**FINAL REPORT**: The complex role of interferons in coordinating anti-viral responses (NN 114423)

## AIMS

The major aim of this project was to identify novel signaling pathways, which have the potential to modulate the outcome of anti-viral and anti-bacterial immune responses. We planned to identify the molecular background of these collaborating or inhibitory mechanisms with the potential to modulate the outcome of anti-microbial responses. Based on our hypothesis the ligand-specific stimulation of pattern recognition receptors (PRR) of dendritic cells (DCs) induce mutual interactions at the level of cytokines and transcription factors. These interactions can induce both parallel and inhibitory effects with the potential to modulate the final outcome of the response. As the expression of some PRRs is restricted to unique cell types, among them myeloid and plasmacytoid DCs, while interferons and cytokines can be produced by many cell types we supposed that the regulation at the level of soluble factors acting mutually on activated cells have an impact on the outcome of the overall response of the cell. Thus we expected that the results of the project will uncover new aspects of immune regulation lead by interferons in concert with other cytokines present in the actual microenvironment of the affected cell.

## RESULTS

### 1. Interferon gamma boosts the nucleotide oligomerization domain 2-mediated signaling pathway in human dendritic cells in an X-linked inhibitor of apoptosis protein and mammalian target of rapamycin-dependent manner

The cytoplasmic nucleotide oligomerization domain 2 (NOD2) receptor recognizes the bacterial cell wall component muramyl dipeptide (MDP). NOD2 ligation initiates the NF-κB and the MAPK cascades. However, administering MDP alone is insufficient to elicit strong cytokine responses in various immune cells, including DCs. Because the simultaneous presence of various microbial products and cytokines in inflamed tissues modulates DC function, we examined how IFNγ, a central modulator of inflammation, affects the NOD2-mediated signaling pathway in human conventional DCs (cDCs). Synergistic stimulation of DCs with MDP and IFNγ increased the expression of CD40, CD80, CD83, CD86, and HLA-DQ proteins as well as significantly elevated the production of pro-inflammatory cytokines IL-1b, IL-6, IL-12, TNF, and anti-inflammatory cytokine IL-10. Furthermore, the simultaneous presence of MDP and IFNγ was necessary to decrease IkBα protein levels. By investigating various mechanisms implicated in MDP and IFNγ-mediated signaling pathways, we revealed that the increased production of pro-inflammatory cytokines is highly dependent on the X-linked inhibitor of apoptosis protein (XIAP) but not on cellular IAP1 and IAP2. We also found that the NOD2 signaling pathway is regulated by the mammalian target of rapamycin (mTOR) but is not affected by phosphatidylinositol-3 kinase or STAT1 inhibition.

Our results demonstrated for the first time that IFNγ boosts NOD2-mediated inflammatory responses in human cDCs. The priming effect of IFNγ on NOD2-induced inflammatory cytokine production requires the expression of XIAP. Moreover, the underlying mechanism of IFNγ-mediated priming is independent of STAT1 and implicates a specific regulation by mTOR. Our finding that the administration of MDP together with IFNγ exerts strong immune stimulatory effects on human cDCs provides a rationale for designing more potent vaccines against microbial pathogens and cancer cells.

*Cell Mol Immunol. 2017 Apr;14(4):380-391.*

## 2. Flagellin increases death receptor-mediated cell death in a RIP1-dependent manner

Efficient adjuvants have the potential to trigger both innate and adaptive immune responses simultaneously. Flagellin is a unique pathogen-derived protein, which is recognized by PRRs as well as by B-cell and T-cell receptors thus providing an important link between innate and adaptive immunity. The aforementioned properties define flagellin as an optimal adjuvant. The induction of immunogenic cell death could be an additional expectation for adjuvants in the context of cancer immunotherapy due to their ability to activate DCs to present tumor antigens through the engulfment of dying cells. The immunostimulatory potential of flagellin in the course of DC and lymphocyte activation is well documented, however the exact mechanism is not fully explored. Based on this limitation we sought to investigate the potential modulatory effects of flagellin on various cell death processes knowing that it plays detrimental roles in regulating the final outcome of various types of immune responses. Here we provide evidence that the pre-treatment of Jurkat T-cells with recombinant flagellin is able to increase the degree of cell death provoked by FasL or TNF-α, and concomitantly increases the cytotoxic potential of phytohemagglutinin activated T-lymphocytes in a TLR5 dependent way. In contrast to these flagellin-mediated effects on the death receptor-induced signaling events, the mitochondrial apoptotic pathway remained unaffected. Furthermore, the cell culture supernatant of wild type *Salmonella enteritidis* bacteria, but not their flagellin deficient variant, was able to enhance the Fas-induced cell death process. To define the molecular mechanisms of flagellin-mediated elevated levels of cell death we were able to detect the upregulation of RIP1-dependent signaling events.

The immunological nature of the various cell death processes can either be tolerogenic, inflammatory or immunogenic. Thus, the various cell death processes have the potential to fundamentally impact the development of degenerative disorders, autoimmune processes, inflammatory diseases and tumors. The results obtained in this study indicate that flagellin not only activates DCs by acting as a PAMP, but at the same time may also direct various cell death pathways towards the RIP-dependent cell death pathway, which may result in enhanced DC-mediated cross-presentation. This unique capability of flagellin designates it as a potent adjuvant, which can be readily harnessed for different therapeutic settings, preferentially in tumor immunotherapies.

*Immunol Lett. 2018 Jan;193:42-50. doi: 10.1016/j.imlet.2017.11.007.*

## 3. The effects of Lactobacterial metabolites on the antiviral functions of mesenchymal stromal cells

Influenza- and Rotaviruses are one of the most common viral disorders with heavy symptoms and serious long-term complications. It is well known that metabolites derived from lactic acid bacteria (LAB) play a role in the protection against flu and rotaviral infections. Mesenchymal stem cells (MSC) localized in every tissue sites of the human body such as the gut and influence directly and indirectly the cellular responses helping the maintenance of the homeostasis in normal conditions. In the case of viral infections MSCs are able to sense the invading viruses and shape the antiviral immune responses. In our *in vitro* system, we pretreated MSC-like (MSCl) cells with the supernatant of *Lactobacillus casei* BL23 and *Lactobacillus reuteri* ATCC PTA-6475 for 24 hours then treated the cells with poly I:C and 3p-hpRNA, respectively. The cells were analyzed phenotypically by flow cytometry as well as we determined the

concentrations of the secreted cytokines and chemokines. In addition, the conditioned media of the MSCl cells were used to culture peripheral blood lymphocytes to analyze the indirect, Tc and Th1 stimulating capacity of the MSCl cells. We showed that the lactobacterial mediators inhibited the CXCL10 and IL-6 production of MSCl cells. Bacterial metabolites could change the expression levels of HLA-ABC, CD86 and certain adhesion molecules on MSCl cell surface. The conditioned media of MSCl cells treated with bacterial supernatants stimulated less amount of IFNγ-producing Th1 and Tc cells.

Collectively, our results show limited antiviral responses mediated by MSCl cells in the presence of LAB components.

*48th Symposium of Hungarian Society for Immunology; 16-18 October 2019, Bükfürdő, Hungary (Poster presentation), Immunológiai Szemle 2019. XI (3) p 38.*

**4. Vessel wall-derived mesenchymal stem cells share similar differentiation potential and immunomodulatory properties with bone marrow-derived stem cells**

This study aimed to investigate the phenotype, differentiation potential, immunomodulatory properties, and responsiveness of vessel wall-derived mesenchymal stem cells (SV-MSCs) to various TLR ligands and pro-inflammatory cytokines, as well as to compare their features to those of their bone marrow-derived counterparts. SV-MSCs were isolated by enzymatic digestion of the saphenous vein vessel wall. Phenotype analysis was carried out by flow cytometry and microscopy, whereas adipogenic, chondrogenic and osteogenic differentiation potentials were tested in in vitro assays. For comparative analysis expression of different stemness, proliferation and differentiation-related genes was determined by Affymetrix gene array. To test the immunomodulatory properties of SV-MSCs, mixed lymphocyte reaction was applied. To investigate their responses to various activating stimuli, SV-MSCs were treated with TLR ligands (LPS, PolyI:C) or proinflammatory cytokines (TNFα, IL-1β, IFNγ), and expression of various early innate immune response-related genes was assessed by qPCR, while secretion of selected cytokines and chemokines was measured by ELISA. The isolated SV-MSCs were able to differentiate into bone, fat and cartilage tissue in vitro. SV-MSCs expressed the most important MSC markers (CD29, CD44, CD73, CD90 and CD105) shared almost identical phenotypic characteristics with bone marrow-derived MSCs (BM-MSCs). Their gene expression pattern and activation pathways were close to those of BMMSCs. SV-MSCs showed immunosuppressive activity inhibiting T lymphocyte proliferation in vitro. Cellular responses to treatments with TLR ligands or pro-inflammatory cytokines, mimicking inflammatory conditions, were comparable in the bone marrow- and saphenous vein-derived MSCs. Namely, similar to BM-MSCs, SV-MSCs secreted increased amount of IL-6 and IL-8 after 12- or 24-hour treatment with LPS, PolyI:C, TNFα or IL-1β, compared to untreated controls. Interestingly, a different CXCL-10 secretion pattern could be observed under inflammatory conditions in the two types of MSCs.

Based on our results, mesenchymal cells isolated from saphenous vein vessel wall fulfilled the ISCT (International Society for Cellular Therapy) criteria, and no significant differences in the phenotype, gene expression pattern and immunomodulatory properties between SV-MSCs and BM-MSCs could be observed. We suppose that SV-MSCs may be useful for regenerative therapeutic applications or tissue engineering purposes.

*Stem Cells International (submitted, Ms ID 8847038)*

# Vessel wall-derived mesenchymal stem cells share similar differentiation potential and immunomodulatory properties with bone marrow-derived stem cells

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## Abstract

**Purpose** This study aimed to investigate the phenotype, differentiation potential, immunomodulatory properties, and responsiveness of vessel wall-derived mesenchymal stem cells (SV-MSCs) to various TLR ligands and pro-inflammatory cytokines, as well as to compare their features to those of their bone marrow-derived counterparts.

**Methods** SV-MSCs were isolated by enzymatic digestion of the saphenous vein vessel wall. Phenotype analysis was carried out by flow cytometry and microscopy, whereas adipogenic, chondrogenic and osteogenic differentiation potentials were tested in *in vitro* assays. For comparative analysis expression of different stemness, proliferation and differentiation-related genes was determined by Affymetrix gene array. To test the immunomodulatory properties of SV-MSCs, mixed lymphocyte reaction was applied. To investigate their responses to various activating stimuli, SV-MSCs were treated with TLR ligands (LPS, PolyI:C) or pro-inflammatory cytokines (TNFα, IL-1β, IFNγ), and expression of various early innate immune response-related genes was assessed by qPCR, while secretion of selected cytokines and chemokines was measured by ELISA.

**Results** The isolated SV-MSCs were able to differentiate into bone, fat and cartilage tissue *in vitro*. SV-MSCs expressed the most important MSC markers (CD29, CD44, CD73, CD90 and CD105) shared almost identical phenotypic characteristics with bone marrow-derived MSCs (BM-MSCs). Their gene expression pattern and activation pathways were close to those of BM-MSCs. SV-MSCs showed immunosuppressive activity inhibiting T lymphocyte proliferation *in vitro.* Cellular responses to treatments with TLR ligands or pro-inflammatory cytokines, mimicking inflammatory conditions, were comparable in the bone marrow- and saphenous vein-derived MSCs. Namely, similar to BM-MSCs, SV-MSCs secreted increased amount of IL-6 and IL-8 after 12- or 24-hour treatment with LPS, PolyI:C, TNFα or IL-1β, compared to untreated controls. Interestingly, a different CXCL-10 secretion pattern could be observed under inflammatory conditions in the two types of MSCs.

**Conclusion** Based on our results, mesenchymal cells isolated from saphenous vein vessel wall fulfilled the ISCT (International Society for Cellular Therapy) criteria, and no significant differences in the phenotype, gene expression pattern and immunomodulatory properties between SV-MSCs and BM-MSCs could be observed. We suppose that SV-MSCs may be useful for regenerative therapeutic applications or tissue engineering purposes.

**Keywords:** vessel wall-derived stem cells, mesenchymal stem cells, gene array, Toll-like receptors, pro-inflammatory cytokines

## Introduction

Regeneration of blood vessels is essential for the homeostasis of vasculature as well as in the restoration of various forms of tissue injury. However, during inflammation or trauma, the endothelial layer of the vessels has limited regeneration potential. Furthermore, in many cases the endothelium itself is responsible for maintaining the inflammation, which could lead to vessel malfunctions and tissue damage [1]. The remodeling of the vasculature is an intricately controlled collaboration among stem/progenitor cells, immune cells and the residual cells of the vessel wall as well [2, 3]. This process is balanced by pro-angiogenic and anti-angiogenic factors secreted by the cells mentioned above, and the vessels could be regenerated by circulating stem cells, endothelial progenitor cells and vessel wall- or endothelium-related progenitor cells in the subendothelial tissue [2-4]. Various cell types and rare cell populations have the properties to differentiate to endothelial cells or to support the vasculogenic processes [5]; however, resident vascular stem/progenitor cells are thought to be a dominant sub-class of the vascular wall cell population involved in vascular homeostasis, repair and pathological processes [3, 6-13]. Mesenchymal stem cells (MSCs) were first described as stromal cells of the bone marrow [14, 15], but in the last decades they were also identified in many other organs and tissues, especially within the perivascular area of large vessels [16-18]. MSCs play a key role in the maintenance of tissue integrity and homeostasis due to their robust differentiation potential into another cell types and their immunomodulatory capacity as well. However, responses of MSCs to microbial stimuli, such as TLR ligands, or to pro-inflammatory cytokines are controversial topics, of which the details are yet to be elucidated.

In this study we made an extended comparison of vessel wall-derived mesenchymal stem cells (SV-MSCs) and bone marrow-derived mesenchymal stem cells (BM-MSCs) regarding their phenotype, differentiation potential, immunomodulatory properties and responsiveness to various activating stimuli.

## Materials and methods

***Bone marrow, saphenous vein and umbilical cord samples.***

Collection of bone marrow, umbilical cord and saphenous vein samples complied with the directive of the Helsinki Declaration were approved by the institutional ethical review board (Medical Research Council) of the Medical and Health Science Center of the University of Debrecen (Ethical protocol numbers: UD MHSC REC/IEC No. 2754-2008, OSTRAT/1210-1/2008/OSTR). Tissue Samples were collected corresponding to the EU Member States' Directive 2004/23/EC on tissue isolation [19].

For the isolation of BM-MSCs, approximately 10 ml of bone marrow aspirate was observed from the donors, which were diluted with saline in 1:3 ratio. The mononuclear cells were recovered by Ficoll Histopaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. The number of live cells was determined by Trypan blue exclusion assay. Bone marrow nucleated cells were plated in 25-$cm^2$ flasks at a density of $2x10^5$ living cells/$cm^2$ and cultured in DMEM-LG medium (DMEM with 1g/L glucose, Gibco/Invitrogen, London, UK), supplemented with 10 % FSC and 1% Antibiotic-Antimycotic Solution (PAA Laboratories GmbH, Pasching, Austria). After 3 - 4 days, the nonadherent cells were removed and the cultures were reefed with fresh medium. Thereafter, the cultures were fed every 3 – 4 days. When cells reached confluence, they were passaged (P1) after 0.025% trypsin-EDTA (both Sigma-Aldrich, Budapest, Hungary) application and replated into new 25-$cm^2$ flasks. For positive BM-MSC control MSCs from bone marrow were purchased from PromoCell (Heidelberg, Germany) and cultured under the same conditions. At passage P5, the cells were tested for antigen expression by flow cytometry, *in vitro* differentiation assays and mycoplasma (Lonza, Basel, Switzerland). Cells positive for Mycoplasma were excluded from the experiments.

Saphenous vein samples were collected from saphenectomies. The samples were collected and transported in ice cold PBS and processed within 4 hours. The vein was cleaned of adipose or connective tissue then cut into small pieces. The segments were washed in PBS, then enzymatically digested by 0.2 mg/mL collagenase type XI (Sigma-Aldrich) dissolved in DMEM-LG medium for 60 minutes at 37 °C. Cells were centrifuged at 1000 rpm for 20 minutes and washed by DMEM-LG medium. After two washing steps, cells were plated and cultured as described for BM-MSC.

The isolation and *in vitro* culture of human umbilical vein endothelial cells (HUVECs) were described elsewhere [20]. Briefly, HUVEC was removed from umbilical cord with 1%

collagenase type XI (Sigma-Aldrich) digestion, and cultured in M199 medium (Sigma-Aldrich) supplemented with 20% FCS (Gibco, London, UK), 1% Antibiotic-Antimycotic Solution (PAA) and 1% L-glutamine (Gibco), in $CO_2$ incubator at 37 °C. After 4-5 days of culturing, when cells reached confluence, they were trypsinized and inoculated into new culture dishes. After 3 passages, the cell monolayers, which reached up to 70%-80% confluence, were used for the experiments.

***Flow cytometry and immunochemistry***

A multiparameter analysis of the surface antigen expression of different MSCs an HUVECs was performed by three-color flow cytometry using different fluorochrome-conjugated antibodies: CD34, CD44, CD45, CD49f, CD73, CD106, CD144, CD147 (All from BD Biosciences, San Jose, CA, USA); CD49a (Biolegend, San Diego, CA, USA), CD14, CD29, CD31, CD36, CD47 CD49b, CD54, CD56, CD69, CD90, CD104, CD105, CD117, CD146, CD166, CXCR4, HLA-DR, PDGFRb, VEGFR2 (All from R&D Systems, Minneapolis, MN, USA) and CD133 (Miltenyi Biotech, Gladbach, Germany). After harvesting the cells with 0.025% trypsin-EDTA, cells were washed with normal medium, then twice with FACS buffer. Cells were incubated with antibodies according the manufacturers' protocol on ice for 30 min than washed again with FACS buffer and fixed in 1% paraformaldehyde (PFA)/PBS and analyzed within 1 day. Samples were measured by FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). Results were expressed as means of positive cells (%) $\pm$SEM. For immunohistochemistry studies, cell cultures were fixed in 4% PFA, then samples were labeled after washing the cells three times in PBS with primary antibodies against iNOS (Calbiochem/Merck, Merck Millipore, Darmstadt, Germany), von Willebrand factor (R&D Systems) and vimentin (Abcam, Cambridge, UK). Cell stainings were visualized with NorthernLights fluorochrome conjugated secondary antibodies (R&D Systems). Actin filaments were stained with phalloidin-TRITC (Sigma-Aldrich). Nuclei were labeled with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) samples mounted with mounting medium -containing Mowiol (Merck) and glycerol in PBS and examined under an Olympus IX81 microscope equipped with a Hamamatsu Orca2 camera.

***In vitro differentiation assays***

Adipogenic, chondrogenic and osteogenic differentiations of MSC were performed by using Gibco's StemPro® Adipogenesis, Osteogenesis and Chondrogenesis Differentiation Kits (Gibco). All differentiations were evaluated as per the manufacture's guide.

***RNA isolation, cDNA synthesis, QPCR and Microarray data analysis***

Total RNA was isolated by TRIzol reagent (Invitrogen). 1.5-2 μg of total RNA were reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)15 primers (Promega, Madison, WI, USA). Gene-specific TaqMan assays (Applied Biosystems) were used to perform QPCR in a final volume of 25 μl in triplicates using AmpliTaq DNA polymerase and ABI Prism 7900HT real-time PCR instrument (Applied Biosystems). Amplification of 36B4 and/or cyclophylin was used as normalizing controls. Cycle threshold values (Ct) were determined using the SDS 2.1 software (Applied Biosystems). Constant threshold values were set for each gene throughout the study. The sequence of the primers and probes are available upon request.

To compare the gene expression profiles of different MSCs Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) were used as described previously [21]. Based on the literature, stem cells related genes were selected and statistical analysis was performed (Oneway ANOVA with Tukey post hoc test and Benjamini-Hochberg FDR) to calculate p value and fold change. To identify the relationships between the selected genes, the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) was used. Excel datasheets containing gene IDs with the assigned gene expression values were uploaded into the program. The Ingenuity Pathways Knowledge Base (IPKB) provided all known functions and interactions which were published in the literature. For the representation of the relationships between the genes, the 'Pathway Designer' tool of the IPA software was used.

***Mixed lymphocyte reaction and mitogen-induced cell proliferation***

Peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll gradient centrifugation (Amersham Biosciences). Mitogen-activated T lymphocyte proliferation was induced by concanavalin A (ConA) or phytohemagglutinin (PHA, all from Sigma-Aldrich) used at a final concentration of 10 μg/mL and 1 μg/mL, respectively, added to $1x10^6$ PBMCs. SV-MSCs were added to $1x10^6$ allogenic PBMCs at $10^4$ and $10^5$ cell numbers and co-cultured for 3 days. On day three, proliferation was detected by a BrDU colorimetric assay directly in the cell culture plate according to the manufacturer's instructions (Roche, Budapest, Hungary).

***In vitro activation of MSC***

To investigate the role of TLR ligands and pro-inflammatory cytokines in MSCs, cells were plated to 24-well plates at $5x10^4$ cell density, then incubated with 1 μg/ml LPS (Sigma-Aldrich), 25 μg/ml PolyI:C (InvivoGen, San Diego, CA, USA), 100 ng/ml TNFα, 10 ng/ml IFNγ and 10 ng/ml IL-1β (all from Preprotech, Rocky Hill, NJ, USA). After the incubation, the supernatant was harvested and kept on -20°C until measurement. For qPCR measurements, cells were plated to 25-$cm^2$ flasks and treated as mentioned above.

***Measurement of cytokine secretion***

Concentrations of secreted IL-6 cytokine as well as IL-8 and CXCL-10/IP-10 chemokines were measured using OptEIA kits (BD Biosciences) according to the manufacturer's protocol. Cell culture media were used as blank samples.

***Statistical analysis***

Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analyses. Normality of distribution of data was assessed by Kolmogorov-Smirnov and Lilliefors tests. Non-normally distributed parameters were transformed logarithmically to correct their skewed distributions. R software was used for hierarchical clustering. Each experiment was performed at least three times and each sample was tested in triplicate. Data are expressed as mean$\pm$SD or SEM. Statistically significant difference was determined with two-way ANOVA analysis when there were more than two groups, for analysis between two groups paired student-t test was used. Significance level was set to 0.95, p values less than 0.05 (*$p<0.05$, ** $p<0.01$, *** $p<0.001$) were considered significant.

## RESULTS

### *Morphology, differentiation potential and phenotype of SV-MSCs*

MSCs isolated from saphenous vein showed similar morphology to bone marrow-derived MSCs (**Figure 1A**). The cultured cells never formed a cobblestone pattern and their size was much larger than that of endothelial cells (HUVECs), which were used as vein endothelial cell controls in our experiments (**Figure 1A**). After 2-3 passages on adherent surface the cells achieved uniform, fibroblast-like morphology, and these cells could be propagated at least for 15 passages without further morphological changes. An MSC type cell should fit the criteria defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) regarding differentiation potential and expression of cell surface markers [22]. In the following experiments, it was examined whether the SV-MSC cultures could be differentiated toward canonical mesodermal (adipogenic, osteogenic, chondrogenic) directions. Bone marrow-derived MSCs and SV-MSCs were differentiated *in vitro* using adipogenic, osteogenic and chondrogenic induction media. Following three weeks of adipogenic differentiation induction, a large number of the SV-MSCs and BM-MSCs showed oil red positive staining, a characteristic for the adipocyte phenotype (**Figure 1B**). In parallel cell cultures, dense calcium deposits were detected after osteogenic differentiation (**Figure 1B**). In sections made from chondrogenic mass culture after 3 weeks of differentiation, metachromasy was observed upon toluidine-blue staining (**Figure 1B**). Both BM-MSC and SV-MSC cultures were positive for vimentin and iNOS; however, none of the cultures showed von Willebrand factor positivity, indicating the absence of endothelial cell contamination (**Figure 1C**).

For a detailed characterization, we compared the expression of cell surface markers on BM-MSCs and SV-MSCs by flow cytometry. As documented in **Table 1**, within the hematopoietic markers no expression of CD34, CD45, CD69, CD133, and the CXCL12 receptor CXCR4 could be detected in the mesenchymal stem cell cultures. A very small percentage of SV-MSCs was positive for CD117/c-kit ($0.02\pm0.02\%$), while none of the BM-MSCs expressed this marker. Neither BM-MSCs nor SV-MSCs expressed HLA-DR antigen-presenting molecule. Due to the possibility of endothelial contamination in SV-SMC cultures, we also investigated the expression of endothelial specific markers. CD31/PECAM, which makes up a large portion of endothelial cells, was absent both in the bone marrow- and saphenous vein-derived MSC cultures. VEGFR2/KDR expression was very low in HUVEC cultures, and was totally absent in MSC cultures. Expression of CD104/integrin β4 was more

typical for the endothelial cells; however, it was also expressed on MSCs. The percentage of CD144/VE-Cadherin positive cells in SV-MSC cultures was in between those of endothelial cells and BM-MSCs (**Table 1**). Any cell that is described as mesenchymal stem cell must fit the criteria defined by ISCT. All the expected markers such as CD73 (ecto-5'-nucleotidase**)**, CD90 (Thy-1) and CD105 (endoglin) could be detected both on BM-MSCs and SV-MSCs. Although CD73 and CD105 were also expressed by endothelial cells, the ratio of CD90 expressing cells was low in the HUVEC cultures (2.86±1.55%), which distanced them from mesenchymal stem cell identity. No statistically significant differences were found in CD147 (Neurothelin) and PDGFRβ expression among the three cell types. None of the ISCT defined markers are exclusively MSC specific; therefore, we further investigated the expression of integrins and other cell adhesion molecules (CAMs), which determine the attachment and the fate of the cells within the tissues. Only the percentage of the melanoma cell adhesion molecule (CD146/MCAM) positive cells was found to be significantly different in bone marrow- (77.54±5.14%) and saphenous vein-derived MSC cultures (7.09±6.56%). Beside CD146 (MCAM), expression of CD54/intercellular adhesion molecule 1 (ICAM-1), CD166/activated leukocyte cell adhesion molecule (ALCAM), CD56/neural cell adhesion molecule (NCAM) and CD44/homing-associated cell adhesion molecule (H-CAM) could be detected in the cell cultures; however, no significant differences were found in their expression. The expression of CD29/integrin (Itg) β1 and CD49a/Itg α1 was similar in BM-MSC, SV-MSC and HUVEC cultures. In contrast, CD49b/Itg α2 was expressed at a lower level on the surface of vessel wall-derived MSCs, and the difference was found to be significant compared to HUVECs ($p=0.0186$), but it was not significant compared to BM-MSCs. The CD49f/Itg α6 is mostly expressed by smooth muscle stem cells, fibroblasts and epithelial cells. MSCs isolated from either bone marrow or saphenous vein vessel wall did not show CD49f positivity (**Table 1**).

Using a cluster analysis on the expression of the above surface markers in the three cell types, we found a clear division of the endothelial cells from the mesenchymal stem cells (**Figure 2**). Results on SV-MSCs from different donors integrated well into the BM-MSC cluster despite inter-donor variability. These observations indicate that our isolation technique with the applied phenotype analysis is suitable to detect mesenchymal stem cells isolated from vessel wall (**Figure 2**).

***Gene expression analysis***

Next, the gene expression profiles of BM-MSCs and SV-MSCs were compared using microarray analyses. Genes related to differentiation and lineage (489 genes), stemness (422

genes), HOX (homeobox), SOCS (suppressor of cytokine signaling) and Notch signaling (380 genes) and cell cycle, oncogenes (242 genes) were collected into functional groups and analyzed. The hierarchical clustering clearly divided the two MSCs groups with different origin in the case of differentiation and lineage, stemness and HOX, SOCS, and Notch signaling custom groups (**Figure 3**). The gene expression profile of SV-MSCs in the cell cycle and oncogenes custom group was not significantly different from that of their BM-MSC counterpart (**Figure 3**); however, several genes related to this biological function group were differentially expressed in MSCs with different origin (**Table 2**). In SV-MSCs expression level of *S100A4* (S100 calcium binding protein A4) was significantly higher (2.8-fold change), whereas that of *SMAD3* (SMAD family member 3) and *CDK6* (cyclin-dependent kinase 6) was significantly lower (-2.6- and -2.2-fold change, respectively) than in BM-MSCs (**Table 2**). Significantly upregulated (≥ 2-fold) genes related to differentiation in SV-MSCs were found to be *PODXL* (podocalyxin-like), *CTSK* (cathepsin K) and *CSF1* (colony stimulating factor 1/macrophage), while *VCAM1* (vascular cell adhesion molecule 1), *ACAN* (aggrecan), *EGR2* (early growth response 2), *TGFB2* (transforming growth factor beta 2), *IGF2* (insulin-like growth factor 2), *BMP2* (bone morphogenetic protein 2), *BDNF* (brain-derived neurotrophic factor), *JAG1* (jagged 1), *INHBA* (inhibin, beta A), *ITGA3* (integrin, alpha 3), *SMAD3*, *HES1* (hairy and enhancer of split 1), *EFNB2* (ephrin-B2), *PTN* (pleiotrophin) and *PDGFA* (platelet-derived growth factor alpha) genes were significantly downregulated (≤ -2-fold). An SV-MSC-specific pattern of stemness could be characterized with high expression of *FGF9* (fibroblast growth factor 9 or glia-activating factor), *ZFPM2* (zinc finger protein, multitype 2), *MME* (membrane metallo-endopeptidase) and *FZD4* (frizzled homolog 4) genes, together with low expression of *LIF* (leukemia inhibitory factor or cholinergic differentiation factor), *MGC20647* (hypothetical protein MGC20647), *CXCL12* (chemokine C-X-C motif ligand 12 or stromal cell-derived factor 1), *MCAM* (melanoma cell adhesion molecule), *ACAN*, *LTBP1* (latent transforming growth factor beta binding protein 1), *BMP2*, *SMAD3*, *ALCAM*, *ITGAV* (integrin, alpha V or vitronectin receptor), *GDF6* (growth differentiation factor 6) and *FGF7* (fibroblast growth factor 7) genes (**Table 2**). In the HOX, SOCS, and Notch signaling superfamily *FGF9*, *IL33* (interleukin 33) and *HOXA11* (homeobox A11) genes were determined as significantly upregulated (≥ 2-fold) ones in SV-MSCs (**Table 2**). Focusing on the expression of MSC-related genes, our microarray data were validated by a qPCR based gene array as well (**Supplementary Figure 1**).

***Immunomodulatory properties of SV-MSCs***

The immunosuppressive properties of MSCs have been extensively studied in the past years, for their promising clinical application potential. In the present study, mitogenic mixed lymphocyte reaction (MLR) was used to test the immunosuppressive properties of SV-MSCs. Human PBMCs from healthy donors were applied as responder cells, and ConA or PHA as mitogenic activators. As expected, PBMCs proliferated in response to ConA or PHA treatment. The addition of SC-MSCs to PBMCs stimulated with either ConA or PHA resulted in a marked reduction of BrDU incorporation (**Figure 4**). However, a statistically significant suppression of the mixed leukocyte reaction by SV-MSCs could be detected only in cell cultures activated with PHA (**Figure 4**).

***Activation of MSCs of different origin***

Although the immunosuppressive function of MSCs is well described, much fewer details are available about their response to pro-inflammatory cytokine exposure or TLR ligand activation, especially in case of vessel wall-derived MSCs. Therefore, in the next series of our experiments, MSCs of different origin were treated with LPS, PolyI:C, TNFα, IL-1β or IFNγ for 12 and 24 hours and expression of various early innate immune response-related genes was investigated. As shown in **Figure 5**, mRNA expression level of a dsRNA sensor *RIG-I* (retinoic acid-inducible gene I) was increased after 12 and 24 hours upon PolyI:C and IFNγ treatment both in BM-MSCs and SV-MSCs. Activation with PolyI:C also induced a marked rise in expression of *MDA5*, another dsRNA sensor of the RIG-I-like receptor family [23]. Induction of both *RIG-I* and *MDA5* gene expression was more robust in BM-MSCs. The expression of *IL-6* gene was increased upon LPS, PolyI:C and TNFα treatments at both time points in both MSC types. *IFNβ* expression was markedly upregulated in the case of PolyI:C activation following 12 and 24-hour treatments in both MSCs of different origin. A robust increase in the gene expression level of CXCL10/IP-10 (interferon gamma-induced protein 10) was observed when MSCs were treated with PolyI:C at both time points (**Figure 5**). Inducible nitrogen-oxide synthase (iNOS) is a key element of MSC-mediated immunosuppression [24]. Expression of *iNOS* was notably induced in SV-MSCs after a 24-hour PolyI:C treatment, whereas its expression level in BM-MSCs remained almost unchanged under the same conditions.

To validate our findings at protein level, secreted cytokine and chemokine concentrations were also determined in MSC cultures upon activation of the cells with TLR ligands or pro-inflammatory cytokines (**Figure 6**). IL-6 was constantly secreted by both types of MSCs under normal conditions. Secretion patterns of IL-6 cytokine and IL-8 chemokine were similar in both MSC cultures. Exposure to LPS, PolyI:C, TNFα, or IL-1β for 12 and 24 hours triggered a

significant increase in the concentrations of both above mentioned secreted mediators, whereas treatments with IFNγ did not modify their production by MSCs of different origin. More intense IL-6 and IL-8 production was observed in BM-MSC than SV-MSC culture (**Figure 6**). Both type of MSCs secreted CXCL10/IP-10 chemokine upon TLR- and cytokine receptor ligation. In contrast to IL-6 and IL-8 levels, SV-MSCs produced more CXCL10/IP-10 in response to activation than BM-MSCs. Based on our measurements, in BM-MSCs cultures PolyI:C and IL-1β stimuli were the most potent inducers of CXCL10/IP-10 production, while SV-MSCs released this chemokine in increased concentrations as a result of any applied activations; however, the changes were statistically significant only when the cells were exposed to the TLR ligands, LPS or PolyI:C (**Figure 6**).

## Discussion

Although arteries and veins differ structurally and functionally, they share certain properties, for instance they contain same cells populating the vessel walls and same mechanisms work to maintain their stability under physiologic condition or to ensure the repair mechanisms leading to the regeneration and recovery after an injury [25]. The cellular components of vessel walls seem to be well characterized, but still several questions remain open regarding the progenitor and stem/stromal cells responsible for the replacement of cells of vessel walls during the lifespan. Endothelial cells and pericytes are two essential components of stable vessel walls [26, 27]; therefore, several prior studies have focused on cell populations that could be potential sources of these cells. In 1973 Schwartz and colleagues detected proliferating endothelial cells upon vascular injury during rodent development [28]. In 1997 Asahara and his colleagues isolated progenitor cells from human blood called putative endothelial progenitor cells, which are able to differentiate into endothelial cells *in vitro*. Results from *in vivo* experiments suggest that these bone marrow-derived circulating cells may contribute to neoangiogenesis in adults [29]. Later, Minasi and colleagues have provided evidence that resident vascular progenitor cells could differentiate into vascular endothelial cells [30]. In 2012, Fang and co-workers published that stem cells with lin-CD31+CD105+Sca1+CD117(c-kit)+ phenotype could be separated by using collagenase from lung blood vasculature and have a capacity to differentiate toward endothelial lineage [31]. These cells called vascular endothelial stem cells (VESCs); however, their existence and cellular identity remained controversial to date. The putative VESCs have the potential to self-renewal and the ability to differentiate into one or more specialized cell types [32]. To reveal the origin of ECs, in 2016, Yu and colleagues carried out *in vivo* experiments showing a robust vessel formation capacity of Procr+ (Protein C Receptor also known as EPCR) VESCs and their active contribution to the development and maintenance of homeostasis in postnatal angiogenesis in mammary gland, skin and retina vasculatures [33]. Several earlier observations also indicated that pericytes can be a source of MSC precursors in multiple organs [34-37], which may explain the phenomenon that MSCs can be isolated from all vascularized organs. However, a recent study has challenged this idea using lineage tracing to demonstrate that pericytes do not behave as tissue-specific progenitors *in vivo*, despite showing MSC potential *in vitro*[38].

In this study we separated MSCs from saphenous vein vessel wall and compared their morphology, phenotype and functions to those of bone marrow-derived MSCs to reveal the differences and similarities, which could be associated with their regulatory role in angiogenesis

or their immunomodulatory properties under physiologic and pathologic conditions. Pericytes and MSCs share morphology, expression of several cell surface molecules, and even differentiation potential *in vitro*; however, MSCs can be characterized by a combination of perivascular (CD146, PDGFRβ) and MSC markers (CD29, CD44, CD73, CD90, CD105) as well as by the lack the expression of hemato-endothelial cell markers (CD31, CD34, CD45, CD144) [39]. According to our findings, the cells isolated from saphenous vein (SV-MSC) showed similar morphology to bone marrow derived MSCs (BM-MSC). The self-renewal, plastic adherent MSCs have been shown to differentiate toward multiple mesodermal lineages including fat, bone and cartilage cells. SV-MSCs cultures could be differentiated toward canonical mesodermal; adipogenic, osteoblastic and chondrogenic directions culturing the cells in the appropriate induction media. Similar to BM-MSCs, the SV-MSCs are also fit to the criteria defined by ISCT, which means that all expected markers were detected. The populations of BM- and SV-MSCs were well identified, they differed from the myoblasts, smooth muscle cell precursors or from the control HUVEC cells. The only significant difference identified was the higher expression of MCAM (CD146) on the surface of BM-MSCs than on that of SV-MSCs. A previous finding that cultured human BM-MSCs upregulate the expression of CD146 in normoxia may explain our observation[40]. Our results provide an evidence that the isolation technique invented by our group is suitable to collect a pure vessel wall-derived mesenchymal stem cell population. To investigate differences and similarities between the gene expression profile of BM-MSCs and SV-MSCs we examined the genes related to differentiation and lineage, stemness, HOX, SOCS, Notch signalling, cell cycle and oncogenes. These data were collected into functional groups to reveal and compare the functional properties of the MSCs with different origin. According to the hierarchical clustering in case of the genes related to the cell cycle and oncogenes custom group we did not detect any significant difference between the BM- and SV-MSCs. In contrast, the expression profile clearly divided the MSCs with different origin into two groups in case of the differentiation and lineage, stemness and HOX, SOCS, Notch signalling groups. Above described differences could be the consequences of the variant origin and localisation of MSCs [41]; functions of BM-MSCs in the bone marrow to support the differentiation and survival of hematopoietic stem cells (HSC) while the SV-MSCs are responsible for the regeneration and wound healing, angiogenesis and neovascularization [42]. The immunosuppressive activity of BM-MSCs is already published in details underlying their importance in the treatment or their possible application in case of a wide array of non-physiologic conditions like autoimmune and inflammatory diseases or cancer. Based on the present study we can state that SV-MSCs also have a potential to suppress the mitogenic

response following the activation of PBMCs. According to these findings the MSCs isolated from vessel walls can also be used as potent immunomodulatory cells. Tottey and colleagues successfully isolated perivascular cells with an increased proliferating capacity under hypoxic conditions. When these cells were exposed to degraded ECM products they exhibited an increased migratory capability [43]. These changes prove the activation of MSCs in the presence of various stimuli such as injury, infection or sterile inflammation resulted in the enhanced secretion of various cytokines, like basic-fibroblast growth factor (b-FGF), chemotactic and mitogenic molecules or vascular endothelial growth factor (VEGF) modulating the angiogenesis [44]. Like many other cells the MSCs also express extra- and intracellular pattern recognition receptors (PRRs). Immunomodulatory functions of MSCs can be influenced by either ligation of PRRs or via exposure to cytokines and other immunomodulatory factors [45]. Response of MSCs to different stimulatory factors determinates the differentiation and functions of neighbouring immune- and not immune cells thus the immune responses themselves [46, 47]. Both BM- and SV-MSCs could be stimulated by PolyI:C leading to the increased expression of RIG-I, MDA5, IL-6, IFN-β, CXCL10/IP-10 and iNOS. However, we detected differences in the intensity of cellular responses following the PolyI:C treatments. BM-MSCs are able to react to PolyI:C to a greater extent by expressing higher levels of RIG-I, MDA-5, IFNβ and CXCL10/IP-10 than SV-MSCs. Both MSC types with different origin could be activated with TLR- and cytokine receptor ligation resulting in up-regulated secretion of IL-6, IL-8 and CXCL10/IP-10, although a treatment with IFN-γ had no effect on their cytokine and chemokine production. Priming of MSCs by PRR ligands to alter their immunomodulatory activity is known to be essential to use these cells in the treatment of various diseases[48]; however, it seems that MSCs with different origin respond to priming stimuli in different ways.

Under physiologic and pathologic conditions MSCs express a wide array of surface markers and produce various factors by which they can communicate with different cell types including immune cells. The immunomodulatory capacity of MSCs may result in the inhibited proliferation of lymphocytes, and suppressed function of activated inflammatory cells. Furthermore, they are able to drive and determine the differentiation of myeloid-derived cells and the polarization of the T cell response [49]. Based on our results, SV-MSCs fulfill the ISCT criteria, and share almost identical phenotypic and functional characteristics with BM-MSCs. Therefore, we presume that SV-MSCs may also be useful for regenerative therapeutic applications.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Author's contributions

Zoltán Veréb and Anett Mázló contributed equally to this work. Attila Bácsi and Éva Rajnavölgyi contributed equally to this work. Conceived and designed the experiments: Zoltán Veréb and Éva Rajnavölgyi. Performed the experiments: Zoltán Veréb, Anett Mázló, Gábor Koncz, Attila Szabó and Szilárd Póliska. Provided materials: Attila Kiss, Krisztina Litauszky, Gábor Koncz, Zoltán Boda, Attila Bácsi. Analyzed the data: Zoltan Veréb, Anett Mázló, Attila Szabó and Szilárd Poliska. Wrote the paper: Zoltan Veréb, Anett Mázló, Attila Bácsi and Éva Rajnavölgyi.

## Acknowledgements

The authors thank Ms. Anikó Gergely Pálné and Erzsébet Nagyné Kovács for their excellent technical assistance.

## Funding

This work was supported by the National Research, Development and Innovation Office (NKFIH PD 132570 to ZV, NN 114423 to ÉR and K 125337 to AB). The work was also supported by GINOP-2.3.2-15-2016-00050 project (AB). The project is co-financed by the European Union and the European Regional Development Fund. The authors acknowledge the "New National Excellence Program" (ÚNKP-17-3 and ÚNKP-18-3) for the co-financing of this project. The work of Zoltán Veréb was supported by the EFOP-3.6.1-16-2016-00008 project (co-financed by the European Union and the European Regional Development Fund), and by the 20391-3/2018/FEKUSTRAT project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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## Figure and table legends

**Figure 1. Morphology and multilineage differentiation potential of vessel wall-derived MSCs**

A) After passage 5 the isolated MSC populations derived from bone marrow (BM) or SV and human umbilical cord vein (HUVEC) exhibited spindle-shaped morphology. B) BM and SV derived MSCs exhibited the capability to differentiate into the three canonical differentiation pathways, such as fat, bone and cartilage. C) *(Cytoskeletal actin labeled by phalloidin-TRITC, Vimentin and iNOS by rabbit monoclonal antibody, visualized by anti-rabbit conjugated with NorthernLights493. Nuclei stained with Hoechst. Original magnification: x200. Data is representative of four experiments.)*

**Figure 2. Hierarchical clustering of surface markers expressed by BM-MSCs and SV-MSCs.**

Robust hierarchical clustering of cell surface molecules' expression divided the stem cells of different tissue origin from the endothelial cells isolated from umbilical cord tissue. SV-MSCs were more closely related to BM-MSC than to endothelial cells (A).

*(Color key represents percentage of positive cells in the in vitro cell cultures,* $N_{HUVEC}=9$, $N_{SV\text{-}MSC}=5$, $N_{BM\text{-}MSC}=12$*)*

**Figure 3. Heatmaps of the differentially expressed genes in BM-MSCs and SV-MSCs.**

Genes related to stemness, HOX, Notch and SOX signaling, differentiation and lineage, cell cycle and oncogenes were selected. The functional cluster analysis of the different expression levels of selected genes show the difference between the cell types suggesting different tissue of origin.

**Figure 4. Immunomodulatory effect of MSC *in vitro***

Vessel wall derived MSCs were capable to inhibit the proliferation of lymphocytes activated by PHA and ConA in a dose dependent manner *in vitro*, however it was significant only in the case of PHA. *(Data shown are mean±SD, N=3).*

**Figure 5. Relative expression levels of selected genes in MSCs derived from bone marrow and saphenous vein in resting and stimulated cells**

Mesenchymal stem cells were treated with 100ng/ml LPS, 25 μg/ml Poly I:C, 100 ng/ml TNFα, 10ng/ml IL-1β or 10 ng/ml IFNγ for 12 and 24 hours as described in Methods. Relative levels of mRNA were measured in triplicates by qPCR and fold changes in gene expression were calculated from the ratio of expression levels in treated and untreated cells as mean±SEM (*N=3 in both cell types*)

**Figure 6. Cytokine secretion by activated MSCs derived from bone marrow and saphenous vein**

IL-6, IL-8, and IP-10 cytokine production of TRL ligands (LPS, Poly I:C), as well as pro-inflammatory cytokines (TNFα, IFNγ, IL-1β) stimulated MSCs. In vitro cultured cells were treated in 12 h and 24 h intervals. *(Data shown are mean±SD; p<0.05 *, p<0.01 **, p<0.001 ***; N=6 for the BMMSCs and N=3 for the SV-MSCs, respectively).*

**Table 1. Detailed phenotypic analysis of SV-MSCs and BM-MSCs**

Expression of surface markers related to different cell types was measured by flow cytometry. The percentage of positive cells in SV-MSC culture was compared to that of BM-MSCs as well as HUVECs, as vein endothelial control.

*(Data are presented as means ± SEM; n=5 for SV-MSC, n=12 for BM-MSC, n=7 for HUVEC. p<0.05 *, p<0.01 **, p<0.001 *** vs. SV-MSCs determined by Student t test.)*

**Table 2. Top up- and down-regulated custom selected genes in SV-MSCs**

Top up and down-regulated genes in SV-MSCs related to stemness, HOX, Notch and SOX signaling, differentiation and lineage, cell cycle and oncogenes were selected by the significance.

**Table 1. Detailed phenotypic analysis of BM-MSCs and SV-MSCs**

| | | BM-MSC | SV-MSC | HUVEC |
|---|---|---|---|---|
| | | Percentage of positive cells (%) | | |
| Hematopoietic markers | CD14 | 0.22±0.11 | 1.37+1.15 | 0+0 |
| | CD34 | 0±0 | 0±0 | 4.62+2.05 |
| | CD36 | 32.51±8.18 | 18.12±5.28 | 36.6+17.60 |
| | CD45 | 0±0 | 0±0 | 0+0 |
| | CD47 | 97.00±0.86 | 96.65±1.55 | 85.06+12.49 |
| | CD69 | 0±0 | 0±0 | 27.24+10.93 |
| | CD133 | 0±0 | 0±0 | 0+012.03 |
| | CD117 | 0±0 | 0.02±0.02 | 81.57+11.26 *** |
| | CXCR4 | 0±0 | 0±0 | 37.37+8.18 ** |
| | HLA-DR | 0±0 | 0±0 | 0.19+0.12 |
| Endothelial markers | CD31 | 0±0 | 0±0 | 96.78+0.82 *** |
| | CD144 | 45.33±12.61 | 61.55±18.18 | 93.91+2.45 |
| | VEGFR2/KDR | 0±0 | 0±0 | 0.75+0.41 |
| | CD104/Integrin β4 | 28.25±12.20 | 34.42±17.82 | 76.42+11.50 |
| MSC/Fibroblast markers | CD73 | 91.99±1.92 | 97.90±0.80 | 97.85+0.94 |
| | CD90/Thy-1 | 89.05±1.49 | 89.68±3.63 | 2.86+1.55 *** |
| | CD105/Endoglin | 82.64±2.56 | 89.62±2.54 | 97.94+0.52 *** |
| | CD147/Neurothelin | 77.33±8.87 | 81.11±13.59 | 98.31+0.91 |
| | PDGF Rβ | 78.01±8.28 | 90.77±3.74 | 54.67+11.90 |
| Cell adhesion molecules | CD29/Integrin β1 | 92.96±1.71 | 97.02±1.87 | 98.77+0.64 |
| | CD44/H-CAM | 87.28±2.87 | 88.66±2.38 | 79.28+5.06 |
| | CD49a | 79.60±7.77 | 94.25±1.55 | 89.44+1.64 |
| | CD49b | 68.52±7.95 | 48.44±12.25 | 85.32+5.42 * |
| | CD49f | 0±0 | 0±0 | 2.21+1.27 |
| | CD54/ICAM | 14.95+8.36 | 19.89+8.59 | 34.29+7.24 |
| | CD56/NCAM | 20.53±8.41 | 19.18±9.10 | 50.33+7.94 |
| | CD146/MCAM | 77.54±5.14 *** | 7.09±6.56 | 96.68+1.02 *** |
| | CD166/ALCAM | 89.57±6.27 | 96.22±2.13 | 98.54+0.45 |

**Table 2. Top up- and down-regulated custom selected genes in SV-MSCs**

| Symbol | Entrez gene name | Fold change | p-value | Molecule type | Group |
|---|---|---|---|---|---|
| **Fold Change up-regulated** | | | | | |
| *S100A4* | S100 calcium binding protein A4 | 2.805862 | 0.0426795 | calcium binding protein | **Cell cycle and oncogenes** |
| *CDH1* | cadherin 1, type 1, E-cadherin (epithelial) | 1.61272 | 0.0221517 | cell adhesion molecule | |
| *BRCA2* | breast cancer 2, early onset | 1.36718 | 0.0158221 | DNA repair | |
| *SMG6* | Smg-6 homolog, nonsense mediated mRNA decay factor (C, elegans) | 1.1617644 | 0.0169296 | enzyme | |
| *FGF9* | fibroblast growth factor 9 (glia-activating factor) | 6.6313844 | 0.0116976 | growth and differentiation factor | **HOX, SOCS, Notch signaling** |
| *IL33* | interleukin 33 | 2.8804057 | 0.0380778 | cytokine | |
| *HOXA11* | homeobox A11 | 2.00343 | 0.0164048 | transcription factor | |
| *BMP4* | bone morphogenetic protein 4 | 1.8365396 | 0.0429056 | growth and differentiation factor | |
| *PODXL* | podocalyxin-like | 4.6124125 | 0.0230397 | cell differentiation | **Differentiation and lineage** |
| *CTSK* | cathepsin K | 2.5564253 | 0.0201834 | lysosomal cysteine protease | |
| *CSF1* | colony stimulating factor 1 (macrophage) | 2.176855 | 0.0155533 | cytokine | |
| *TGFB3* | transforming growth factor, beta 3 | 1.8547666 | 0.0230397 | growth and differentiation factor | |
| *NRP1* | neuropilin 1 | 1.7981821 | 0.0058021 | membrane-bound coreceptor | |
| *GDF10* | growth differentiation factor 10 | 1.6743402 | 0.0155533 | growth and differentiation factor | |
| *FGF9* | fibroblast growth factor 9 (glia-activating factor) | 6.6313844 | 0.0143408 | growth and differentiation factor | **Stemness** |
| *ZFPM2* | zinc finger protein, multitype 2 | 5.1527076 | 0.0195738 | transcription factor | |
| *MME* | membrane metallo-endopeptidase | 3.1862447 | 0.0245681 | enzyme | |
| *FZD4* | frizzled homolog 4 (Drosophila) | 2.4862442 | 0.0143408 | receptor | |
| *ACVRL1* | activin A receptor type II-like 1 | 1.8575617 | 0.0143408 | enzyme | |
| **Fold Change down-regulated** | | | | | |
| *SMAD3* | SMAD family member 3 | -2.579574 | 0.0108465 | transcriptional modulator | **Cell cycle and oncogenes** |
| *CDK6* | cyclin-dependent kinase 6 | -2.243104 | 0.0237225 | enzyme | |
| *KRAS\|LYRM5* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog \| LYR motif containing 5 | -1.901833 | 0.0461384 | proto-oncogene | |
| *TGFB1* | transforming growth factor, beta 1 | -1.731404 | 0.0158221 | growth and differentiation factor | |
| *RARA* | retinoic acid receptor, alpha | -1.566421 | 0.0337291 | nuclear receptor | |
| *HUS1* | Checkpoint protein HUS1 | -1.287702 | 0.0108465 | genotoxin-activated checkpoint complex | |

| | | | | | |
|---|---|---|---|---|---|
| *SUN1\|C7orf 20* | Sad1 and UNC84 domain containing 1 \| chromosome 7 open reading frame 20 | -1.263087 | 0.0108465 | nuclear envelope protein | |
| *PURA* | purine-rich element binding protein A | -1.253684 | 0.0108465 | multi-functional DNA- and RNA-binding protein | |
| *CDON* | Cdon homolog (mouse) | -1,5534242 | 0,0691849 | cell surface receptor | **HOX ,SOCS, Notch signaling** |
| *HOXA2* | homeobox A2 | -1,6179696 | 0,0280393 | transcription factor | |
| *SNAI1* | snail homolog 1 (Drosophila) | -1,6272678 | 0,0442530 | transcription factor | |
| *PYGO1* | pygopus homolog 1 (Drosophila) | -1,6621072 | 0,0924799 | | |
| *NOTCH2* | Notch homolog 2 (Drosophila) | -1,6733813 | 0,0177410 | transmembrane protein | |
| *MAML2* | mastermind-like 2 (Drosophila) | -1,6876673 | 0,0481715 | transcriptional co-activator | |
| *VCAM1* | vascular cell adhesion molecule 1 | -17,354261 | 0.0058021 | | **Differentiation and lineage** |
| *ACAN* | aggrecan | -5,5746202 | 0.0192411 | | |
| *EGR2* | early growth response 2 | -4,387574 | 0.0191151 | | |
| *TGFB2* | transforming growth factor, beta 2 | -3,9135396 | 0.0058021 | | |
| *IGF2\|INS-IGF2* | insulin-like growth factor 2 (somatomedin A) \| INS-IGF2 readthrough transcript | -3,4904327 | 0.0192411 | | |
| *BMP2* | bone morphogenetic protein 2 | -3,314717 | 0.0230696 | | |
| *BDNF* | brain-derived neurotrophic factor | -3,2864723 | 0.0155533 | | |
| *JAG1* | jagged 1 (Alagille syndrome) | -3,0462105 | 0.0058021 | | |
| *INHBA* | inhibin, beta A | -2,8667028 | 0.0422998 | | |
| *ITGA3* | integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) | -2,775381 | 0.0155533 | | |
| *SMAD3* | SMAD family member 3 | -2,5795743 | 0.0078861 | | |
| *HES1* | hairy and enhancer of split 1, (Drosophila) | -2,220433 | 0.0192411 | | |
| *EFNB2* | ephrin-B2 | -2,1113176 | 0.0358564 | | |
| *PTN* | pleiotrophin | -2,1063795 | 0.0155533 | | |
| *PDGFA LOC100132 080* | platelet-derived growth factor alpha polypeptide \| hypothetical LOC100132080 | -2,0340111 | 0.0155533 | | |
| *LIF\|MGC20 647* | leukemia inhibitory factor (cholinergic differentiation factor) \| hypothetical protein MGC20647 | -9.517681 | 0.0154874 | | **Stemness** |
| *CXCL12* | chemokine (C-X-C motif) ligand 12 \| chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) | -8.499458 | 0.0414186 | | |
| *MCAM* | melanoma cell adhesion molecule | -5.909656 | 0.0080718 | | |
| *ACAN* | aggrecan | -5.5746202 | 0.0193145 | | |
| *LTBP1* | latent transforming growth factor beta binding protein 1 | -4.3929467 | 0.0398936 | | |
| *BMP2* | bone morphogenetic protein 2 | -3.314717 | 0.0236414 | | |
| *SMAD3* | SMAD family member 3 | -2.5795743 | 0.0087946 | | |
| *ALCAM* | activated leukocyte cell adhesion molecule | -2.1179285 | 0.0080718 | | |
| *ITGAV* | integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51) | -2.0977302 | 0.0143408 | | |
| *GDF6* | growth differentiation factor 6 | -2.054666 | 0.0427049 | | |

| | | | | | |
|---|---|---|---|---|---|
| *FGF7* | fibroblast growth factor 7 (keratinocyte growth factor) | -2.0363815 | 0.049803 | | |
| *FGFR2* | fibroblast growth factor receptor 2 | -1.8906314 | 0.0324610 | | |

**Figure 1.**

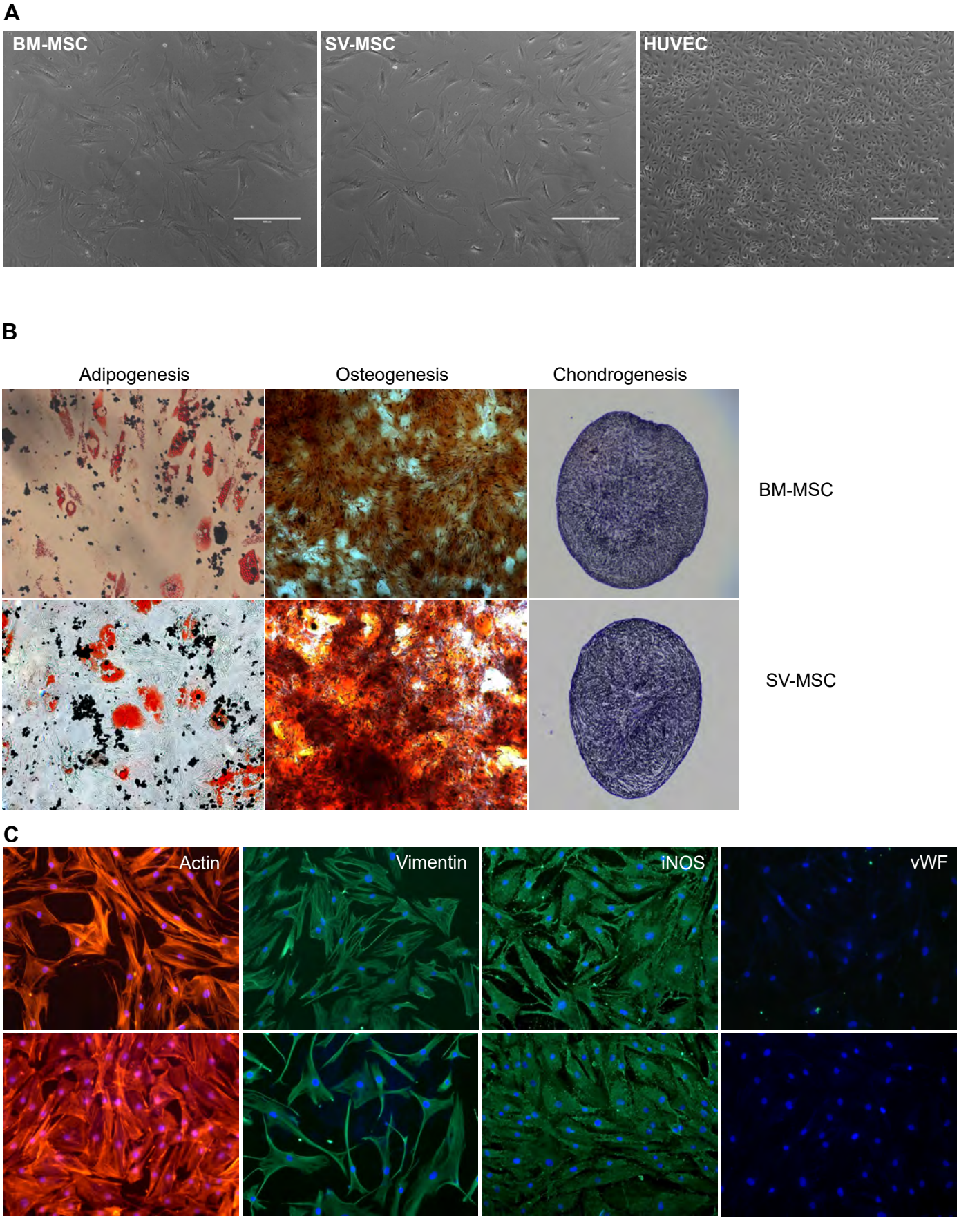

A
BM-MSC
SV-MSC
HUVEC
B
Adipogenesis
Osteogenesis
Chondrogenesis
BM-MSC
SV-MSC
C
Actin
Vimentin
iNOS
vWF
BM-MSC
SV-MSC

**Figure 2.**

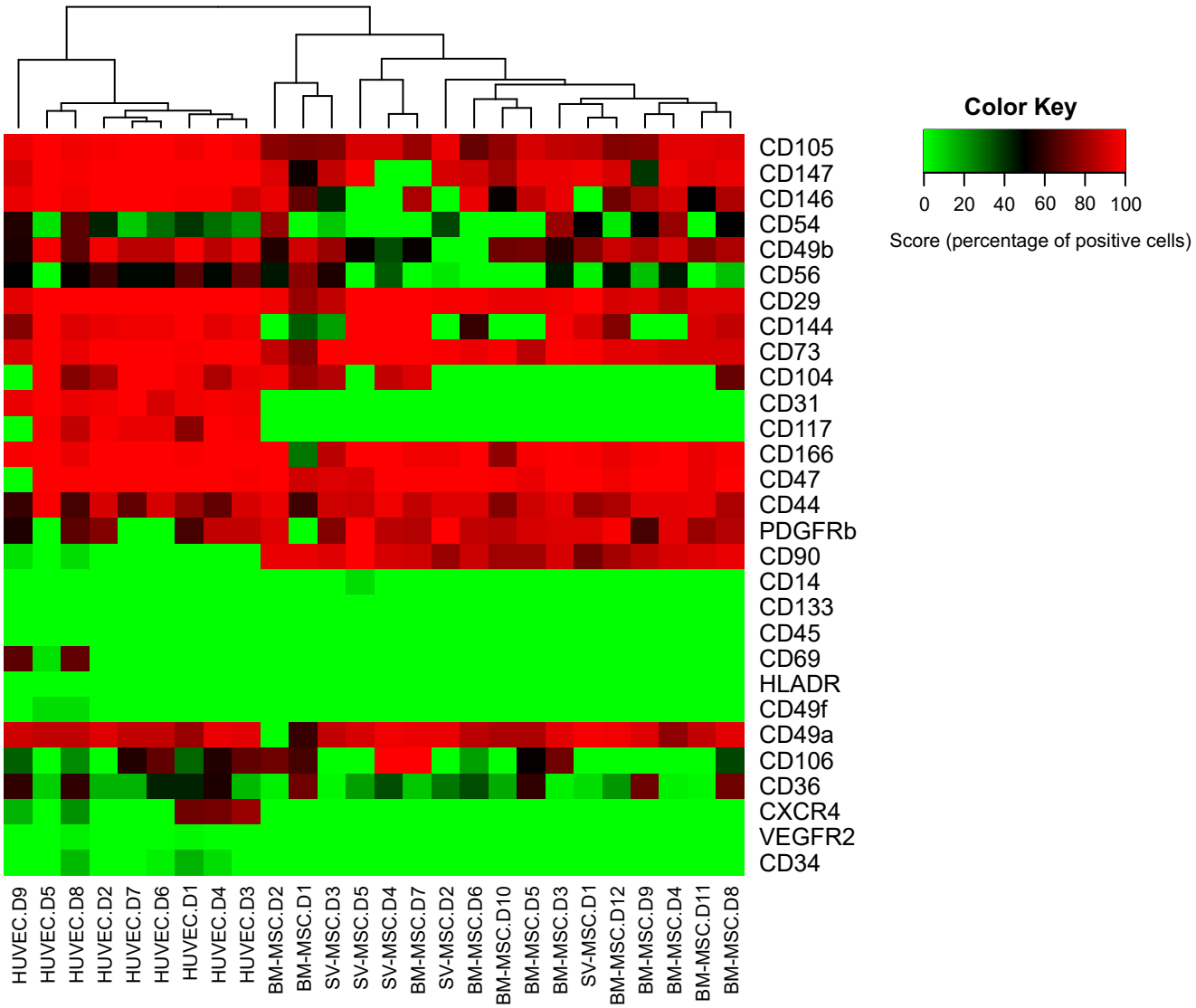

Color Key
0 20 40 60 80 100
Score (percentage of positive cells)
CD105
CD147
CD146
CD54
CD49b
CD56
CD29
CD144
CD73
CD104
CD31
CD117
CD166
CD47
CD44
PDGFRb
CD90
CD14
CD133
CD45
CD69
HLADR
CD49f
CD49a
CD106
CD36
CXCR4
VEGFR2
CD34
HUVEC.D9
HUVEC.D5
HUVEC.D8
HUVEC.D2
HUVEC.D7
HUVEC.D6
HUVEC.D1
HUVEC.D4
HUVEC.D3
BM-MSC.D2
BM-MSC.D1
SV-MSC.D3
SV-MSC.D5
SV-MSC.D4
BM-MSC.D7
SV-MSC.D2
BM-MSC.D6
BM-MSC.D10
BM-MSC.D5
BM-MSC.D3
SV-MSC.D1
BM-MSC.D12
BM-MSC.D9
BM-MSC.D4
BM-MSC.D11
BM-MSC.D8

**Figure 3.**

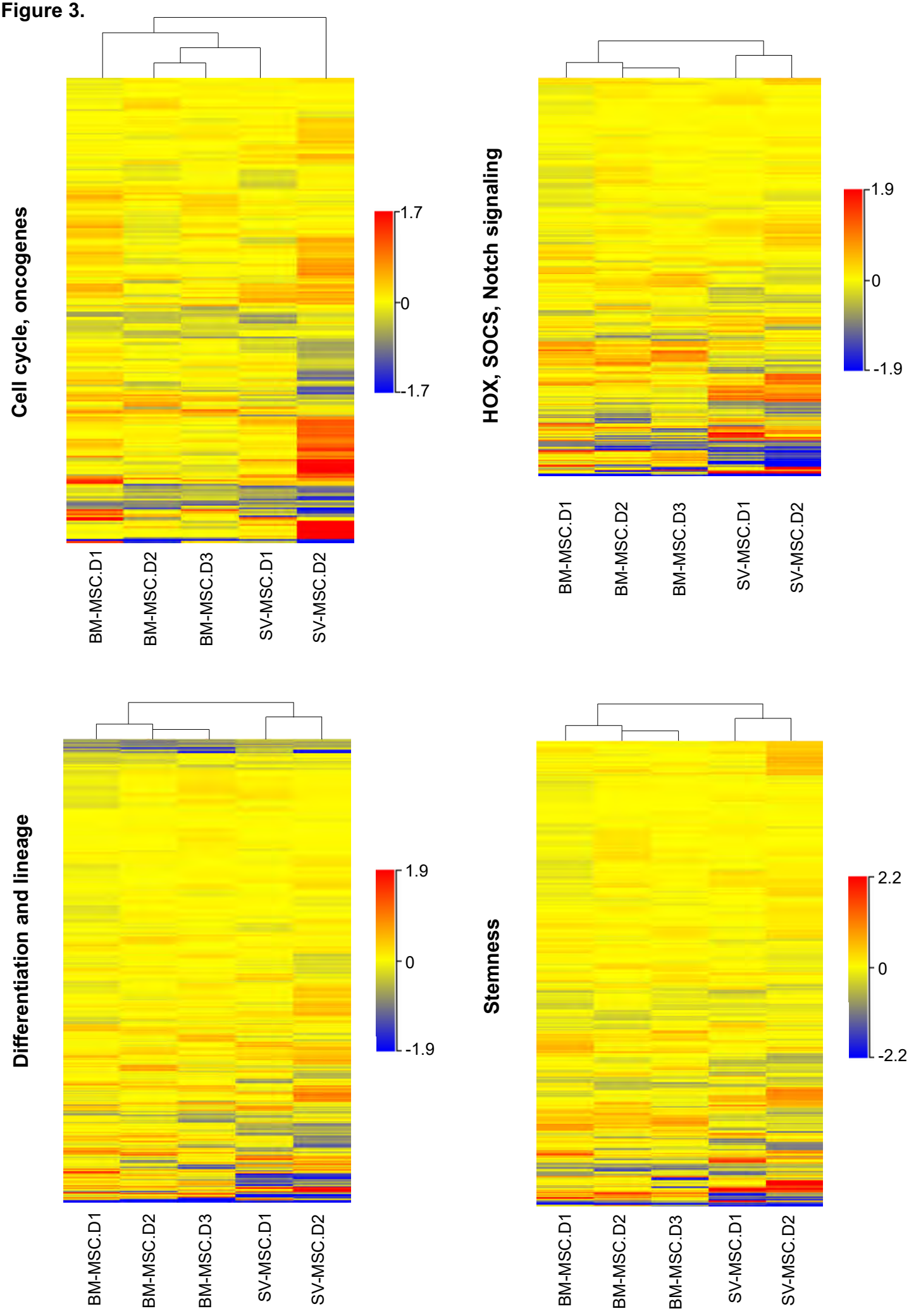

Cell cycle, oncogenes
1.7
0
-1.7
BM-MSC.D1
BM-MSC.D2
BM-MSC.D3
SV-MSC.D1
SV-MSC.D2
HOX, SOCS, Notch signaling
1.9
0
-1.9
BM-MSC.D1
BM-MSC.D2
BM-MSC.D3
SV-MSC.D1
SV-MSC.D2
Differentiation and lineage
1.9
0
-1.9
BM-MSC.D1
BM-MSC.D2
BM-MSC.D3
SV-MSC.D1
SV-MSC.D2
Stemness
2.2
0
-2.2
BM-MSC.D1
BM-MSC.D2
BM-MSC.D3
SV-MSC.D1
SV-MSC.D2

**Figure 4.**

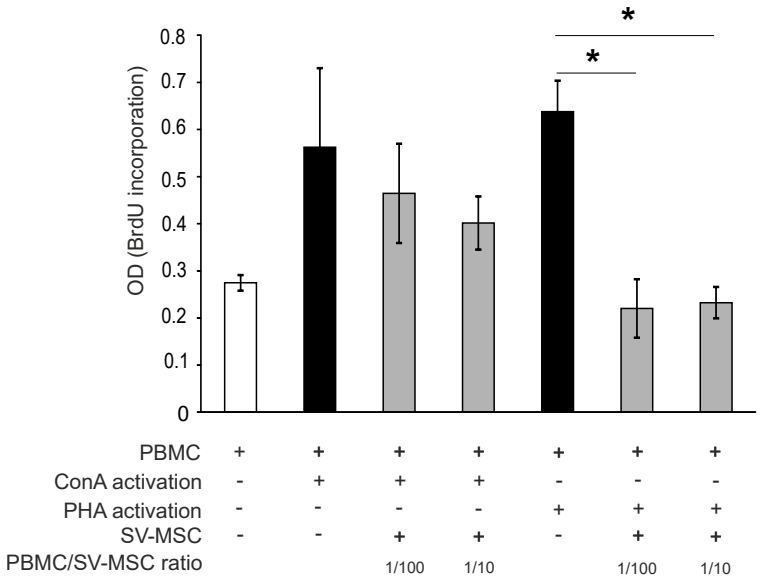

*
*
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD (BrdU incorporation)
PBMC + + + + + + +
ConA activation - + + + - - -
PHA activation - - - - + + +
SV-MSC - - + + - + +
PBMC/SV-MSC ratio 1/100 1/10 1/100 1/10

**Figure 5.**

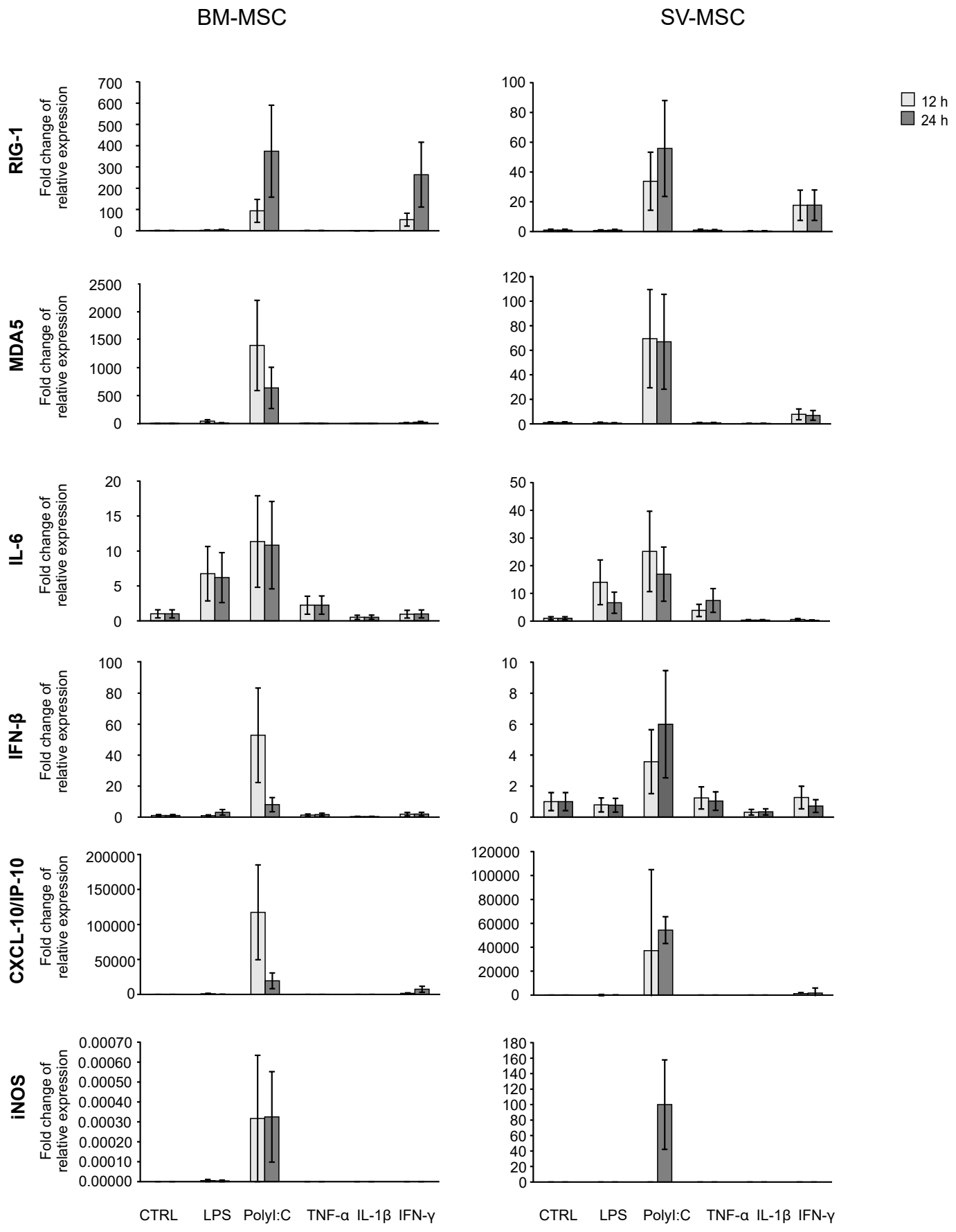
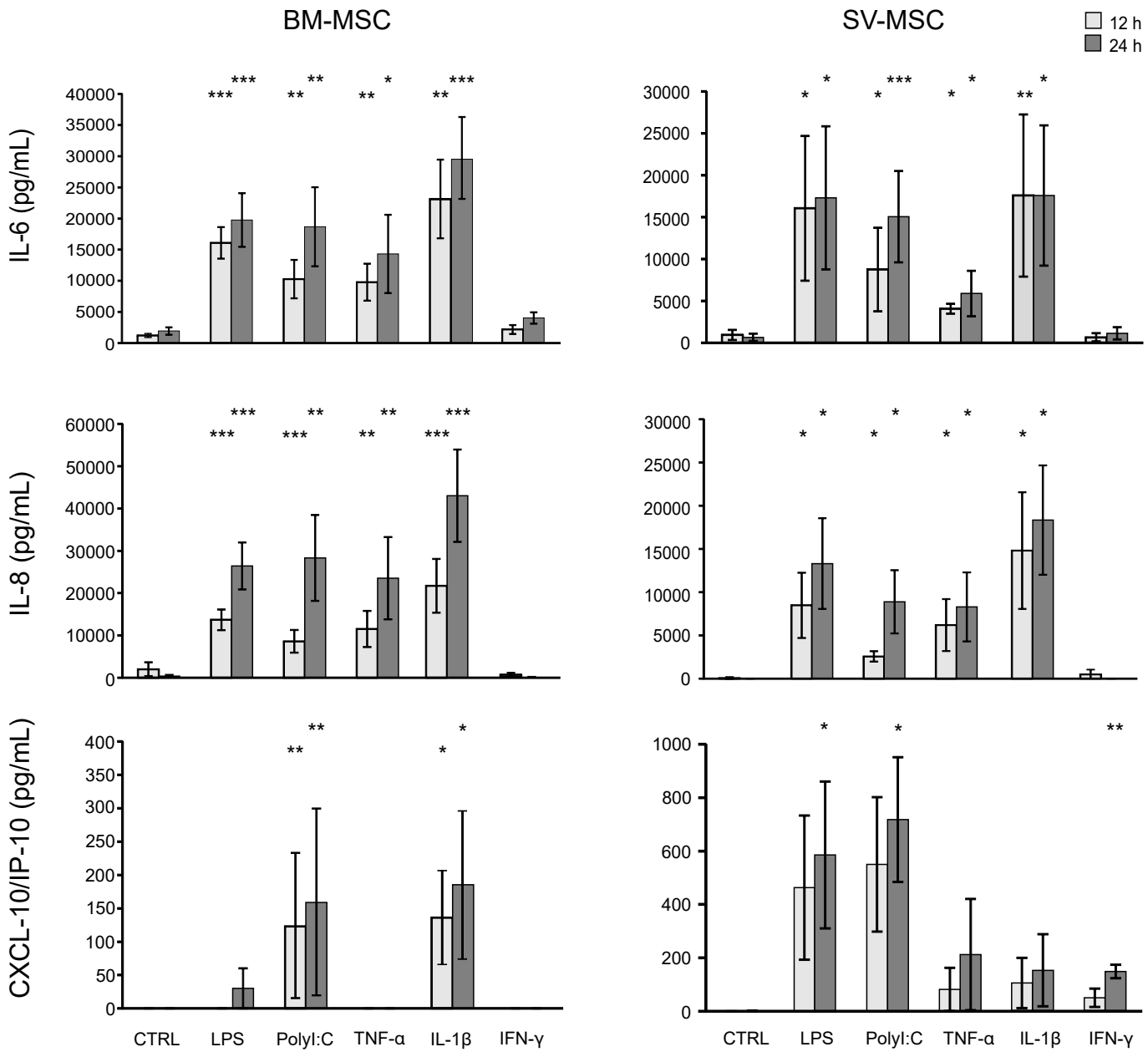

BM-MSC
SV-MSC
12 h
24 h
RIG-1
MDA5
IL-6
IFN-β
CXCL-10/IP-10
iNOS
Fold change of relative expression
CTRL
LPS
PolyI:C
TNF-α
IL-1β
IFN-γ

**Figure 6.**


BM-MSC
SV-MSC
12 h
24 h
IL-6 (pg/mL)
IL-8 (pg/mL)
CXCL-10/IP-10 (pg/mL)
CTRL
LPS
PolyI:C
TNF-α
IL-1β
IFN-γ

# Supplementary information

## Materials and methods

Mesenchymal stem cell-related gene expression profile was studied with the Human Mesenchymal Stem Cell PCR Array (SABiosciences). Total RNA was isolated by Trizol reagent, and then purified on RNeasy Mini kits (Qiagen) after DNase I digestion. RT2 First Strand kit was used to perform first strand cDNA synthesis. Real-time PCR measurement was performed on the ABI Prism 7900 platform. Data were analyzed and statistical significance was calculated by SABiosciences on-line software.

**Supplementary Figure 1.**

Heatmap of differently expressed genes related to MSC biology. The clustering showed that cells isolated from the saphenous vein are MSCs, according the selected 84 transcripts.

The mesenchymal stem cell related gene expression patterns were compared between SV-MSCs and BM-MSCs using Human Mesenchymal Stem Cell RT² Profiler™ PCR Array. The array includes 84 selected genes, which have previously been shown to play role in pluripotency and self-renewal of mesenchymal stem cells.

**Supplementary figure 1.**