

In vitro characterization of umbilical cord-derived MSCs supplemented with PLAY[®]: A potential FBS substitute

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SUMMARY

Recent interest in the wound healing and regenerative properties of mesenchymal stem cells (MSCs) has prompted further investigation to elucidate their regenerative properties. Characterized by their ability to differentiate into various cell lineages, such as osteocytes, chondrocytes, and adipocytes, alongside promoting local angiogenesis and chemotaxis at the wound site, MSCs have proven to reduce the stipulated healing time. The myriad sources they originate from, including bone marrow and umbilical cord, underscore their wide range of clinical applicability. Extending the time for MSC senescence by using alternative media in a manner that preserves their stem-cell-like nature may be a viable option of achieving optimum cell numbers required for clinical protocols. Furthermore, various culture conditions have varied effects on MSCs, therefore, standardising MSC growth conditions may help to produce MSCs with verified potency, which could be then extrapolated to clinical conditions. The presence of potential contaminants within commonly used cell culture media such as fetal bovine serum (FBS) curbs the scope of MSC implementation in tissue regeneration. Our current study aimed to continue to investigate the effects of blood derivative PLAY[®] on trends in MSC culture across various experimental parameters in comparison to FBS. Our results demonstrated that PLAY[®] consistently outperformed FBS across all the experimental metrics studied.

INTRODUCTION

Mesenchymal stem cells (MSCs) have recently gained attention in the field of tissue engineering and regenerative medicine for their therapeutic properties including differentiation and regenerative potential, immune modulation and migratory capacity. The major three criteria required for their use in therapeutics, as listed by the International Society for Cell and Gene Therapy, are MSCs should (a) possess spindle-shaped fibroblastic-like morphology and plastic adherence, (b) be positive (>95%) for surface antigens CD105, CD90 and CD73, and (c) be capable of differentiating into chondrocytes, adipocytes, and osteocytes (1-6). MSCs' ability to proliferate *in vitro* across several passages while retaining their self-renewal capacity, alongside possessing intrinsic immunomodulatory and regenerative properties, emphasizes their therapeutic effect in wound healing and tissue homeostatic applications (7, 8). They have been

employed in the treatment of diseases such as multiple sclerosis, autoimmune diseases, and orthopaedic conditions (9). In addition, alternative treatment options to treat chronic ulcers, non-healing fractures, and scarless wound recovery have been made possible with the advances in MSC research (10-12). This reparative effect is exhibited through both paracrine and direct signaling involving the release of bioactive molecules at the wound site, as demonstrated both *in vitro* and *in vivo* in humans (13). Consequently, MSCs prevent the progression of a chronic wound state by promoting the wound healing and proliferative phase via the reduction of inflammatory cytokines (TNF- α and IFN- γ) and the upregulation of anti-inflammatory cytokines (IL-10 and IL-4) (8, 14, 15). MSC expression of VEGF, bFGF, and HGF also promotes re-epithelialization and angiogenesis at the wound site (16).

MSCs can be extracted from bone marrow, umbilical cord, adipose tissue, and dental pulp, among various other sources (17-19). The source of MSCs dictates their various properties. Umbilical cord-derived MSCs (UC-MSCs) are considered to have a more potent proliferative, immunomodulatory, and self-renewal capacity compared to MSCs derived from other tissues (20, 21). Furthermore, recent evidence has demonstrated the safety of UC-MSCs in clinical usage and immunocompromised patients (22, 23). Major histocompatibility complex I and II (MHC I and MHC II) and human leukocyte antigen (HLA) are proteins present on the surface of cells which represent the genetic uniqueness of an individual. HLA matching is mandatory for tissue and bone marrow transplantation and is required to ensure graft acceptance. Interestingly, MSCs have been shown to evade allo-rejection due to their lack of MHC I and MHC II expression. It is due to the lack of such markers on the surface of MSCs that allows them to be used in patients across HLA types. Additionally, their ability to promote immunosuppression by preventing chronic inflammation, allows UC-MSCs to be suitable for a wide range of applications, including premature ovarian failure, graft vs. host disease, and in diabetic wound healing (20, 24).

Considering that UC-MSCs are extracted from the umbilical cord at relatively low quantities, their expansion *in vitro* is necessary to facilitate their applications in translational medicine. However, this practice itself has inherent drawbacks because the cells undergo replicative senescence (i.e., shortening of the telomere length) progressively as the passages increase (25). This shows the crucial need for effective media supplements which preserve UC-MSCs' regenerative capabilities for a large number of passages.

Fetal Bovine Serum (FBS) is currently the conventional media supplement for the *in vitro* expansion of MSCs. FBS

contains various components, some of which are proteins, carbohydrates, hormones, electrolytes, and enzymes. Although it contains the necessary growth factors for MSC metabolism, proliferation, and growth, it is unsafe for clinical usage as it is prone to contamination with viruses, endotoxins, and mycoplasma (26-30). Moreover, animal welfare concerns and ethical implications bolster the need for xeno-free, human blood-derived media supplement alternatives. Additionally, it is highly important to expand FBS-MSCs (UC-MSCs' grown in FBS) *in vitro* to achieve clinically relevant cell numbers. However, FBS-MSCs have shown to become senescent at middle passages (P10), thereby hampering the clinical utilization of these cells (31).

PLAY[®], a blood-derived cytokine concentrate, may be a suitable alternative to FBS. PLAY[®] may also prevent chromosomal aberrations and preserve genomic stability, making it an ideal candidate for further study (25, 32). Additionally, this alternative blood-derived media supplement may enhance the *in vitro* expansion of MSCs, improving their application in clinical settings.

We hypothesized that culturing MSCs in media supplemented with PLAY[®] would increase cell growth, maintain pluripotency, cell morphology, and maintain the functionality of MSCs by allowing differentiation into adipocytes, chondrocytes, and osteocytes at later passages compared to culture with FBS. Our results demonstrated that PLAY[®] consistently outperformed FBS across all the experimental metrics studied.

RESULTS

This study aimed to assess the effect of UC-MSCs when grown in media supplemented with either 10% PLAY[®] or 10% FBS. We quantified the results in terms of cell numbers, population doubling time, pluripotent gene expression, and differentiation ability of both experimental groups, (a) UC-MSCs grown in FBS (FBS-MSCs) and (b) UC-MSCs grown in PLAY[®] (PLAY[®]-MSCs), at passages P5, P10, P15, and P20.

UC-MSCs grown in PLAY[®] maintain their morphology across passages

UC-MSCs were grown in media supplemented with either 10% FBS or 10% PLAY[®] for 7 days, and at each passage, were imaged using a wide field microscope to characterize their morphology. PLAY[®]-MSCs showed fibroblastic morphology with a high number of colony-forming units. Moreover, they maintained their morphology through P20. In our previous study we demonstrated that FBS-MSCs showed an elongated, flat, irregular morphology at P10 which could indicate senescence (33). Thus, we saw the preservation of overall cell culture quality as observed by cell morphology when grown in media supplemented with PLAY[®] (Figure 1).

Cell number and population doubling time of PLAY[®]-MSCs are maintained across passages

UC-MSCs were grown in media supplemented with either 10% FBS or 10% PLAY[®] for 7 days. These cells were harvested and counted using trypan blue to obtain cell numbers at each passage. The harvested cell numbers were used to theoretically calculate the population doubling time of the cells using the prescribed equation.

PLAY[®]-MSCs had faster growth kinetics, with a much

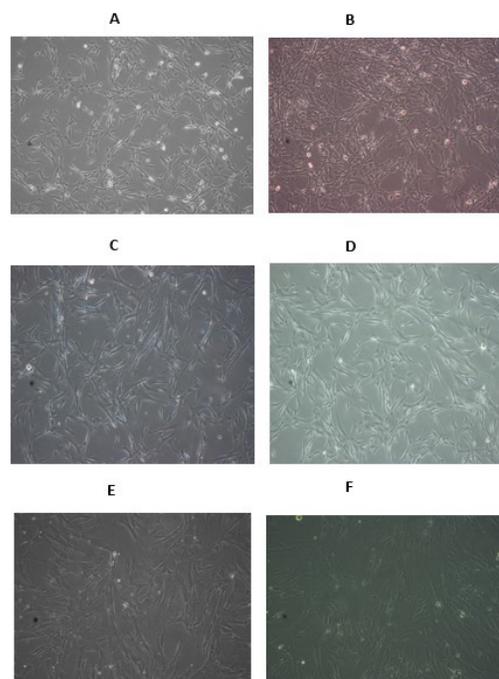


Figure 1. Morphology of UCMSCs grown in either 10% PLAY[®] or 10% FBS. A-D Representative inverted microscopic images (10X) displayed spindle-shaped fibroblastic morphology of PLAY[®]-MSCs at all passages studied (P5, P10, P15, and P20). E-F FBS-MSCs at P5 also displayed spindle-shaped morphology but at P10, these cells lost spindle-shaped morphology as seen by the enlarged, flat, and irregular shape.

higher proliferation rate in comparison to FBS-MSCs. PLAY[®]-MSCs cell numbers drastically decreased from P5 to P10 and thereafter maintained similar cell numbers until P20 (Figure 2). This contrasts starkly with the trend observed in FBS-MSCs, which had decreased cell counts in all the passages as compared to PLAY[®]-MSCs. Although the difference in cell numbers between PLAY[®]-MSCs and FBS-MSCs was not significant at P10 ($p > 0.05$), there was a significant difference at P15 ($p < 0.05$). FBS-MSCs were not subcultured after P15 as there were not enough cells to passage. The trends in population doubling time reflect those in cell number in that PLAY[®]-MSCs doubling time were much faster across experimental passages (P5, P10, P15, P20) in comparison to FBS-MSCs. Similar to the trend observed in cell numbers, the difference in doubling time between PLAY[®]-MSCs and FBS-MSCs was not significant at P5 or P10 ($p > 0.05$), but a significant difference was observed at P15 ($p < 0.01$).

PLAY[®]-MSCs express pluripotency markers

The stemness nature of UC-MSCs was determined as a function of pluripotency markers (*SOX2*, *OCT4*, *NANOG*) and was measured by RT-PCR. PLAY[®]-MSCs expressed these three markers through P20. *SOX2* and *NANOG* were highly expressed in P5 PLAY[®]-MSCs as compared to FBS-MSCs ($p < 0.05$). Although PLAY[®]-MSCs expression gradually decreased at later passages, there was no significant difference between late passage PLAY[®]-MSCs (P20) and early passage FBS-MSCs (P5). In the case of *OCT4*, a significant increase in the expression levels was observed at late passage (P20) of PLAY[®]-MSCs in comparison to early passage FBS-MSCs (P5).

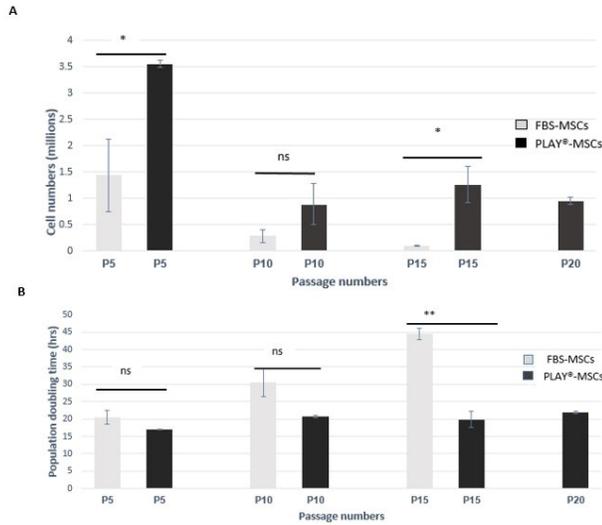


Figure 2. Growth kinetics of UCMSCs grown in either 10% PLAY® or 10% FBS. (A) Cell numbers obtained via trypan blue exclusion (n=3) **(B)** Population doubling time (n=3). Data values are represented by mean ± SD. *p < 0.05, **p < 0.01, ns=not significant.

(Figure 3). Due to lower cell numbers and thus lower sample concentration of FBS-MSCs at P10, gene expression analysis for this experimental group was not performed further P10.

PLAY®-MSCs retain tri-lineage differentiation

UC-MSCs were grown in media supplemented with either 10% FBS or 10% PLAY® until 70% confluency was attained. We then added of one of three differentiation media (adipocyte, osteocyte and chondrocyte) and incubated cells for 21 days with periodic changing half media every three days. These cells were then evaluated for their differentiation potential in terms of staining and specific gene expression analysis.

We evaluated the ability of PLAY®-MSCs to differentiate into adipocytes, osteocytes, and chondrocytes at passages P5, P10, P15, and P20 in comparison to FBS-MSCs at P5. Due to a very slow growth rate of FBS-MSCs from P10, these cells were not assessed for its differentiation potential. PLAY®-MSCs cultured in adipocyte differentiation medium exhibited adipocyte specific morphological changes as evidenced by the accumulation of oil droplets when stained with oil red O (stain specific for adipocyte), thus indicating the adipocyte differentiation. Similarly, PLAY®-MSCs directed to differentiate into osteocytes also showed distinct morphological changes pertaining to osteocytes and stained positive for alizarin red (stain specific for osteocyte), as indicated by the presence of red calcium deposits. PLAY®-MSCs were also differentiated into chondrocytes, as evidenced by the presence of chondrocyte specific blue proteoglycan-producing pellets when stained with alcian blue **(Figure 4)**, thus confirming the chondrocyte differentiation. Therefore, from our results, PLAY®-MSCs seemed to have similar levels of differentiation capacity (as that seen in FBS-MSCs at P5) at all 4 timepoints (P5, P10, P15 and P20) **(Figure 5)**.

We evaluated PLAY®-MSCs for their adipogenic, osteogenic, and chondrogenic markers (*FABP4*, *SPP1*, and *ACAN*) after exposure to the differentiation media for 21 days, by semi quantitative RT-PCR. Our results indicated the stable expression of these markers at passages P5, P10, P15, and

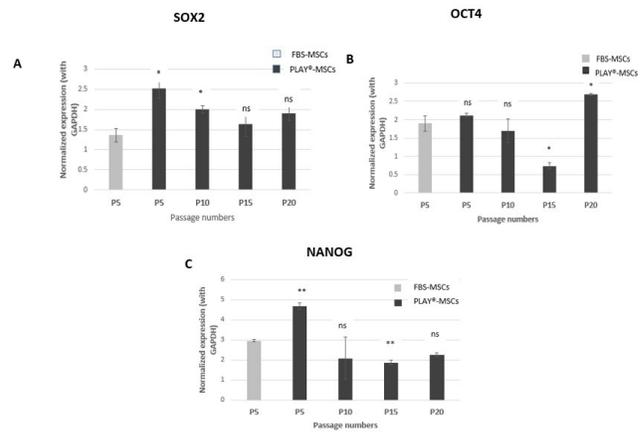


Figure 3. Pluripotency gene expression analysis by semi-quantitative RT-PCR. Normalized expression (with *GAPDH*) of **(A)** *SOX2*, **(B)** *OCT4*, and **(C)** *NANOG* of PLAY®-MSCs at P5, P10, P15, and P20 (n=2) in comparison to control (P5-FBS-MSCs). Data values are represented as mean ± SD. *p < 0.05, **p < 0.01, ns=not significant in comparison to FBS-MSCs at P5.

P20 of PLAY®-MSCs. We saw no significant difference in the expression of *FABP4* (adipogenic marker) between P5 FBS-MSCs and P5, P10, P15 and P20 of PLAY®-MSCs **(Figure 6)**. Even though we could see a significant decrease in the expression levels of *SPP1* (osteogenic marker) (p < 0.01 and p < 0.001) and *ACAN* (chondrogenic marker) (p < 0.05) at P5, P10, P15 and P20 of PLAY®-MSCs in comparison to the control (FBS-MSCs at P5), our results showed the ability of PLAY®-MSCs to differentiate into three lineages in the presence of induction medium (as per ISCT criteria).

DISCUSSION

Our results demonstrated the efficacy of our biological concentrate PLAY®, a human blood derivative, in terms of supporting MSCs in culture and providing optimal growth conditions for the cells to retain their stemness and growth potential. Our results illustrated that PLAY® not only maintains, if not enhances, MSC morphology and cell growth but also promotes stemness and differentiability, thereby preserving and strengthening their potential regenerative capabilities in comparison to FBS. Our results for FBS-MSCs align with the research conducted by Gu *et al.*, which revealed a higher percentage of senescent cells at middle and late passages compared to early passages (P11 and P17 vs. P4) (31).

Researchers have been exploring alternative supplements for growing cells for several decades. The earliest cultures utilized human serum to grow cells, which was replaced with more viable sources such as horse serum and bovine serum. With the advent of regenerative medicine, where cells are used as therapeutic agents, the issue of uniformity of cells and their immunogenicity is a matter of intense discussion. The typical requirement for therapeutic use is around 1 million cells/kg body weight (34). This demands a reliable source of serum/growth factors and a cost-effective alternative to fulfill the demand. Other important considerations include the immunogenicity of MSCs grown in xenogenic supplement such as FBS, which is currently the staple supplement. It is crucial to consider supplement source and growth medium conditions when cultivating MSCs because MSCs gradually lose their stemness during subsequent passages, and that

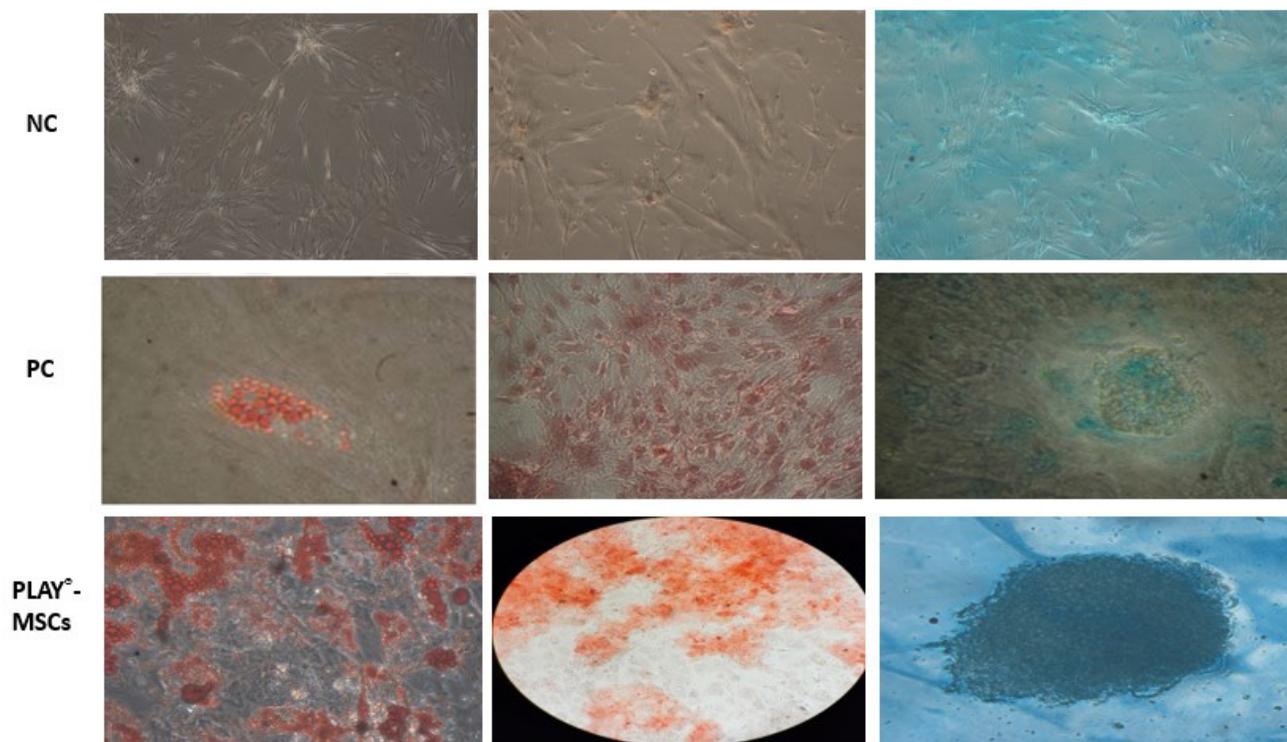


Figure 4. Differentiation potential of UC-MSCs expanded in media supplemented with either 10% PLAY® or 10% FBS. Representative microscopic images were taken at 10X magnification. Adipogenic-differentiated PLAY®-MSCs stained with oil red indicating the presence of lipid droplets, osteogenic-differentiated PLAY®-MSCs stained with alizarin red indicating the presence of calcium deposits, and chondrogenic-differentiated PLAY®-MSCs stained with alcian blue indicating the presence of proteoglycan-producing pellets to confirm differentiation potential. NC: Negative control (PLAY®-MSCs without the respective differentiation medium), PC: Positive control (Bone marrow derived MSCs at P5 exposed to respective differentiation medium).

poses a severe limitation on their expansion potential. PLAY® offers a reliable, cost-effective, and ethically viable solution to grow and expand MSCs, as evidenced by our results.

Antoninus *et al.*, evaluated the potential of human platelet lysate as an alternative xeno-free media in enhancing the proliferation rates of umbilical-cord derived mesenchymal stem cells (UC-MSCs) in comparison to FBS culture condition. Their results demonstrated a much lower population doubling time for UC-MSCs grown in platelet lysate than in FBS culture conditions especially at higher passages. Additionally, both the groups at late passage (P8) showed the tri-lineage differentiation (35). Our study also explored an alternative xeno-free media (human blood-derivative product (PLAY®)) in the expansion of UC-MSCs *in vitro* in comparison to the conventional supplement FBS. Interestingly, PLAY® enabled the UC-MSCs to grow till passage 20 in comparison to FBS which allowed cells to grow stably till P7 (Data not shown). Although UCMSCs grown in PLAY® showed the tri-lineage differentiation potential till passage 20, the same was not shown in FBS culture conditions from P10 due to its relatively slow growth rate. Therefore, large-scale *in vitro* expansion of UC-MSCs in PLAY® may serve as a viable option to achieve the optimum cell numbers required for clinical protocols.

Our study revealed PLAY®'s capability to boost cell proliferation beyond passage 12 when compared to one study which also demonstrated that growth factor concentrates obtained from blood can be substituted instead of FBS in the growth of UC-MSCs in culture. In this particular study, it was

observed that the ability to maintain these cells over several passages was a challenge since these cells could not be prolonged over a maximum of 12 passages, in some cases. Cells stop proliferating and begin to show signs of senescence, leading to a decrease in the proliferation index (36).

Further studies can implicate the translational ramifications of these findings and determine if the regenerative potential suggested by PLAY®-MSCs are reflected *in vivo*. Additionally, observing the onset of senescence, expression of flow cytometric markers specific to MSCs beyond P20 and studying the functional aspects especially in terms of its immunomodulatory property at P5, P10, P15 and P20 of PLAY®-MSCs could also constitute the material of prospective studies.

MATERIALS AND METHODS

Preparation of PLAY®

PLAY® is a proprietary product of International Stem Cell Services Limited (iCREST). It is an active biological concentrate generated from human blood that is a rich natural source of growth factors and bioactive modulators. Human blood was taken from healthy donors with the individual's informed consent.

Culturing of UC-MSCs

UC-MSCs were obtained from Lonza. UC-MSCs at P4 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with either 10% Fetal Bovine

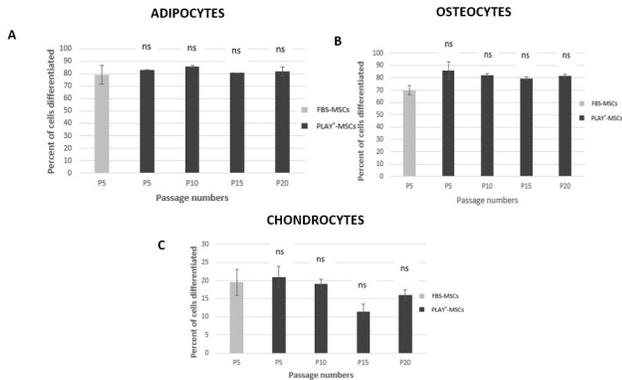


Figure 5. Differentiation potential of UC-MSCs expanded in media supplemented with either 10% PLAY® or 10% FBS. Adipogenic, osteogenic, and chondrogenic differentiation of PLAY®-MSCs and FBS-MSCs expressed as percentage when stained with oil red O (adipocytes), alizarin red (osteocytes) and alcian blue (chondrocytes) (n=2). Data values are represented as mean ± SD. p=ns is considered non-significant in comparison to FBS-MSCs at P5.

Serum (Gibco) or 10% PLAY® and 1% antibiotic-antimycotic solution (HiMedia Laboratories). Cells were seeded at a density of 0.01×10^6 cells per well in a 6-well plate. All cultures were maintained at 37°C in 5% CO₂ incubator. The medium was changed every third day. Cells were harvested at 80% confluency using 0.25% trypsin-EDTA (HiMedia Laboratories), counted, and checked for their viability by trypan blue exclusion method (HiMedia Laboratories) using a hemocytometer (37). The cells were reseeded and subcultured till P20. The morphological changes of the passages studied (P5, P10, P15, and P20) were microscopically observed (CKX41 Inverted microscope) at 10X magnification.

Cell number and population doubling time

Cell numbers at each passage for both the groups were counted using the trypan blue exclusion method and the population doubling time was calculated using the formula taken from previous studies:

$$\frac{(t - t_0) \cdot \log 2}{\log(N - N_0)}$$

where t indicates the time of harvesting the cells and t₀ the initial time both represented in hours. N indicates the number of cells harvested at each passage, and N₀ represents the initial cell number seeded.

RNA Extraction and cDNA synthesis

Total RNA of PLAY®-MSCs and FBS-MSCs was extracted using Nucleospin® RNA Isolation Kit (Takara Bio Inc) as per manufacturer's instructions. The concentration and purity of RNA was quantified using a spectrophotometer (Thermo Scientific) and was determined using the formula below:
RNA concentration (µg/ml) = (OD260) x (dilution factor) x (40µg RNA/ml).

RNA samples each of 1000ng were used for cDNA synthesis using a RT-PCR kit (Takara Bio Inc) following the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

For the amplification of pluripotency genes (SOX2, OCT4

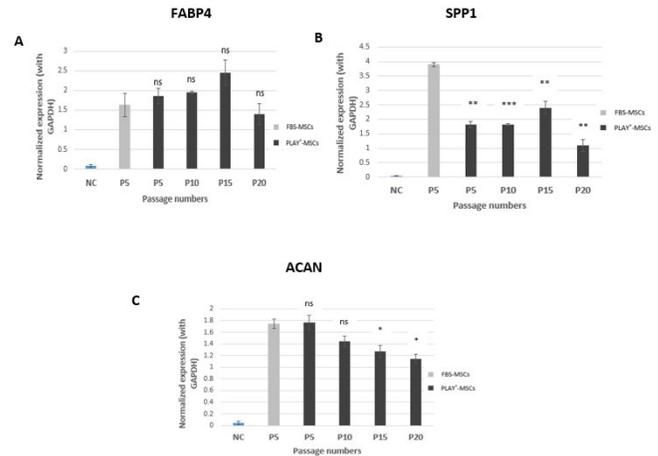


Figure 6. Adipogenic, osteogenic and chondrogenic differentiation marker expressions of PLAY®-MSCs and FBS-MSCs. Semi quantitative RT-PCR revealed the presence of (A) adipogenic marker (Fatty acid binding protein 4 (FABP4)), (B) osteogenic marker (Osteopontin (SPP1)), and (C) chondrogenic marker (Aggrecan (ACAN)) at all passages studied (P5, P10, P15, and P20) (n=2). Data values are represented as mean ± SD. NC-Negative control (PLAY®-MSCs without differentiation medium) *p < 0.05, **p < 0.01 and ***p < 0.001, ns=not significant in comparison to FBS-MSCs at P5.

and NANOG), 55 ng of cDNA sample was used, and PCR was carried out using JumpStart™ Taq DNA Polymerase (6 µL) (Sigma-Aldrich) with 1 pmol of forward and reverse primer for each gene (Table 1). A final volume of 12.5 µL for each reaction was obtained using molecular grade water.

Gene	Primer Sequences	Reference
GAPDH	F- GGTCGGAGTCAACGGATTTGGTCG R- CCTCCGACGCCTGCTTACCAC	38
NANOG	F- GCTGGTTGCCTCATGTTATTATGC R- CCATGGAGGAAGGAGAGGAGAGA	39
SOX2	F-GCCGAGTGGAACTTTTGTCTG R- GCAGCGTGTACTTATCCTTCTT	40
OCT4	F-GACAACAATGAGAACCTTCAGGAGA R-TTCTGGCGCCGGTTACAGAACCA	41

Table 1. Forward and reverse primers sequences used for PCR of pluripotency markers.

Gel Electrophoresis

A 2% agarose gel was used to resolve the PCR products and was observed under a UV-transilluminator (Biobee Tech). ImageJ software was used to assess and quantify the target gene expression, and the results were normalized to GAPDH.

Tri-lineage differentiation assay

0.01×10^6 cells of 10% PLAY®-MSCs of P5, P10, P15, and P20 passages were seeded in a 24-well plate. These cells were cultured in HiAdipoXLT™ Adipocyte Differentiation Medium, HiOsteoXLT™ Osteocyte Differentiation Medium, or HiChondroXLT™ Chondrocyte Differentiation Medium (HiMedia Laboratories) under standard cell culture conditions (37°C, 5% CO₂) for 18 days. Differentiation medium was prepared according to the manufacturer's instructions and 1% antibiotic antimycotic solution was added to each medium. The medium was replaced after every 72 hours.

Cells were observed for the morphological changes using an inverted light microscope (CKX41 Inverted microscope) at 10X magnification.

Differential staining

After 18 days of incubation with the respective differentiation medium, the cells were then fixed using freshly prepared 4% paraformaldehyde in 1X PBS (phosphate buffered saline) for 15 minutes and were then washed with 1 mL of normal saline. Cells were then stained for cell-specific markers: alizarin red for osteocytes, oil red O for adipocytes, and alcian blue for chondrocytes. 100 µL of each stain was added to the cells and incubated for 10 minutes at room temperature. The stained cells were imaged using an inverted microscope (CKX41 Inverted microscope) at 10X magnification.

Differential gene expression

After inducing differentiation of MSCs at P5, P10, P15, and P20, PLAY[®]-MSCs and FBS-MSCs were analyzed for their differential gene markers. Total RNA was extracted, cDNA synthesis was performed, and semi-quantitative PCR was carried out for the following genes: Fatty acid binding protein 4 (*FABP4*) for adipocytes, osteopontin (*SPP1*) for osteocytes, and aggrecan (*ACAN*) for chondrocytes (Table 2).

Gene	Primer Sequence
<i>GAPDH</i>	F-GGTCGGAGTCAACGGATTTGGTCG R- CCTCCGACGCCTGCTTACCAC
<i>FABP4</i>	F- ATGCTTTTTGTAGGTACCTGG R- CTCTCTCATAAACTCTCGTG
<i>SPP1</i>	F- GCCGAGGTGATAGTGTGGTT R- TGAGGTGATGTCCTCGTCTG
<i>ACAN</i>	F- TGCATTCCACGAAGCTAACCTT R- GACGCCTCGCCTCGCCTTCTTGAA

Table 2: Forward and reverse primers sequences for PCR of differentiation markers.

Statistical Analysis

All data obtained were from a minimum of two independent experiments. The data values are represented as mean ± SD. Unpaired two-tail t test function from Excel software was used to determine the statistical significance between the two groups (FBS-MSCs and PLAY[®]-MSCs). *p < 0.05, **p < 0.01 and ***p < 0.001 were considered significant.

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