

Different Phenotypes and Chondrogenic Responses of Human Menstrual Blood and Bone Marrow Mesenchymal Stem Cells to activin A and TGF- β 3

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Research

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Abstract

Background: Due to its low capacity for self-repair, articular cartilage is highly susceptible to damage and deterioration, which leads to the development of degenerative joint diseases such as osteoarthritis. Menstrual blood-derived mesenchymal stem cells (MenSCs) are much less characterized compared to bone marrow mesenchymal stem cells (BMMSCs). However, MenSCs seem an attractive alternative to classical BMMSCs due to ease of access and broader differentiation capacity. The aim of this study was to evaluate chondrogenic differentiation potential of MenSCs and BMMSCs stimulated with transforming growth factor β (TGF- β 3) and activin A, member of the TGF- β superfamily of proteins.

Methods: MenSCs (n=6) and BMMSCs (n=5) were isolated from different healthy donors. Expression of cell surface markers CD90, CD73, CD105, CD44, CD45, CD14, CD36, CD55, CD54, CD63, CD106, CD34, CD10, Notch1 was analysed by flow cytometry. Cell proliferation capacity was determined using CCK-8 proliferation kit. Adipogenic differentiation capacity was evaluated according to Oil-Red staining, osteogenic differentiation - Alizarin Red staining. Chondrogenic differentiation (Activin A and TGF- β 3 stimulation) was induced *in vitro* and *in vivo* (subcutaneous scaffolds in nude BALB/c mice) and investigated by histologically and by expression of chondrogenic genes (collagen type II, aggrecan). Activin A protein production was evaluated by ELISA.

Results: MenSCs exhibited a higher proliferation rate, as compared to BMMSCs, and a different expression profile of several cell surface markers. Activin A stimulated collagen type II gene expression and glycosaminoglycan synthesis in TGF- β 3 treated MenSCs but not in BMMSCs, both *in vitro* and *in vivo*, although the effects of TGF- β 3 alone were more pronounced in BMMSCs *in vitro*.

Conclusion: These data suggest that activin A exerts differential effects on the induction of chondrogenic differentiation in MenSCs vs. BMMSCs, which implies that different mechanisms of chondrogenic regulation are activated in these cells. Following further optimisation of differentiation protocols and the choice of growth factors, potentially including activin A, MenSCs may turn out to be a promising population of stem cells for the development of cell-based therapies with the capacity to stimulate cartilage repair and regeneration.

Trial registration: Not applicable.

Introduction

Human articular cartilage has a poor capacity for intrinsic repair weak ability to restore its lesions leading to the development of progressive degenerative diseases such as osteoarthritis (OA), which is one of the most common form of arthritis across the world [1]. Currently, there is no effective treatment for OA, although cell-based therapies using mesenchymal stem cells (MSCs) seem promising approaches for cartilage regeneration. However, thus far, there MSC-based therapies that can effectively restore damaged cartilage in OA [2][3][4]. Furthermore, due to complications associated with the collection of bone marrow samples, which is an invasive procedure, and the poor yield of MSCs, alternative sources of cells are

needed in order to overcome technical issues in BMMSC isolation, for instance, adipose, amnion and umbilical cord tissue [2][5][6]. Menstrual blood is a unique body fluid, a renewable and sustainable source of multipotent stem cells (MenSCs) for regenerative medicine, which may originate from different subpopulations of endometrium, as reviewed [7][8]. These cells are of great interest due to their ease of access, as their collection does not require any complicated procedures and the permissions of ethics authorities, as well as invasive surgical procedures [9][10]. Moreover, MenSCs were demonstrated to differentiate towards a wider range of tissues, including neurogenic, cardiomyogenic and even hepatogenic cell lineages, as compared to BMMSCs [11][12][13][14]. Chondrogenic differentiation of MenSCs has been reported in several studies, suggesting that MenSCs could be a suitable candidate for cartilage tissue engineering as they have been reported to produce and accumulate extracellular matrix (ECM) [14][15]. However, the expression of collagen 2A1 mRNA was exclusively detectable in differentiated BMMSCs, whereas it was not observed in differentiated MenSCs [14], suggesting that molecular mechanisms regulating chondrogenic differentiation of MenSCs may differ in these two cells and the classical protocol applied for BMMSCs might not be suitable for MSCs of other origins [16][17]. Classical chondrogenesis stimulating factors, such as TGF- β and bone morphogenetic protein-2 (BMP-2), may not stimulate differentiation in MenSCs [18][19][20] and application of different stimulating factors may be needed.

Several studies suggest that a member of the TGF- β superfamily activin A could play a role in the early stages of MSC chondrogenesis [21][22]. Activin A induces the expression of octamer-binding transcription factor 4 (Oct4), Nanog, Nodal, proto-oncogene protein Wnt3, fibroblast growth factor 8 (FGF-8) and is necessary for the maintenance of self-renewal and pluripotency of MSCs [21]. Furthermore, enhanced production of activin A was demonstrated in OA cartilage, as compared to the healthy one, while it suppressed aggrecanase-mediated cleavage of aggrecan [23]. This suggests activin A to be a protective factor for OA development, and a potential candidate for future cartilage repair strategies.

The aim of this study was to evaluate the chondrogenic differentiation potential of MenSCs and BMMSCs stimulated with TGF- β 3 and activin A by histochemical staining with safranin-O and determining collagen II and aggrecan gene expression in cell pellets. We also stimulated chondrogenic differentiation of MenSCs and BMMSCs on atelocollagen/Polylactic acid (PLLA) scaffolds and subcutaneously inserted them into nude BALB/c mice for 9 weeks. The isolated constructs with cells were analyzed histologically by glycosaminoglycan (GAG) protein expression. In addition, we evaluated activin A secretion in proliferating BMMSCs and MenSCs and during chondrogenic differentiation in pellets.

The key focus of this study was to compare chondrogenic differentiation of both cell types, however, we also compared stem cell properties of MenSCs and BMMSCs by characterizing the expression of cell surface markers (CD90, CD73, CD105, CD44, CD45, CD14, CD36, CD55, CD54, CD63, CD106, CD34, CD10, Notch1), their proliferation capacity and potential to differentiate into two other MSC lineages – adipogenic and osteogenic.

Materials And Methods

Cell isolation and culture

Menstrual blood samples were collected from six healthy women aged between 20–40. About 5–10 mL of menstrual blood was collected by donors using sterile silicone cups (iCare) inserted into vagina during second day of menstrual cycle. In laboratory facilities mononuclear cells were separated using Ficoll-Paque PLUS (Stem Cell Technologies) density gradient centrifugation (30 min, room temperature, 400 *g*) and washed out two times in phosphate buffered saline (PBS) (Sigma Aldrich), centrifuged 10 min, 600 *g*. Collected MenSCs were seeded into tissue culture flasks (Gibco, Life Technologies) with low glucose (1 g/L) Dulbecco's modified Eagle medium (DMEM) (Merck Millipore) supplemented with 10% fetal bovine serum (FBS) (Merck Millipore), 1% penicillin/streptomycin (Gibco, Life Technologies), 1 ng/mL FGF2 (Applied Biological Materials) (later referred as "complete medium"), and cultured in 37 °C incubator with 5% CO², saturated humidity. Medium was changed twice a week and after cells reached their confluence (~ 80%) they were detached using trypsin-EDTA 0.25% solution (Gibco, Life Technologies), calculated (CASY, Omni Life Science) and sub-cultured. Human BMMSCs were isolated from bone marrow tissues, remaining after surgical procedures according to the established protocols as previously described [24]. There were five bone-marrow donors aged between 50–60. BMMSCs were cultured under the same conditions as MenSCs. All procedures using human tissues in this study were approved by the local bioethics committee, permission No. 158200-14-741.

The experiments were performed using MenSCs and BMMSCs at early passages (P) P2 to P3.

Proliferation assay

MenSCs and BMMSCs were seeded into 12-well plates (SPL, Life Sciences) at density 5000 cells/well in a complete medium. Cell proliferation was determined at days 1, 5, 8 and 12 by measuring it with cell counting kit – 8 (CCK-8) (Dojindo) according to manufacturer's instructions. The medium was collected to 96 well plate (Orange Scientific) and absorbance of reduced formazan dye was quantified using spectrophotometric quantification (SpectroMaxi3, Molecular Devices) (absorbance, 450 nm).

Scratch assay

Scratch assay was performed to evaluate cell migration capacity. MenSCs and BMMSCs were seeded into T25 flasks (Gibco, Life Technologies) and grown to full confluence. Later a straight scratch of the cell monolayer was performed with a 1 mL pipet tip. The cell scratch was photographed under a phase-contrast microscope at 0, 8, 24 and 48 hours, and cell migration abilities were evaluated by the size of area of migrated cells inside the scratch.

Immunophenotypic characterization of cells

Surface marker analysis was performed by harvesting the cells, washing them with cytometer buffer (PBS + 2% bovine serum albumin (BSA) (Biological Industries)) 5 min at 600 *g*, and incubating with the specific labelled antibodies in cytometer buffer for 20 min at 4 °C. Antibodies used for the experiment

were against human cell surface cluster of differentiation CD antigens CD90, CD73, CD105, CD44, CD45, CD14, CD36, CD55, CD54, CD63, CD106, CD34, CD10, Notch1 purchased from BD Pharmingen (BD Biosciences, San Jose, CA, USA), R&D Systems (Minneapolis, MN, USA) and Biolegend (San Diego, CA, USA). In all experiments, matching isotype antibodies were used as negative controls. Data (10.000 events) were collected using a flow cytometer FACS Aria II (BD Biosciences) and analyzed on FACS Diva analysis software (BD Biosciences).

Adipogenic and osteogenic differentiation

For adipogenic differentiation, MenSCs and BMMSCs were seeded into a 12 well plate, at a density of 60 000 cells/well. Differentiation was induced in sub-confluent cells using adipogenic medium, consisting of low glucose (1 g/L) DMEM medium, 1% penicillin/streptomycin, 20% FBS, 1 μ mol/L dexamethasone (Sigma Aldrich), 60 μ mol/L indomethacin (Sigma Aldrich) and 50 μ mol/L 3-isobutyl-1-methylxanthine (IBMX) (Biosource) for 21 day. After that, lipid droplets in cells were stained with Oil Red-O (Carl Roth) and visualized in an inverted light microscope. Quantitative analysis was performed by dissolving lipid droplets in 70% isopropanol solution (Eurochemicals), after which the released oil-red dye was collected into 96 well plate and measured absorbance using spectrophotometer (absorbance, 520 nm).

For osteogenic differentiation MenSCs and BMMSCs were also seeded into a 12 well plate, at a density 40 000 cells/well. Osteogenic differentiation medium was added after the cells reached their sub-confluence, consisting of high glucose (4.5 g/L) DMEM medium (Gibco, Life Technologies), 1% penicillin/streptomycin, 10% FBS, 0.1 μ mol/L dexamethasone, 50 μ g/mL ascorbic acid and 10 mmol/L β – glycerophosphate (Santa Cruz) for 21 day. Osteogenesis was evaluated by light microscopy, staining the cells with Alizarin Red S (Carl Roth). Quantitative analysis was evaluated by dissolving the calcium hydroxyapatite crystals, stained with Alizarin, in 10% cetyl-pyridinium chloride (Sigma Aldrich). The dissolved solution was then collected into 96 well plate and absorbance measured using spectrophotometer (absorbance, 562 nm).

Chondrogenic differentiation using activin A and TGF- β 3 in vitro

Chondrogenesis was induced using a protocol used by State Research Institute Centre for Innovative medicine. Chondrogenic medium was composed of high glucose (4.5 g/L) DMEM medium, 1% penicillin/streptomycin, 1% insulin-transferrin-selenium (Gibco Life Technologies), 350 μ M L-proline (Carl Roth), 0.1% dexamethasone, 170 μ M ascorbic acid-phosphate (Sigma Aldrich) and 10 ng/mL TGF- β 3 (Gibco, Life Technologies), or 50 ng/mL activin A (Merck, Millipore). Briefly, 250 k of MenSCs or BMMSCs were added in 15 mL tubes (Gibco, Life Technologies), centrifuged 5 min at 600 *g* and cultivated in chondrogenic medium. The tubes with cells were divided into 4 groups: 1 – Control (chondrogenic medium without growth factors); 2 – activin A; 3 – TGF- β 3; 4 – TGF- β 3 + activin A. Double growth factor group was activin A-stimulated only once at the start of differentiation, while during second medium change on day 2 – only TGF- β 3 was added. This protocol was chosen based on previous studies suggesting the efficacy of activin A at the early stages of chondrogenic differentiation [21]. Each

treatment was applied in technical triplicates. The production of ECM in cell pellets was evaluated macroscopically and histologically (Safranin O staining).

Chondrogenic differentiation using activin A and TGF- β 3 in vivo

Chondrogenic differentiation of MenSCs and BMMSCs was also stimulated in atelocollagen/PLLA constructs, which were inserted into six nude mice (BALB/c). For this purpose, 100 000 cells were mixed with 100 μ L of atelocollagen gel (Koken) for 10 min according to manufacturer's recommendations, transferred to PLLA (R&D) and incubated for 2 h in 37 °C incubator with 5% CO₂. After that cell constructs were transferred into 20 mL tubes and chondrogenic differentiation medium was added. The constructs with cells of each donor were divided into three groups: 1 – Control; 2 – activin A and 3 – TGF- β 3, where control – cells incubated in chondrogenic medium without growth factors, activin A – cells incubated in chondrogenic medium with activin A (50 ng/mL), which was added only once, at the beginning of the differentiation (at day 2 it was replaced with TGF- β 3), TGF- β 3 – cells incubated in chondrogenic medium with TGF- β 3 (10 ng/mL). Chondrogenesis was induced for 21 days. The medium was changed three times a week. After the end of differentiation, the cell constructs were subcutaneously implanted into BALB/c nude mice (n = 6), under general anesthesia with isoflurane (Sigma Aldrich). Each donor's cell constructs were inserted into the same mouse. The constructs with cells remained in mice for 9 weeks. After that period, the mice were sacrificed using diethyl ether (Sigma Aldrich) and the constructs were carefully removed. Every construct was divided into equal two parts, where one part was used for histological analysis and the other for GAG protein analysis (Biocolor).

GAG analysis in cell atelocollagen/PLLA constructs

Half of the collected cell atelocollagen/PLLA constructs were transferred into acetic acid solution (50 nM) (Sigma Aldrich) and homogenized using stainless steel beads in homogenizer (300 sec, 3200 rpm). After homogenization, all procedures were performed on ice. The homogenized solution was transferred to Eppendorf and pepsin (10 ng/mL) (Wako) was added for 48 h, 4 °C in a rotor spin. After 48 h, the solution was neutralized using Tris-buffered saline (TBS) buffer, consisting of 1 M Tris, 2 M sodium chloride and 50 mM calcium chloride solution. Elastase (1 mg/mL) (Wako) was added overnight, 4°C in a rotor spin. The following morning, the samples were centrifuged at 910 *g*, 5 min, 4°C. The protein levels were standardized using Lowry method. The GAG levels were measured spectrophotometrically using blyscan™-sulphated GAG assay (Biocolor), according to manufacturer's recommendations, as previously described [25].

Histology of chondrogenic differentiation cell pellets

For histochemical and immunohistochemical analysis, chondrogenic differentiation pellets were fixed in 10% neutral formalin and embedded into paraffin. 3-micrometer sections were deparaffinized and processed for standard staining with safranin-O (Sigma Aldrich).

RNA extraction from cell pellets

At the end of chondrogenic differentiation period, the cell pellets were collected, flash-frozen in liquid nitrogen and stored at -70 °C. Frozen samples were homogenized by ultrasonication (Bandelin Sonopuls) in lysis buffer (Qiagen, 74104) and RNA was extracted according manufacturer's protocol. RNA concentration and purity were measured with the SpectraMax i3 (Molecular Devices, USA).

RT-qPCR

RNA was reverse-transcribed with Maxima cDNA Synthesis kit including dsDNase treatment (Thermo Scientific). RT-qPCR reaction mixes were prepared with Maxima Probe qPCR Master Mix (Thermo Scientific) and TaqMan Gene expression Assays (*RPS9* - Hs02339424_g1, *B2M* - Hs00984230_m1, *COL2A1* - Hs01060345_m1, *ACAN* - Hs00153936_m1 (Thermo Scientific)) and ran on the Agilent Aria MX instrument (Agilent Technologies) in technical triplicates starting with denaturation step for 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C denaturation and 60 s for annealing and extension. Relative levels of gene transcripts were calculated by subtracting the threshold cycle (Ct) of the normalizer (the geometric mean of the two housekeeping genes – RPS9 and B2M) from the Ct of the gene of interest, giving the dCt values which were subsequently transformed to 2^{-dCt} values and multiplied by 1000 to scale-up for better graphical representation.

Quantification of activin A from chondrogenic pellet supernatants and monolayer by ELISA

Activin A protein production was evaluated during chondrogenic differentiation of MenSCs and BMMSCs and during cell cultivation in monolayer. Supernatants were collected from the pellets incubated with TGF- β 3 and control during chondrogenic differentiation, and from monolayer cultures during 8th and 12th days. Activin A protein levels were detected using activin A duo set ELISA (R&D Systems) according to the manufacturer's protocol. Activin A levels secreted during monolayer culture were normalized according to upgrowing number of cells by precise value of proliferation. It was implemented by calculating the ratio of a secreted activin A to proliferation value.

Statistical analysis of studies

The Student's t-test was used to calculate statistical difference among MenSCs and BMMSCs data in proliferation, immunophenotypic, GAG analysis, gene analysis and ELISA assays. A $P \leq 0.05$ was considered as statistically significant.

Results

MenSCs and BMMSCs proliferation and migration capacity

The proliferation capacity of MenSCs was significantly higher than BMMSCs (Fig. 1-A). During the first 3 days of culture, there were no significant differences in cell proliferation, however, from the 5th day,

MenSCs proliferation started to increase and on day 12 the difference reached statistical significance compared to BMMSCs (MenSCs 0.828 (+/- 0.201), BMMSCs 0.286 (+/- 0.085)) (P value 0.022).

In scratch assays, after 48 hours complete wound closure was observed in MenSCs, but not in BMMSCs (Fig. 1-B).

These results suggest that proliferation and migration of MenSCs are higher than BMMSCs.

Surface marker expression in MenSCs and BMMSCs

In order to determine and compare immunophenotypical profiles of MenSCs and BMMSCs, the cells were stained with antibodies against MSC surface markers (CD44, CD73, CD90, CD105), hematopoietic (CD14, CD34, CD36, CD45) and pluripotential stem cell markers (CD10, CD54, CD55, CD63, CD106, Notch1) and analysed by flow cytometer (Fig. 2).

MenSCs and BMMSCs were both positive for classical MSC markers – CD44, CD73, CD90, CD105, where more than 95% of total cell population was positive, and negative for hematopoietic stem cell markers – CD14, CD34, CD36, CD45 (less than 10% of total cell population was positive). Expression of CD55, CD54, CD10 and Notch1 was statistically significantly higher in MenSCs, as compared to BMMSCs, whereas CD63 in BMMSCs.

MenSCs and BMMSCs ability to differentiate into osteoblasts and adipocytes

To evaluate mesodermal lineage potential, MenSCs and BMMSCs were induced to differentiate towards adipogenic and osteogenic direction (Fig. 3). The results demonstrate that MenSCs and BMMSCs are able to differentiate into adipogenic and osteogenic lineage cells. However, adipogenic differentiation capacity was significantly stronger in BMMSCs (3.22 ± 1.39), than in MenSCs (2.41 ± 0.13), as they formed more lipid droplets. However, osteogenic differentiation, was significantly higher in MenSCs (38.86 ± 10.71) than in BMMSCs (19.74 ± 7.32).

Secretion of activin A during proliferation and chondrogenic differentiation of MenSCs and BMMSCs

During 12 days of cell culture in monolayer (Fig. 4-A), the level of activin A was increasing in supernatants, and during 8 and 12 days it was significantly higher in MenSCs (757 ± 79 pg/mL, and 2064 ± 312 pg/mL, respectively), as compared to BMMSCs (396 ± 187.51 pg/mL and 586 ± 359.2 pg/mL, respectively). P values: 8th day – 0.018; 12th day – 0.028. We next wanted to determine, whether the higher levels of activin A are associated with more intensive its secretion in MenSCs or rather with the higher numbers of those cells due to the more intensive proliferation. To investigate that, activin A secretion was normalized according to the upgrowing cell activity, measured by CCK-8, which essentially represents cell numbers (Fig. 4-B). After normalisation, secretion of activin A still remained significantly

higher in MenSCs as compared to BMMSCs on days 8th and 12th in monolayer:(8th day - MenSCs 2622.8; BMMSCs 2066.7, $P = 0.027$; 12th day: -MenSCs 2493.1; BMMSCs 2046.7, $P = 0.031$).

Stimulation of chondrogenic differentiation in MenSCs and BMMSCs with activin A and/or TGF- β 3

After analysis of activin A secretion in monolayer, both cell types were stimulated to differentiate into chondrogenic lineage using TGF- β 3 and cell supernatants were collected for activin A secretion analysis. During the 3rd day of chondrogenic differentiation in pellet system, the levels of activin A are presented in Fig. 5. It was significantly higher in control cells and cells, stimulated with TGF- β 3 on 3rd day of chondrogenic induction in MenSCs (control – 82.32 ± 2.94 pg/mL; TGF- β 3 – 63.21 ± 1.47 pg/mL), as compared to BMMSCs (control 5.88 ± 2.94 pg/mL and TGF- β 3 102.9 ± 1.47 pg/mL), where $P \leq 0.001$ for controls and $P \leq 0.05$ for TGF- β 3. BMMSCs maintained production of activin A on 3rd day of differentiation only in combination with TGF- β 3 (102.9 ± 1.47 pg/mL), as compared to control (5.88 ± 2.94 pg/mL) ($P \leq 0.05$). Noteworthy, MenSCs control cells, which were incubated without any growth factors, produced higher quantities of activin A than cells stimulated with TGF- β 3, where in contrast, BMMSCs maintained activin A secretion only under stimulation with TGF- β 3. These differences might be associated with activin A-dependent mechanisms specific for MenSCs. During the later 19 days of chondrogenic induction of both, MenSCs and BMMSCs, the secretion of activin A was very low and practically non-detectable (data not shown).

According to histological staining of pellet sections with safranin-O, different responses to TGF- β 3 and activin A were observed in MenSCs and BMMSCs (Fig. 6). Combination of activin A and TGF- β 3 stimulated proteoglycan synthesis in MenSCs, while stimulation with either activin A or TGF- β 3 alone had no clear effect on chondrogenic response in those cells. In BMMSCs, accumulation of proteoglycans was observed in both, activin A or TGF- β 3-stimulated groups of BMMSCs, however, the effects of TGF- β 3 were more pronounced.

At gene expression level, a weaker ability to differentiate into chondrogenic lineage was observed in MenSCs, as compared to BMMSCs (Fig. 7 and Fig. 8). Collagen type II (*COL2A1*), which is one the major collagen type in articular cartilage, gene expression was significantly lower in TGF- β 3 and TGF- β 3 with activin A groups of MenSCs, as compared to these two groups of BMMSCs ($P \leq 0.001$). However, application of TGF- β 3 alone or in combination with activin A significantly upregulated *COL2A1* expression in MenSCs and the combination of activin A and TGF- β 3 was significantly stronger than the effect of TGF- β 3 alone (Fig. 7). Activin A had no substantial effect on *COL2A1* gene expression in MenSCs.

Low levels of aggrecan (*ACAN*) gene expression was detected in all 4 conditions of MenSCs (Fig. 8), as compared to substantially higher levels of its expression in BMMSCs. *ACAN* expression statistically significantly increased only in BMMSCs and only after stimulation with TGF- β 3 or a combination of activin A with TGF- β 3.

BMMSCs possessed a strong ability to differentiate into chondrogenic lineage with TGF- β 3, according to *COL2A1* and *ACAN* gene expression (Fig. 7 and Fig. 8). In opposite to MenSCs, combination of activin A with TGF- β 3 in BMMSCs had no additional effect on the expression of the genes tested.

Chondrogenesis of MenSCs and BMMSCs in vivo

According to safranin-O staining, higher accumulation of ECM proteins was observed in samples of combined activin A and TGF- β 3 treatment, as compared to control in both cells (Fig. 9, B).

GAG synthesis was also observed in higher content in samples treated with combination of TGF- β 3 and activin A as well as single factors, in both MenSCs and BMMSCs (Fig. 10). However, statistically significant differences were observed only in MenSCs, where GAG production was higher in TGF- β 3 stimulated group and additionally more pronounced if the combination of TGF- β 3 + activin A was used. Although there were no significant differences between MenSCs and BMMSCs groups, MenSCs stimulated with TGF- β 3 + activin A samples produced more GAGs, as compared to samples of BMMSCs under the same stimulation.

Discussion

MSCs have been a focus in regenerative medicine due to their high potential to differentiate towards several lineages including osteoblasts, chondrocytes and adipocytes. Firstly, MSCs were discovered in bone marrow, while later they were isolated from almost all human tissues, including bone marrow, adipose tissue, umbilical cord blood, placenta [26][27][28][6][29] and human menstrual blood [8][30][31]. The collection of menstrual blood samples does not require invasive procedures, which highlights a key advantage of MenSCs in regenerative medicine over BMMSCs [10]. MenSCs are increasingly investigated in different studies, and are known to be similar to BMMSCs [8][32][33]. Furthermore, due to a high potential to differentiate into several different lineages, MenSCs have been proposed for treatment of critical limb ischemia, stroke, type I diabetes mellitus, Parkinson's disease and other neurodegenerative disorders in experimental disease models [14][9]. Despite those advantages of MenSCs as compared to MSCs derived from other tissues, their characteristics have not been extensively studied so far. For instance, potential application of MenSCs for cartilage regeneration still requires additional studies on chondrogenic differentiation capacity. One of the usually used growth factors in chondrogenic differentiation of BMMSCs is TGF- β , however, other types of MSCs, for instance isolated from adipose tissue, may respond better to other growth factors, for instance BMP-2, -4 or -7 [34][35]. The growth factor activin A is important in numerous cell processes, as well as the early stages of chondrogenesis [21]. Mechanisms of action of activin A and TGF- β s appear similar, they both induce phosphorylation of Smad2 and Smad3 during chondrogenesis [36]. However, the role of activin A in chondrogenic differentiation is controversial, as some studies propose that it antagonises chondrogenesis and suppresses the expression of chondrogenic genes [37][38].

Furthermore, activin A is very important in regulating the female menstrual cycle and has various functions in reproduction [39][40][41][42], suggesting that it may modulate other functional

characteristics of MenSCs, including differentiation.

In this study, MenSCs and BMMSCs were isolated and characterized and compared by MSC surface marker expression (flow cytometry), proliferation abilities (CCK-8, spectrophotometry) and MSC trilineage differentiation capacity (osteogenic, adipogenic, chondrogenic). We observed higher proliferation and migration in MenSCs compared to BMMSCs, which is similar to the results obtained in other studies, where MenSCs and BMMSCs proliferation was investigated [32][43].

Expression of typical MSCs surface markers (CD44, CD73, CD90, CD105), hematopoietic stem cell markers (CD14 CD34, CD36, CD45), and pluripotent stem cell markers (CD10, CD54, CD55, CD63, Notch1) were analysed for characterisation of MenSCs phenotype [44][45][46][47][48].

Expression of CD55, CD54, CD10 and Notch1 surface markers in MenSCs was significantly higher, as compared to BMMSCs, whereas expression of CD63 was higher in BMMSCs (Fig. 2). CD10 is a membrane metallo-endopeptidase, also referred as neprilysin, which is involved in different cell signalling pathways influencing cell migration, angiogenesis, tumorigenesis and immunomodulation [44]. Moreover, CD10 has also been a marker of highly proliferating cells, as well as calcifying cells [49], which was also observed for MenSCs in the present study. CD55 is a complement decay-accelerating factor, also a MSC marker used to identify endoderm in early embryonic development [50]. CD54 is an intercellular adhesion molecule-1 (ICAM-1), which was shown to be involved in the osteogenic and adipogenic differentiation of MSCs [51]. Notch1 is a member of the Notch family of receptors and plays a significant role in mediating BMP9-induced osteogenic differentiation in MSC [52]. Notch is also known to be associated with enhanced MSC proliferation [53], which might be associated with high regenerative abilities of endometrium and could represent an advantage in regenerative medicine when large amounts of cells are needed.

Even though the main focus of this study was chondrogenic differentiation in both MenSCs and BMMSCs we also compared their potential to differentiate into adipogenic and osteogenic lineages. MenSCs showed weak adipogenic differentiation capacity, which is in agreement to the data obtained in other studies [32][43]. Moreover, the weak adipogenic differentiation capacity was also observed in amniotic fluid, placenta, umbilical cord and umbilical cord blood MSCs [54][55], which are of similar origin with MenSCs. This is in contrast to classical trilineage differentiation of BMMSCs. However, as described above, potential of MenSC to differentiate into neurogenic, cardiomyogenic and even hepatogenic cell lineages [11][12][13] confirm their advanced differentiation potential, while the weaker adipogenesis probably indicate different differentiation profile rather than lack of stem cell properties. Furthermore, osteogenic differentiation capacity of MenSCs was significantly stronger as compared to BMMSCs (Fig. 3), which to the best of our knowledge, has not been demonstrated previously. Stronger osteogenic differentiation capacity than in BMMSCs has been already reported for MSCs derived from placental and amniotic fluid [56][57][58], which are the ones of the closest origin to MenSCs, therefore, might share some functional similarities.

The levels of secreted activin A were measured during cell proliferation in monolayer and their chondrogenic differentiation. Higher levels of activin A were secreted by MenSCs in monolayer (Fig. 4), as compared to BMMSCs, and the elevated levels remained until 3rd day of chondrogenic differentiation in both – non-stimulated control and TGF- β 3 group of MenSC (Fig. 5). No significant chondrogenic response to activin A has been previously reported in BMMSCs [37][38], which is in agreement with results obtained in this study. However, this is the first report of MenSCs stimulated to differentiate into chondrogenic lineage using activin A. Noteworthy, the expression of chondrogenic genes indicates weaker ability MenSCs to differentiate into chondrogenic lineage, in comparison to the levels in BMMSCs.

The response to activin A during chondrogenic differentiation was also different in MenSCs and BMMSCs. In MenSCs, the effects of activin A alone were negligible and only in combination with TGF- β 3 resulted in a substantial increase of *COL2A1* expression. Even though *COL2A1* and *ACAN* gene expression was stronger in BMMSCs after stimulation with TGF- β 3, activin A had no significant effect on the expression of these genes neither alone, nor in combination with TGF- β 3. Positive safranin-O staining was observed in histological samples of activin A-treated BMMSCs pellets, suggesting stimulatory effects of this factor on chondrogenic differentiation. In BMMSCs stimulated with a combination of activin A with TGF- β 3, the expression of *COL2A1* gene (Fig. 7) as well as the ECM protein content observed in histology samples (Fig. 6) were not significantly different, as compared to a single TGF- β 3 stimulation.

The *in vitro* chondrogenesis studies using activin A were followed by an *in vivo* study. The technology of Atelocollagen/PLLA construct application was chosen due to its biocompatibility and efficacy for stimulation of BMMSCs chondrogenic differentiation *in vivo* [59][60]. Chondrogenesis of MenSCs and BMMSCs in constructs was induced for 3 weeks *in vitro*, followed by their subcutaneous insertion into nude BALB/C mice. Histological evaluation of the samples (Fig. 9), as well as GAG analysis (Fig. 10) indicated that the additional stimulation with activin A, prior to switching to TGF- β 3, upregulated the production of ECM proteins in MenSCs, as compared to the controls or TGF- β 3 stimulated samples. Somewhat similar tendencies, although less expressed were observed in BMMSCs. These data are in contrast to the results obtained during *in vitro* cultures, where potential of MenSCs to differentiate into chondrogenic lineage was lower, as compared to BMMSCs. It is likely that longer stimulation is needed for MenSCs to effectively induce chondrogenic responses, as compared to BMMSCs, as *in vivo* study lasted an additional 63 days. Furthermore, paracrine effects in both cell types might also play a role in stimulating host cell response and their migration, which induces differentiation of MSCs [61]. MenSCs produce some unique factors, as demonstrated by others and in the present study, and seem less responsive to cytokine activation and express less immunosuppressive molecules, as compared to BMMSCs [10]. The differences in secretion of various paracrine factors might result in the disparities of host cell responses to MenSCs and BMMSCs *in vivo*, which in turn led to more expressed chondrogenic induction. It should also be considered that application of the scaffolds may play a role in stimulation of ECM production in MSCs, as previously demonstrated [15][62]. Although it might appear that collagen-based scaffolds are essential for stimulating chondrogenesis in MenSCs, further studies are needed to support their use. Moreover, there are many soluble factors in mouse body fluids that may differently

foster chondrogenic differentiation of implanted cells. Taken together, combined *in vitro* and *in vivo* data suggest that MenSCs differently respond to external stimuli and show distinct functional activity from BMMSCs. Nevertheless, it is likely that they are capable of switching to chondrogenesis, but this process takes longer time and the choice of suitable combinations of the factors to guide these cells and orchestrate the differentiation process are needed.

Conclusions

The results presented in this original investigation demonstrate that MenSCs share many similar phenotypic properties with BMMSCs, but they exhibit several differences as well. The proliferation rate and osteogenic differentiation capacity of MenSCs is higher than of BMMSCs, while adipogenic differentiation capacity is lower. Expression of surface markers CD55, CD54, CD10 and Notch1 were significantly higher in MenSCs, while of CD63 was lower, as compared to BMMSCs. Secreted levels of activin A were significantly higher in MenSCs as compared to BMMSCs on days 8 and 12 of cultivation in monolayer. The potential of BMMSCs to differentiate into the chondrogenic lineage was more pronounced under stimulation with TGF- β 3 *in vitro*. In contrast, the efficacy of activin A was higher in chondrogenic differentiation of MenSCs, as compared to BMMSCs, both *in vivo* and, particularly, *in vitro*. These data suggest that activin A is differently involved in the induction of chondrogenic differentiation in MenSCs vs. BMMSCs, which implies distinct pathways of chondrogenesis regulation in these cells. Following further optimisation of the differentiation protocol and growth factor choice, potentially including activin A and extending the duration of growth factor stimulation, MenSCs may indeed turn out to be a promising population of stem cells for the further development as a key component of cell-based therapies for stimulating cartilage repair and regeneration.

Abbreviations

Aggrecan

ACAN;

Bone marrow MSCs

BMMSCs;

Bone morphogenetic protein-2

BMP-2;

Cell counting kit 8

CCK-8;

Collagen Type II Alpha 1 Chain gene

COL2A1;

Dulbecco's modified Eagle medium

DMEM;

Extracellular matrix

ECM;

Fibroblast growth factor 8
FGF-8;
Glycosaminoglycan
GAG;
Menstrual blood-derived MSCs
MenSCs;
Mesenchymal stem cells
MSCs;
Osteoarthritis
OA;
Polylactic acid
PLLA;
Transforming growth factor β
TGF- β ;

Declarations

All procedures using human tissues in this study were approved by "Vilnius Regional Committee for Biomedical Research Ethics", permission No. 158200-14-741-257.

All procedures using mice in this study were carried out at Tokyo university hospital, and were approved by "Animal Experimentation Committee, Graduate School of Medicine and Faculty of Medicine, The University of Tokyo" (approval number: P15-019).

Consent for publication:

Not applicable

Availability of Data and Materials:

The data supporting these findings can be found at State Research Institute Centre for Innovative Medicine, Department of Regenerative medicine.

Author Contributions:

Writing-Original Draft Preparation and study design – I. Uzieliene and E. Bernotiene; donor patient selection and enrollment G.Kvederas; cell isolation, chondrogenesis, proliferation, ELISA studies, as well as scratch assay, osteogenic/adipogenic differentiation – Z. Tachtamisevaite, G. Rakauskiene and I. Uzieliene, Surface marker analysis – I. Uzieliene; Gene expression study – E. Bagdonas; conceptualization of the manuscript – E. Bernotiene; critical revision - E. Bernotiene, A.Mobasheri; In vivo study design,

conceptualization – A. Hikita, K. Hoshi, I. Uzieliene; In vivo study, chondrogenesis on Atelocollagen/PLLA scaffolds, GAG analysis – T. Sakamoto and I. Uzieliene; editing – all authors.

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Conflicts of Interest:

The authors do not declare any conflicts of interest.

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Figures

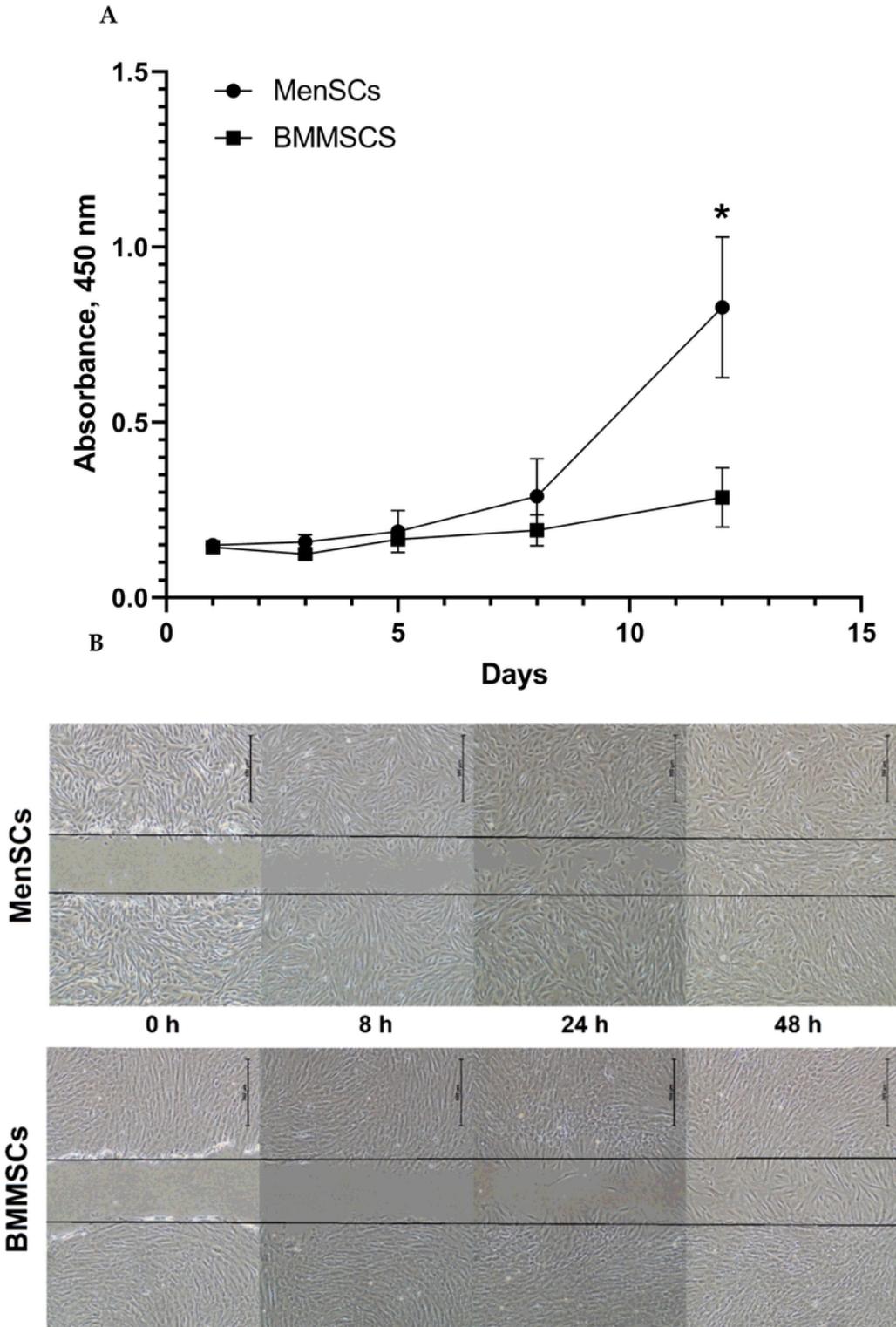


Figure 1

A – Proliferation of MenSCs and BMMSCs in monolayer, during 1, 3, 5, 8, 12 days of culture. Measured using CCK-8 viability and cytotoxicity assay. The absorbance of reduced formazan dye, produced by living cells, is presented. Absorbance measured at 450 nm. * $P \leq 0.05$. B – Scratch assay. MenSCs and

BMMSCs migration after mechanically disrupted monolayer with a sterile 1 ml pipet tip, obtained at different time points (0, 8, 24 and 48 h). Scale bar 500 μm .

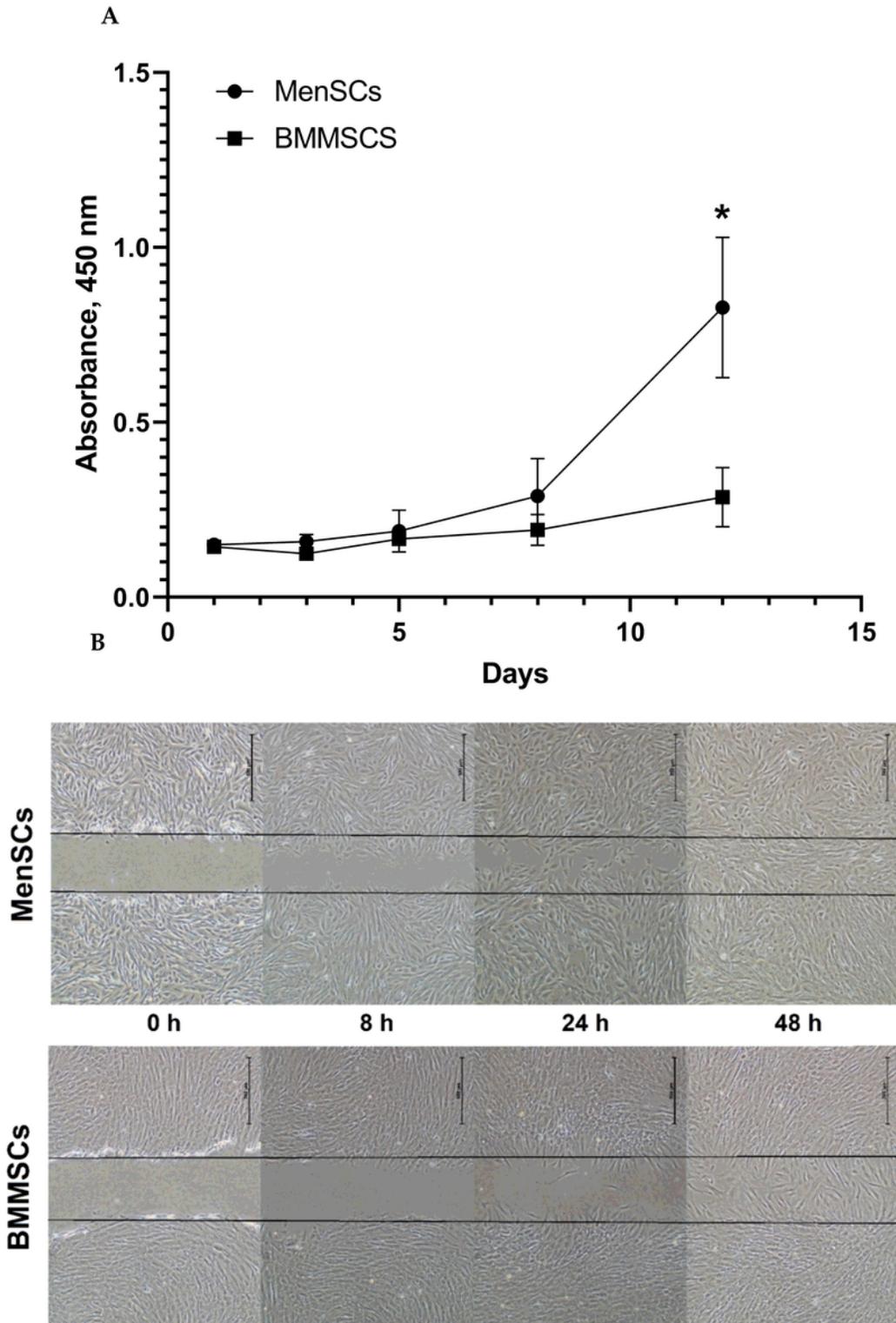


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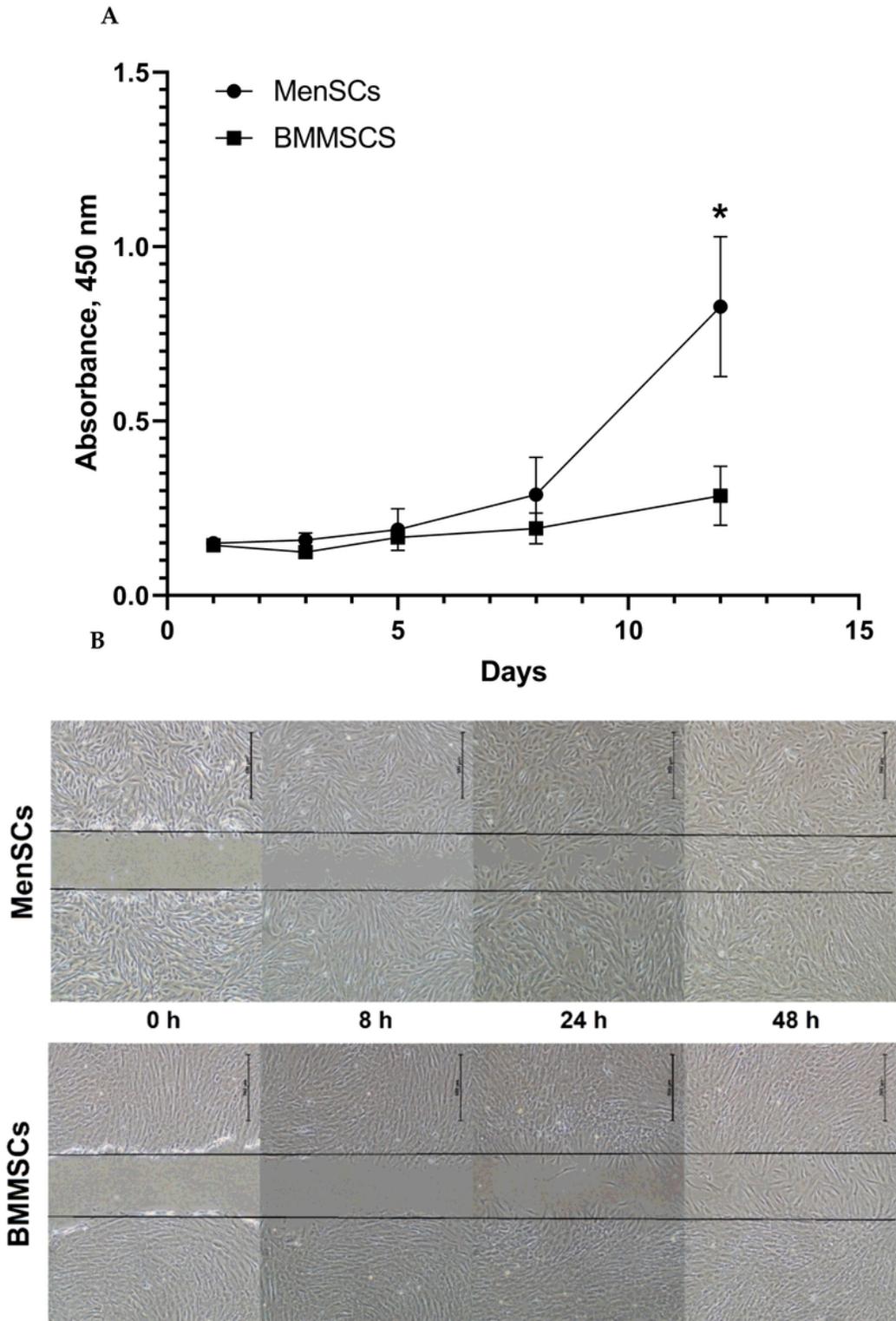


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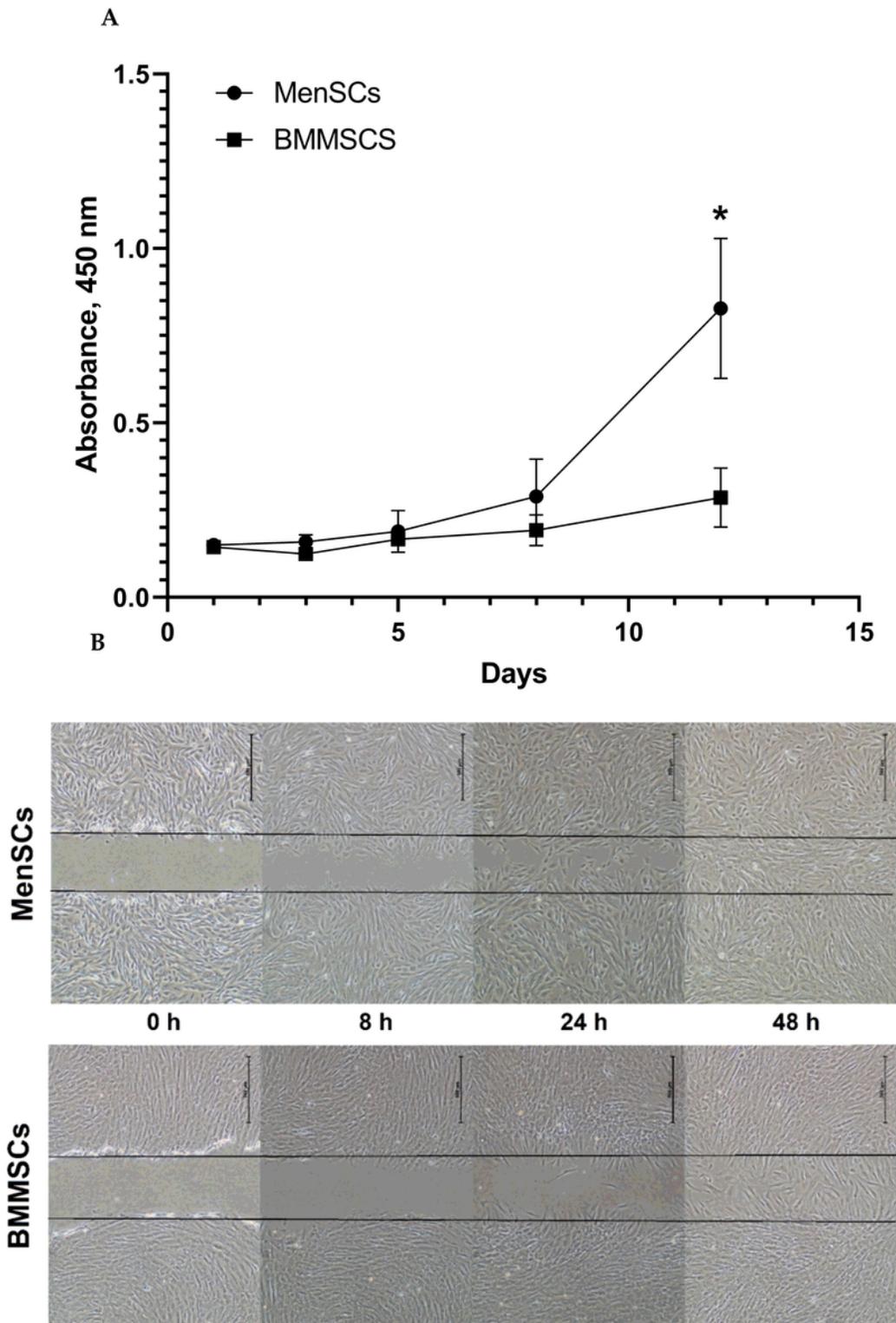


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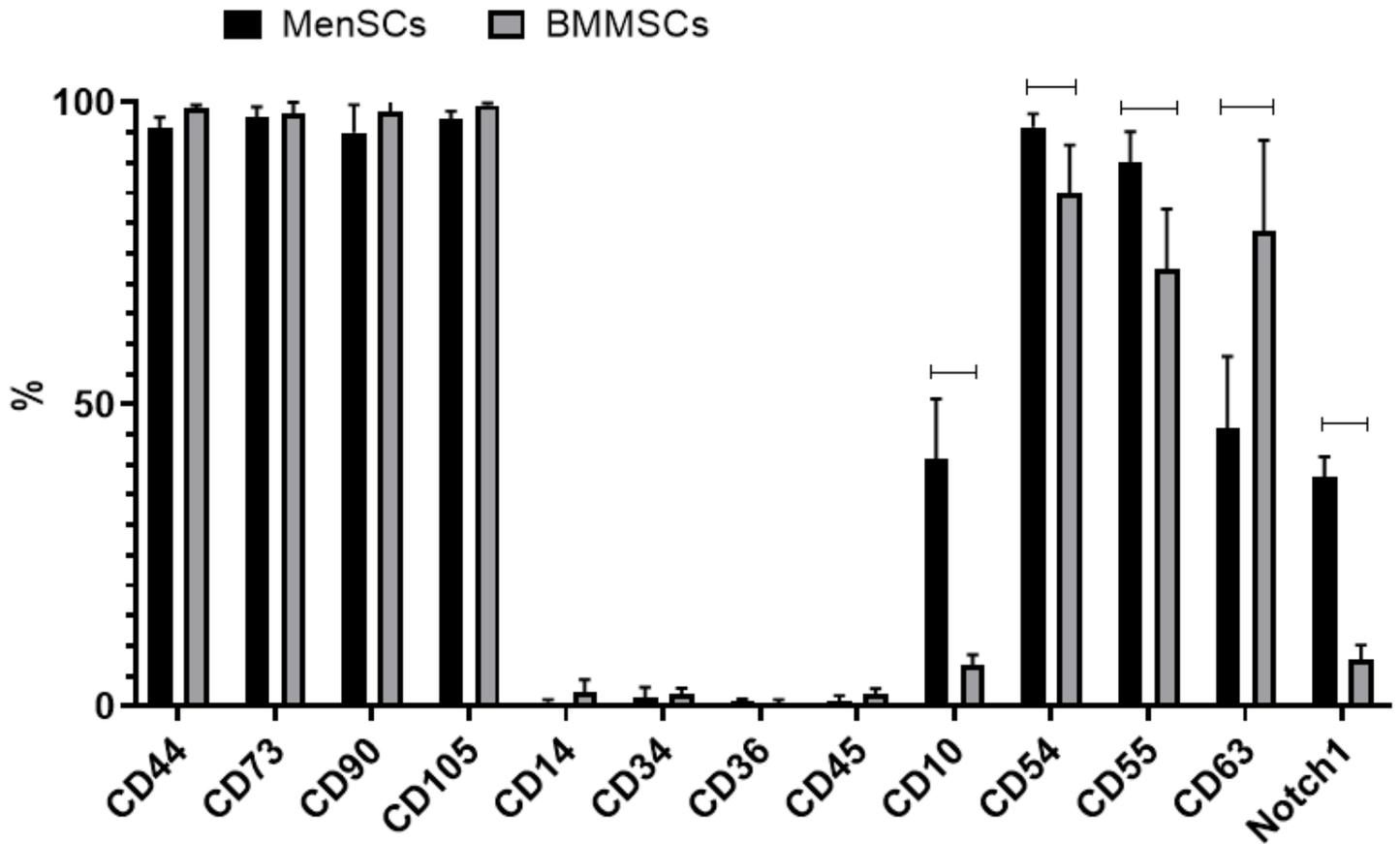


Figure 2

Expression of surface markers (CD44, CD73, CD90, CD105, CD14, CD34, CD36, CD45, CD10, CD54, CD55, CD63, Notch1) in MenSCs and BMMSCs. Flow cytometry analysis. % - percentage of cell population positive for indicated marker. Horizontal bars represent $P \leq 0.05$

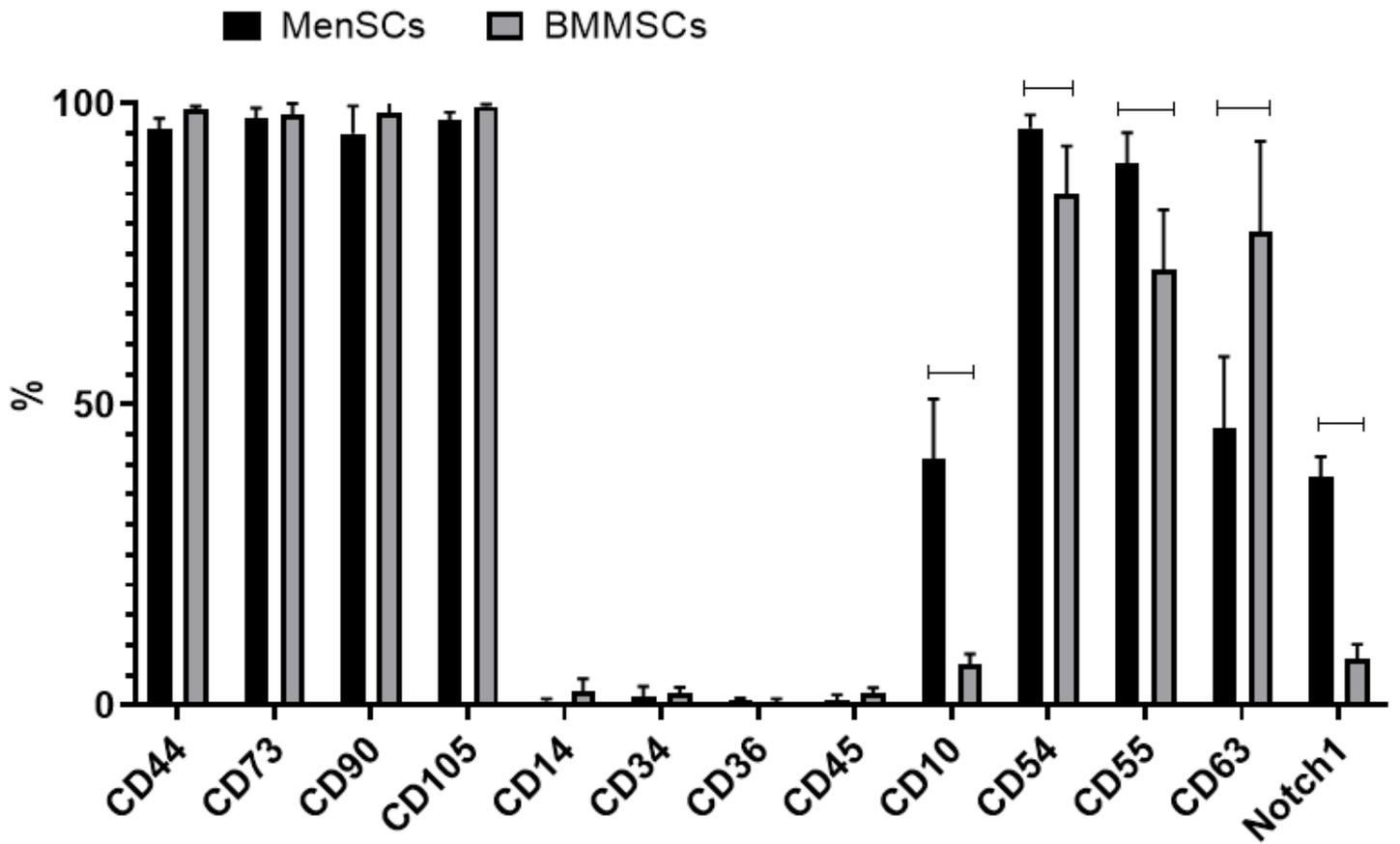


Figure 3

Expression of surface markers (CD44, CD73, CD90, CD105, CD14, CD34, CD36, CD45, CD10, CD54, CD55, CD63, Notch1) in MenSCs and BMMSCs. Flow cytometry analysis. % - percentage of cell population positive for indicated marker. Horizontal bars represent $P \leq 0.05$

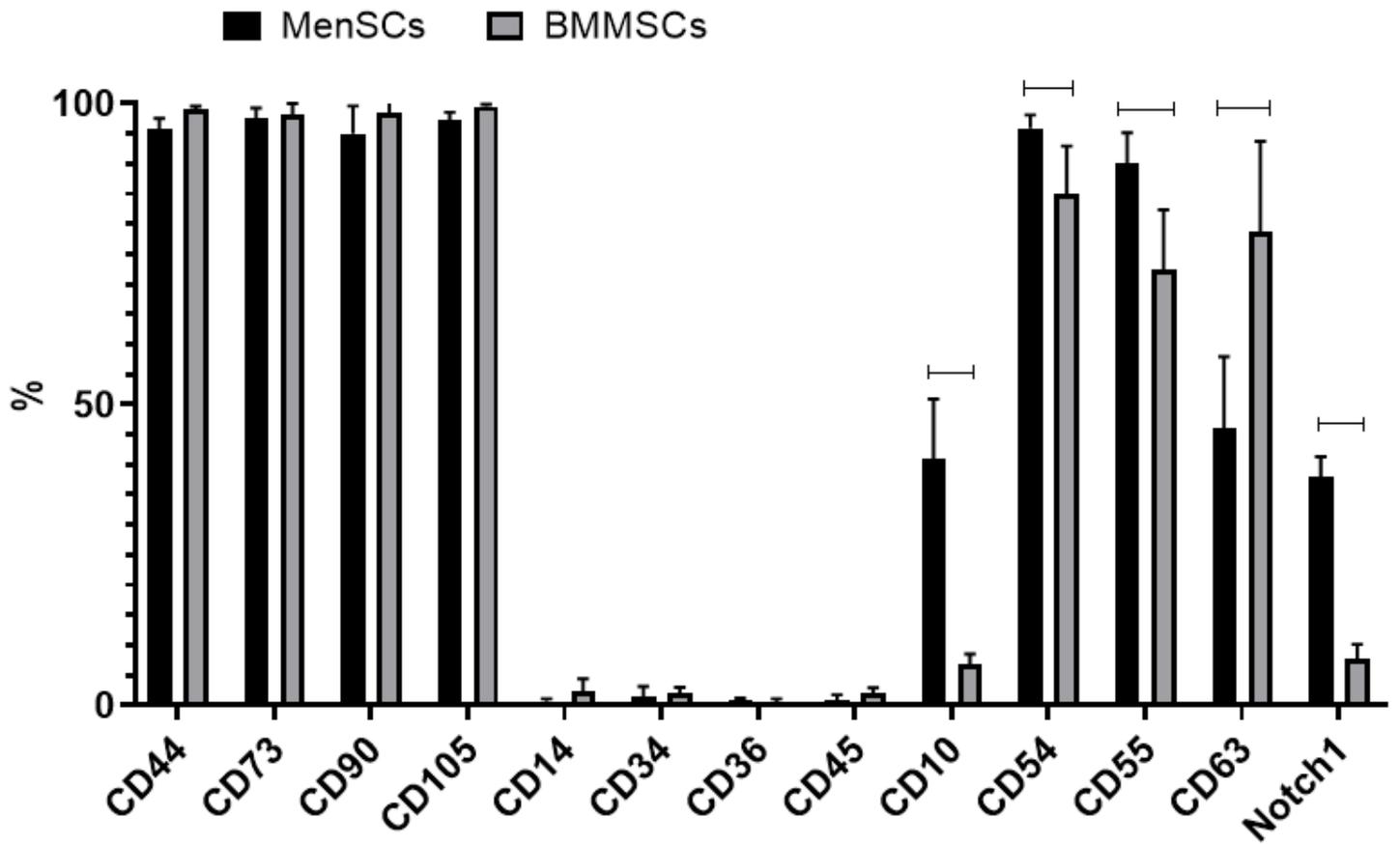


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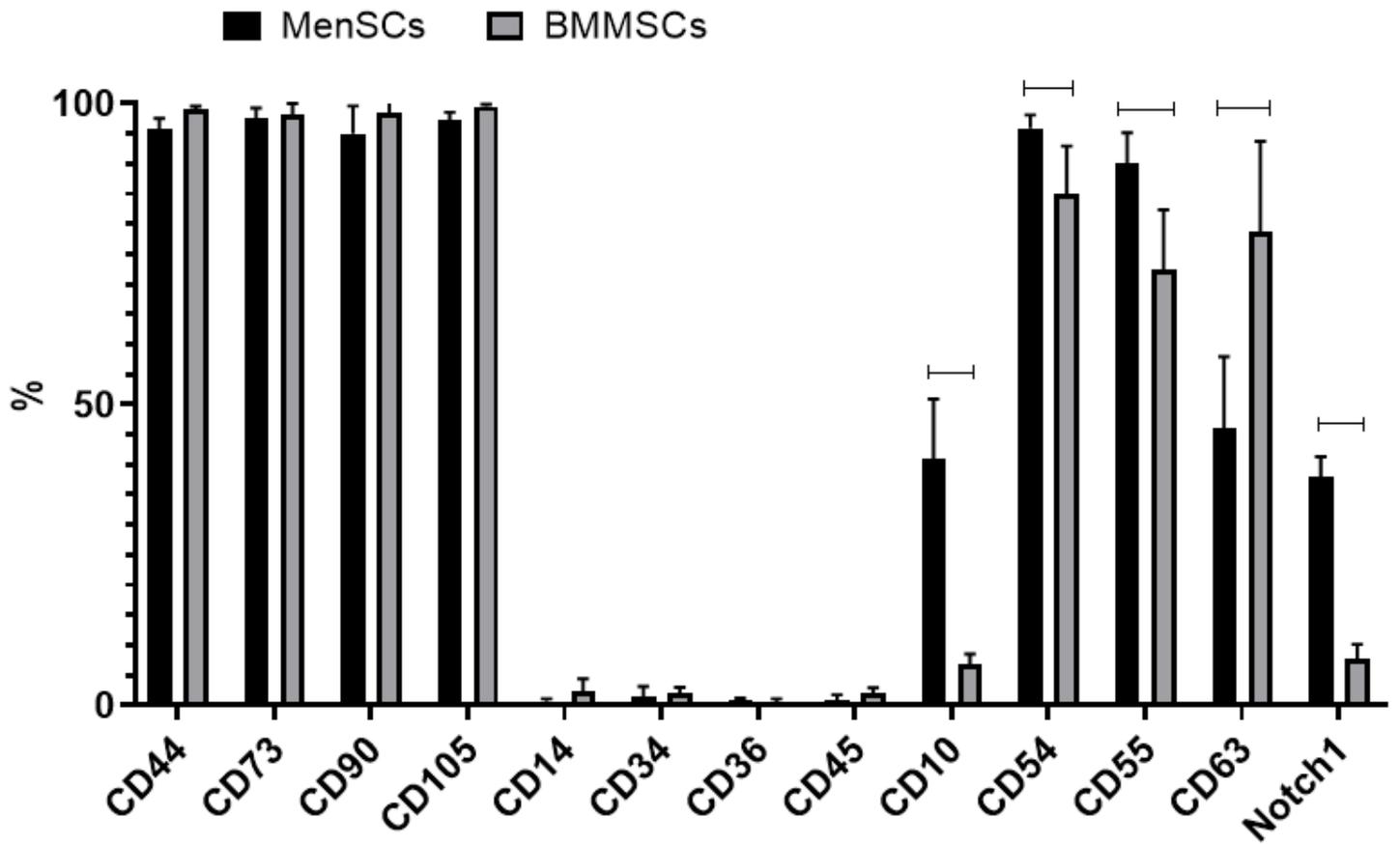


Figure 3

Expression of surface markers (CD44, CD73, CD90, CD105, CD14, CD34, CD36, CD45, CD10, CD54, CD55, CD63, Notch1) in MenSCs and BMMSCs. Flow cytometry analysis. % - percentage of cell population positive for indicated marker. Horizontal bars represent $P \leq 0.05$

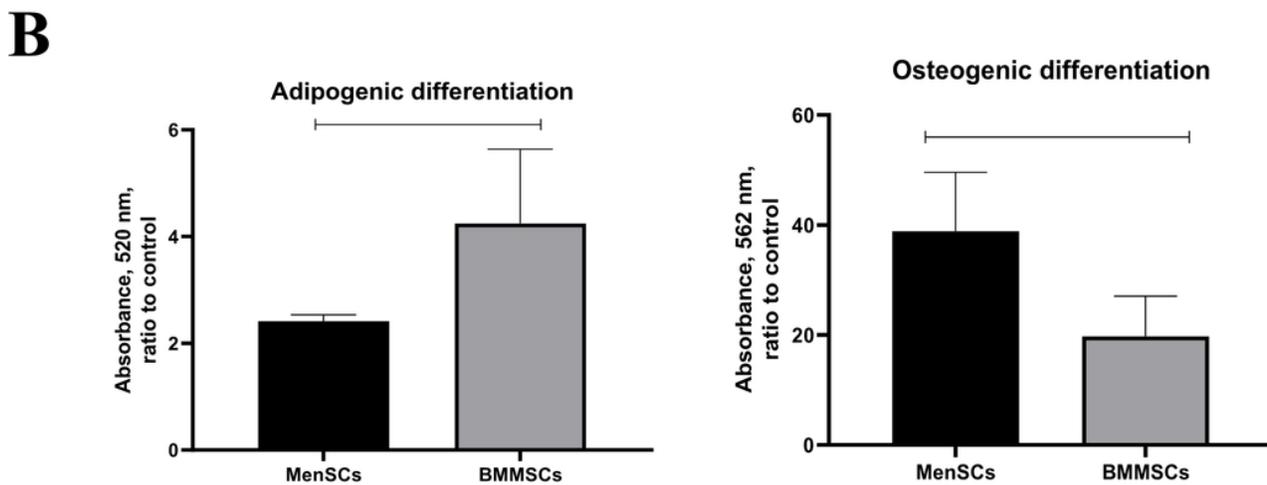
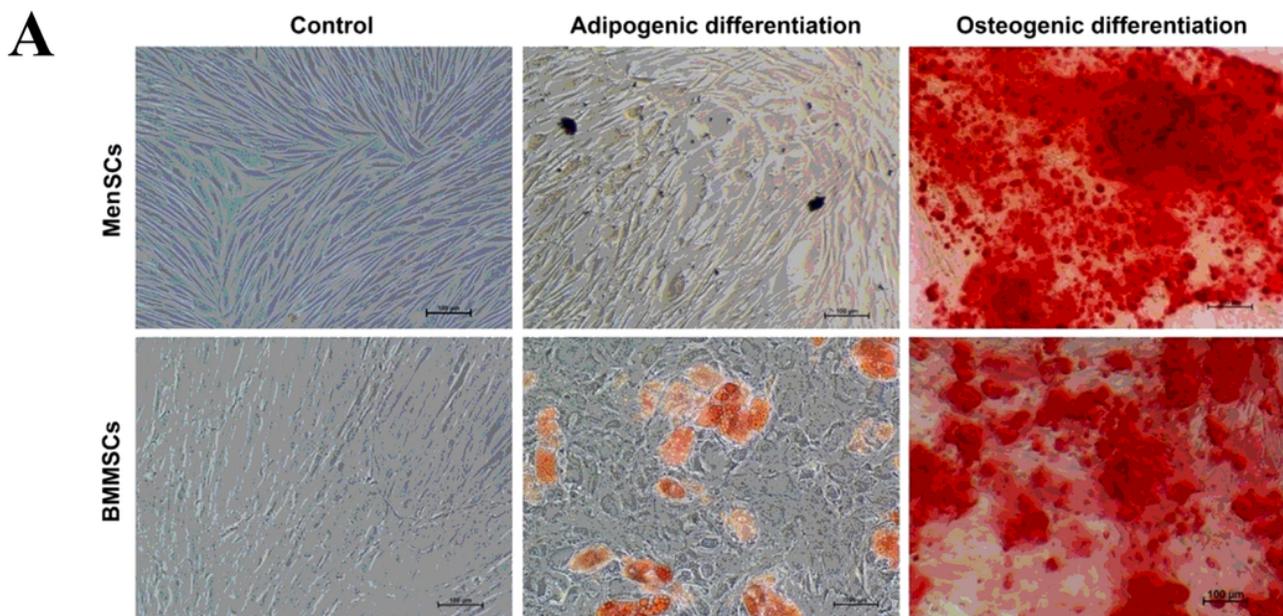


Figure 4

A – Adipogenic and osteogenic differentiation analysis of MenSCs and BMMSCs, staining the cells with oil red, and alizarin, respectively. Control cells were incubated using standard complete DMEM medium and stained with the same dyes after differentiation ended. The cells were cultivated in adipogenic/osteogenic medium for 21 day. Scale bar 100 μm . B – Quantitative adipogenic and osteogenic differentiation analysis of MenSCs and BMMSCs (21 day), dissolving lipid droplets (stained with oil-red) in isopropanol and calcium hydroxyapatite crystals (stained with alizarin) in cetyl-pyridinium chloride solutions. Absorbance measured at 520 nm and 562 nm, respectively. Graphs presented as ratio to control. Horizontal bars represent $P \leq 0.05$

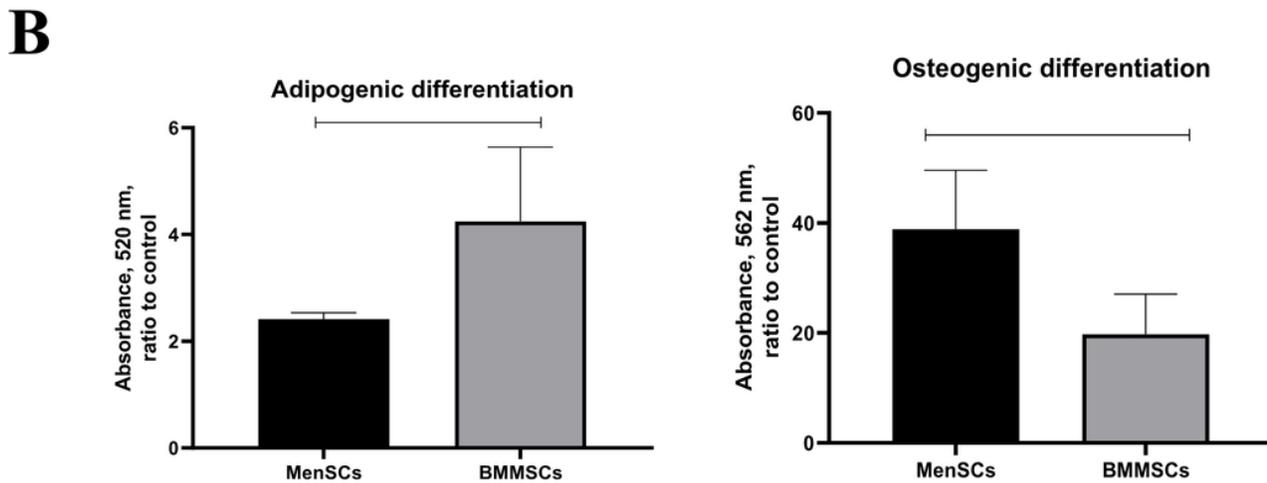
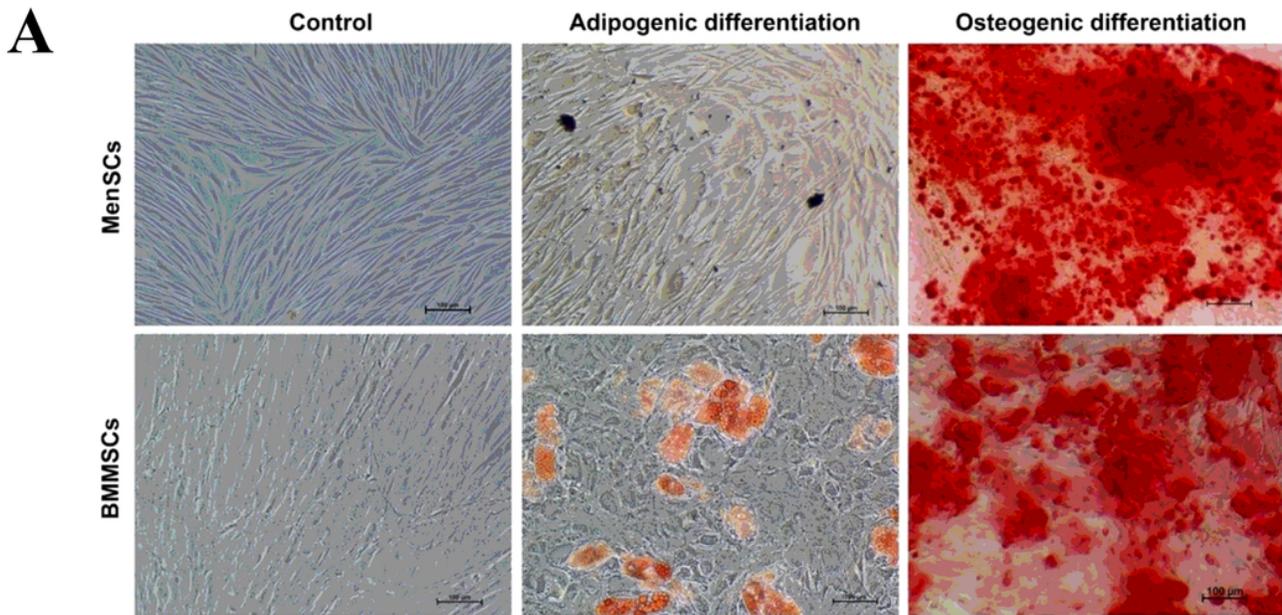


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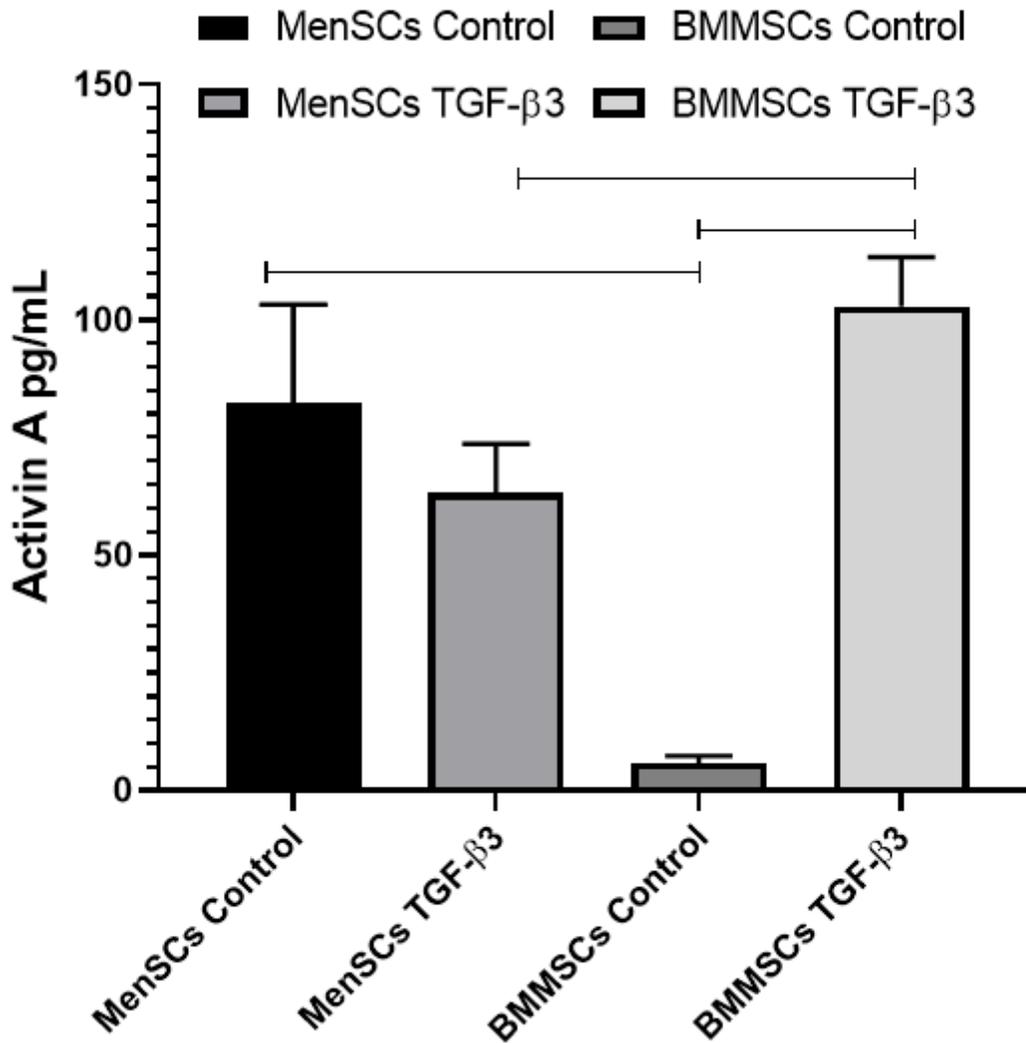


Figure 5

Levels of secreted activin A after 3 days of chondrogenic differentiation in MenSCs and BMMSCs in incomplete chondrogenic medium (control) or incomplete chondrogenic medium + TGF-β3 (10 ng/mL). Pellet cultures, ELISA assay. Horizontal bars represent $P \leq 0.05$

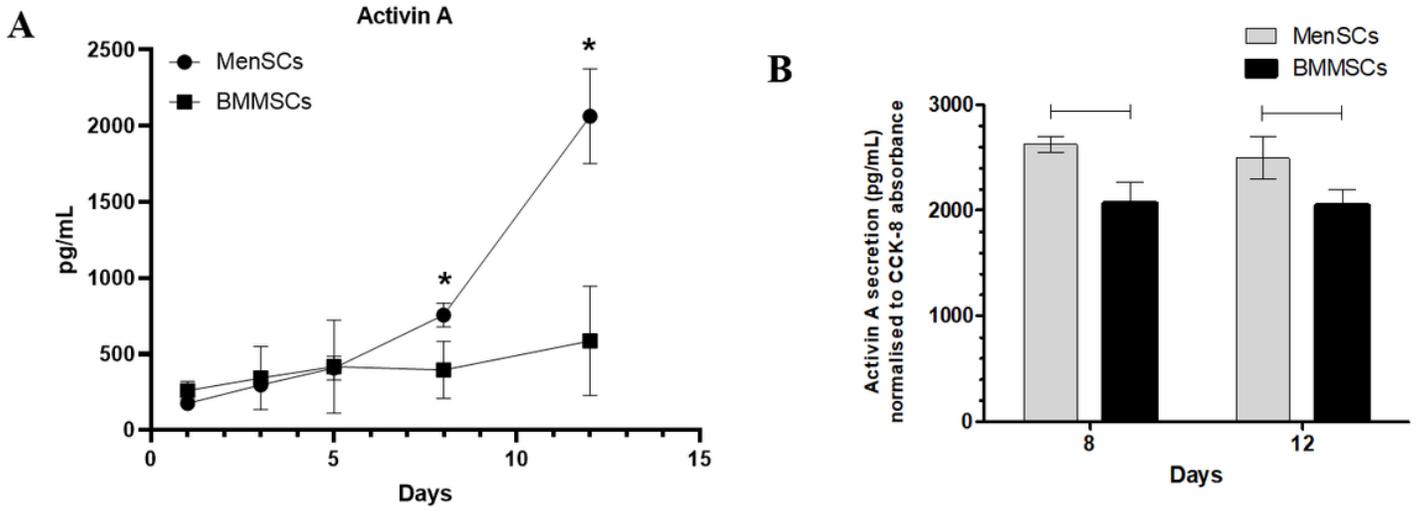


Figure 5

A – Levels of activin A in supernatants of MenSCs and BMMSCs monolayer cultures on days 1, 3, 5, 8, 12 of cell proliferation in complete DMEM medium. ELISA assay. * $P \leq 0.05$. B – Secreted levels of activin A, normalised according to CCK-8 absorbance of MenSCs and BMMSCs on days 8 and 12. Horizontal bars represent $P \leq 0.05$

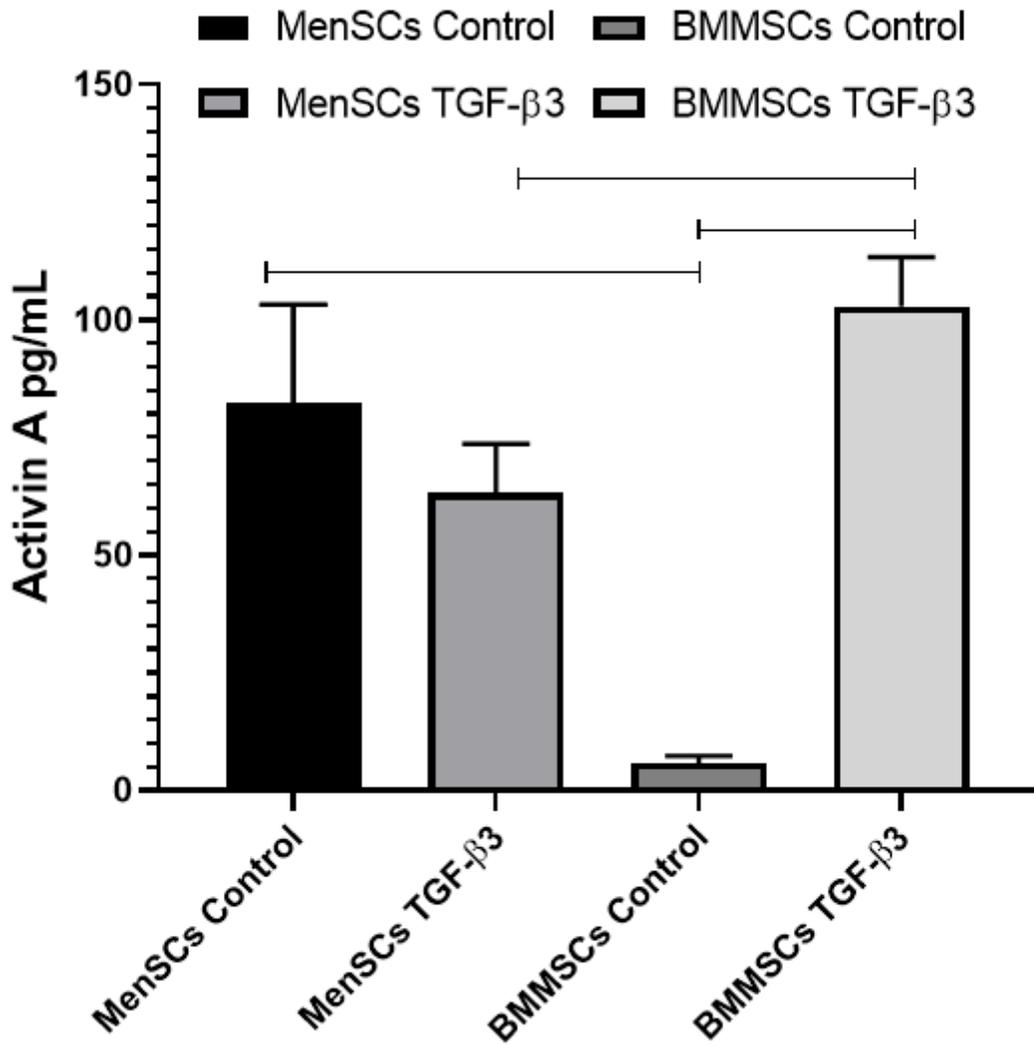


Figure 6

Levels of secreted activin A after 3 days of chondrogenic differentiation in MenSCs and BMMSCs in incomplete chondrogenic medium (control) or incomplete chondrogenic medium + TGF- β 3 (10 ng/mL). Pellet cultures, ELISA assay. Horizontal bars represent $P \leq 0.05$

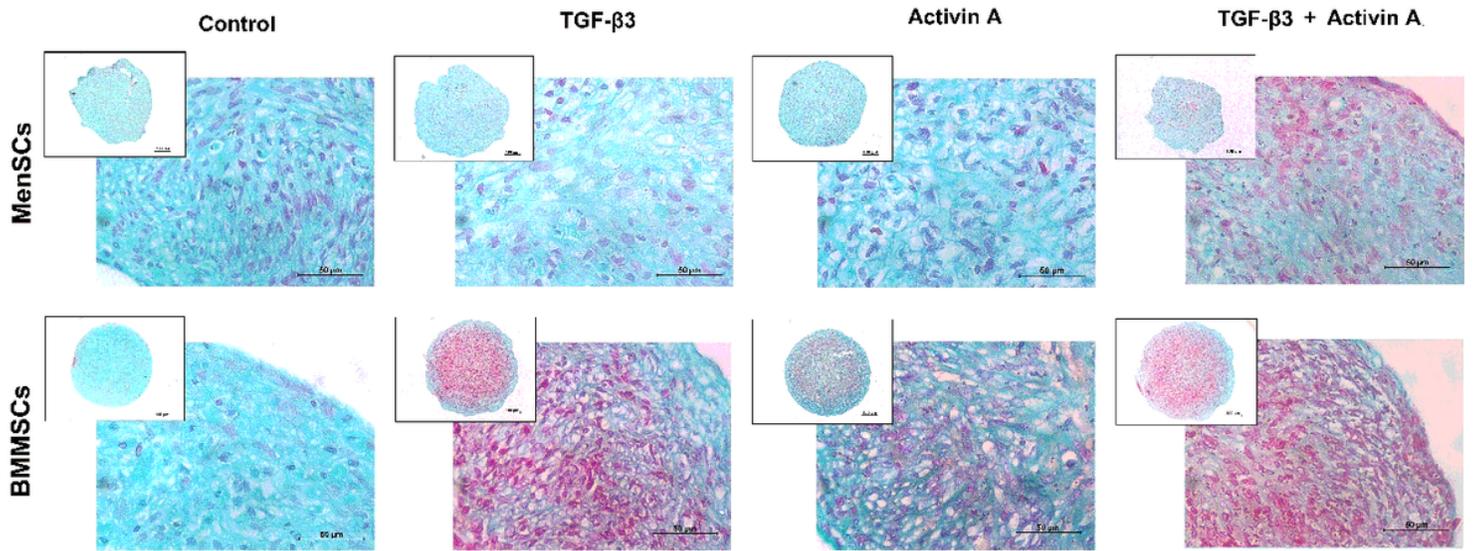


Figure 7

Chondrogenic differentiation of MenSCs and BMMSCs stimulated with activin A (50 ng/mL) and TGF- β 3 (10 ng/mL). Histological analysis of cell pellets (Safranin-O staining after 21 days of chondrogenic induction). Control cells were cultivated in chondrogenic medium without growth factors. Scale bars 100 μ m and 500 μ m.

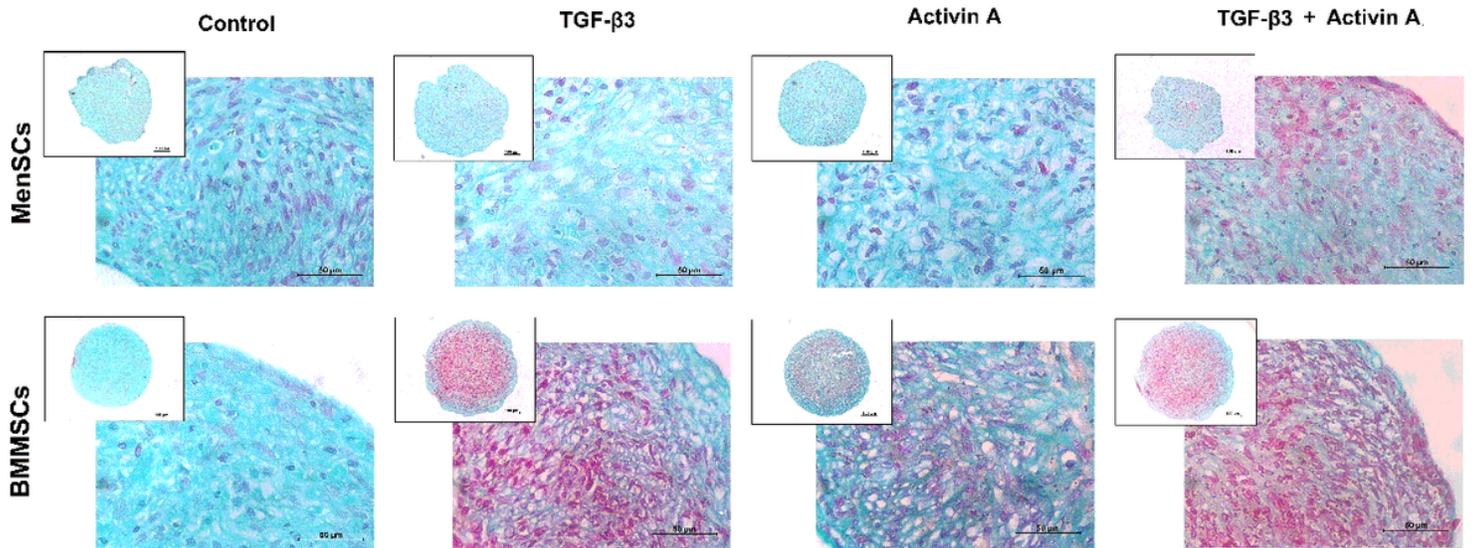


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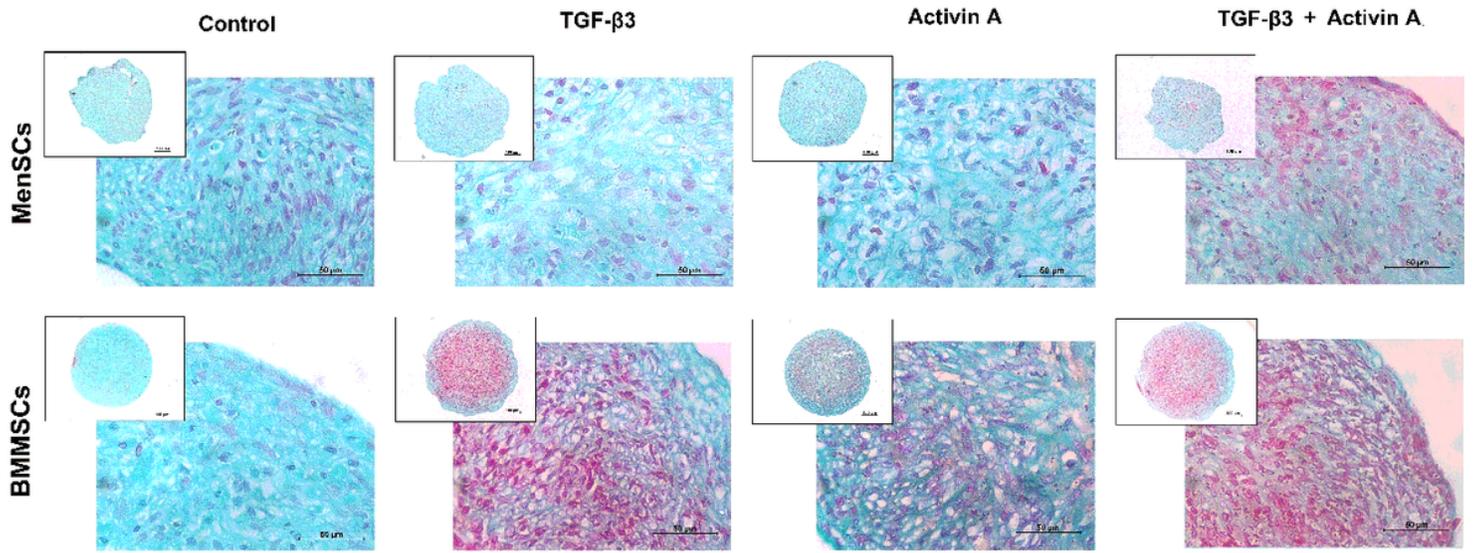


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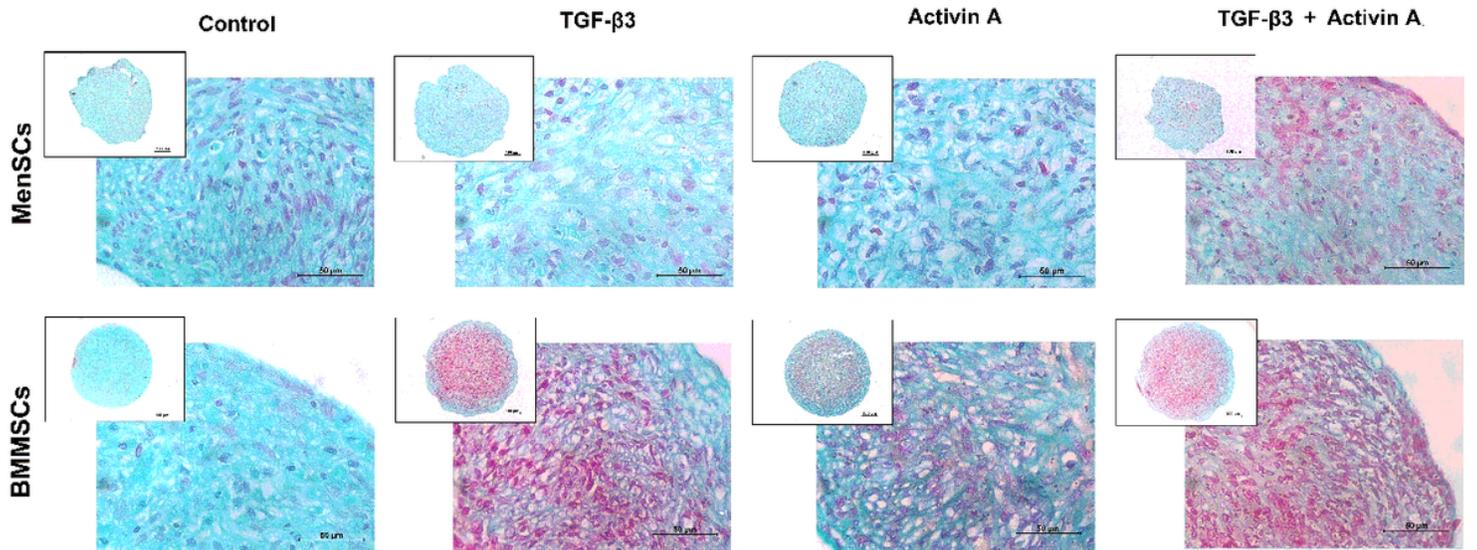


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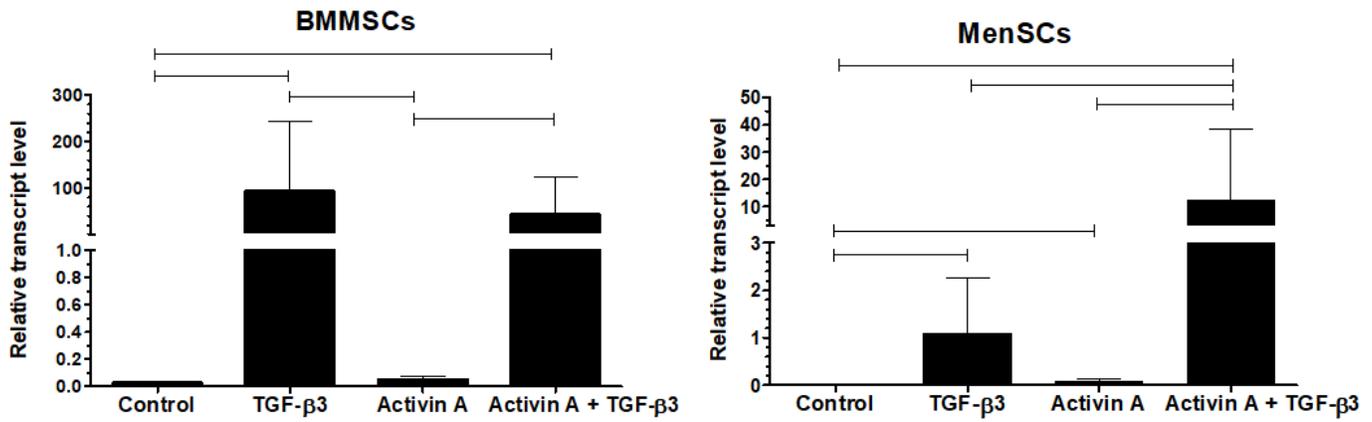


Figure 8

Collagen type 2 (COL2A1) gene expression in MenSCs and BMMSCs after stimulating chondrogenic differentiation for 21 days with activin A (50 ng/mL), TGF-β3 (10 ng/mL) and both TGF-β3 and activin A. Control cells were cultivated in the same chondrogenic medium without growth factors. Relative transcript level after normalization to geometric mean of B2M and RPS9 housekeeping genes. Horizontal bars represent $P \leq 0.05$

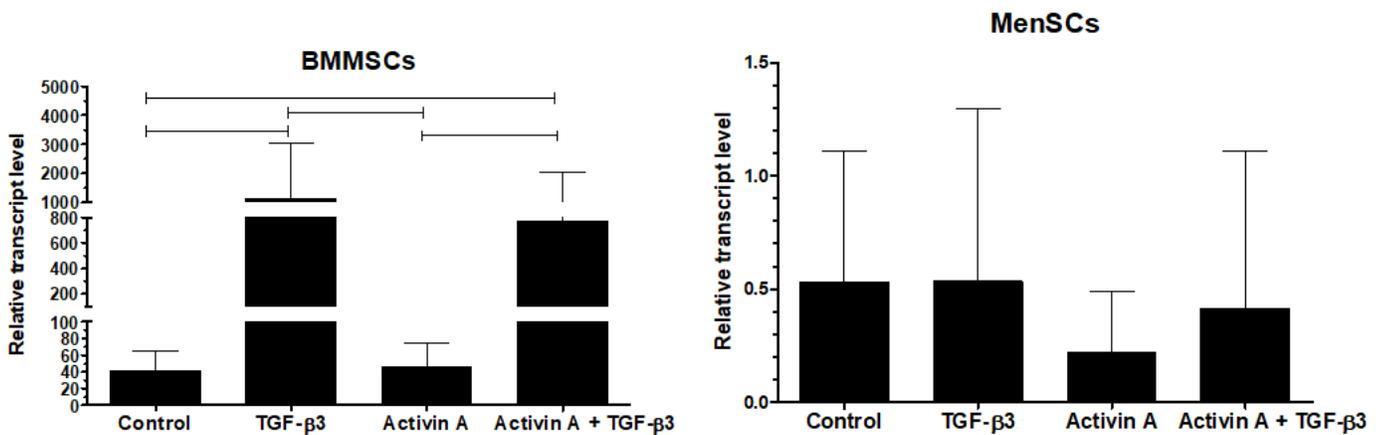


Figure 8

Aggrecan (ACAN) gene expression in MenSCs and BMMSCs after stimulating chondrogenic differentiation for 21 days with activin A (50 ng/mL), TGF-β3 (10 ng/mL) and both TGF-β3 and activin A. Control cells were cultivated in the same chondrogenic medium without growth factors. Relative transcript level after normalization to geometric mean of B2M and RPS9 housekeeping genes. Horizontal bars represent $P \leq 0.05$

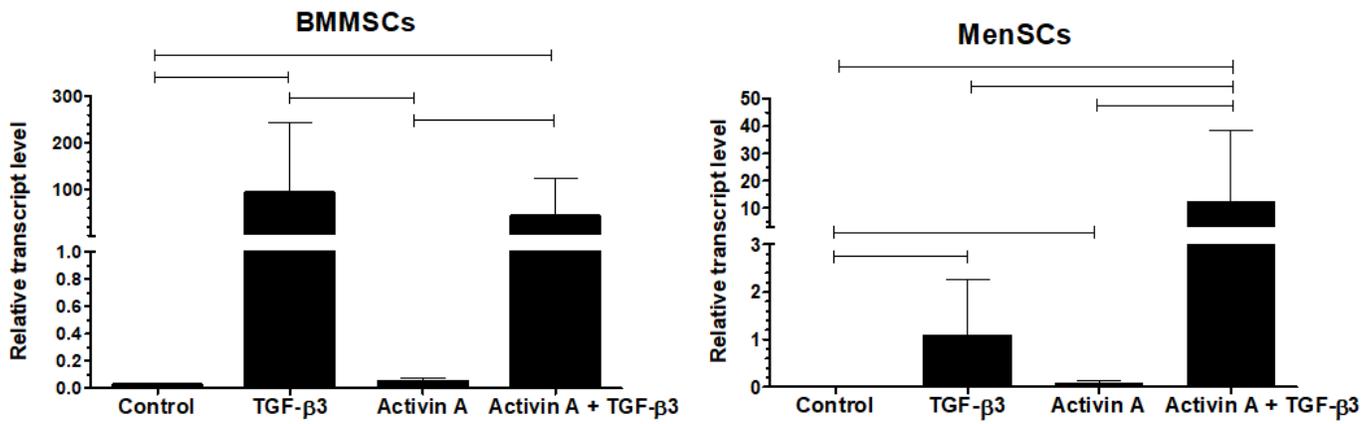


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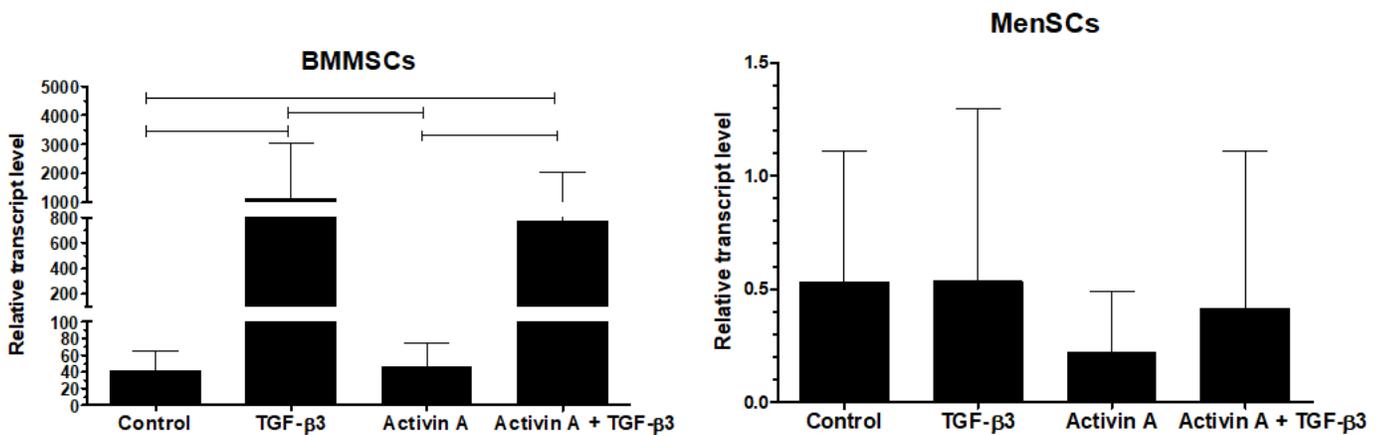


Figure 9

Aggrecan (ACAN) gene expression in MenSCs and BMMSCs after stimulating chondrogenic differentiation for 21 days with activin A (50 ng/mL), TGF-β3 (10 ng/mL) and both TGF-β3 and activin A. Control cells were cultivated in the same chondrogenic medium without growth factors. Relative transcript level after normalization to geometric mean of B2M and RPS9 housekeeping genes. Horizontal bars represent $P \leq 0.05$

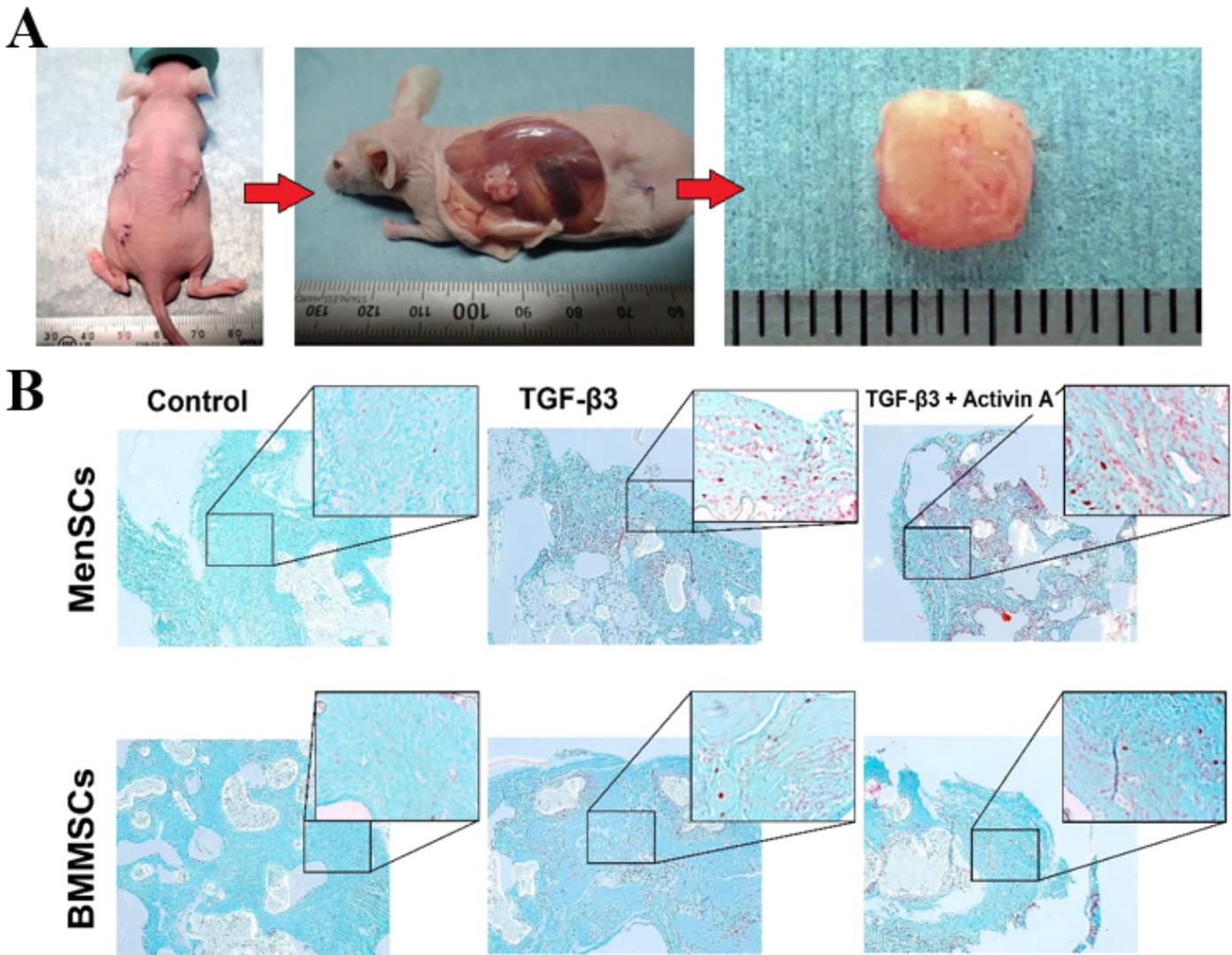


Figure 9

Atelocollagen/PLLA constructs with MenSCs and BMMSCs, stimulated for 2 days with activin A (50 ng/mL), followed by for 21 day with TGF- β 3 (10 ng/mL) and subcutaneous insertion into mice for 9 weeks. A – experimental mice and construct loaded with cells, B – Histological analysis of constructs after 9 weeks in vivo. Control constructs were differentiated under the same conditions, excluding growth factors. Safranin-O staining. 100x and 400x magnification.

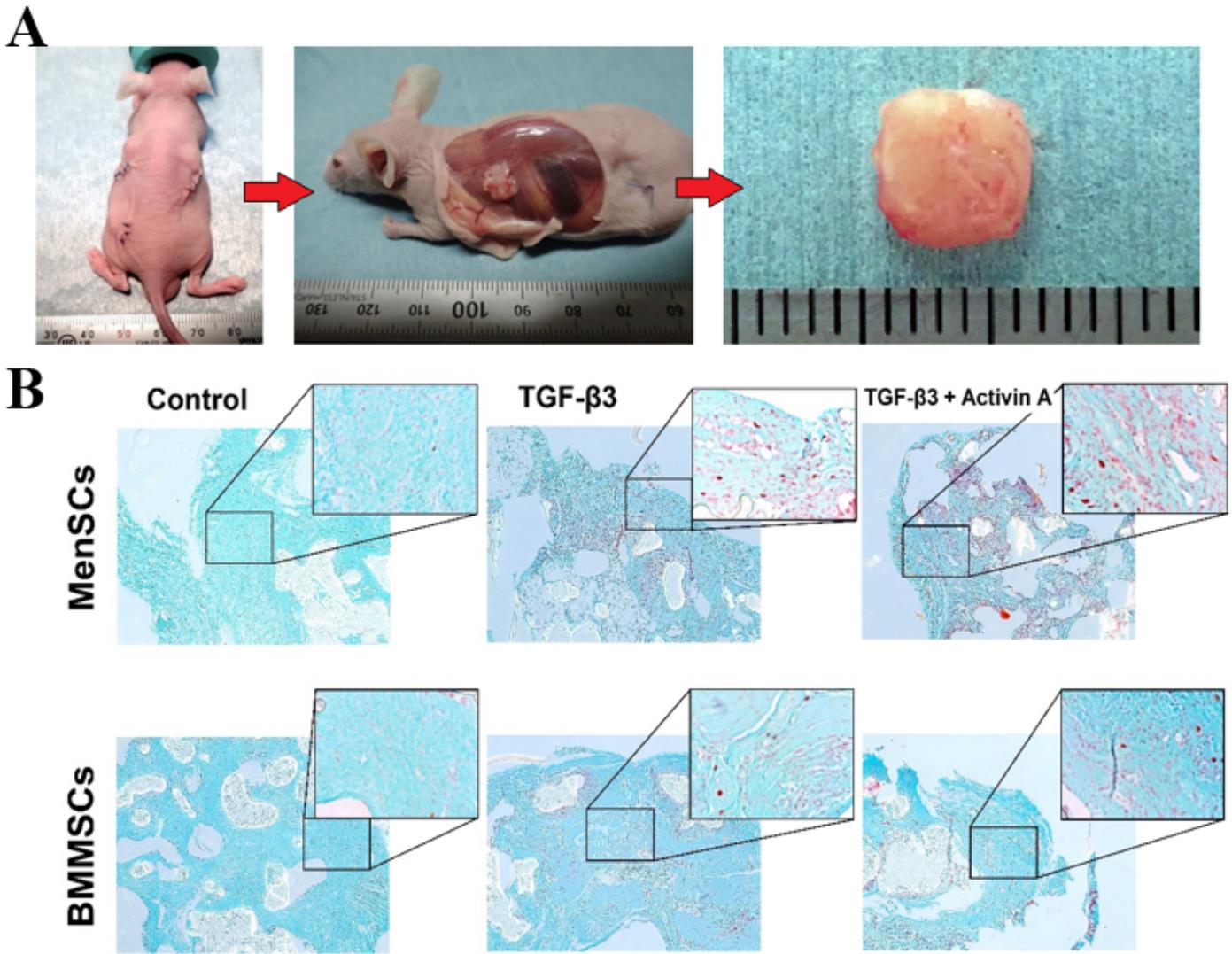


Figure 10

Atelocollagen/PLLA constructs with MenSCs and BMMSCs, stimulated for 2 days with activin A (50 ng/mL), followed by for 21 day with TGF- β 3 (10 ng/mL) and subcutaneous insertion into mice for 9 weeks. A – experimental mice and construct loaded with cells, B – Histological analysis of constructs after 9 weeks in vivo. Control constructs were differentiated under the same conditions, excluding growth factors. Safranin-O staining. 100x and 400x magnification.

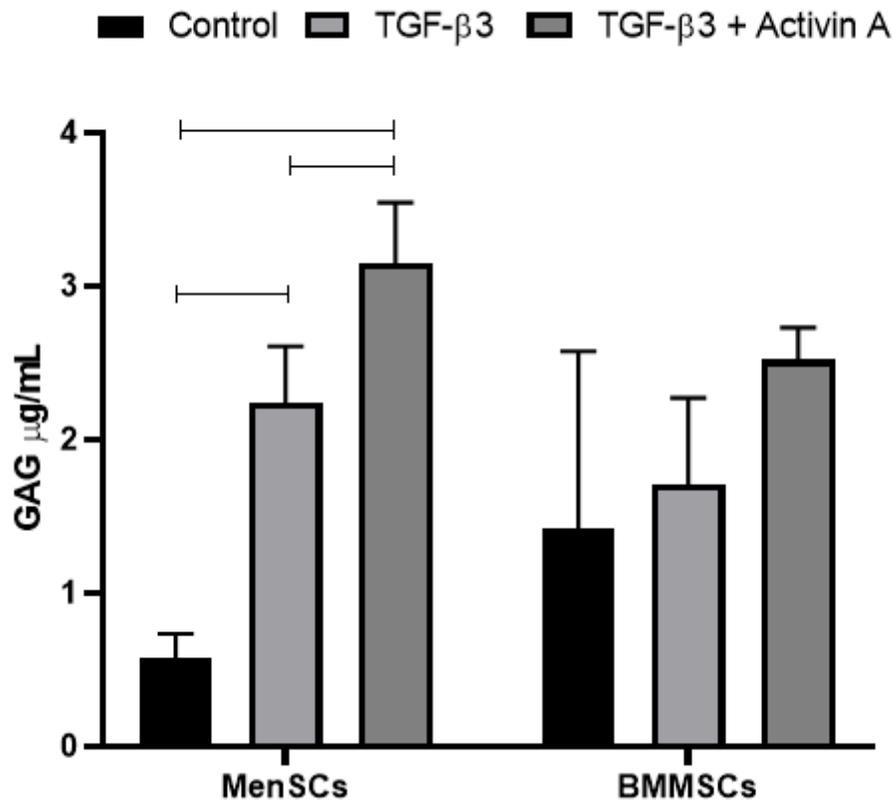


Figure 11

Production of GAGs in atelocollagen/PLLA constructs by MenSCs and BMMSCs, treated for 2 days with activin A (50 ng/mL), followed by 21 day with TGF-β3 (10 ng/mL) and subcutaneous insertion into mice for 9 weeks, spectrophotometrically measured by GAG assay. Control constructs were differentiated under the same conditions, but without growth factors. Horizontal bars represent $P \leq 0.05$

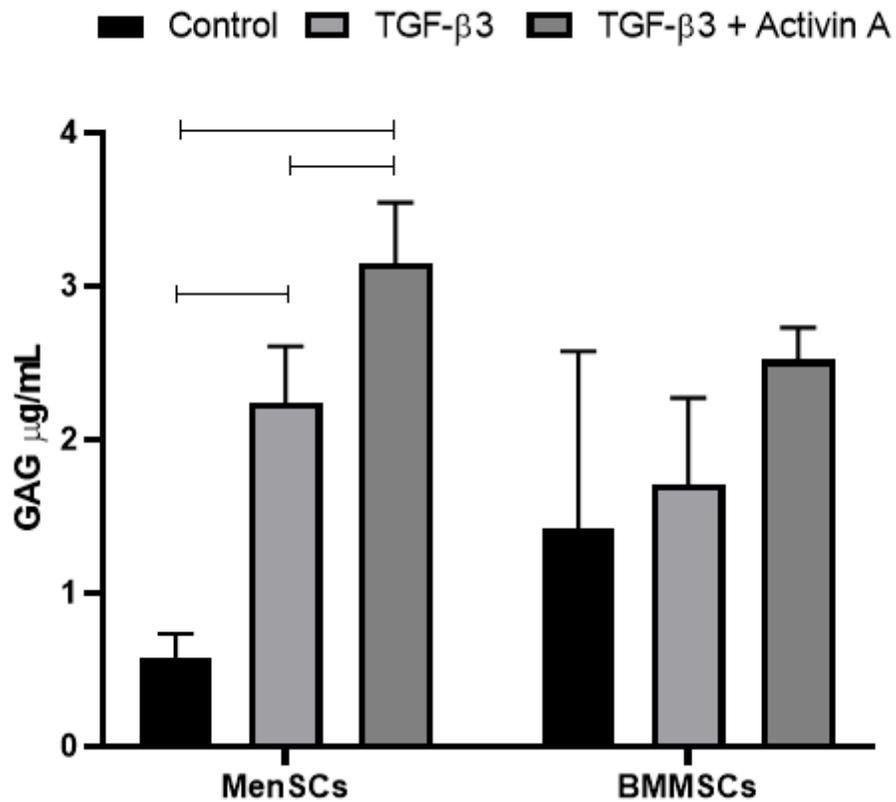


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