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Original Article

Isolation and optimization of mice skeletal muscle satellite cells using preplating method and culture media substitution

Nuredin Bakhtiari*

Department of Biochemistry, Faculty of Basic Sciences, Young Researchers and Elite Club, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran

Abstract

Introduction: Satellite cells are known as the main regenerative cell type in skeletal muscles. Our study established a modified digestion and preplating method for the isolation of slow or weak adherent cells for the enrichment of satellite cells. Low-survival rate of these primary stem cells prompted us to address whether cell culture medium substitution might change cell viability status.

Methods: Skeletal muscle from 10-day-NMRI mice was gently isolated, dissected and digested by collagenase type I, IV and dispases. The isolated cells were verified by cellular (immunocytochemistry and flow-cytometry) and molecular (real-time PCR) techniques and the results were compared with sub-cultured cells (non-preplated cells) to determine the efficiency of preplating technique as a common isolating procedure of satellite cells. All data were analyzed using SPSS 16 and One Away ANOVA test.

Results: The isolated cells exhibited a close gene expression pattern with satellite cells for self-renewal and fusion phases. The findings revealed that Pax7 as a self-renewal marker was expressed ~ 201.4 times higher than sub-cultured-group. Moreover, the findings obviously indicated that substitution of α-MEM to DMEM cell culture medium improves the survival rates of the cells.

Conclusion: Our results recommend that preplating technique is a useful procedure for the isolation of satellite cells. In addition, it seems that substitution of culture medium paves the way for investigators to seek various therapeutic methods for skeletal muscle-related disorders such as skeletal muscle atrophy (SMA), amyotrophic lateral sclerosis (ALS), sarcopenia, diabetes and aging.

Keywords:
Skeletal Muscle;
Satellite cells;
Preplate technique;
PAX7

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***Correspondence to:**
N. Bakhtiari
Tel: +98 9181336236
Email:
Nuredin.bakhtiary@modares.ac.ir

Abbreviations:
PP1, Preplating 1; PP2, Preplating 2; SC1, Subcultured 1; SC2, Subcultured 2; nM, μM, mM and M nano Molar, micro Molar, milli Molar and Molar; MyH, Myosin heavy chain; α-actin, α-sarcomeric actin; β-actin, β-sarcomeric actin; IBMX, Isobutyl methyl xanthin; MAP-2, Microtubule-associated Protein-2; M-cad, M-cadherin; βtub, βtubulin 3

Introduction

The function of skeletal muscle tissue is affected by myopathies, muscular dystrophies such as Duchenne muscular dystrophy (DMD), aging, exposure to myotoxic agents, sharp or blunt trauma, ischemia, and excessive hot or cold temperatures (Cai et al., 2004; Riboldi et al., 2005). Satellite cells are the progenitor populations of quiescent mononucleated stem cells in skeletal muscle which reside between the basal lamina and plasmalemma of muscle fibers (Usas and Huard, 2007; Sacco et al., 2008). In

response to muscle injury, they become activated via proliferation and fuse together to form new myofibers (Hawke and Garry, 2001; Yiou et al., 2003; Siegel et al., 2009). On the other hand, satellite cells also can differentiate into other lineages, such as osteoblastic, adipocytic (Peng and Huard, 2004) and neural cells (Arsic et al., 2008).

In recent studies, satellite cells have been identified by expression of a self-renewal signal, Pax7 and regulators of myogenic commitment, such as MyoD. The differentiation of satellite cells is regulated by Pax7 downstream pathways and upregulation of MyoD in the early stages of regenerative myogenesis. (Otto et al., 2009). Similarly, the expression of M-cadherin is increased during myogenesis in quiescent satellite cells following injury (Hawke and Garry, 2001). Moreover, satellite cells have a common marker with endothelium cells, CD34 (Zammit et al., 2006a), that is down regulated during muscle regeneration (Ieronimakis et al., 2010).

For prospective enrichment of satellite cells and depletion of other cellular lineages such as fibroblasts from muscle tissue, preplate technique has been developed which separates cells based on different adherence propensity to gelatin-based substrates (Danisovic et al., 2008). In the present study, we isolated the satellite cells by a modified preplate technique from mouse skeletal muscle on the basis of their adhesion to gelatin-coated flasks (Lavasani et al., 2013) and optimized their viability by exchanging of growth culture medium. Then we analyzed the isolated cells and their potential for maturation by characterizing the gene expression profile using molecular and cellular assays.

Materials and methods

Digestion of skeletal muscle

The 10-day-*NMRI* mice were obtained from Pasteur institute of Iran. Upon sacrificing animals, skeletal muscles were isolated from fore and hind feet. Non-muscle tissues were gently removed and muscle tissue was treated with Trypsin (0.25% Trypsin/1 mM EDTA) (Gibco) for 4 min. For complete digestion, tissue was incubated in enzymatic dissociation solution composed of collagenase type1 (Gibco), collagenase type 4 (Gibco) 0.1% and dispase 1.75 unit/mg (Gibco), overnight at 4 °C. After 24 hours, the sample was again incubated for 3.5 hours at 37 °C on a shaker at 250 rpm and filtered through a 70-µm mesh (BD Falcon). The suspension was centrifuged at 1250 rpm for 5 min and the supernatant was removed. Finally, the effect of preplating on the enrichment of satellite cells was assessed by comparison between preplated culture and a subculture group as control without preplating (Lavasani et al., 2013). All animal procedures were approved by the institutional animal care and use committee of the endocrinology and metabolism research center, Tehran University of Medical Sciences.

Isolation of satellite cells by preplating

The single-cell suspension was cultured by adding medium consisting of α-MEM (Gibco) with 10% FBS. The cells were incubated at 37°C and after 1.5 hours, the first preplate protocol was performed by adding of PBS-EDTA (Sigma). After centrifugation of detached cells at 1250 rpm for 5 min, the cells were resuspended with medium consisting of α-MEM with 10% FBS and were plated into a gelatin (Sigma)-coated flask (Lavasani et al., 2013). The procedure was repeated every day and the enriched cells from 6^{th} preplating round were used for detection by real-time PCR, flow-cytometry and immunostaining.

Characterization of skeletal muscle enriched cells

Molecular technique

Real-time PCR was done for β-actin as internal control along with Pax7, MyoD, α-actin, MyH, M-cadherin and CD34 as skeletal muscle markers on 1^{th}, 6^{th} and 12^{th} days. The primer sequences are listed in Table 1.

Extraction of RNA, cDNA synthesis and real-time PCR assay

Trizol reagent (Sigma) was used to isolate total RNA and according to the manufacturer's instructions (Fermentas), cDNA was synthesized with M-MuLV reverse transcriptase (RT) and random hexamer primers. The PCR reactions were started at 94º for 3 min and followed by 35 cycles of 30s at 94º, 45s at 62º, 45s at 72º and an extension time of 7 -10 min at 72º, using 0.5 µl of cDNA product (Kunwar et al.,

Table1: the list of used primers

Primers	Forward (5'-3')	Reverse (5'-3')	Tm
m-Myh2	GCTTACTTACCAGACAGAAGAAG	TGTTCCTCAGCCTCCTCAG	62
m-MyoD	CTGATGGCATGATGGATTAC	GACACAGCCGCACTCTTC	62
α- actin	GTGGCTATTCCTTCGTGAC	GCAGACTCCATACCGATAAAG	62
m-cadherin	AGGTGGTGGCTGTGTATAATC	GCCTCCATAAAGAACTCATCC	62
m-CD34	GCCAATAGCACAGAACTTCC	GCCAGCAGAACTCCAGAGG	62
m-Pax7	GCGTAAGCAGGCGGAG	ATGGTTGATGGCGGAAG	62
B-actin	GTCCTCTCCCAAGTCCACAC	GGGAGACCCAAAAGCCTTCAT	60

2011). Real-time PCR reactions were performed using Maxima^{TM} SYBR Green/ROX Real-Time PCR Master Mix (Fermentas) and Rotor-gene Q software (corbett) for data analysis of threshold cycle average. Gene expression levels were calculated based on the ΔΔCt method (Beekman et al., 2012).

Cellular techniques

Flow-cytometry analysis

Flow-cytometry was done for CD34, Pax7, M-cadherin, MHC fast and MHC slow on 12th day. First, the isolated cells were cultured and then detached with trypsin, spun down, washed, counted (about 10,000 cells), and divided into two groups (preplated and subcultured groups). Cells were blocked with 0.5% BSA and fixed with 1% paraformaldeide at 4 ºC. Optimal amounts of rat anti–mouse mAbs were predetermined and added directly to each tube for 1 hour at 4 ºC. Each preplated tube received PE-conjugated secondary Ab for 1hour at 4 ºC. For intracellular Abs Triton-X100, 0.1%, was used at 4 ºC for 4 min.

Immunostaining assay

The isolated satellite cells were determined by immunostaining of Pax7 (R&D systems), Myosin Heavy Chain (MHC, SIGMA), M-cadherin (Santa Cruz biotechnology) and CD34 (eBioscience). The cells were fixed with 4% paraformaldehide (SIGMA). Triton X100 (SIGMA) 1% was added and the samples were kept at room temperature (for intracellular antigens only). For blocking step, we used rat serum 5% for Pax7, MHC, CD34 and goat serum 5% (Merck) for M-cadherin, 45min at room temperature. Then without washing, first antibodies were added (diluted in BSA (Merck)/PBS 0.2%), and incubated at 4°C for an overnight. Tween 20 (Merck) 0.1% was added to wash the samples and followed by adding secondary antibody (diluted in BSA/PBS 0.2%), 3 hours at room temperature. The cells also were stained with DAPI (SIGMA), and analyzed with an invert fluorescent microscope (Nikon TE-2000).

In Vitro differentiation

Osteogenic differentiation

The cultured cells from 6th preplating round were treated with bone-morphogenesis proteins (Dexamethasone 10^{-7} M, Beta-Glycerol-Phosphate 10 mM and Ascorbic Acid bi-Phosphate 50 µg/ml) (Lavasani et al., 2013).

Adipogenic differentiation

The conditioned medium with adipogenic-inducing agents including IBMX 0.5 mM, Dexamethasone 10^{-7} M, Insulin 66 nM and Indomethacine 0.2 mM were used for adipogenic differentiation (Lavasani et al., 2013).

Neural differentiation

The inducing agents to neural lineage were Isobutyl methyl xanthin: IBMX 0.5 mM, Forskollin 10 µM and Retinoic Acid 10^{-6} M. The differentiated cells were presented by immuno-cytochmistry for specific markers of neural, Microtubule-associated Protein-2 (Leinco Technologies) and βtubulin 3 (abcam) (Lavasani et al., 2013).

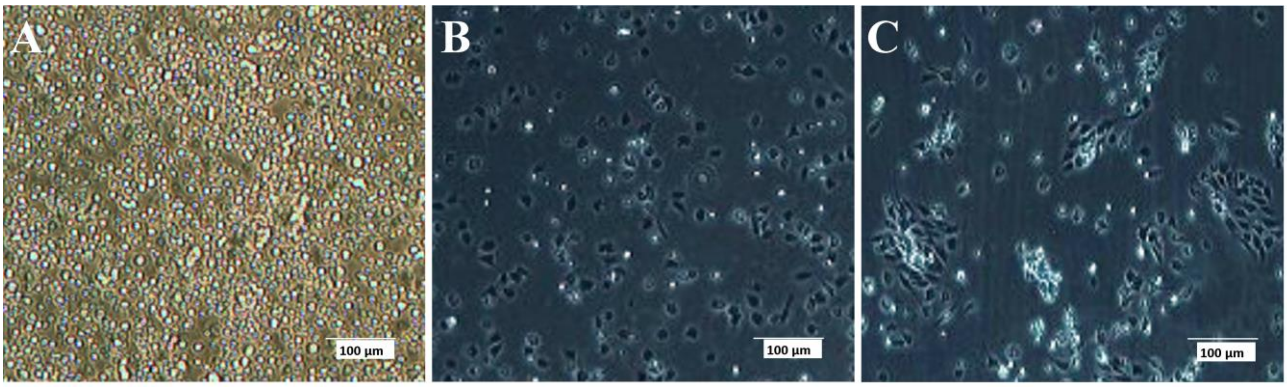

Fig.1. (A) The all cells of Skeletal Muscle after digestion period, (B) The remaining cells after first preplating, (C) The closely-packed appearance similar to cuboidal cells. Scale bar is 100 µm

Statistical Analysis

Statistical analysis was done by using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). Mann – Whitney was used to assess differences between means of satellite cells as test group and subcultured cells as control group. Each molecular and cellular assay was repeated at least for 3 times. A P-value of less than or equal 0.05 was considered statistically significant and data were presented as mean ± standard error.

Results

The enriched satellite cells have in vitro- specific properties

After enzymatic tissue digestion as described above, the cells were very highly dissociated (Fig. 1. A). After performing of second preplate round, the remaining cells had the closely-packed appearance similar to epithelial cells in (Fig. 1. B-C). The other cell lineages like neural and adipose cells were completely removed after third preplate. By the end of rounds of 4^{th} and 5^{th} preplates what remained were the very differentiating cells to large and multi-nuclear cells, the specific morphology of the adult skeletal muscle cells (FIG 2. A-B). By further enrichment, after 6^{th} preplate, cells showed higher proliferation potential and owing to this, the growth factors like EGF (20 ng/ml) and bFGF (10 ng/ml) (Arsic et al., 2008) had no significant impression on the proliferative potency of these cells and their differentiation occurred on 12-15^{th} day (Fig. 2. C-D). Although, by applying 2% horse serum (Smith et al., 2009) to the culture media, the number of committed muscle cells increased and cell fusion was observed after 3-4 days (Fig 2. E). On the other hand, the subcultured group had high proliferative potency but much little propensity to give rise to the adult muscle cells (Fig 2. F-G).

Satellite cells have specific gene expression profile

In this study, we investigated the efficiency of preplate technique for the isolation of satellite cells. The analysis by Real-Time PCR was performed to compare gene expression of preplated cells as test group and subcultured cells as control group on 1^{th}, 6^{th} and 12^{th} day after digestion of skeletal muscle tissue and was normalized with β-actin (the housekeeping gene in skeletal muscle cells). The comparison between separated groups (test and control) in FIG.3 shows that among the targeted genes, on day 6^{th} (the relative expression of 6^{th} day vs 1^{th} day), Pax7 had the significant high expression level in preplated group (Mann-Whitney Test, $p \leq 0.050$). Besides, except for CD34 and M-cadherin, other skeletal muscle markers, MyoD, MyH and α-sarcomeric actin were significantly upregulated in preplated group (Mann-Whitney Test, $p<0.037$, $p<0.037$ and $p<0.037$ respectively). The analysis was repeated at 12^{th} day and revealed that all markers, except CD34 and α-sarcomeric actin, were down-regulated. (Mann-Whitney Test, $p< 0.037$ for MyoD, $p< 0.050$ for MyH, and $p<0.050$ for M-cadherin, not significant about Pax7). These results demonstrate preplate technique could purify satellite cells and the obtained cells on 6^{th} preplating can be induced to proliferate more with less fluctuation of differentiation markers (MyH and M-cadherin) during *in vitro*

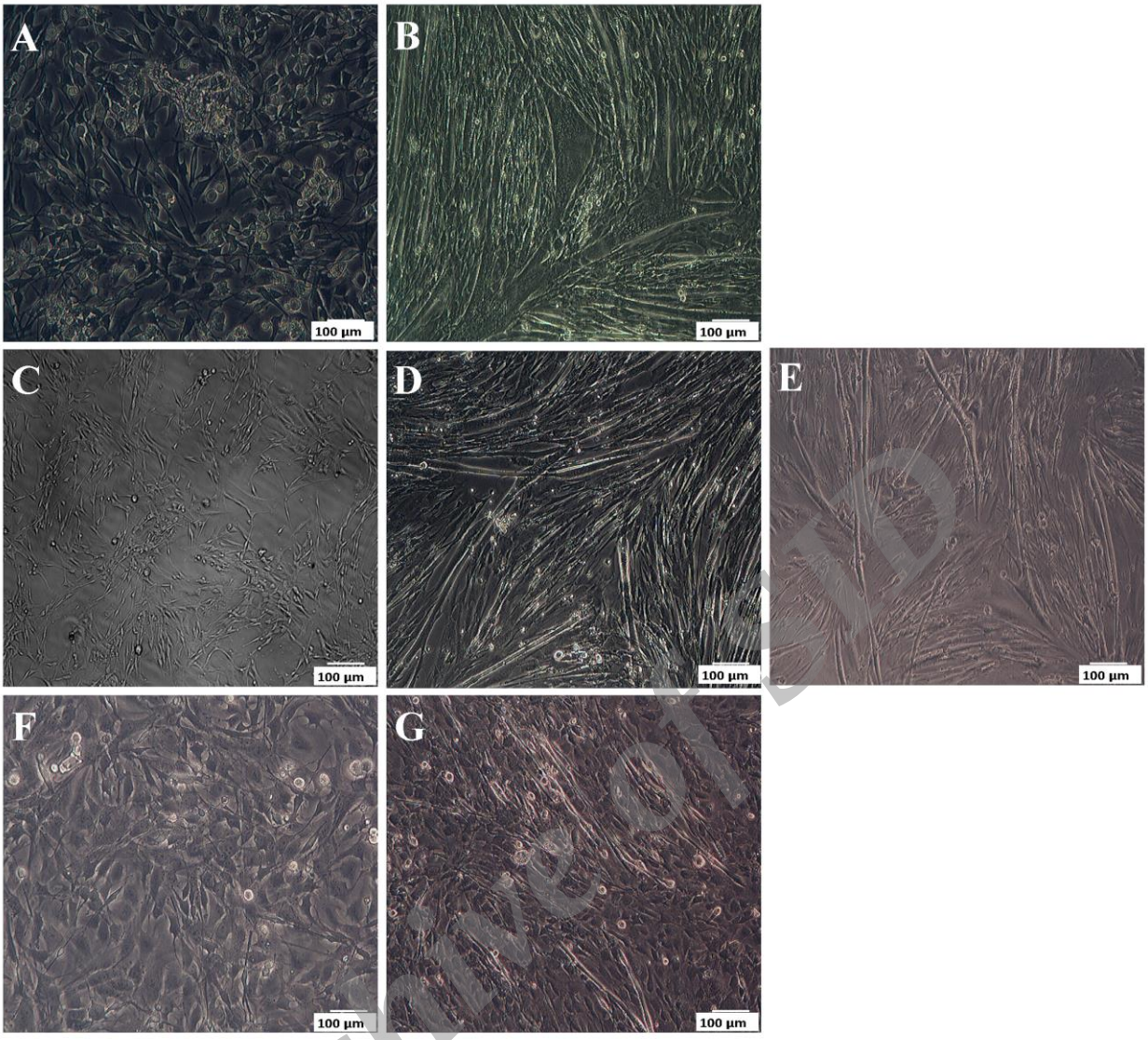

Fig.2. (A) The resulted cells from 5th preplating, (B) The spontaneous differentiation of the cells of fifth preplating, (C) The resulted cells from 6th preplating, (D)The spontaneous differentiation of cells of 6th preplating, (E) The differentiation and fusion of cells after 6th preplating with 2% of HorseSerum, (F) The subcultured cells of Control group contemporary with preplating groups (G) The spontaneous differentiation of subcultured cells of Control group by forming multi-nuclear cells of skeletal muscle after 12 days. Scale bar is 100 µm

cultivation until 12th day. Absolutely, these cells need more time and use if more effective differentiating factors like horse serum for giving rise to multinuclear myocytes.

Furthermore, subcultured cells of control group showed no expression patterns of satellite cell markers after 6 days of cultivation, but they presented the maturation markers after 12 days. This has been summarized in FIG.4 as a schematic view of preplate technique with the results of real-time PCR assay.

Flow-cytometry and immunostaining assays could specify Myosin-Heavy Chain (fast isoform) expression level

For determination of protein variants including several Myosin-Heavy-Chain isoforms (fast type 1 and slow type 2), CD34, M-cadherin and Pax7, we used Flow cytometry and immunostaining for preplated group (the enriched cells on 12th day after digestion) and subcultured group (mostly adherent cells). All markers were negative for both preplated and subcultured groups, except MHC fast (5.46% and 28.5% for preplated and subcultured respectively) (FIG.5). In addition, semi-quantitive immunostaining on 12th day for MHC fast, CD34, M-Cadherein and Pax7 confirmed the results of flow-cytometry by being

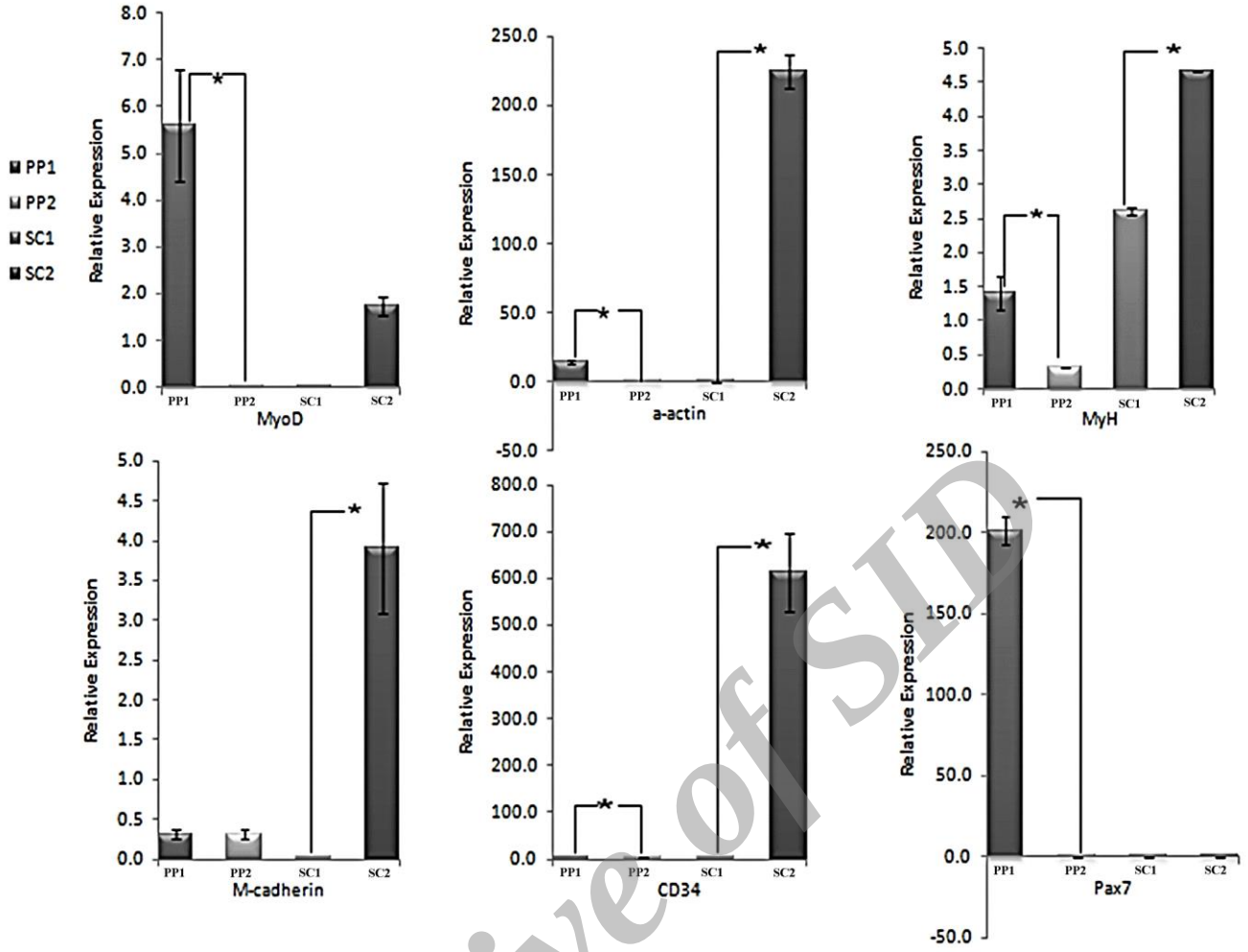

Fig.3. The Real-Time PCR shows relative expression of Markers for satellite cells (preplated and subcultured cells). PP1;preplated cells at 1th day, PP2; preplated cells at 12th day, SC1; subcultured cells at 1th day, SC2;subcultured cells at 12th day. All data were analyzed using one A way ANOWA * p<0.05, **p<0.01, ***p<0.001.

positive for MHC and negative for other markers (FIG.6). WinMDI 2.8 software (Scripps Institute, CA) was used to create the histograms.

Satellite cells are able to differentiate to *mesodermal* and *ectodermal lineages*

Adult stem cells and progenitors are multipotent, so they can give rise to other lineage cells (Garry and Olson, 2006). After using the induction media and following staining techniques, we observed their osteogenic, adipogenic and neuronal differentiation potency. These observations demonstrate that satellite cells are multipotent and capable of forming osteocytes, adipocytes and neural cells. However, the degree of differentiation capability of enriched cells had varied between these lineage cells. So, the isolated cells were expanded and incubated in an adipogenic medium for 21 days, osteogenic medium for 8 days and neural medium for 6 days. Adipogenic differentiation was weaker than osteogenic lineage detected by observation of the calcium-rich crystals for osteogenic differentiation and Oil Red O staining for determination of neutral triglycerides (FIG.7. A-B). For neuronal differentiation of these cells, we used the induction media and documented their ability to convert into neural cells by immunostaining of specific markers of neural, Microtubule-associated Protein-2 (MAP-2) and βtubulin 3 (Baharvand et al., 2007). (FIG.8. A-F).

Discussion

Satellite cells offer several advantages over other adult stem cell populations in therapeutic applications such as easy access, unlike brain and bone marrow. Moreover, small amount of this tissue is sufficient to

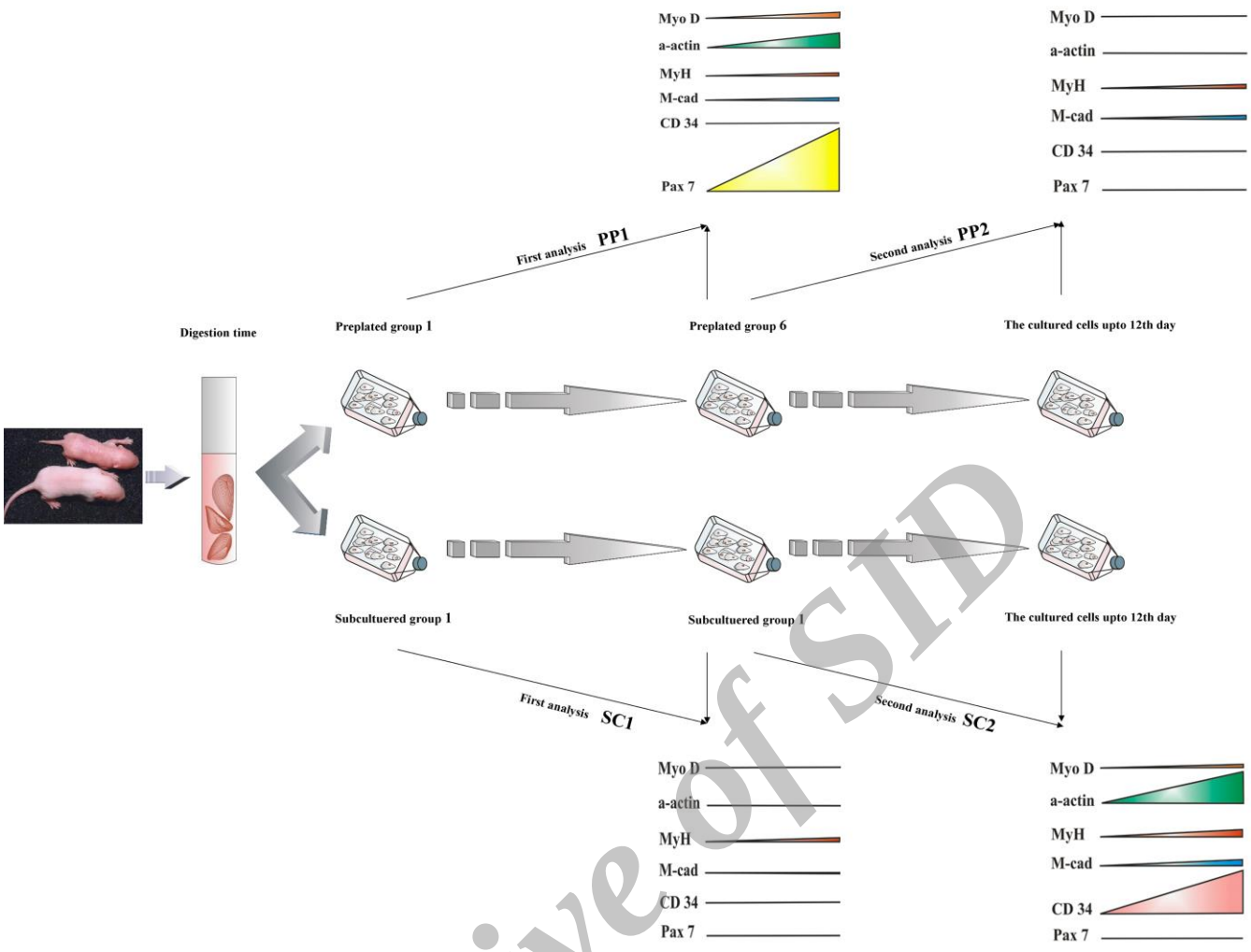

Fig.4. The summary of preplate technique along with the gene expression of isolated cells in comparison with subcultured cells. Here, molecular assay has specified the efficiency of our modified preplate protocol. The enriched cells of preplated group had the expression pattern of satellite cells on 6th preplate and showed a small augmentation just about MyH and M-cadherin by following cultivation untill 12th day. Nevertheless, the subcultured cells of Control group had not shown the expression of self-renewal markers except the upregulation of maturing genes which are considered as the rest of differentiated cells in skeletal muscle tissue.

achieve high number of cells in order to apply stem cell therapy (Ūsas et al., 2011). More importantly, the markers of satellite cells have been approximately determined. The transcription factor, Pax7 acts as a cell survival signal for maintenance of satellite cells and among the cell surface markers, M-cadherin is expressed in quiescent, activated and proliferating satellite cells. CD34 is not specific to satellite cells, but it has been reported that quiescent satellite cells mostly are CD34 positive. Along these markers, MyoD is compatible with proliferation and activation of satellite cells (Morgan and Partridge, 2003; Zammit et al., 2006b). Thus, increasing level of MyoD induces satellite cell differentiation from quiescent form to multi-nuclear cells by increasing the expression of myosin heavy chain (MyH) and α-sarcomeric actin (Hawke et al., 2007). These markers are useful for characterization of committed and activated satellite cells.

The objective of this study was the assessment of preplate technique as a common technique for enrichment of satellite cells. For this approach, we needed a digestion protocol for a complete dissociation of skeletal muscle tissue to single cells. The protocols were used in the previous studies did not possess suitable efficacy for obtaining *in vitro* muscle cells. Here, we offer a modified protocol which makes it easier to digest skeletal muscle tissue into alive single cells with similar gene expression pattern to satellite cells. This modification was required for improvement of preplate technique, because the enriched cells by using other agents

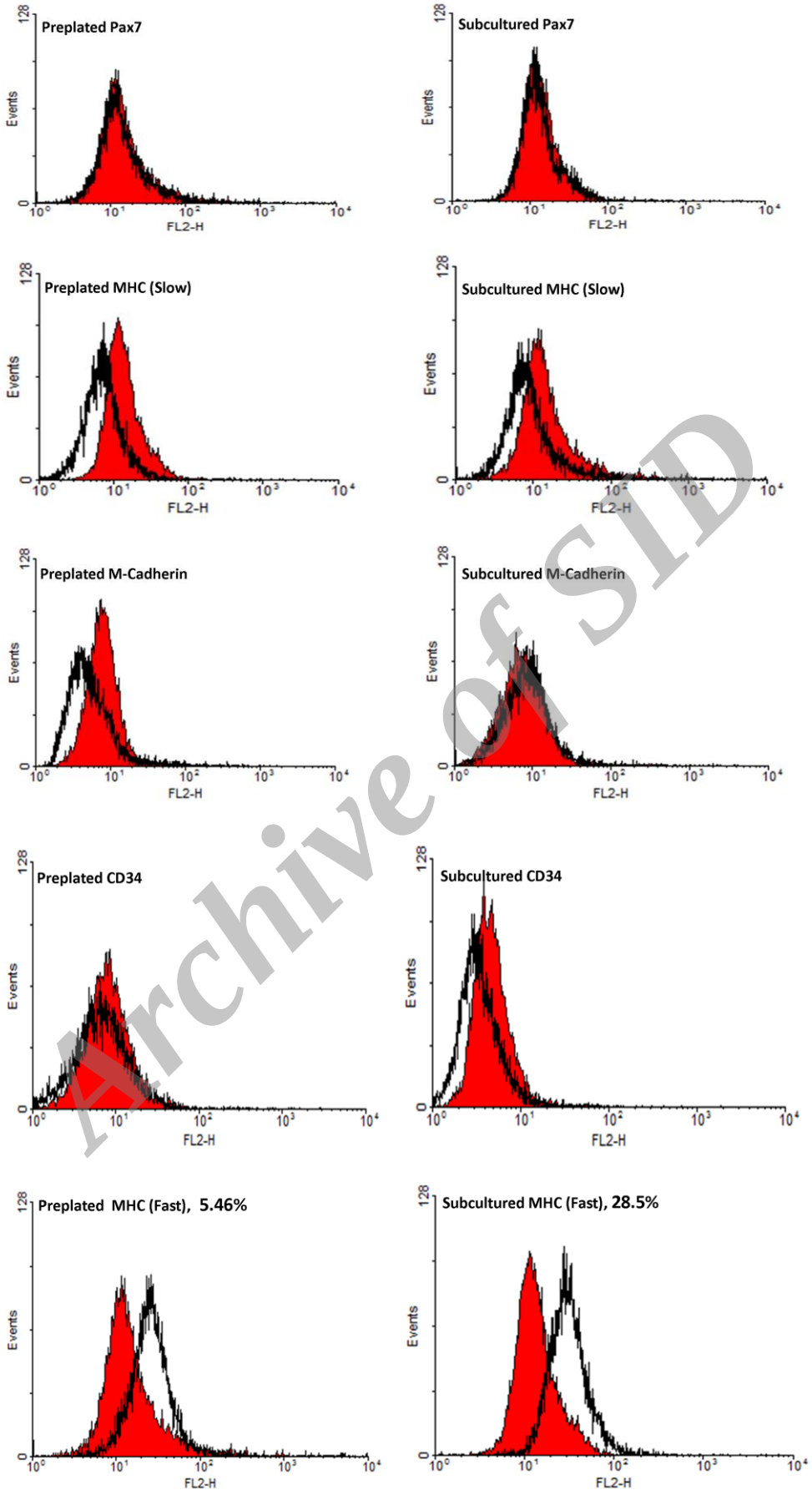

Fig.5. Representative Flow-Cytometry data on 12th day: For all markers of Skeletal Muscle cells, preplated and subcultured cells were negative but for MHC fast, were 5.46% and 28.5% respectively.

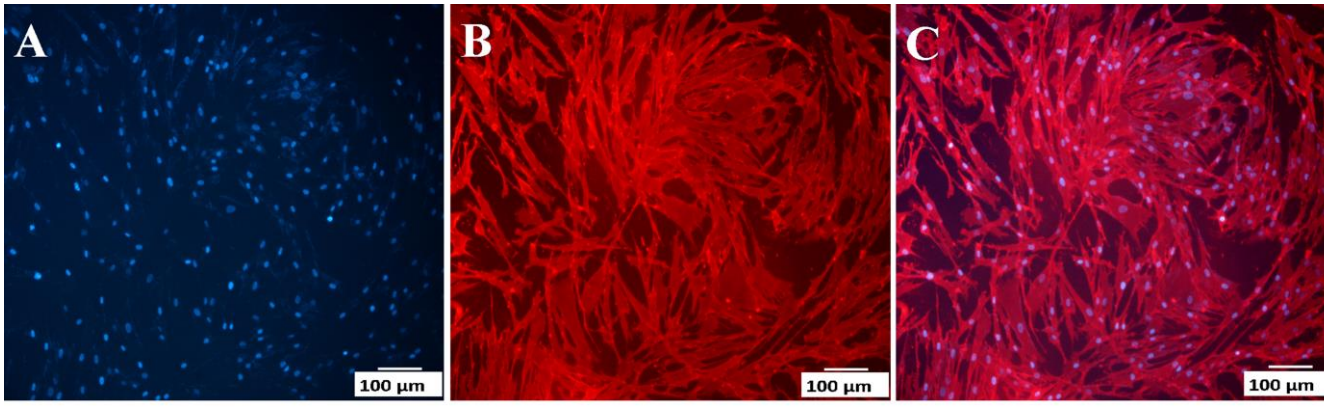

Fig.6. Immunofluorescence analysis of muscle-derived slow-adherent cells on 12th day to Myosin-Heavy-Chain. (A) DAPI, (B) MHC and (C) Cells were co-stained with MHC and DAPI for visualize nuclei (blue) related to MHC. Scale bar is 100 µm

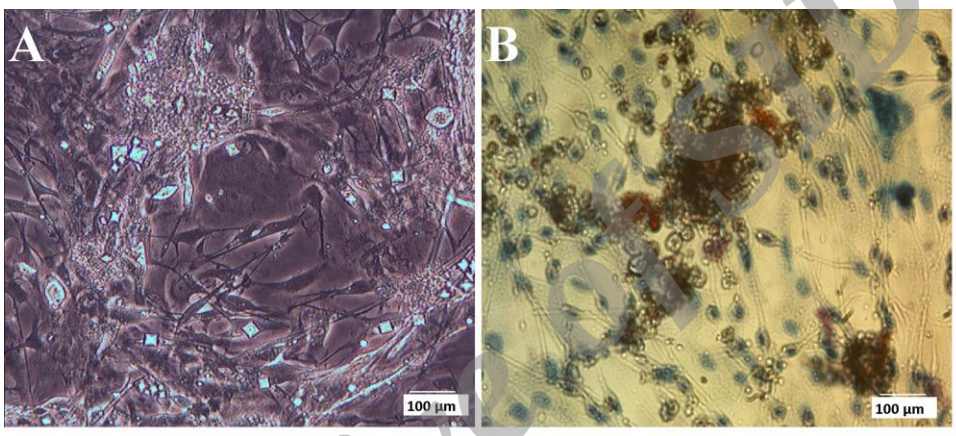

Fig.7. Skeletal Muscle slow-adherent cells characterized by differentiation into Mesodermal lineage. (A) Forming of Calcium-rich crystals from bone differentiation and (B) Oil Red O for staining of neutral triglycerids with Trypan Blue stain for presentation of vital cells. Scale bar is 100 µm

such as trypsin, PBS or using coated or non-coated conditions by collagen or gelatin, were not adequate to purify the cells with the expression profile of satellite cells. We used PBS-EDTA to transfer the low adherent cells and the gelatin-coated condition for complete removal of the adherent cells. In this study, we gradually shift the application time of PBS-EDTA from 30 second in the first preplating to 4 minute in the last one. That is because the slow adherent cells become matured gradually and thereupon show more potent adhesion.

Here, the cells of skeletal muscle tissue were separated into 2 groups: subcultured and preplated cells. The subcultured group was used as control group without preplate steps and possessed all type of muscle cells by different adhesive behavior. The result of this group was compared with preplated group for the assessment of efficacy of our modified preplate technique.

The gene expression of preplated group after 6th step, showed high expression level of self-renewal gene, Pax7. On the other hand, M-cadherin is important for fusing the satellite cells to form multi-nuclear cells (Hollnagel et al., 2002; Pavlath, 2010), but has low expression in quiescent phase of satellite cells. In our study, the low-level expression of M-cadherin continued upto 12th day. Eventually, by removing of non-muscle cells and further purification of muscle cells, the level of differentiation markers including MyoD, MyH and α-sarcomeric actin declined after 6th preplate stage and preserved the quiescent state pattern upto 12th day of serial cultures. Furthermore, the low-level expression of CD34 on 6th stage of preplate technique and its upregulation on 12th day supports the quiescent phase of enriched cells after 2 weeks (Ieronimakis et al., 2010). Conversely, on 6th preplate round after *in vitro* cultivation of subcultured cells, Pax7 was absent, along with the high level of MyH. We could postulate that perhaps satellite cells were removed by

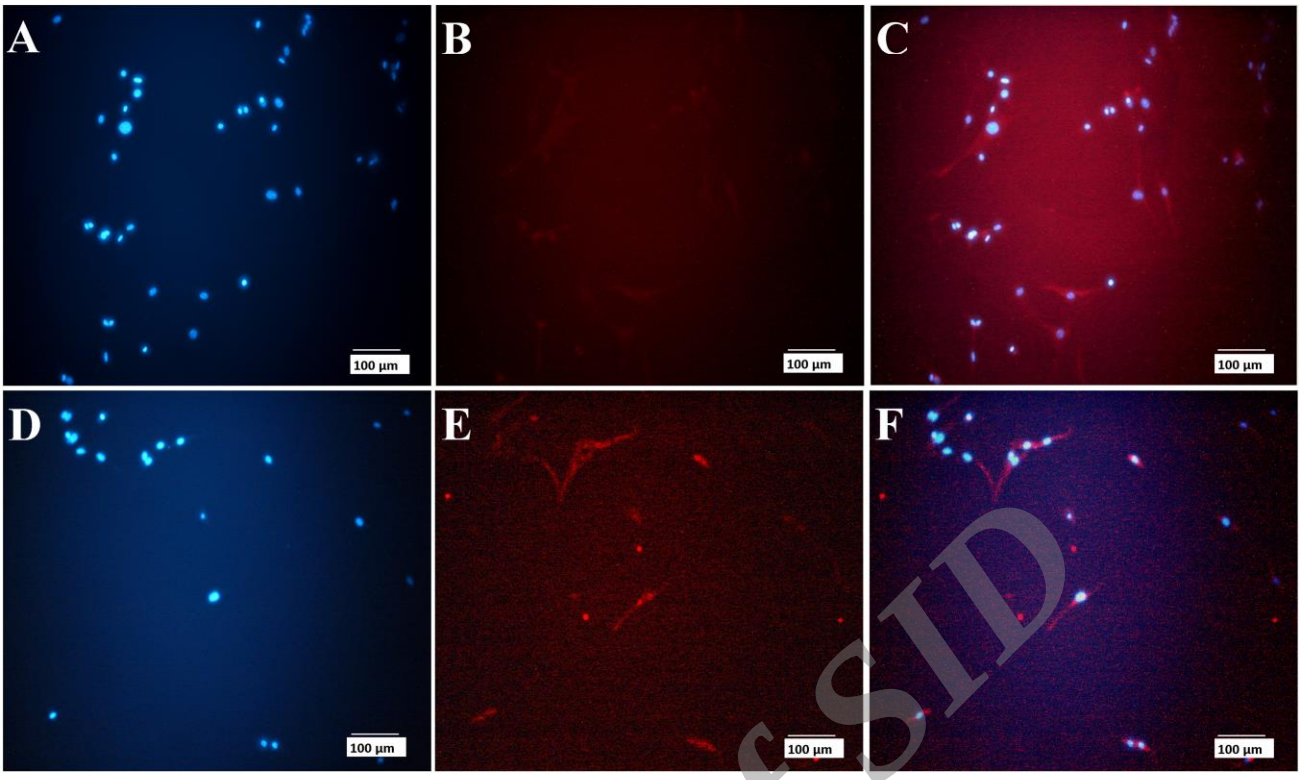

Fig.8. Differentiation of satellite cells into Ectodermal lineage. (A-F) Neural differentiation: immunostaining detection of enriched cells to neural markers: (A-B) β-tubulin 3 (βtub) and (C) Cells were co-stained with β-tubulin 3 and DAPI for visualize nuclei (blue) related to βtubulin 3, (D-E) Microtubule-associated Protein-2 (MAP-2) and (F) Cells were co-stained with MAP-2 and DAPI for visualize nuclei (blue) related to MAP-2. Scale bar is 100 µm

further cultivation and moreover, the matured muscle cells remained in control group.

Additionally, Flow-cytometry compared the preplated cells with subcultured cells on 6th and 12th days. The results, showed the absence of all specific markers on 6th day and higher expression level of myosin (fast) in subcultured cells on 12th day.

Taken together, these results show the enriched cells of 6th preplate were closer to quiescent phase and the down-regulation of differentiation markers continued upto 12th day. Besides, *in vitro* studies of last preplated cells with low potency to differentiate, propose the cells of 6th preplate are muscular stem cells (Sinanan et al., 2006). Furthermore, the ability of differentiation to multiple lineage cells depict the enriched cells had low ability to adipogenic cells but higher tendency to osteogenic and neural cells. These data supports the multipotent potential of isolated cells and their stem cell identity.

Conclusion

In conclusion, the assessment of particular protein markers, especially about the paired box transcription factor, Pax7 and some surface markers loke CD34 need more elaborate assays such as western-blot analysis. But real-time PCR as a molecular assay showed that our modified preplate protocol could be suggested for sufficient enrichment of satellite cells. In addition, we showed that cell culture medium replacement from α-MEM to DMEM drives cells to immortalization and makes these cell s more suitable for more evaluation by investigators to treat skeletal-muscle related disorders such as SMA, ALS, sarcopenia, diabetes and aging.

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Conflict of Interest

The author of this manuscript confirms that there are

no known conflicts of interest associated with this publication.

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