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Effect of expansion media and fibronectin coating on growth and chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells

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In the field of regenerative medicine, considerable advances have been made from the technological and biological point of view. However, there are still large gaps to be filled regarding translation and application of mesenchymal stromal cell (MSC)-based therapies into clinical practice. Indeed, variables such as cell type, unpredictable donor variation, and expansion/differentiation methods lead to inconsistencies. Most protocols use bovine serum (FBS) derivatives during MSC expansion. However, the xenogeneic risks associated with FBS limits the use of MSC-based products in clinical practice. Herein we compare a chemically defined, xenogeneic-free commercial growth medium with a conventional medium containing 10% FBS and 5 ng/ml FGF2. Furthermore, the effect of a fibronectin-coated growth surface was investigated. The effect of the different culture conditions on chondrogenic commitment was assessed by analyzing matrix deposition and gene expression of common chondrogenic markers. Chondrogenic differentiation potential was similar between the FBS-containing α MEM and the chemically defined medium with fibronectin coating. On the contrary, the use of fibronectin coating with FBS-containing medium appeared to reduce the differentiation potential of MSCs. Moreover, cells that were poorly responsive to in vitro chondrogenic stimuli were shown to improve their differentiation potential after expansion in a TGF- β 1 containing medium. In conclusion, the use of a xenogeneic-free medium provides a suitable alternative for human bone marrow MSC expansion, due the capability to maintain cell characteristic and potency. To further improve chondrogenic potential of BMSCs, priming the cells with TGF- β 1 during expansion is a promising strategy.

Abbreviations

α MEM	Minimum essential medium Eagle, alpha modification
ANOVA	Analysis of variance
FGF2	Basic fibroblast growth factor
BMSCs	Bone-marrow MSCs
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EthD-1	Ethidium homodimer-1
FBS	Foetal bovine serum
MSCs	Mesenchymal stromal cells
PDT	Population doubling time

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SMAD	Small mother against decapentaplegic
TGF- β	Transforming growth factor, beta
XFSM	Xeno-serum-free medium

The regenerative medicine field requires a cell source for either tissue engineering or secretome production. Ideally, any novel therapy should be efficient, fast, and cost-effective, maintaining the original physiological functions in the process^{1,2}. Given this ambitious expectation for the development of *in vitro* tissues, there is also an increasing research interest for fabrication of customized, patient specific implants, while other branches in this field are focusing on the use of cell products, such as secretome or extracellular vesicles, as a future strategy for tissue healing³. Many of these processes require an initial step of primary cell expansion. Regenerative medicine typically uses patient's autologous cells, seeds them on suitable support materials, induces cell differentiation through the addition of specific growth factors and then uses the matured construct or the cell secretome to induce healing or for a total replacement of the tissue^{4,5}.

Although research in this field is rapidly progressing, there are still numerous and fundamental questions to be answered: which is the optimal cell type⁶, how many cells are needed⁷, and what is the most suitable method of cell isolation, proliferation and differentiation that can guarantee a high yield of commitment into a stable phenotype. These factors are highly affected by donor variability^{8–10}, as well as the culture methods^{11,12}.

So far, research in this field has tested countless sources of mesenchymal stromal cells (MSCs) from different sites such as bone marrow, dental pulp¹³, adipose tissue¹⁴, and synovial membrane¹⁵, demonstrating that it is possible to isolate and differentiate them towards different phenotypes. However, most of the techniques described use fetal bovine serum (FBS) to support cell growth during expansion. While on one hand it supports cell growth and differentiation, on the other hand FBS is an animal-derived product, complicating the translation of any biotech therapy¹⁶. Studies have highlighted the possibility that proteins and peptides contained in FBS can be incorporated into cells during the transfection process, consequently causing a rejection of transplanted cells^{17,18}. Moreover, FBS is an ill-defined culture media component, being a heterogeneous solution of growth factors and proteins that are not controllable and are subject to batch-to-batch variation. This variability between different lots of the same product is recognized to influence cell proliferation and phenotype¹⁹.

In this study, we therefore aim to compare the growth and differentiation of primary human bone marrow MSCs (BMSCs) expanded in standard FBS-containing α MEM medium or in a commercial serum-free medium. The FBS containing medium was further supplemented with Fibroblast Growth Factor -2 (FGF2) as this factor is known to maintain cells in a more stem-like state and promotes proliferation^{20–22}. The combination of MSC-screened FBS with FGF2 is generally considered to be the "gold standard" expansion medium for primary human MSCs. We further investigated the potential of Transforming Growth Factor (TGF)- β priming in monolayer cultures to enhance BMSC chondrogenic potential. In addition, according with previous studies performed on MSCs derived from adipose tissue, bone marrow, and umbilical cord²³, we tested whether using fibronectin to coat the culture surface, in combination with the different media, can improve chondrogenic differentiation of primary human BMSCs.

Results

Effect of different expansion protocols on BMSC growth and morphology. As expected, media composition did not affect cell viability, as assessed by Live/Dead staining. The presence or absence of a fibronectin coating also had no apparent effect, even though the combination of fibronectin coating with α MEM/FBS/FGF2 appears to result in a lower number of cells (Fig. 1).

All cells in the presence of a chemically defined medium (xeno/serum free medium—XFSM) or α MEM exhibited a fibroblast-like and spindle-shaped morphology with long cell processes (Fig. 2).

Cell size was measured in two consecutive passages: the results showed no significant variation of the average cell diameter for BMSCs expanded in the different conditions, with a higher variability observed in α MEM/FBS/FGF2 groups (Fig. 3).

The population doubling time (PDT) was similar for cells grown in XFSM or in α MEM/FBS/FGF2, with no statistically significant differences among the groups (Fig. 4A). The PDT was calculated in the range 24–72 h for the cells to be in the exponential phase of cell growth. The growth curve also showed a similar trend, with no difference between the groups between 24 and 72 h (Fig. 4B). As expected, the cells reached a growth plateau at day 7, with similar cell numbers for all groups.

Chondrogenic differentiation of BMSCs expanded in α MEM/FBS/FGF2 or XFSM. After comparing cell growth in different media, we focused on the chondrogenic differentiation potential of BMSCs after expansion in different conditions. We used two different types of donors that can be classified as good responders ($n = 3$) or poor responders ($n = 2$), based on histological results of cells expanded with the standard medium (α MEM/FBS/FGF2, no fibronectin coating, Fig. 5A–C).

Chondrogenic differentiation was evaluated at 21 days by Safranin-O/Fast Green staining of pellet cryosections (Fig. 5A). The histological evaluation for all donors expanded with XFSM did not show any difference compared to α MEM/FBS/FGF2 medium, although there was a tendency towards a stronger intensity for the matrix deposition and internal pellet structure either with or without fibronectin. Pellets from all groups had comparable diameters (data not shown). However, pellets from BMSCs expanded with α MEM/FBS/FGF2 on a fibronectin coating showed in some cases (donors #1, #4 and #5) a considerable dispersion and poor matrix organization compared to the other groups, indicating a greater variability.

The use of XFSM with BMSC from non-responsive donors improved the size of differentiated pellets, but not the intensity for Safranin-O staining.

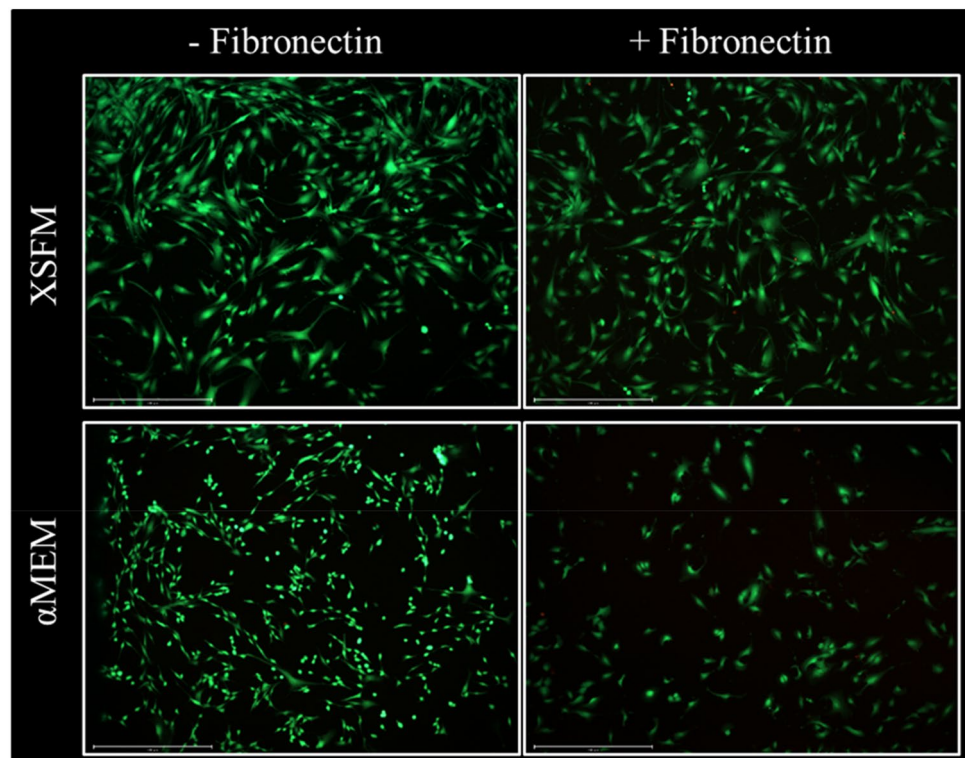


Figure 1. Live/Dead assay of BMSCs seeded on a glass chamber slide, with or without fibronectin coating, and cultured with α MEM/FBS/FGF2 or with a chemically defined medium (XSFM) for 72 h. Green: Calcein-AM that stains live cells; red: ethidium homodimer for dead cells. Scale bar (bottom left in each panel): 200 μ m. Figure representative of $n = 5$ biological replicates.

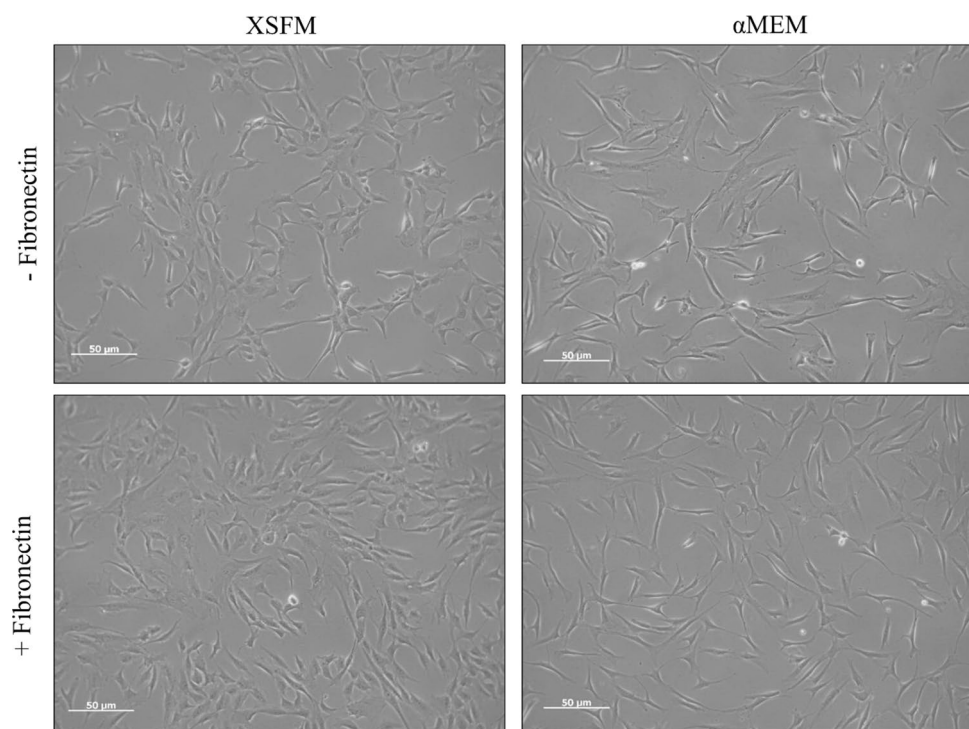


Figure 2. Representative images of BMSCs seeded on tissue culture plastic, with or without a 5 μ g/ml fibronectin coating and cultured with α MEM/FBS/FGF2 or XSFM for 72 h. The Figure is representative of $n = 5$ biological replicates.

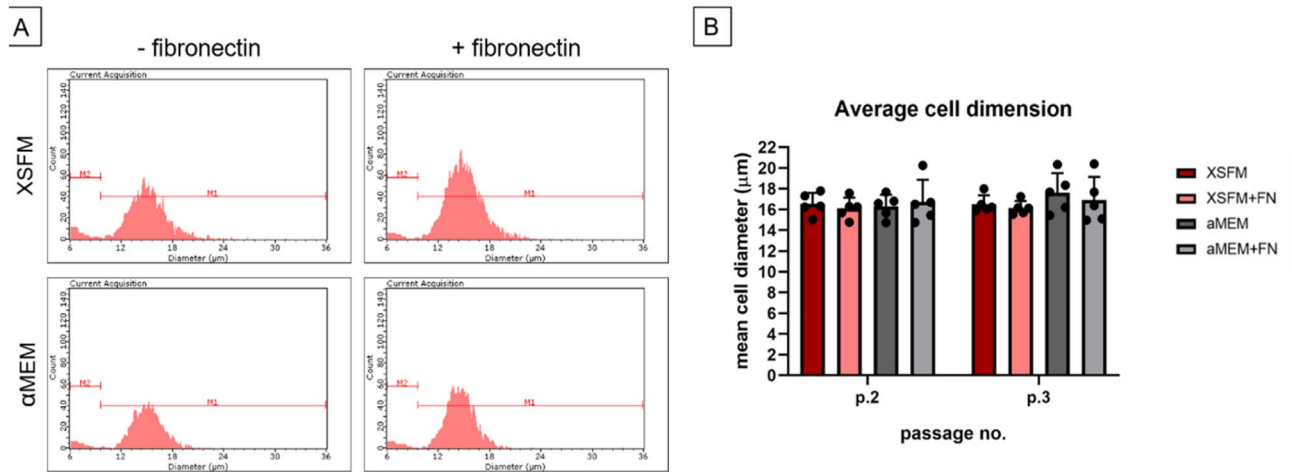


Figure 3. Analysis of cell dimension of BMSCs grown in αMEM/FBS/FGF2 or XSFM, with or without fibronectin coating. (A) Examples of histograms that can be obtained with using Scepter 2.0 for cell counting representative for 1 donor. X-axis reports cell diameter in μm, while the y-axis reports the count of cells. M1 gate includes intact cells larger than 9.6 μm, while M2 includes cell debris (6–9.6 μm). The average cell dimension for each cell population in (B) is calculated from M1. The comparison between growth conditions revealed no significant difference in cell average cell diameter (n = 5 donors).

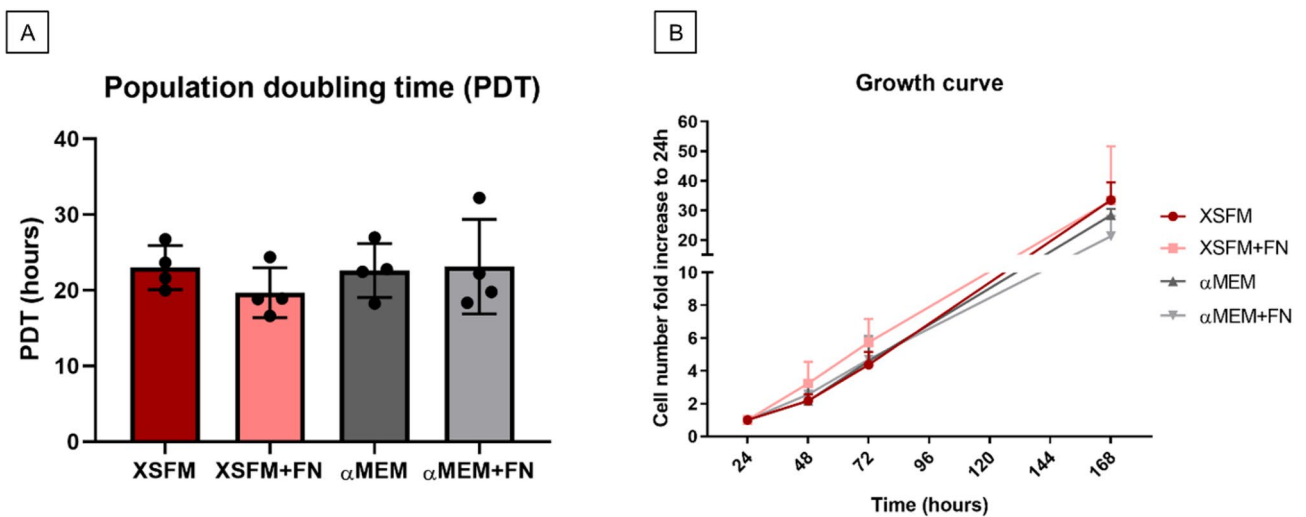


Figure 4. Cell growth and metabolic activity of BMSC cultured in αMEM/FBS/FGF2 (grey shades) or XSFM (red shades) up to 168 h (7 days). Tissue culture plastic was either pre-coated with 5 μg/ml fibronectin (light color lines and bars) or not (dark color lines and bars). (A) Comparison of BMSC doubling time (range 24–72 h) among the different groups, expressed in hours. (B) Growth curve indicating the average cell fold increase referred to 24 h after cell adhesion. n = 4 donors.

To determine the quality of matrix produced in hBMSC pellets, immunofluorescence staining for type II and I collagen was performed. The use of XSFM or αMEM medium did not influence the deposition of type II collagen within the donors. Donors #1 and #2, as previously observed with Saf-O/Fast-Green staining (Fig. 5), were less responsive to the differentiation induced by TGF-β1, and immunofluorescence confirmed that there was almost no production of type II collagen. The other 3 donors did not show differences in type II collagen staining after cell expansion with different media (Fig. 6A). Type I collagen staining also did not show any particular influence of the expansion medium or fibronectin coating on matrix deposition. Interestingly, the less-responsive donors showed a higher staining intensity for type I collagen compared to the other donors (Fig. 6B).

Gene expression analysis on chondrogenic markers. Histological evaluation showed that the use of XSFM medium during in vitro expansion led to similar differentiation when compared to medium containing serum and 5 ng/ml FGF2. We further investigated the expression of common molecular markers associated with chondrogenic differentiation. Analysis of cartilage related genes expression (*COL2A1*, *COL1A1*, *COL10A1*, *ACAN*, *PRG4* and *SOX9*, Fig. 7A–G) showed no difference between groups, although a positive trend in XSFM expanded cells compared to αMEM/FBS/FGF2, except for *PRG4* (Fig. 7F), can be observed. Similarly, αMEM/

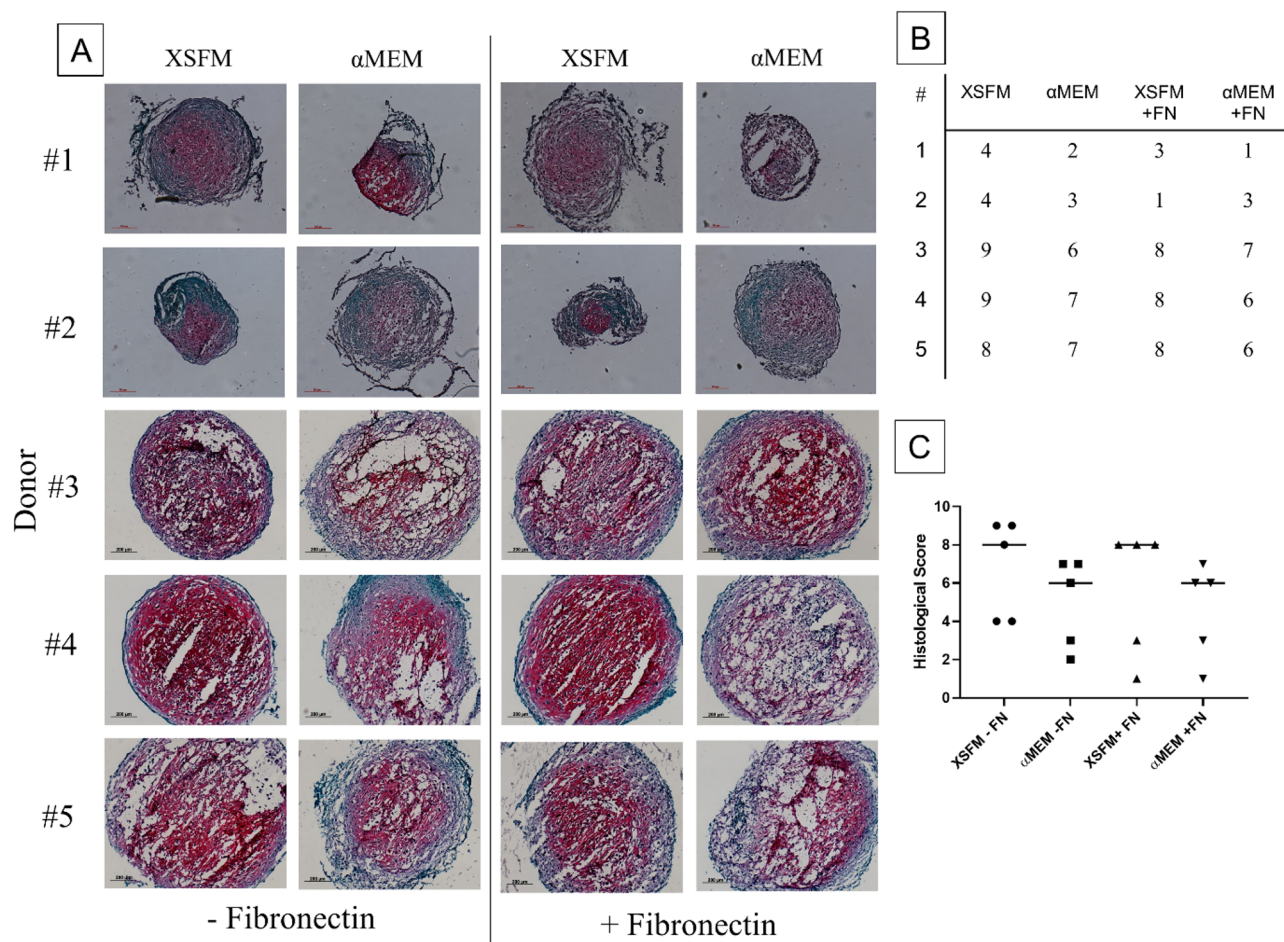


Figure 5. Analysis of the chondrogenic potential of BMSCs expanded in different cell culture media (n = 5 donors). **(A)** Representative images of pellet cryosections stained by Safranin-O/Fast Green: the intensity of Safranin-O (red) staining is directly proportional to the extracellular proteoglycan content, while Fast Green solution (green) stains collagen fibers. Scale bar (bottom left in each picture) = 200 μ m. **(B)** The table indicates the histological score assigned to each pellet (1- undifferentiated; 10- very good differentiation). Grading of the safranin O staining was ranked on a scale of 1–10 according to the Bern Score²⁴. **(C)** Comparison of the histological scores among the groups for all donors.

FBS/FGF2 tends towards an increase in *COL2A1/COL10A1* ratio, but this did not reach significance (Fig. 7D). The expression of cartilage hypertrophy-associated markers, namely *COL10A1* and *MMP13* (Fig. 7C,H), were also similar between different groups, with no statistically significant differences. We further studied the genes associated to osteogenic differentiation, *RUNX2*, *IBSP* (Fig. 7I,K) and the balance of chondrogenesis and osteogenesis through *RUNX2/SOX9* ratio (Fig. 7J). Results showed no significant differences among groups of media and the use or not of fibronectin for *RUNX2* expression and *RUNX2/SOX9* ratio. The expression of *IBSP* (Fig. 7K) showed an increased trend for the group α MEM/FBS/FGF2 compared to XSFM, but it was not statistically significant.

Overall, the results from histological evaluations, as well as from gene expression analysis, showed that cells in a chemically defined medium displayed similar subsequent chondrogenic differentiation.

Non-responsive donor priming. In a separate experiment, we tested the effect of adding TGF- β 1 to the standard expansion medium on subsequent chondrogenesis, in the attempt to improve differentiation of poorly responsive cells. Specifically, poorly responsive cells were expanded in α MEM/FBS/FGF2 as previously described. However, in the final 5 days of expansion, TGF- β 1 was added either at increasing concentration (1 to 10 ng/ml) or at 10 ng/ml. Cell priming with gradual increase or constant concentration of TGF- β 1 during the expansion in monolayer in α MEM strongly improved chondrogenic differentiation (Fig. 8A) in terms of qualitative scoring, compared to cells expanded without the addition of TGF- β 1 in monolayer cultures (n = 2). These findings show that the use of TGF- β 1 during cell expansion can be beneficial for chondrogenesis and can have a pivotal role in priming non-responsive donors.

As previously observed by Saf-O/Fast-Green staining, BMSCs cultured with or without TGF- β 1 differently responded to chondrogenic differentiation in pellet culture. Indeed, the use of 1–10 ng/ml and 10 ng/ml TGF- β 1 significantly improved the yield of type collagen II production in non-responsive donors, specifically in the

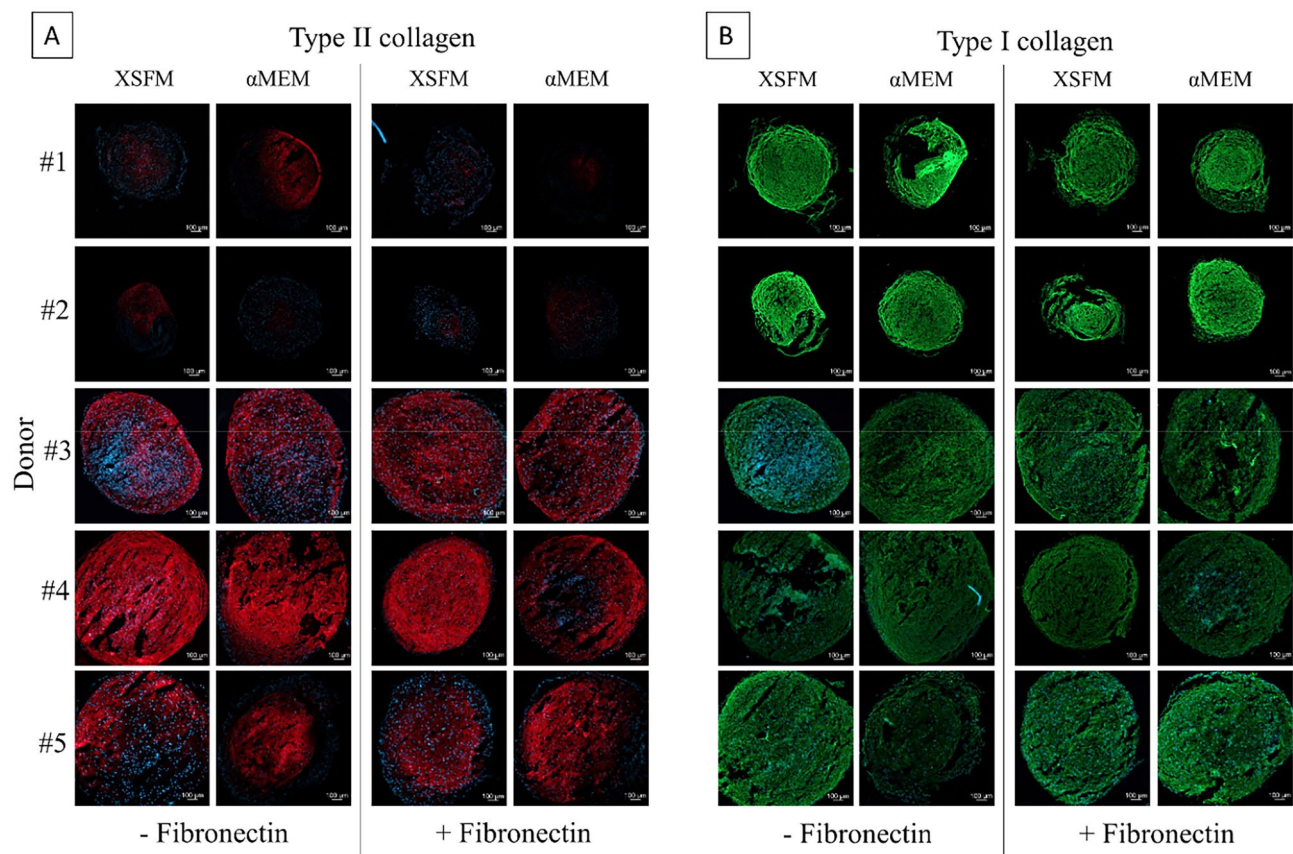


Figure 6. Immunohistological analysis of the chondrogenic potential of BMSCs expanded in different cell culture media ($n = 5$ donors). Pellet cryosections stained for type II (A) and type I collagen (B). The intensity of fluorescent secondary antibody is directly proportional to the production of different collagen content, while DAPI (blue) stains cell nuclei. Scale bar (bottom right in each picture) = 100 μm .

picture donor #1 is shown (Fig. 8B). Previously we observed (Fig. 6A,B) that the donors with a poor type II collagen deposition had a high type I collagen content; here the non-responsive donors primed using TGF- β 1 did not show a massive change in the type I collagen staining intensity, with a slight reduction in fluorescence intensity in the 10 ng/ml TGF- β 1 group (Fig. 8C).

Discussion

Human-derived mesenchymal stem cells (MSCs) are a promising cell source for clinical applications in regenerative medicine, due their relative ease of harvest²⁵. However, one of the most difficult tasks in cell manufacture is controlling their quality.

FBS has been used as a primary component of cell expansion media for decades^{26,27}, principally because it promotes adhesion and cellular expansion. However, due to several problems associated with batch-to-batch variability, uncontrolled composition that may vary from supplier to supplier, as well as the risk of zoonosis and transmissions of proteins not desired at the cellular level, its use is still challenging in a Good Manufacturing process (GMP) environment. One of the reasons why the application of MSCs in regenerative medicine appears to be limited is due to the non-standardized expansion protocols used in various labs. Methods involving the use of platelet lysates have been proposed²⁸, but also this alternative is affected by innumerable variables, mostly associated to the donor variation²⁹. In our study we have shown that the use of a chemically defined medium without FBS (XSFM) has the same performance as the gold standard medium containing FBS and 5 ng/ml FGF2 starting with the same condition of cells isolated and then cryopreserved in the presence of FBS. This suggests XSFM medium as a clinically relevant alternative.

Fibronectin coating has been proposed as a beneficial method to enhance MSC adhesion onto plastic surfaces³⁰. Fibronectin does not limit cell growth in XSFM medium; on the contrary, cells expanded in αMEM/FBS/FGF2 were negatively affected in terms of growth.

We investigated the expression of main chondrogenic markers after 21 days of differentiation, concluding that the use of XSFM medium did not modify the cell potency, indeed no significant difference in the expression of the main chondrogenic markers was observed compared with αMEM/FBS/ 5 ng/ml FGF2.

Duan et al. previously demonstrated that TGF- β and BMP rapidly mobilizes additional TGFBRs from intracellular stores to the cell surface, increasing the abundance of cell-surface TGFBRs in HaCaT and A549 cell lines, priming the downstream TGF- β signaling³¹. Based on this report and on our previous work on the TGFBR expression profile³², we investigated the potential of late addition of TGF- β 1 into the expansion medium as a

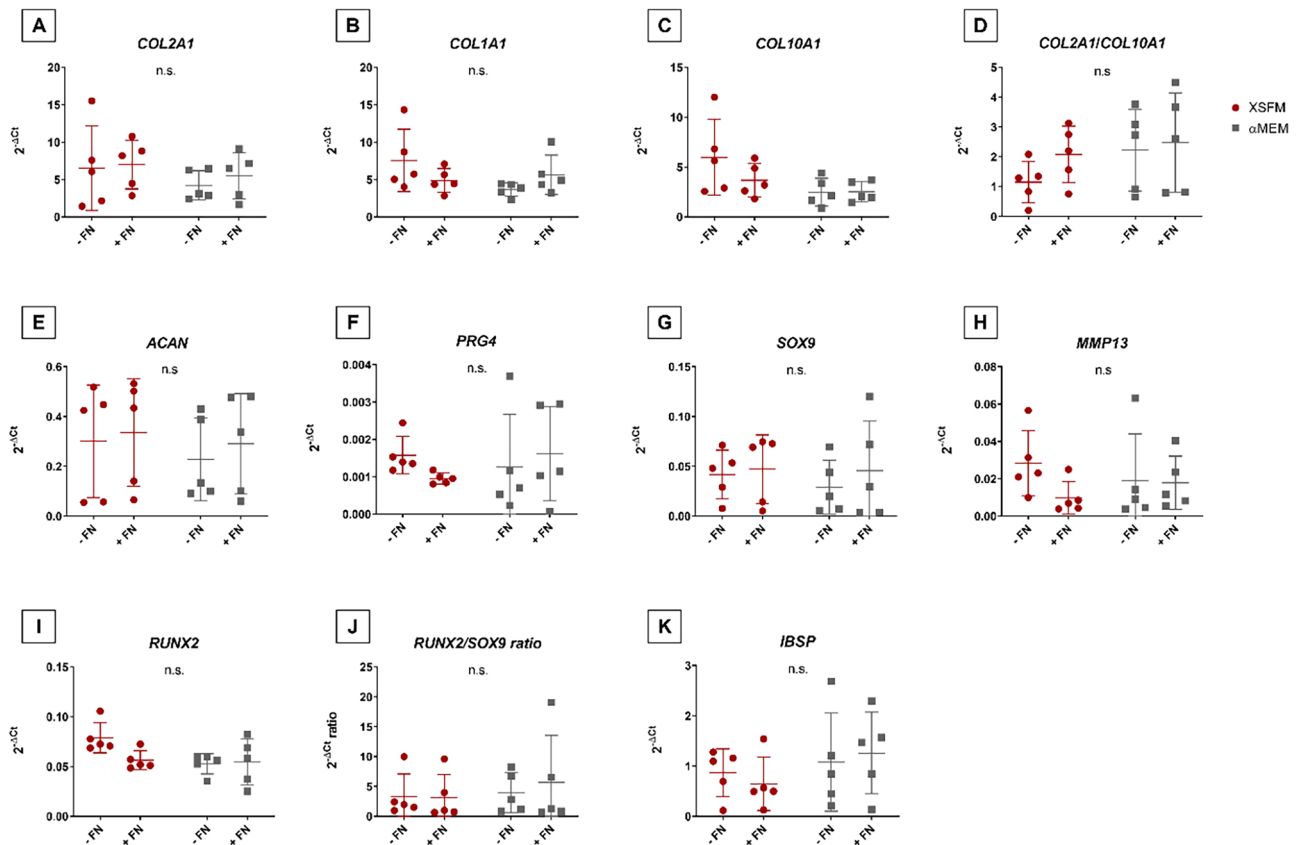


Figure 7. Culture conditions during monolayer expansion of BMSCs modulate differently the expression of chondrogenic markers during differentiation. After expansion in either α MEM/FBS/FGF2 or XFSM (with or without fibronectin coating), cells were cultured for 21 days in chondrogenic medium containing 10 ng/ml of TGF- β 1. The expression of (A) *COL2A1*, (B) *COL1A1*, (C) *COL10A1*, (D) *COL2A1/COL10A1* ratio, (E) *ACAN*, (F) *PRG4*, (G) *SOX9*, (H) *MMP13*, (I) *RUNX2*, (J) *RUNX2/SOX9* ratio and (K) *IBSP* were normalized to ribosomal protein large, P0 (*RPLP0*). The gene expression levels are represented as $2^{-\Delta Ct}$ (mean \pm SD; $n = 5$). Grey bars represent scatterplot of different donor measurements in BMSC cultured in α MEM, red bars represent scatterplot of different measurements in BMSC cultured in XFSM medium, FN is the presence (+) or absence (–) fibronectin coating during the BMSC expansion. Data are expressed as mean \pm SD.

means to improve chondrogenic differentiation. Poorly responsive cells, after expansion in our standard medium, were grown again in α MEM/FBS/FGF2 and in the final week of expansion either increasing concentrations of TGF- β 1 or a constant 10 ng/ml TGF- β 1 was added. We observed that there was a significant enhancement of chondrogenic differentiation in terms of matrix deposition. This enhancement was similar to that seen in previous studies using siRNA against *TGFBR2*³², thus offering a powerful method to modulate MSC phenotype that is easily applied. This also demonstrates that cell phenotype can be modified even in late stages of expansion. Therefore, TGF- β 1 addition during the expansion process can prime chondrogenic differentiation, probably by enhancing the downstream pathways associated with Small Mother Against Decapentaplegic (SMAD) proteins. Indeed, other studies have highlighted the role of TGF- β signaling associated with recruitment of the ALK5 (*TGFBR1*) receptor, with the consequent activation SMAD 2/3 pathway, while excess of TGF- β can lead to activation of ALK1 (*ACVRL1*), promoting the activation of SMAD 1/5/8³³. We know from previous studies that these receptors are present on MSCs and are essential for chondrogenesis³⁴.

Furthermore, it has been shown that increasing TGF- β concentration shifts the balance of ALK signaling from ALK5 (SMAD 2/3) at low dose to ALK1 (SMAD 1/5/8) at higher doses³⁵. Further analysis is ongoing to understand the possible mechanism during the priming of MSC and the role of TGF- β 1 in the expansion medium.

The main limitation of this study resides in the use of previously banked BMSCs. As described in the materials and methods section, our procedure of BMSC isolation is highly standardized to minimize manipulation-dependent sources of variation. The procedure involves the use of a tested batch of MSC-qualified FBS and the cryopreservation at Passage 1 in an FBS/DMSO solution. For this reason, this study was conducted with cells that were exposed to xenoderivatives during the isolation phase and first duplications in vitro. Future studies will be focused on analyzing more in detail the effect of different media composition during the isolation phase, where the presence of a fibronectin coating might be essential, and the overall performance of cells which were never treated with any animal-derived products.

In summary, this report describes how the use of xenogeneic serum-free medium during BMSC expansion provides the same performance compared to FBS containing medium with respect to chondrogenic commitment.

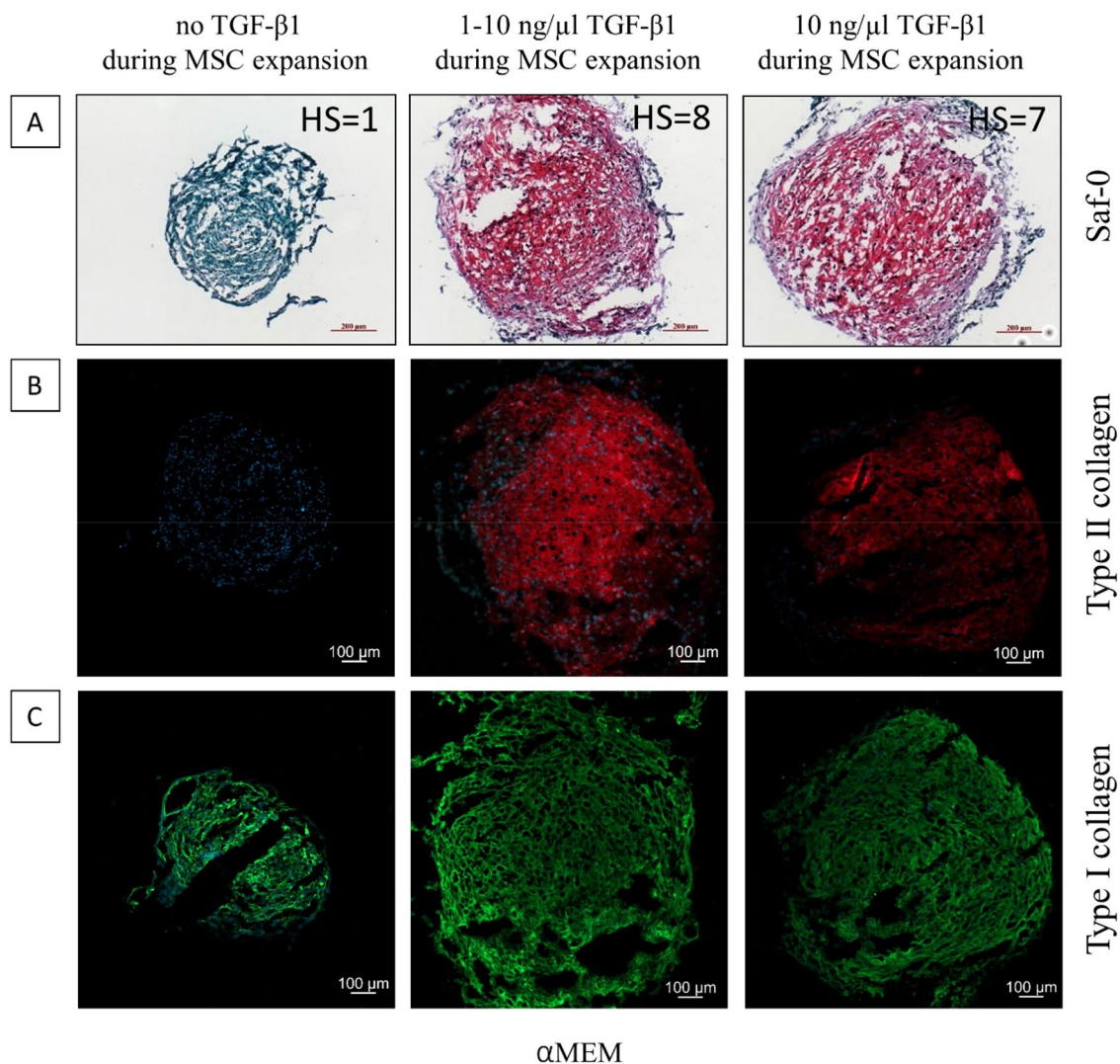


Figure 8. Representative images of Safranin-O/Fast Green staining on chondrogenic pellets of poorly responsive cells ($n=2$ donors); here it is shown donor #1 (A). BMSCs were cultured in α MEM/FBS/FGF2 with different concentrations of TGF- β 1 during cell expansion. The intensity of Safranin-O (red) staining is directly proportional to the proteoglycan content inside the pellet, while Fast Green counterstains collagen fibers. Cells in monolayer were expanded in the presence of increasing concentration of TGF- β 1 (1–10 ng/ml) or with constant 10 ng/ml of TGF- β 1 before the induction of chondrogenesis. All the cells were induced to chondrogenic differentiation using the same medium containing 10 ng/ml TGF- β 1. Each image includes the histological score (HS) assigned on the top right corner. Scale bar for $10\times$ Objective = $200\ \mu\text{m}$ (bottom-right in each panel). (B, C) Immunohistological analysis of the chondrogenic potential of BMSCs expanded in α MEM supplemented or not with 1–10 ng/ml or 10 ng/ml TGF β during MSC expansion. Pellet cryosections stained for type II (B) and type I (C) collagen. The intensity of staining is directly proportional to the collagen content, while DAPI (blue) stains cell nuclei. Scale bar (bottom left in each picture) = $100\ \mu\text{m}$.

Furthermore, the use of TGF- β 1 during the later stages of cell expansion strongly improves the yield of differentiation in those donors defined as less responsive to TGF- β 1 stimulation. With the vision of clinical translation, the use of serum-free medium could pave the way to future application in the clinical scenario by allowing chemically defined cell culture, thus limiting variations among different FBS batches. In addition, a xenogeneic serum-free medium avoids a potential risk for developing zoonosis in patients treated with cultured cells. The use of serum-free medium that has the same performance as the gold standard represents a step further for the use of cells, tissue engineered constructs and cell related products that can be suitable for the treatment of pathological and traumatic situations.

Methods

All methods were performed in accordance with the relevant guidelines and regulations.

Isolation and expansion of human mesenchymal stem cells from bone marrow. Bone marrow from 5 different donors was harvested from vertebral body after signed informed consent and full ethical approval (Ethic -Commission, Albert-Ludwigs-University Freiburg, Germany, AN-EK-135/14). Fresh bone marrow was diluted 1:4 and layered on top of Histopaque-1077 solution (Sigma-Aldrich), in a proportion of 2.6 ml of Histopaque per ml of undiluted marrow. After centrifugation at 800 g for 20 min, the mononuclear cell-containing interface was recovered, and cells were counted using the Cell Sceptor 2.0 Automated Cell Counter (Millipore). Isolated cells were seeded at a density of 50,000 cells/cm² into 300 cm² tissue culture flasks in Minimum Essential Medium Eagle, Alpha Modification (αMEM; Gibco, Thermo Fisher, Zürich, Switzerland) containing 10% batch-tested FBS for MSC expansion (PAN-Biotech, Aidenbach, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco), and 5 ng/ml recombinant human basic Fibroblast Growth Factor (FGF2, Fitzgerald Industries International, Acton, MA, USA). Cells were maintained at 37 °C in 5% CO₂, 95% humidity atmosphere. After 4 days, non-adherent hematopoietic cells were removed to select the BMSC population. Medium was then refreshed every 2nd day. Cells at passage 1 were frozen in 8% dimethyl sulfoxide (DMSO) and 92% FBS.

After thawing, and during the cell expansion phase, two different media were compared. One was αMEM medium containing 10% FBS and 5 ng/ml FGF2 described above for cell isolation (αMEM/FBS/FGF2), while the second was a chemically defined medium (PRIME-XV MSC Expansion XFSM, Fujifilm Irvine Scientific—hence XFSM). Where specified, tissue culture plastic was coated with a fibronectin solution (5 µg/ml fibronectin in PBS—Fujifilm Irvine Scientific, Santa Ana, CA, USA) according to the manufacturer's protocol.

In summary, BMSCs were expanded under the following conditions:

- αMEM/FBS/ 5 ng/ml FGF2
- αMEM/FBS/ 5 ng/ml FGF2 with fibronectin coating
- XFSM
- XFSM with fibronectin coating.

Cells were cultured until passage 3 in the different conditions. At each passage, the cells were counted, and their size measured using the Cell Sceptor 2.0 Automated Cell Counter (Millipore), which uses the Coulter principle for particle analysis. The average cell size (expressed as diameter) was recorded and analyzed as a quantitative morphological cell parameter.

In a separate experiment, αMEM/FBS/FGF2 medium was supplemented with the addition of increasing TGF-β1 concentrations (Fitzgerald, using 1 ng/ml for 48 h, 5 ng/ml for the following 48 h, then 10 ng/ml for the final 24 h) or constant 10 ng/ml TGF-β1 for the final 5 days before chondrogenic commitment, in order to improve chondrogenesis of BMSCs.

A schematic of the experimental design is represented in Supplementary Fig. 1.

Live/dead assay. Viability of cells seeded at 3000/cm² on glass chamber slides (Falcon) was determined by Live/Dead staining. After 72 h, cells were stained with 2 mM Calcein AM (Invitrogen, Thermo Fisher) and 4 mM ethidium homodimer-1 (EthD-1, Invitrogen, Thermo Fisher) in culture medium for 30 min and immediately evaluated by confocal microscopy (LSM800, Zeiss AG, Feldbach, Switzerland).

Cell counting and population doubling. Proliferation of BMSCs expanded according to different protocols was measured by counting cells with a Trypan Blue exclusion test after 24 h, 48 h, 72 h and 7 days after seeding at p.2. Briefly, cells were seeded at a density of 3000/cm² in 6-well tissue culture plates and cultured with αMEM or XFSM medium, with or without a fibronectin coating. After cell detachment with 0.05% Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco), the cells were counted in a Neubauer hemocytometer after a two-fold dilution in Trypan Blue.

The population doubling time (PDT) was calculated using the following formula:

$$PDT = (t_2 - t_1) \times \frac{\log 2}{\log \frac{N_2}{N_1}}$$

where *t* is time (hours), *N* is the number of cells, and *N*₂ and *N*₁ represent the number of cells at *t*₂ (72 h) and *t*₁ (24 h), respectively. The PDT was calculated in the range 24–72 h for the cells to be in the exponential phase of cell growth.

At 72 h, cell cultures were imaged for a qualitative assessment of cell morphology.

Chondrogenic differentiation. Chondrogenic differentiation of BMSCs was performed in 3D pellet culture. 2 × 10⁵ BMSCs per pellet were seeded in V-bottom 96-well plates (Corning, Corning, NY, USA). Cells were centrifuged for 5 min at 400 g in either chondropermissive (for undifferentiated control) or chondrogenic medium. Chondropermissive medium contained Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/l glucose (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco), 1% non-essential amino acids (Gibco), 1% ITS+ (Corning); chondrogenic medium was obtained with the addition of 100 nM dexamethasone (Sigma-Aldrich, Buchs, Switzerland), 10 ng/ml TGF-β1 (Fitzgerald Industries International), and 50 µg/ml ascorbic acid-2 phosphate (Sigma-Aldrich). The medium was replaced every second day and pellets were harvested for further analyses after 21 days.

Real-time quantitative PCR analysis. Total RNA was isolated from adherent hBMSC cells during passaging and from 3D chondrogenic pellets at day 0 (cells before cell seeding) and after 21 days of differentiation using TRI Reagent Solution (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. RNA quantity was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher). For reverse transcription, TaqMan Reverse Transcription reagents (Applied Biosystems, Thermo Fisher) were used. The reverse transcription reaction was initiated at 25 °C for 10 min, followed by 30 min at 42 °C and ended by 5 min at 85 °C. qPCR reactions were set up in 10 µl reaction mixtures containing TaqMan Gene Expression Master Mix (Applied Biosystems, Thermo Fisher), gene-specific assays (Supplementary Table 1), Diethyl pyrocarbonate (DEPC)-H₂O and 10 ng of cDNA template. qPCR analysis was performed in a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher). The reaction program was set up as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s followed by an annealing/extension step at 60 °C for 1 min. Triplicates were used for each target gene (technical replicates). The relative expression of *SOX9*, *ACAN*, *MMP13*, *COL2A1*, *COL10A1*, *COL1A1*, *PRG4*, *SOX9*, *RUNX2* and *IBSP* during chondrogenic differentiation was determined using the $2^{-(\Delta\Delta Ct)}$ method, with ribosomal protein large, P0 (*RPLP0*) as reference gene and day 0 RNA as the calibrator sample.

Histological staining. *Safranin-O/ fast green.* At day 21, pellets were fixed in 70% methanol, then transferred to sucrose-PBS 5% solution overnight, before being embedded in cryocompound (Thermo Fisher). Cryosections were cut with a thickness of 8–10 µm. For Safranin-O staining, samples were first stained with Weigert's Haematoxylin for 10 min, followed by 6 min with Fast Green and 15 min with Safranin-O solutions. After dehydration with increasing concentrations of ethanol and xylene, samples were coverslipped with Eukitt mounting medium and dried overnight.

The histological sections were observed using Zeiss AxioPlan 2 Microscope (Zeiss Microscopy GmbH, Jena, Germany) with objective 10X/0.50. Pictures were acquired using RGB camera 1X (16 bit) and AxioCam Software (Zeiss Microscopy GmbH, Göttingen, Germany). Grading of the safranin O staining was on a scale of 1–10 as assessed according with the Bern Score²⁴.

Immunofluorescence. Cryosections were washed for 10 min with dH₂O to remove the cryocompound, transferred to absolute methanol for 20 min and washed twice in 0.01% Tween 20 in PBS (PBS-T). Enzymatic treatment by 1U/ml Hyaluronidase (Sigma-Aldrich, H3506) and 0.25 U/ml Chondroitinase ABC (Sigma-Aldrich, C2905) in PBS-T for 30 min at 37 °C allowed the digestion of matrix. After washing in PBS-T, pellets were transferred in blocking solution containing 5% horse serum (Vector laboratories, S-2000) in PBS-T for 30 min at room temperature. Primary antibody anti-type II collagen (4 µg/mL, CIICI, see acknowledgement section) and anti-type I collagen (1:400, Origene Acris, R1038) were added over night at 4 °C. Slides were washed with PBS-T, then the secondary antibodies were added, 4 µg/ml anti Rabbit Alexa Fluor 488 against type I collagen (Thermo Fisher, A-11008) and 5 µg/ml anti Mouse Alexa Fluor 660 against type II collagen (Thermo Fisher, A-21055) for 1 h at 37 °C. After washing with PBS-T, the nuclei were counterstained with 2-(4-Amidinophenyl)-1H-indole-6-carboxamide (DAPI) 2.5 µg/mL and then cover slipped with ProLong mounting solution (Thermo Fisher, P10144).

Statistical analysis. A total number of 5 donors were used for chondrogenesis experiments, while 4 were used for cell counting/population doubling calculations. Statistical analysis was performed using GraphPad Prism 7.03 software. Non-parametric two-way Analysis of Variance (ANOVA) with Tukey's multiple comparison test were applied, with $p < 0.05$ considered as statistically significant. A two-way ANOVA was used to evaluate the distributions and homogeneity variance in the groups.

Data availability

All data and materials used in the analysis are available to any researcher for purposes of reproducing or extending the analysis.

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Author contributions

V.B.—Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. E.D.B.—collection and assembly of data, data analysis and interpretation, manuscript writing. J.K.—Provision of study material or patients. M.A.—Financial support, final approval of manuscript. M.J.S.—Conception and design, financial support, final approval of manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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