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Differentiated Adipose-Derived Stem Cells for Bladder Bioengineering

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Abstract

Objective

To characterize and differentiate adipose-derived stem cells (ADSCs) to functional smooth muscle cells (SMCs) as an alternative cell source for bladder engineering.

Materials and Methods

Rat ADSCs were differentiated into SMCs for 1 to 6 weeks using induction medium. The changes in contractile genes and proteins expression were investigated by Real time PCR, FACS, and western blot (WB) at different time points. In addition, spontaneous and carbachol-induced contractions of engineered SMC tissue at different stages were investigated to define the optimal duration of induction.

Results

We found that ADSCs differentiated into SMCs lose their capacity for expansion and their contractile phenotype changing to a synthetic phenotype over time. Highest levels of calponin, smoothelin, and MyH11 expression were observed in ADSCs induced for 3 weeks. Cells acquired typical SMC morphology when contractile proteins were expressed. However, SMC morphology was lost with reduction of contractile proteins, especially smoothelin and MyH11. The maximal spontaneous and carbachol-induced contraction of differentiated ADSC was after 3 weeks.

Conclusions

Our study demonstrates that ADCSs are a suitable cell source for engineering tissues that require functional and contractile SMCs. An induction time of 3 weeks appears to be sufficient for ADSC differentiation to contractile SMCs suitable for urologic tissue engineering.

Introduction

Over the last decades elaborate methods using gastrointestinal tissues for bladder reconstruction have been developed. However, there is still significant short and long-term complications associated with this strategy, including stone formation, mucus, infection and even cancer development has been reported [1,2]. Therefore, significant efforts have been made to engineer bladder tissues using autologous cells and biomaterials [3]. One of the remaining challenges is the availability of easy accessible healthy smooth muscle cells (SMCs) for *in vitro* expansion.

Several groups isolated SMCs directly from biopsies of the diseased organs, such as bladders or vessels [4,5]. However, taking a biopsy from a targeted organ has several drawbacks including morbidity caused by complex surgery and limited sample size. Furthermore, the SMCs derived from diseased organs possess and maintain pathologic characteristics *in vitro* [6]. Mature SMCs isolated from healthy sources have shown limited proliferation capacity and usually lose their contractile phenotype followed by a change to a synthetic form during their *in vitro* expansion [4]. Therefore, alternative cell sources must be considered for the functional bioengineering of SM tissue. Candidate cells include embryonic [7] and adult stem cells [8]. The ethical issues have limited the use of embryonic stem cells. Adipose-derived stem cells (ADSCs) have

potential advantages in tissue engineering applications because of their easy accessibility, abundant availability and multipotency. In culture, they have remarkable developmental plasticity, including the ability to undergo multilineage differentiation and self-renewal [9]. Therefore, ADSCs seem to represent an excellent alternative for the bioengineering of smooth muscle.

Other laboratories have published data on the differentiation of ADSCs into SMCs [10-13]. In a recent study, our group established a reliable small animal model for hypocontractile bladder and demonstrated that ADSCs support the early restoration of bladder voiding with improved voiding pressures and molecular expression of contractile proteins after cell therapy [14]. Although, ADSCs can support the regeneration of damaged bladder tissue, using pre-differentiated contracting cells may have a better impact on the regenerated tissue functionality.

Previous studies have reported the use of growth factors such as angiotensin, sphingosylphosphorylcholine, Transforming Growth Factor b1, and Transforming Growth Factor b3 [13,15] using different induction periods. To facilitate rapid clinical translation in near future a smooth muscle inductive medium (MCDB-131 medium) without growth factors was developed for SMC differentiation [7,8]. However, SMCs generated from ADSCs following these protocols were differentiated for the duration of 6 weeks *in vitro* and then used for *in vivo* experiments without demonstrating the maintenance of a contractile phenotype [8]. Compared with other cell types, little is known about the necessary baseline level of differentiation of ADSCs into SMCs and their functional characteristics. The optimal cell type for bioengineered smooth

muscle should exhibit the SMC phenotype and have the ability to contract. Differentiated SMCs can vary between two specific phenotypes: synthetic and proliferative or contractile and quiescent [16]. SMCs that exhibit a contractile phenotype are characterized by the high expression of contractile proteins including smooth muscle actin (SMA), calponin, caldesmon, SM22, smoothelin and MyH11 [17,18]. It is reasonable to assume that the optimal conditions for the differentiation of ADSCs into SMCs have not been well defined. Therefore, this study investigated the differentiation potential of ADSCs into SMCs at different stages of differentiation with respect to protein expression and cell contractility. The SMC phenotype was assessed on a molecular level by examining the expression of the lineage-specific markers SMA, calponin, smoothelin and MyH11 using different techniques. In addition, the study tested the functional capacity of differentiated SMCs to contract and relax spontaneously and upon pharmacologic stimulation in an *in vitro* system.

Materials and Methods

Isolation of adipose-derived stem cells *in vitro*

To isolate ADSCs, subcutaneous adipose tissue of adult male Lewis rats (n= 10) was excised from their inguinal regions, cut into small pieces and cells were isolated according to the established protocols [11,19]. All of the experiments were carried out using cells that were between passages 3 and 5.

Cell characterization and multilineage plasticity

Expanded cells were characterized by immunostaining and fluorescence-activated cell sorting (FACS) with lineage-specific markers CD44, CD29, Stro-1, SSEA-1 and CD34 [20]. To further demonstrate the multipotency of the isolated

ADSCs a differentiation towards the adipocyte and neuron and smooth muscle lineages using established protocols and lineage-specific factors were performed [21].

Undifferentiated ADSCs were differentiated into SMCs by incubation in SMC inductive MCDB 131 medium (Sigma, Buchs, Switzerland) plus 1% FBS and 100 U/mL heparin for 1 to 6 weeks as described previously [10]. The entire experiment was repeated at least three times with three different samples.

Cell proliferation

To evaluate cells proliferation and viability during the differentiation process, ADSCs were cultured in SMC induction medium for 1 to 6 weeks. The cell proliferation reagent WST-1 (Roche, Penzberg, Germany) was used according to the manufacturer's protocol. This whole experiment was repeated at least three times and samples were analyzed in triplicate.

Real-time PCR

Total RNA was isolated using the SV Total RNA Isolation System kit (Promega, Dübendorf, Switzerland) according to the manufacturer's protocol. RNA was reverse transcribed with random primers (High-Capacity cDNA reverse transcription, Life Technologies, Zug, Switzerland). Pre-designed primers for rat smoothelin (Rn01453105-m1), calponin (Rn00582058-m1) and MyH11 (Rn01530326-m1) were purchased from Life Technologies (Zug, Switzerland). GAPDH (Rn01775763-g1) was used to normalize cDNA concentrations. All the values for mRNA expression were compared with the standard time point of 6

weeks. The entire experiment was repeated at least three times and samples were analyzed in triplicate.

Flourescence-activated cell sorting

Flow cytometry was performed on ADSCs and differentiated cells between 1 and 6 weeks of differentiation. Cells were immunolabeled with hyaluronan receptor (CD44, Millipore, Hessen, Germany), beta-1 integrin (CD29, BioLegend, Fell, Germany), Stro-1 (Invitrogen, Lucerne, Switzerland) and hematopoietic marker (CD34, BD Biosciences, Allschwil, Switzerland) for ADSCs. The differentiation of ADSCs into SMCs was confirmed with the SMC markers anti-calponin (Sigma, Buchs, Switzerland), anti-smoothelin (Santa Cruz, Heidelberg, Germany), anti-MyH11 (Santa Cruz), and anti-alpha-smooth muscle actin (Sigma, Buchs, Switzerland).

Immunofluorescent staining

ADSCs were cultured on Lab-Tek chamber slides (Thermo Scientific, Nunc, Switzerland) in growth medium or inductive medium for the required time. The indirect immunostainings were performed at 4°C overnight using the following primary antibodies for the ADSCs: CD44, CD29, Stro-1, stage-specific embryonic antigen-1 (SSEA-1) and CD34. The neurogenic differentiation was confirmed using mouse anti-neurofilament 68 (Sigma, Buchs, Switzerland) and mouse anti-beta tubulin Isotyope III (Sigma, Buchs, Switzerland) antibodies. The differentiation was confirmed with SMC markers as mentioned above. The slides were incubated with the secondary antibodies: goat anti-mouse FITC (BD Biosciences, Allschwil, Switzerland), goat anti-rabbit FITC (Vector lab, Servion,

Switzerland) or Cy3-conjugated goat anti-mouse antibody (Sigma) at room temperature for 1 h. The slides were counter stained with DAPI (4',6-diamidino-2-phenylindole, Sigma). The slides were analyzed with a Leica fluorescence microscope (CTR 6000). Negative control consisted of secondary antibody only and primary antibody was omitted. For negative controls only secondary antibodies were added.

Western blotting

Cells were washed with cold PBS supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Allschwil, Switzerland) and lysed with lysis buffer. Twenty-five micrograms of each sample's protein lysate was loaded on a 12% gel (Bio-Rad, Cressier, Switzerland) and WB was performed according to the manufacturer protocol. The primary antibodies were anti-calponin, anti-smoothelin, anti-MyH11and anti-GAPDH (Sigma, Buchs, Switzerland).

Gel contraction assay

To measure the spontaneous and active contractility of the differentiated ADSCs, small tissues with cells in collagen were formed and a contraction assays were performed *in vitro* [22] [23]. SMCs isolated from rat bladder were used as positive controls and undifferentiated ADSCs, a prostate cancer cell-line (DU145) and collagen discs without cells as negative controls. The floating tissue-like discs was incubated in a medium containing 2% serum at 37°C for the indicated time periods. A digital camera at a fixed distance was used to take pictures. The area of the discs was calculated with ImageJ software (NIH, Bethesda, MD).

Animal ethics

Rat fat tissue was obtained in accordance with the animal ethics guidelines.

Results

Characterization of ADSCs and multilineage differentiation

The ADSCs proliferated quickly in culture medium, forming a uniform confluent monolayer and showed fibroblast-like spindle shape morphologies in phase-contrast microscopy (Figure 1). The phenotype was confirmed by immunostaining with lineage-specific markers CD29, CD44, Stro-1, SSEA-1 and CD34 (Figure 1A, b-f). These results were confirmed by FACS analysis with the same markers (Figure 1C). At passage 3, ADSCs exhibited high positive expression of the lineage-specific markers CD29 (65% ± 5.6), CD44 (79.2% ± 6.4), Stro-1 (54.5% ± 8) and were negative for CD34 (4.6% ± 1.1). For these experiments, only the ADSCs with the expression higher than 65% were used. Adipogenesis was confirmed 2 weeks post-induction by Oil Red O staining, showing intracellular lipid droplets of variable sizes, indicative of lipid accumulation (Figure 1B, a-b). Using neuron-specific culture conditions, the cells were differentiated into neurons. The differentiated cells exhibited neuron-like morphologies, and were confirmed with the neuron-specific markers beta-III tubulin and neurofilament 68 (Figure 1B, 1c, 1d, 1e, 1f).

Adipose-derived stem cell differentiation into smooth muscle

The ADSC cells under differentiation conditions showed an increase in proliferation rate up to 3 weeks and a decrease after 4-6 weeks (Figure 2A). The induction of ADSCs in defined media elicited their differentiation into SMCs

as demonstrated by a gradual increase in the mRNA expression of calponin, MyH11, and smoothelin between 1 and 4 weeks followed by a decrease after 5 and 6 weeks of differentiation (Figure 2B, C, D). The maximum significant increase of calponin, MyH11 and smoothelin was observed after 3 weeks compared to 6 weeks of differentiation. Similarly, the FACS data showed a gradual and significant increase in calponin ($P=0.0002$), smoothelin ($P<0.0001$) and SMA ($P<0.0001$) proteins after 3 weeks of differentiation compared to undifferentiated ADSC. The gradual increase expression of MyH11 was observed after 3 weeks. Comparing the expression of contractile proteins at the different time-points the most prominent and significant induction was observed over 3 weeks, followed by a significant decrease after 6 weeks (Figure 2 E, F, G, H). The same pattern was observed using immunofluorescent staining (Figure 3A). Smooth muscle induction media induced differentiation of the ADSCs into a SMC phenotype in which the differentiated ADSC demonstrated expression of SMC-specific proteins, including calponin, smoothelin and MyH11. Smoothelin, the key contractile protein, was reduced after 5 and 6 weeks of differentiation. The cytoplasmic expression of MyH11 completely disappeared, and only slight perinuclear staining of this protein was observed. Cells were stained positive for SMA protein at all time-points. As shown by immunofluorescent, when cells were differentiated, they acquired spindle-shape morphology and grew in hill-and-valley pattern similar to what was observed in bladder tissue derived primary SMCs. This morphology was lost after 4-6 weeks of differentiation. The WB analysis showed increased levels of SMC contractile proteins, smoothelin (115 KD), calponin (34 KD) and MyH11

during weeks 1-4 and a reduction in their levels after 5-6 weeks of differentiation (Figure 3B).

Contractility of smooth muscle cells differentiated from adipose-derived stem cells

We measured the contractile ability of the cells in small bioengineered tissue discs. Tissues with the differentiated ADSCs displayed a maximal degree of spontaneous contraction after 3 weeks of differentiation. The incorporation of differentiated SMCs reduced the size of the tissue discs in a time-dependent manner up to 3 weeks, indicating good contractile tone (Figure 4A). However, the tissues containing ADSCs that had been differentiated for 5 and 6 weeks or undifferentiated ADSCs contracted to a lesser extent. Furthermore, the effect of contractile stimuli such as carbachol on the contraction of tissues with differentiated SMCs, ADSCs and primary SMCs (Figure 4B). The highest active contraction upon carbachol (10 μM) stimulation was observed in ADSCs differentiated for 3 weeks demonstrating an additional 38% contraction, which was comparable to tissue-isolated SMCs. Neither undifferentiated ADSCs nor ADSCs differentiated for more than 4 weeks showed any contraction upon carbachol stimulation (Figure 4C).

Discussion

Tissue engineering offers the possibility of creating functional bladder tissues that may replace a removed or malfunctioning bladder. Autologous cells can be injected directly, or a scaffold with cells can be implanted in the patient with a specific disease to achieve tissue regeneration [24]. The corner stone of cell-

based therapies is an adequate source of healthy and functional cells. The most important characteristic of SMCs for functional bioengineering is their contractile phenotype. Some groups isolated autologous SMC from biopsies [6] and observed a decreased contractile response in cultured neuropathic compared with normal SMCs. These results were consistent with *in vivo* studies, in which neuropathic and myelodysplastic bladders generated less contraction than normal bladders in urodynamic studies [25]. These findings imply that altered intracellular contractile machinery is present in diseased SMCs, which make them less attractive for use in tissue engineering. The ease of isolation of ADSCs and their multipotency make them an attractive stem cell source for use in bladder engineering. The focus of the present study was to evaluate the optimal induction time of ADSCs for differentiation into SMCs. We investigated the time-dependent expression of contractile proteins that generates the diversity of phenotypes ranging from immature cells to SMCs.

This study demonstrated for the first time that the functionality of SMCs differentiated from ADSCs is time-dependent, and shorter induction times might be beneficial with regard to functionality and expression of contractile proteins and cell growth. This has potential benefits for future application since shorter incubation time lowers the costs and minimizes the risk of contamination. SMA [24] is an early SMC marker, whereas calponin, MyH11, and smoothelin are highly restricted to contractile, functional SMCs [16]. Smoothelin is a constituent of the cytoskeleton and co-localizes with actin stress fibers [26], whereas calponin is an actin-binding troponin-like protein that forms an integral part of the actin-linked contractile machinery in SMCs [27]. The importance of

smoothelin expression was studied in normal and overactive human bladders [28]. It has been shown that its expression is rapidly down-regulated in primary cultures of SMCs and its transcription is restricted to SMCs with contractile phenotypes. The up-regulation of uterine smoothelin during pregnancy has been reported indicating smoothelin levels directly reflect the potential of SM to contract [29].

Here we demonstrated that ADSCs acquired SMCs phenotype as evidenced by the expression of specific genes and proteins upon induction *in vitro*. At mRNA level, ADSCs exhibited a baseline expression of SM markers, similar to other pluripotent stem cells. Upon induction, the highest expression of calponin and the late markers smoothelin and MyH11, which are only present in contractile, mature SMCs, was observed in cells induced for 3 weeks. With respect to protein expression, the differentiated cells showed an increase in calponin, smoothelin and MyH11 during weeks 1 to 4 and a down-regulation of MyH11 and smoothelin after 5 and 6 weeks. It is known that smoothelin and MyH11 can distinguish SMCs from myofibroblasts or muscle at the synthetic stage [16]. Our results are consistent with a larger body of evidence showing the importance of contractile protein expression [30,31]. Using immunofluorescence, we demonstrated that the cells acquired typical SMC morphology when all of the contractile proteins were expressed during differentiation. However, the cells could only maintain their SMC phenotype during the first 3 weeks. The SMC morphology was lost after 4 weeks of differentiation in parallel to a reduction of all contractile proteins, especially smoothelin and MyH11. The expression of SMA protein was observed during the differentiation process with an increase

over time. As an early marker of SMC differentiation, SMA is often accepted to characterize the preliminary SMC phenotype [18] . However, expression of SMA can often be observed in myofibroblasts and fibroblasts. Hence, its expression alone does not provide definitive evidence for the SM lineage. Here, we clearly demonstrated that induction times longer than 3 weeks decreases the expression of contractile proteins and leads to phenotypic changes. Cells differentiated more than 4 weeks lost their SMC morphology and increased in size. To confirm that the phenotypic changes and contractile proteins expression has an effect on functionality of the cells, small tissues were bioengineered *ex vivo* and their contractility assessed. Indeed, cells differentiated for more than 4 weeks showed reduced spontaneous or stimulated contractility.

The use of differentiated ADSC towards functional SMCs described here not only serves as an easy accessible cell source for urologic tissue engineering but could also be used as a good non-immortalized SM cell source for *in vitro* gene expression studies, growth factors and drug testing.

Conclusion

The present study demonstrated a time-dependent gain and loss of variable phenotypic and functional markers of cell differentiation of murine ADSCs differentiated into SMCs. Our data indicates that cells after 3 weeks of differentiation express smooth muscle phenotype for urologic tissue engineering applications clearly superior to cells differentiated for 6 weeks.

Figure 1. Characterization of rat ADSCs.

A-a: Phase-contrast image showing ADSCs at third passage. **b-f:** Immunofluorescent staining of ADSCs**.** The cells were stained with anti-CD44, anti-CD29, anti-CD34, and Stro-1 and SSEA-1 antibodies, respectively. The staining of the cells was detected using fluorescein isothiocyanate-conjugated (green color) secondary antibody and DAPI (blue). Scale bar = 10 μm

B: Rat ADSCs cells are multipotent. **a.** Phase-contrast images of differentiated ADSC with the characteristics and morphology of adipocytes. **b.** Lipid clusters were generated in ADSCs after 2 weeks of induction in adipogenic medium as indicated by Oil Red O staining.

c: Neurogenic differentiation of ADSCs. Phase-contrast images show the neuron-like morphology of the cells, which is indicated by arrows. **d.** WB analysis showing the expression of the 46 KD beta-III tubulin proteins. **e and f.** The cells were stained with anti-beta-III tubulin and anti-neurofilament 68 antibodies. The staining was detected with a CY3-conjugated secondary antibody (red) and DAPI.

C. Characterization of isolated ADSCs. Primary isolated ADSC's were stained for CD34, CD44, Stro-1 and CD29 characterized by FACS.

Figure 2. Proliferation of ADSC and time-dependent SMC-specific mRNA and protein expression upon differentiation.

A. Cell proliferation rate of ADSC differentiated 1 to 6 weeks. **B-D.** Induction of the gene expression of the SM markers smoothelin, calponin and MyH11 determined by real-time PCR with GAPDH as the control gene. *$p<0.02$. **E-H.**

FACS. Undifferentiated ADSCs and differentiated cells were stained with calponin, smoothelin, SMA and MyH11. All data represent the SEM of 3 triplicate experiments.

Figure 3. Immunostaining of ADSCs during weeks 1 to 6 of differentiation. Fluorescence images of ADSCs differentiated to SMCs for 1-6 weeks. The cells were immunostained for the SM markers SMA, calponin, and MyH11 (red-CY3), smoothelin (green-FITC) and nucleus staining DAPI. Scale bar = 10μm.
B. WB analysis of SMC specific protein expression.

Figure 4. Smooth muscle-like contractile responses of differentiated ADSCs.

A. Spontaneous contraction. ADSCs were differentiated weekly, the differentiated SMCs and the controls embedded in collagen gel. **B.** Stimulated contraction. Collagen gels were exposed to 10 μM carbachol for 12 and 24 hours. **C.** Percentage of active gel size decrease upon carbochol stimulation. Data represent the SEM of 3 triplicate experiments.

Animals Ethics

Rat fat tissue was obtained in accordance with the Animal Ethics Committee.

Conflict of interest

The authors declare that they have no conflict of interest.

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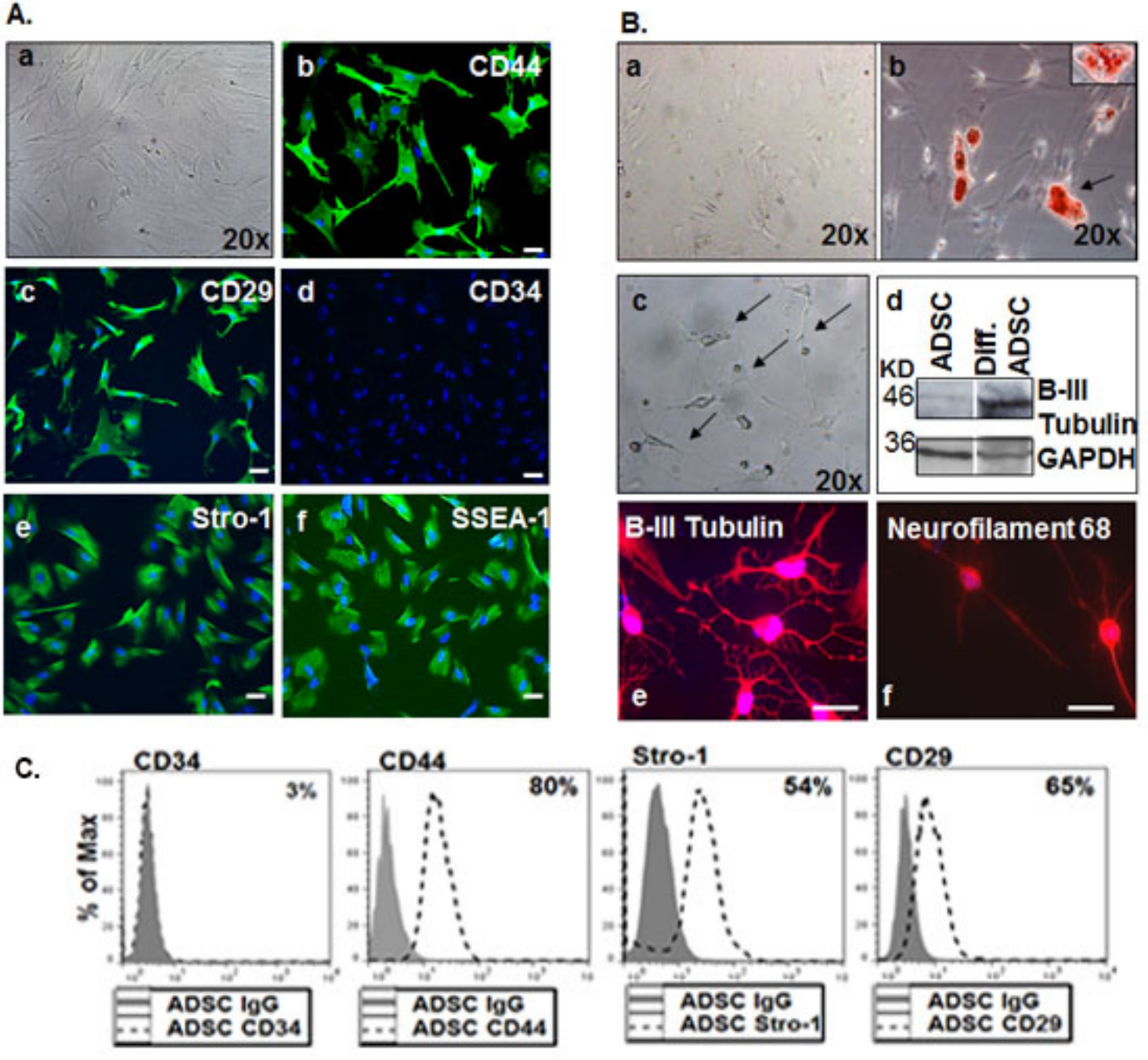
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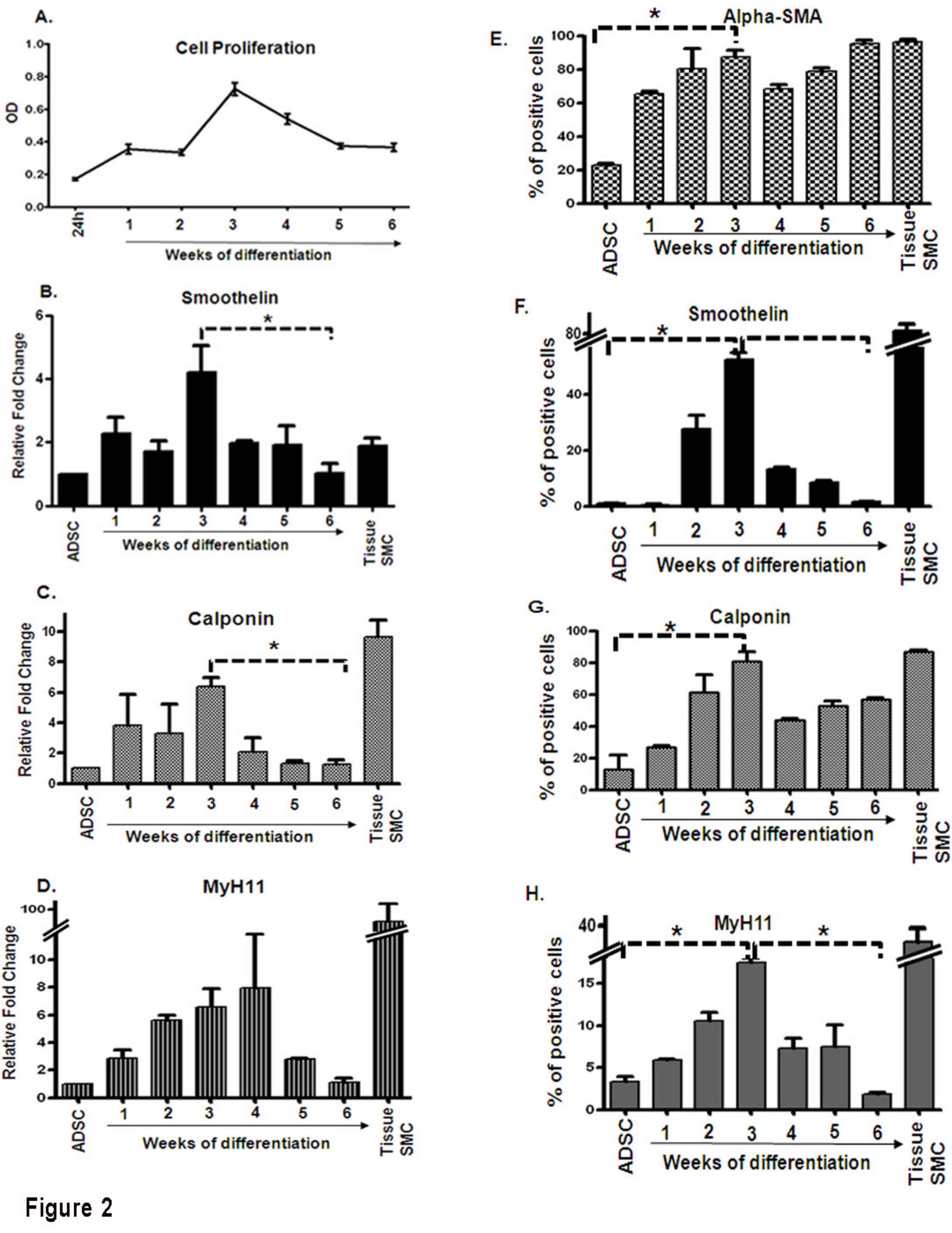
Figure 1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Weeks of differentiation

Figure 2

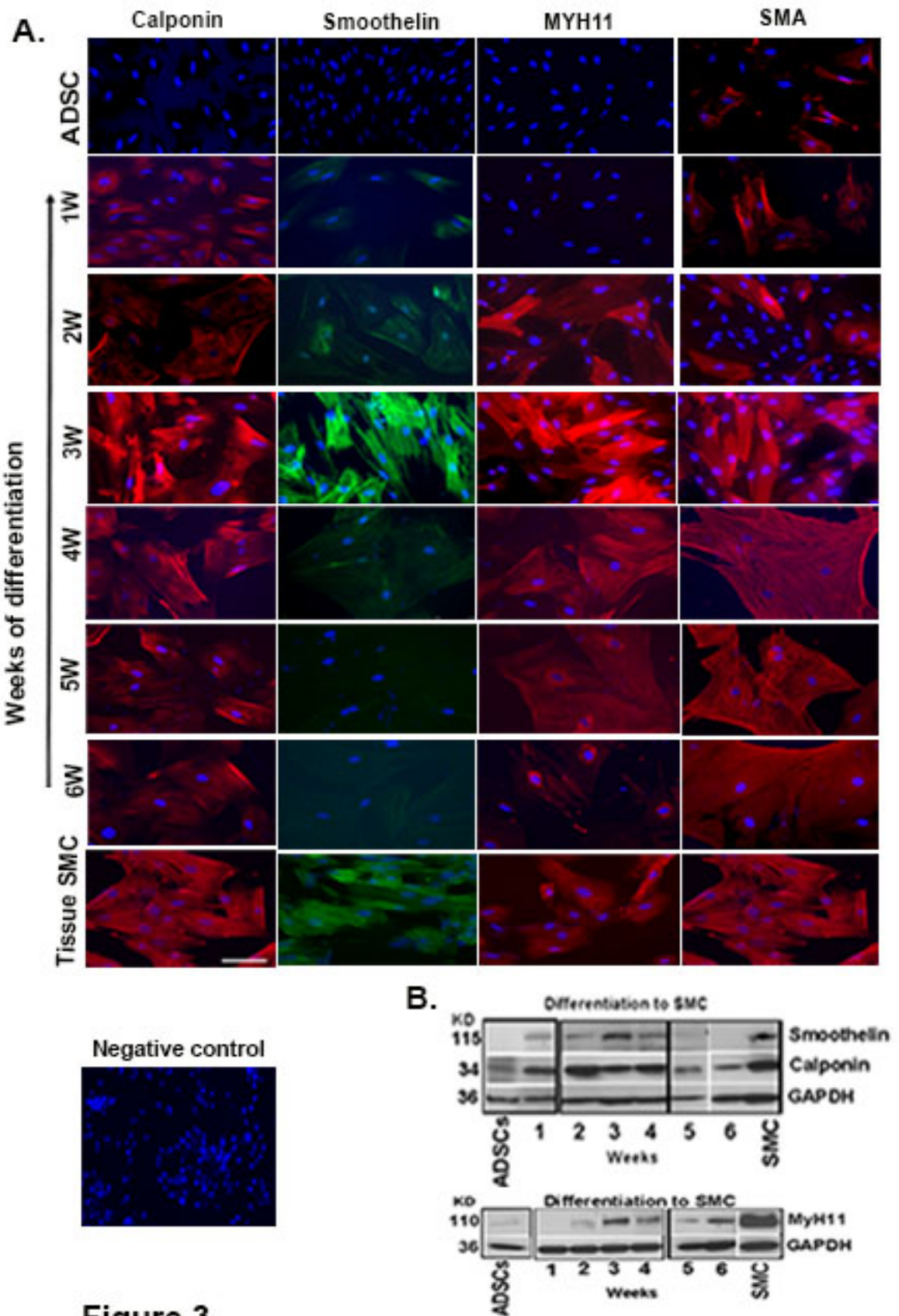
A.
Calponin
Smoothelin
MYH11
SMA
ADSC
1W
2W
3W
4W
5W
6W
Tissue SMC
Weeks of differentiation
Negative control
B.
Differentiation to SMC
KD
115
34
36
Smoothelin
Calponin
GAPDH
ADSCs
1
2
3
4
5
6
SMC
Weeks
Differentiation to SMC
KD
110
36
MyH11
GAPDH
ADSCs
1
2
3
4
5
6
SMC
Weeks

Figure 3.

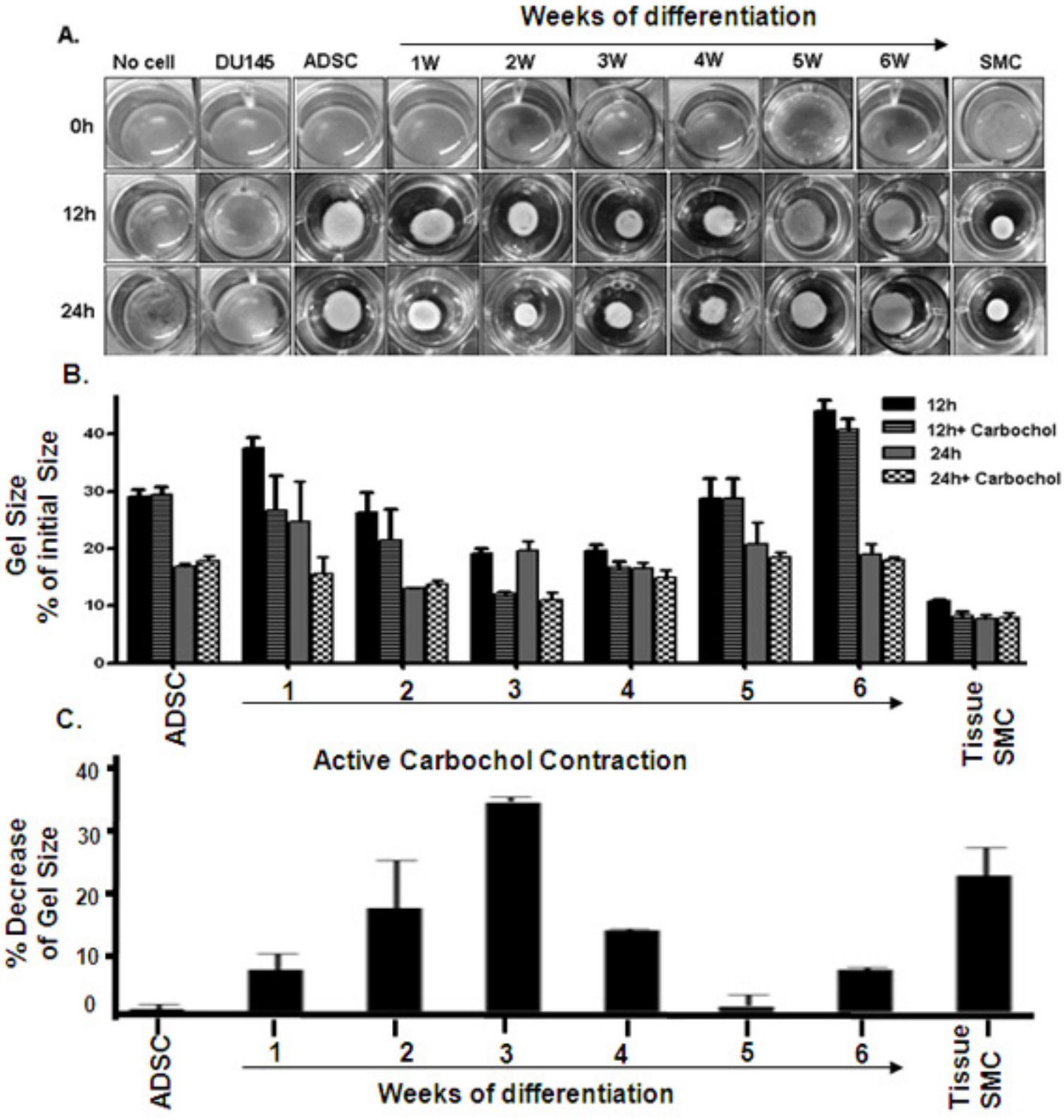
A.
Weeks of differentiation
No cell
DU145
ADSC
1W
2W
3W
4W
5W
6W
SMC
0h
12h
24h
B.
Gel Size
% of initial Size
12h
12h+ Carbochol
24h
24h+ Carbochol
ADSC
1
2
3
4
5
6
Tissue SMC
C.
Active Carbochol Contraction
% Decrease of Gel Size
ADSC
1
2
3
4
5
6
Tissue SMC
Weeks of differentiation

Figure 4