influence on the expression of all the three adipogenic marker genes. As a result, early appearance of adipoblast-like cell morphology by day 3, better progress of differentiation by day 12, no presence of osteogenic cells and no undifferentiated MSC observed by day 21 differentiated cells of M199 grown pMSC. The reason could be attributable to the presence of some pro-adipogenic factors such as retinyl-acetate @ 0.14 mg/L (0.43μM), the vitamin E (α-tocopherol) @ 0.01mg/L (23.2nM) and a purine base Xanthine @ 0.344 mg/L (2.26μM) in M199. The vitamin E at 50μM concentration is known to induce adiponectin and PPARγ expression in mice3T3 cells-L1 pre-adipocyte (Landrier et al., 2012). The tocopherol based regulation of PPARγ expression is shown to be mediated through the increased concentrations of endogenous ligand 15d-PGJ2 concentrations (Landrier et al., 2012). Xanthine is the precursor of Iso-butyyl methyl Xanthine (IBMX), a phosphodiester inhibitor that raises the cAMP and protein kinase-A levels to require for the PPARγ activation (Scott et al., 2011). Xanthine also gets converted into uric acid by Xanthine oxidoreductase resulting in hyperuricemia, a condition known to be associated with adiposity in humans (Cheung et al., 2007). At 1mM concentration, Xanthine helps in the maintenance of stemness by suppression of asymmetric cell kinetics and promotes the multiplication of adult stem cells (Paré and Andsherley, 2011). It is not known however if low concentrations of Xanthine in M199 and αMEM/M199 contribute towards better adipogenic differentiation which might need separate experimentation for confirmation. Besides, the vitamin B12 @10μM is proved to attenuate the adipogenesis process by activating Wingless-type (Wnt) signals by turning on Wnt10b and related proteins in 3T3-L1 pre-adipocyte cells (Bellner et al., 2015). The αMEM and αMEM/M199 both contained the vitamin B12 @ 1.36 mg/L (1μM) and 0.68 mg/L (0.5μM), respectively. In our study, the αMEM grown pMSCs had a problem of adipogenesis, whereas, the αMEM/M199 grown cells showed better differentiation and had a different pattern of C/EBPα and ApN expressions.

The presence of osteogenic cells along with adipogenic cells in aDMEM and aDMEM/M199 grown pMSC during differentiation, although indicated the osteogenic potential of the cells is not a desirable outcome for research and other application. The down-regulation of PPARγ and C/EBPα in aDMEM and aDMEM/M199 media at passage 10 coincided with the up-regulation of pre-osteogenic marker Collagen 1 expression in these media (data submitted elsewhere). The reason for the presence of osteoblast and osteocyte-like cells, along with predominant adipogenic cells could be attributable to the above reason. The different cell phenotypes at the end of 21 days differentiation period in αMEM/aDMEM media than in αMEM and aDMEM grown MSC indicated a differential response and acquisition of divergent properties of the cells towards adipogenesis when two media were combined.

The PPARγ and C/EBPα together regulate adipogenesis by inducing the expression of mainly the adiponectin (ApN) and other genes (Menssen et al., 2011). Overexpression of ApN promotes lipid accumulations and stimulation of insulin for greater glucose uptake from the blood through increased expression of glucose transporter (GLUT4) (Fu et al., 2005). The pre-adipocytes aid hematopoietic stem cells for blood cell formation, while the mature adipocytes produce less colony-stimulating factor-1 and alter haematopoiesis by changing the expression of cytokines (Zhang et al., 2020). Synthesis of ApN increases with adipocyte differentiation. However, the pre-adipocytes direct ApN towards a paracrine feedback loop to block the fat cell formation by the induction of cyclooxygenase-2 (COX-2) and prostaglandins (Yokota et al., 2002). Interestingly, the osteoblast differentiation is stimulated by ApN in the COX-2 dependent manner by activating AdipoR1 and p38 MAPK to promote c-Jun, which is essential for osteogenic differentiation (Lee et al., 2009). The downregulation of ApN expression in aDMEM, αMEM/M199, aDMEM/M199 and αMEM/aDMEM media grown MSC might reflect the inhibition of this process and was visible in differentiated cells. The ApN expression in αMEM and M199 media were similar. However, the pMSC grown in M199 differentiated better to adipogenic cells than αMEM by day 21 of differentiation. This can be explained by the fact that pMSC grown in M199 had up-regulated PPARγ and C/EBPα than αMEM due to the compositional differences and the up-regulation was maintained even by passage 10. The up-regulated expressions of PPARγ and C/EBPα give rise to several adipogenic transcription cascades by the induction of pro-adipogenic factors.
such as the Krüppel-like factors (KLFs), early growth response protein-2 (KROX20) and sterol regulatory element-binding protein-1c (SREBP-1c) that promote adipogenesis (Rosen and MacDougald, 2006).

As a result, M199 had shown better differentiation. Our results were thus in agreement with the findings of (Adamzyk et al., 2013) which states that the differentiation potential of MSC might vary with the changes in the composition of the pre-culture medium. On the contrary, (Hagmann et al., 2013) did not observe any change in osteogenic and adipogenic differentiation potential of bone marrow MSC cultured in different basal media. It has been clear from this study that the M199, αMEM/M199 grown MSC responded to the best adipogenesis compared to the intermediate in αMEM/aDMEM and aDMEM/M199 and the least in αMEM and aDMEM grown pMSC. The reduction in PPARγ and C/EBPα gene expression (p<0.05) in both aDMEM and aDMEM/M199 grown cells at passage 10 compared to passage 5 indicated the differentiation potentials of MSC might not be consistent with the increase in passage numbers.

We have seen a high and low expression of CD90 and CD14 in different media derived cells. The CD90 in MSC controls the expression of collagen-I and osteonectin in osteoblast-like cells (Wiesmann et al., 2006) and block adipogenesis by dampening the expression of PPARγ (Woeller et al., 2015). We did not observe any relationship of adipogenic differentiation with the high and low expression of CD90 and CD14 by the endpoint PCR analysis. Nevertheless, the observed effect on adipogenic differentiation was due to the influence of basal media since the cells were grown in the same medium although had different cell marker expression patterns in three different pigs, had similar adipogenic differentiation patterns.

CONCLUSIONS

The results of this study indicated basal media influenced the expression of adipogenic marker genes and differentiation potential of porcine bone marrow MSC. The pMSC grown in M199 and αMEM/M199 were the best; the αMEM/aDMEM and aDMEM/M199 were intermediate and the αMEM, aDMEM were the least capable of adipogenesis as assessed by 21 days of differentiation period. Different levels of pro and anti-adipogenic factors present in the basal media might have contributed to the observed differences in adipogenesis of the cultured cells.

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Conflict Of Interest

The authors declare no conflict of interest for this study.

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REFERENCES

Adamzyk, C., Emonds, T., Falkenstein, J., Tolba, R., Jahnen-Dechent, W., Lethaus, B., Neuss, S. 2013. Different Culture Media Affect Proliferation, Surface Epitope Expression, and Differentiation of Ovine MSC. Stem Cells International, 2013:1–13.

Arora, M. 2013. Cell culture media: a review. Materials methods, 3.

Ayatollahi, M., Salmani, M. K., Geramizadeh, B., Tabei, S. Z., Soleimani, Andsanati, M. H. 2012. Conditions to improve expansion of human mesenchymal stem cells based on rats samples. World Journal of Stem Cells, 4(1):1–8.

Bellner, L., Nichols, A., Pandey, V., Vanella, L., Gilliam, C., Gup, N. A. 2015. Effects of vitamin B12 and nutrients on adipogenesis-adipogenic markers in 3T3 cells. The Federation of. Experimental Biology, 29(1):996–1002.

Bosch, P., Pratt, S. L., Stice, S. L. 2006. Isolation, Characterization, Gene Modification, and Nuclear Reprogramming of Porcine Mesenchymal Stem Cells1. Biology of Reproduction, 74(1):46–57.

Cao, J., Ou, G., Yang, N., Ding, K., Kream, B. E., Hamrick, M. W., Isales, C. M., Shi, X.-M. 2015. Impact of targeted PPARγ disruption on bone remodeling. Molecular and Cellular Endocrinology, 410:27–34.

Cheung, K. J., Tzameli, I., Pissios, P., Rovira, I., Gavrilova, O., Ohtsubo, T., Chen, Z., Finkel, T., Flier, J., Friedman, J. M. 2007. Xanthine-oxidoreductase is a regulator of adipogenesis and PPARgamma activity. Cell Metabolism, 5(2):115–128.

Duque, G. 2008. Bone and fat connection in aging bone. Current Opinion in Rheumatology, 20(4):429–434.

Ferrari, C., Olmos, E., Balandras, F., Tran, N., Chevalot, I., Guedon, E., Marc, A. 2014. Investigation of Growth Conditions for the Expansion of Porcine Mesenchymal Stem Cells on Microcarriers in Stirred Cultures. Applied Biochemistry and
Biotechnology, 172(2):1004–1017.

Fu, Y., Luo, N., Klein, R. L., Garvey, W. T. 2005. Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. Journal of Lipid Research, 46(7):1369–1379.

Gordon, S., Pittenger, M., McIntosh, K., Peter, S., Archambault, M., Young, R. 2005. Tendon regeneration using mesenchymal stem cells. Tendon Regeneration Using Mesenchymal Stem Cells, pages 313–320.

Guo, X., Bai, Y., Zhang, L., Zhang, B., Zagidullin, N., Carvalho, K., Du, Z., Cai, B. 2018. Cardiomyocyte differentiation of mesenchymal stem cells from bone marrow: new regulators and its implications. Stem Cell Research & Therapy, 9(1):44–44.

Hashmani, K., Branch, M., Sidney, L., Dhillon, P., Verma, M., McIntosh, O., Hopkinson, A., Dua, H. 2013. Characterization of corneal stromal stem cells with the potential for epithelial transdifferentiation. Stem Cell Research & Therapy, 4(3):75–75.

James, A. W. 2013. Review of signalling pathways governing MSC osteogenic and adipogenic differentiation. Scientifica.

Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K. T. 2006. Adiponectin and adiponectin receptors in insulin resistance, diabetes and the metabolic syndrome. The Journal of Clinical Investigations, 116(7):1784–1792.

Kannan, S., Dhara, S. K., Ghosh, J. 2019. Porcine Mesenchymal Stem Cell Derivation by Plating Bone Marrow Cells Directly and After Erythrocyte Lysis. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 89(2):565–573.

Landrier, J.-F., Marcotorchino, J., Tourniaire, F. 2012. Lipophilic Micronutrients and Adipose Tissue Biology. Nutrients, 4(11):1622–1649.

Lee, H. W., Kim, S. Y., Kim, Y., Lee, E. J., Choi, Kim, J. B. 2009. Adiponectin stimulates osteoblast differentiation through induction of COX2 in mesenchymal progenitor cells. Stem Cell, 27(9):2254–2262.

Lee, K. D., Kuo, T. K., Whang-Peng, J., Chung, Y. F., Lin, C. T., Chou, S. H., Chen, J. R., Chen, Y., Lee, O. K. 2004. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology, 40(6):1275–1284.

Maun, H. R., Jackman, J. K., Choy, D. F., Loyet, K. M., Staton, T. L., Jia, G., Viji, R. 2019. An allosteric antitryptase antibody for the treatment of mast cell-mediated severe asthma. Cell, 179(2):417–431.

Menssen, A., Häupl, T., Sittering, M., Delorme, B., Charbord, P., Ringe, J. 2011. Differential gene expression profiling of human bone marrow-derived mesenchymal stem cells during adipogenic development. BMC Genomics, 12(1):461–461.

Moseti, D., Regassa, A., Kim, W.-K. 2016. Molecular Regulation of Adipogenesis and Potential Anti-Adipogenic Bioactive Molecules. International Journal of Molecular Sciences, 17(1):124–124.

Muruganandan, S., Roman, A. A., Sinal, C. J. 2009. Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: Cross talk with the osteoblastogenic program. Cellular and Molecular Life Sciences, 66(2):236–253.

Otto, T. C., Lane, M. D. 2005. Adipose Development: From Stem Cell to Adipocyte. Critical Reviews in Biochemistry and Molecular Biology, 40(4):229–242.

Paré, J., Andsherley, J. L. 2011. Culture environment-induced pluripotency of SACK-expanded tissue stem cells. Journal of Biomedicine and Biotechnology, pages 312457–312457.

Patel, Y. M., Lane, M. D. 2000. Mitotic Clonal Expansion during Preadipocyte Differentiation: Calpain-mediated Turnover of p27. Journal of Biological Chemistry, 275(23):17653–17660.

Pfaffl, M. W. 2004. Relative quantification. Real-time PCR, 63:63–82.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, Marshak, D. R. 1999. Multilineage potential of adult human mesenchymal stem cells. Science, 284(5411):143–147.

Portsalka, K. J., Leferink, A., Groen, N., Fernandes, H., Moroni, L., van Blitterswijk, C., de Boer, J. 2012. Endothelial Differentiation of Mesenchymal Stromal Cells. PLoS ONE, 7(10):e46842–e46842.

Rooney, G. E., McMahon, S. S., Ritter, T., Garcia, Y., Moran, C., Madigan, N. N., Flügel, A., Dockery, P., O’Brien, T., Howard, L., Windebank, A. J., Barry, F. P. 2009. Neurotrophic Factor–Expressing Mesenchymal Stem Cells Survive Transplantation into the Contused Spinal Cord Without Differentiating into Neural Cells. Tissue Engineering Part A, 15(10):3049–3059.

Rosen, E. D., MacDougald, O. A. 2006. Adipocyte differentiation from the inside out. Nature Reviews Molecular Cell Biology, 7(12):885–896.
Sanjurjo-Rodríguez, C., Castro-Viñuelas, R., Hermida-Gómez, T., Fernández-Vázquez, T., Fuentes-Boquete, I. M., de Toro-Santos, F. J., Díaz-Prado, S. M., Blanco-García, F. J. 2017. Ovine Mesenchymal Stromal Cells: Morphologic, Phenotypic and Functional Characterization for Osteochondral Tissue Engineering. *PLOS ONE*, 12(1):e0171231–e0171231.

Scott, M. A., Nguyen, V. T., Levi, B., James, A. W. 2011. Current Methods of Adipogenic Differentiation of Mesenchymal Stem Cells. *Stem Cells and Development*, 20(10):1793–1804.

Sidney, L. E., Branch, M. J., Dua, H. S., Hopkinson, A. 2015. Effect of culture medium on propagation and phenotype of corneal stroma-derived stem cells. *Cytotherapy*, 17(12):1706–1722.

Simon, J., Vigne, P., Eklund, K. M., Michel, A. D., Carruthers, A. M., Humphrey, P. P., Frelin, Barnard, E. A. 2001. Activity of adenosine diphosphates and triphosphates on a P2Y(T)-type receptor in brain capillary endothelial cells. *British Journal of Pharmacology*, 132(1):173–182.

Toda, H., Tsuji, M., Nakano, I., Kobuke, K., Hayashi, T., Kasahara, H., Takahashi, J., Mizoguchi, A., Houtani, T., Sugimoto, T., Hashimoto, N., Palmer, T. D., Honjo, T., Tashiro, K. 2003. Stem Cell-derived Neural Stem/Progenitor Cell Supporting Factor Is an Autocrine/Paracrine Survival Factor for Adult Neural Stem/Progenitor Cells. *Journal of Biological Chemistry*, 278(37):35491–35500.

Ullah, I., Subbarao, R. B., Rho, G. J. 2015. Human mesenchymal stem cells - current trends and future prospective. *Bioscience Reports*, 35(2):191–191.

Vacanti, V., Kong, E., Suzuki, G., Sato, K., Canty, J. M., Lee, T. 2005. Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. *Journal of Cellular Physiology*, 205(2):194–201.

Vaidyanathan, S., Salahudeen, A. A., Sellers, Z. M., Bravo, D. T., Choi, S. S., Batish, A., Le, W., Baik, R., de la O and Milan P. Kaushik, S., Galper, N., Lee, C. M., Teran, C. A., Yoo, J. H., Bao, G., Chang, E. H., Patel, Z. M., Hwang, P. H., Wine, J. J., Milla, C. E., Desai, T. J., Nayak, J. V., Kuo, C. J., Porteus, M. H. 2020. High-Efficiency, Selection-free Gene Repair in Airway Stem Cells from Cystic Fibrosis Patients Rescues CFTR Function in Differentiated Epithelia. *Cell Stem Cell*, 26(2):161–171.e4.

Wakitani, S., Saito, T., Caplan, A. I. 1995. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle and Nerve*, 18:1417–1426.

Wang, Y. 2016. Differentiation of Bone Marrow Mesenchymal Stem Cells in Osteoblasts and Adipocytes and Its Role in Treatment of Osteoporosis. *Medical Science Monitor*, 22:226–233.

Watcharat, K., Korchunjit, W., Buranasinsup, S., Taylor, J., Rittruechai, P., Wongtawan, T. 2017. MEM α Promotes Cell Proliferation and Expression of Bone Marrow Derived Equine Mesenchymal Stem Cell Gene Markers but Depresses Differentiation Gene Markers. *Journal of Equine Veterinary Science*, 50:8–14.

Wiesmann, A., Bühring, H.-J., Mentrup, C., Wiesmann, H.-P. 2006. Decreased CD90 expression in human mesenchymal stem cells by applying mechanical stimulation. *Head and Face Medicine*, 2:8–8.

Woeller, C. F., O’Loughlin, C. W., Pollock, S. J., Thatcher, T. H., Feldon, S. E., Phipps, R. P. 2015. Thy1 (CD90) controls adipogenesis by regulating activity of the Src family kinase, Fyn. *The FASEB Journal*, 29(3):920–931.

Yokota, T., Meka, C. R., Medina, K. L., Igarashi, H., Comp, P. C., Takahashi, M., Nishida, M., Oritani, K., ichiro Miyagawa, J., Funahashi, T., Tomiyama, Y., Matsuzawa, Y., Kincade, P. W. 2002. Paracrine regulation of fat cell formation in bone marrow cultures via adiponectin and prostaglandins. *Journal of Clinical Investigation*, 109(10):1303–1310.

Zhang, J., Liu, Y., Yin, W., Hu, X. 2020. Adipose-derived stromal cells in regulation of hematopoiesis. *Cellular & Molecular Biology Letters*, 25(1):1–11.