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RESEARCH ARTICLE

Micro-aggregates do not influence bone marrow stromal cell chondrogenesis

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Abstract

Although bone marrow stromal cells (BMSCs) appear promising for cartilage repair, current clinical results are suboptimal and the success of BMSC-based therapies relies on a number of methodological improvements, among which is better understanding and control of their differentiation pathways. We investigated here the role of the cellular environment (paracrine vs juxtacrine signalling) in the chondrogenic differentiation of BMSCs. Bovine BMSCs were encapsulated in alginate beads, as dispersed cells or as small micro-aggregates, to create different paracrine and juxtacrine signalling conditions. BMSCs were then cultured for 21 days with TGFβ_3 added for 0, 7 or 21 days. Chondrogenic differentiation was assessed at the gene (type II and X collagens, aggrecan, TGFβ, sp7) and matrix (biochemical assays and histology) levels. The results showed that micro-aggregates had no beneficial effects over dispersed cells: matrix production was similar, whereas chondrogenic marker gene expression was lower for the micro-aggregates, under all TGFβ conditions tested. This weakened chondrogenic differentiation might be explained by a different cytoskeleton organization at day 0 in the micro-aggregates.

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Keywords bone marrow stromal cells; mesenchymal stem cells; chondrogenesis; cell–cell interactions; micro-aggregates; hydrogel

1. Introduction

Articular hyaline cartilage only possesses a limited self-repair capacity. Most tissue damage, caused either by wear and tear or trauma, will not be healed but replaced by fibrocartilage. This tissue has inferior biochemical and biomechanical properties compared to native hyaline cartilage, altering the function of the joint and ultimately leading to severe pain (Ahmed and Hincke, 2010; Nesic *et al*., 2006). Surgical approaches are commonly proposed to promote the healing of cartilage damage. They present, however, several limitations linked to the cell/tissue source and may lead to the formation of fibrocartilage rather than hyaline cartilage (Ahmed and Hincke, 2010; Khan *et al*., 2010). Alternative sources of cells/tissues are therefore needed to regenerate cartilage damage.

One of the most promising sources is bone marrow stromal cells (BMSCs) (Gregory *et al*., 2005; Khan *et al*., 2010; Krampera *et al*., 2006; Prockop, 1997). As these cells are isolated from the bone marrow, no cartilage tissue harvesting is required and the tissue source is exempt from degenerative cartilage disease. BMSCs also possess a high proliferative rate, allowing the regeneration of large defects. Many studies have established that BMSCs can differentiate *in vitro* into chondrocytes (Muraglia *et al*., 2000; Halleux *et al*., 2001; Pittenger *et al*., 1999). The patient's condition can affect BMSC proliferation and differentiation: age and osteoarthritis have been reported to reduce the chondrogenic potential of BMSCs (Murphy *et al*., 2002); although other studies report that these factors do not influence BMSC chondrogenesis (Dudics *et al*., 2009; Scharstuhl *et al*., 2007). BMSCs have been used to repair cartilage lesions in numerous animal models

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(Guo *et al*., 2004; Uematsu *et al*., 2005) but also in humans (Wakitani *et al*., 2002; Nejadnik *et al*., 2010). Although the results are promising, the repair tissues were not completely composed of hyaline cartilage (Matsumoto *et al*., 2010). However, it is the general belief that, with further advancements, BMSC-based therapies will eventually be helpful in clinics.

One important direction for improvement is to better understand and control the differentiation pathway leading BMSCs to fully differentiated and functional chondrocytes. For many years, BMSC differentiation has been induced by various cocktails of biochemical factors (Augello and De, 2010) and more and more evidence indicates that their biomechanical environment can control their differentiation (Potier *et al*., 2010). Other than applied exogenous stimulation, direct communication of cells with their environment can also affect their behaviour. For example, cells can respond to different substrate stiffness, for BMSCs, by adapting their differentiation pathways (Engler *et al*., 2006; Pek *et al*., 2010) or, for chondrocytes, their chondrogenic phenotype (Sanz-Ramos *et al*., 2013; Schuh *et al*., 2010). However, so far, few studies have focused on the relationship between cell–cell communication and BMSC differentiation, when adhesion of cells to each other may also provide important clues to control BMSCs, as shown with the osteogenic differentiation pathway (Tang *et al*., 2010).

The aim of this study was, therefore, to modulate the cell–cell interactions between BMSCs and evaluate the impact on BMSC *in vitro* chondrogenesis. In order to create different cell–cell interactions, BMSCs were seeded into hydrogel either as dispersed cells, where interactions rely on paracrine signalling, or as micro-aggregates, where interactions rely on paracrine and juxtacrine signalling. Micro-aggregates, rather than micromass, were used to promote cell–cell contact locally. Indeed, it has been shown that micromass culture, used to mimic the condensation phenomenon of mesenchymal cells during development (Bobick *et al*., 2009), leads to heterogeneous distribution of the cartilaginous matrix (Barry *et al*., 2001; Mackay *et al*., 1998; Schmitt *et al*., 2003; Murdoch *et al*., 2007), most likely due to mass transport limitations within the micromass. Downscaling from micromass (200 000–250 000 cells) to micro-aggregates (50–300 cells) should overcome these mass transport issues. In fact, micro-aggregate culture has already been shown to be superior to micromass for BMSC chondrogenesis, with a more homogeneous differentiation and matrix deposition observed (Markway *et al*., 2010). Finally, the effects of cell–cell interactions on BMSC chondrogenesis could be attenuated by the presence of exogenous growth factors (e.g. TGFβ_3) in the culture medium. We therefore used different patterns of TGFβ stimulation (0, 7 or 21 days) to assess the influence of different cellular environments [dispersed cells (DC) vs micro-aggregates (MA)] on BMSC chondrogenesis.

2. Materials and methods

2.1. Bovine BMSC isolation and expansion

Bovine BMSCs were isolated from three cows (8–12 months old, all skeletally immature), in accordance with local regulations. Bone marrow was aspirated from the pelvis and immediately mixed 1:1 with high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium (hgDMEM; Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml heparin (Sigma, Zwijndrecht, The Netherlands) and 3% penicillin–streptomycin (Lonza, Basel, Switzerland). Bone marrow samples were then centrifuged (300 × *g*, 5 min) and resuspended in growth medium: hgDMEM +10% fetal bovine serum (FBS; Gibco Invitrogen; batch selected for BMSC growth and differentiation) +1% penicillin–streptomycin. BMSCs were isolated by adhesion (Friedenstein *et al*., 1970; Kon *et al*., 2000; Potier *et al*., 2007). Cells were seeded in flasks (using 7–10 ml medium:bone marrow mix per 75 cm^2) and, after 4 days, the medium was changed. BMSCs were then expanded up to P1 (passage at 5000 cells/cm^2) before freezing [70–80% confluence; in 90% FBS/10% dimethylsulphoxide (Sigma)]. A fresh batch of BMSCs was thawed and cultured up to P4 for each experiment (each passage at 5000 cells/cm^2). Cells from each donor were cultured separately. Bovine BMSCs (n = 4) isolated and expanded following these protocols showed successful chondrogenesis using the micromass approach (Johnstone *et al*., 1998) (as shown with safranin O staining).

2.2. Production of agarose chips

Custom-made PDMS stamps, with a microstructured surface consisting of 2865 rounded pins with a diameter of 200 μm and a spacing of 100 μm, were produced. The stamps were sterilized with alcohol and placed in a six-well plate, microstructured surface up. Warm ultra-pure agarose solution [Gibco Invitrogen; 3% in phosphate-buffered saline (PBS)] was poured on the stamps, centrifuged for 1 min at 2500 rpm and incubated for 30 min at 4°C. The agarose chips were then separated from the stamps, cut to size to fit in a well of a 12-well plate, covered with PBS and kept at 4°C until use (Rivron *et al*., 2012).

2.3. Formation of micro-aggregates and alginate seeding

At passage 5, BMSCs were used to seed: (a) alginate beads (dispersed cells; DC); or (b) agarose chips (micro-aggregates; MA) (Figure 1). For the DC condition, BMSCs were resuspended in 1.2% sodium alginate (Sigma) solution (in 0.9% NaCl; Merck, Darmstadt, Germany) at a concentration of 7×10^6 cells/ml. The cell + alginate suspension was slowly forced through a 22G needle and added dropwise to a 102 mM $CaCl_2$ (Merck) solution

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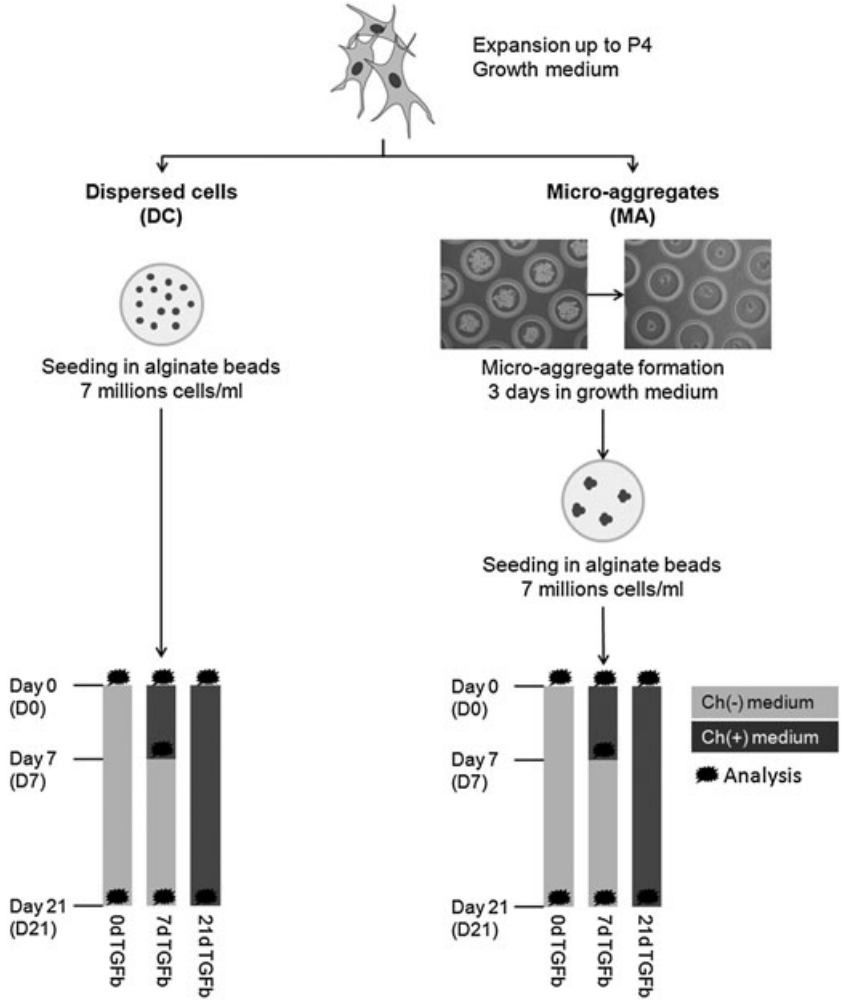

Figure 1. Experimental design. Bovine BMSCs (n = 3) were expanded up to P4 in hgDMEM + 10% FBS + 1% P/S (growth medium). Cells were then used to seed either alginate beads at 7 million cells/ml (dispersed cells; DC) or agarose chips cast on PDMS stamps (micro-aggregates; MA). BMSCs on agarose chips were cultured for 3 additional days in growth medium to allow the cells to form micro-aggregates. Those were then used to seed alginate beads at 7 million cells/ml. After seeding in alginate, BMSCs (DC or MA) were cultured for 3 weeks in hgDMEM + 1% P/S + 0.1 μM dexamethasone + 1% ITS-1$^+$ + 1.25 mg/ml BSA + 50 μg/ml ascorbic acid 2-phosphate + 40 μg/ml L-proline + 100 μg/ml sodium pyruvate (Ch$^-$ medium). This medium was supplemented with 10 ng/ml TGFβ_3 (Ch$^+$ medium) for 0, 7 or 21 days. At D0 and D21, cell viability was characterized by live/dead staining; cell morphology by histology (phalloidin, anti-vinculin and anti-pan-cadherin staining); produced matrix by histology (Alcian blue staining); biochemical assays [glycosaminoglycan (GAG) and DNA content]; and cell phenotype was characterized by qRT–PCR (types II and X collagens, sox9, aggrecan, TGFβ and sp7)

(Guo *et al*., 1989; Jonitz *et al*., 2011). Beads were incubated for 10 min at 37°C to polymerize and were then rinsed three times in NaCl 0.9% and twice in hgDMEM + 1% penicillin–streptomycin. For the MA condition, BMSCs were resuspended in growth medium at 2×10^5 cells/ml and 750 μl cell suspension was used per agarose chip (with PBS previously removed) to produce the micro-aggregates. Seeded chips were centrifuged for 1 min at $200 \times g$ to force the cells to the bottom of the microwells; 3 ml growth medium was then slowly added and the cells were cultured for an additional 3 days in growth medium to allow cell aggregation. Micro-aggregates were then collected, flushing the agarose chips with growth medium, and used to seed alginate beads at a final concentration of 7×10^6 cells/ml, as described for the DC condition.

2.4. Culture

Seeded beads (with either DC or MA) were cultured for 3 weeks in Ch$^-$ medium [hgDMEM + 1% penicillin–streptomycin + 0.1 μM dexamethasone (Sigma) + 1% ITS-1$^+$ (Sigma) + 1.25 mg/ml bovine serum albumin (BSA; Sigma) + 50 μg/ml ascorbic acid 2-phosphate (Sigma) + 40 μg/ml L-proline (Sigma) + 100 μg/ml sodium pyruvate (Gibco Invitrogen)] (Mackay *et al*., 1998). This medium was supplemented with 10 ng/ml (TGFβ_3; (Peprotech, Rocky Hill, NJ, USA) (Barry *et al*., 2001) (Ch(+) medium) for 0, 7 or 21 days (Figure 1). BMSCs were cultured under 5% CO_2 and 2% O_2 (Markway *et al*., 2010); six beads/well, of a six-well plate containing 3 ml medium, were cultured.

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2.5. Cell viability

At days 0 and 21, beads (n = 3 beads/donor/group) were washed in PBS and incubated in 10 μM calcein AM (Sigma)/10 μM propidium iodide (Gibco Invitrogen) solution (in PBS) for 1 h at 37°C. Cells were then imaged in the centres of the beads at a depth of 200 μm, using a confocal microscope (CLSM 510 Meta, Zeiss, Sliedrecht, The Netherlands).

2.6. Cell morphology and adhesion

At days 0 and 21, beads (n = 3 beads/donor/group) were embedded in cryo-compound (Tissue-Tek® OCT™; Sakura, Alphen aan den Rijn, The Netherlands) and snap-frozen in liquid nitrogen; 50 μm-thick cryosections were cut in the middle of the beads. The sections were then thawed, fixed for 30 min at room temperature (RT) in buffered formalin 3.7% (Merck), rinsed in PBS and incubated for 5 min at RT in Triton 1.5% in PBS. The sections were rinsed in PBS and stained with TRITC–phalloidin (Sigma; 1 μM in PBS + 1% BSA) for 2 h at RT. The sections were then rinsed in PBS, counterstained with DAPI for 15 min at RT (Sigma; 100 ng/ml in PBS), rinsed in PBS and MilliQ water, air-dried and mounted in Entellan (Merck). The stained sections were observed using a confocal microscope.

Morphometric analyses to determine cluster areas and numbers of cells/cluster were conducted on these images, using Zen 2012 software (Zeiss). For each group, 25 clusters or cells were analysed. Stained clusters or cells were manually outlined and the corresponding area determined. Cell numbers/cluster were also counted manually.

Immunostaining for vinculin and pan-cadherin was conducted on 10 μm-thick cryosections. The sections were thawed, fixed for 10 min at RT in buffered formalin 3.7%, rinsed in PBS and incubated for 10 min at RT in Triton 0.5% in PBS. After blocking in 3% BSA for 1 h, the sections were incubated for 1 h at RT with monoclonal mouse anti-vinculin antibodies (Sigma), diluted at 1:400, or with monoclonal mouse anti-cadherin antibodies (Abcam; Cambridge, UK), diluted at 1:100, in 3% BSA. The sections were then washed three times in PBS and incubated for 1 h at 38°C with Alexa 488-conjugated goat anti-mouse antibodies (Molecular Probes; Bleiswijk, The Netherlands), diluted 1:300 in PBS. The stained sections were then rinsed three times and mounted with Mowiol. For both stainings, human cardiomyocyte progenitor cells grown on coverslips were used as a positive control. Both antibodies are known to work with bovine material.

2.7. Cartilaginous matrix formation and cell proliferation

At days 0 and 21, five beads/donor and group were pooled and digested in papain solution [150 mM NaCl (Merck), 789 μg/ml L-cysteine (Sigma), 5 mM $Na_2EDTA{\cdot}2H_2O$ (Sigma), 55 mM $Na_3citrate{\cdot}2H_2O$ (Sigma) and 125 μg/ml papain (Sigma)] at 60°C for 16 h. Digested samples were then used to determine their content of sulphated glycosaminoglycans (sGAG), as a measure of proteoglycans, and DNA. sGAG content was determined using the dimethyl methylene blue (DMMB) assay, adapted for alginate presence (Enobakhare *et al*., 1996). Shark cartilage chondroitin sulphate (Sigma) was used as a reference and digested with empty alginate beads (i.e. alginate concentration identical for references and experimental samples). DNA content was measured using the Hoechst dye method (Cesarone *et al*., 1979), with a calf thymus DNA reference (Sigma). For the 7 days of TGFβ_3-treatment group, the beads were also analysed at day 7.

At days 0 and 21, beads (n = 3 beads/donor/group) were also embedded in cryo-compound and snap-frozen in liquid nitrogen. 10 μm thick cryosections were cut in the middle of the beads. The sections were then thawed, incubated for 5 min in 0.1 M $CaCl_2$ at RT and fixed in buffered formalin 3.7% for 3 min at RT. The sections were then rinsed in 3% glacial acetic acid (Merck) and stained in Alcian blue solution (Sigma; 1%, pH 1.0, for alginate presence) for 30 min at 37°C. The sections were then rinsed in 0.05 M $CaCl_2$ and counterstained with nuclear fast red solution (Sigma) for 7 min at RT. The stained sections were rinsed in 0.05 M $CaCl_2$ before mounting in Mowiol (Merck) and were observed using a brightfield microscope (Observer Z1, Zeiss).

2.8. Gene expression

At days 0 and 21, nine beads/donor and group were pooled, snap-frozen in liquid nitrogen and stored at –80°C until RNA isolation. Frozen beads were placed in between a 316 SS 8 mm bead and a custom-made lid, placed in a 2 ml Eppendorf tube, and were disrupted for 30 s at 1500 rpm (Micro-dismembrator; Sartorius, Göttingen, Germany). RNA was then extracted using TRIzol® (Gibco Invitrogen) and purified using an RNeasy mini-kit (Qiagen, Venlo, The Netherlands). The quantity and purity of the isolated RNA were measured by spectrophotometry (ND-1000, Isogen, De Meern, The Netherlands) and integrity by gel electrophoresis. Absence of genomic DNA was validated by end-point PCR and gel electrophoresis using primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Total RNA (300 ng) was then reverse-transcribed (M-MLV; Gibco Invitrogen) and the gene expression levels of *sox9*, aggrecan, type II collagen, *TGFβ*, type X collagen and *sp7* (also known as *Osterix*) were assessed with SYBR green qPCR (iCycler; Biorad, Hercules, CA, USA) (see Table 1 for primer list). *18S* (PrimerDesign Ltd, Southampton, UK) was selected as a reference gene from three genes (*RPL13A*, *GAPDH* and *18S*) as the most stable gene throughout our experimental conditions. Expression

Table 1. Primer sequences for target and reference genes used in RT–qPCR assays

Gene	Accession No.*	Sequence 5′–3′	Product size (bp)	Source**
RPL13a	NM_001076998	CTGCCCCACAAGACCAAG TTGCGAGTAGGCTTCAGAC	140	BD
GAPDH	NM_001034034	GGCGTGAACCACGAGAAGTATAA CCCTCCACGATGCCAAAGT	119	van Dijk *et al*. (2011)
SOX9	AF278703	ACGCCGAGCTCAGCAAGA CACGAACGGCCGCTTCT	70	Shintani *et al*. (2007)
COL2A1	NM_001113224	TGGCTGACCTGACCTGAC GGGCGTTTGACTCACTCC	187	BD
ACAN	NM_173981	CCAACGAAACCTATGACGTGTACT GCACTCGTTGGCTGCCTC	107	Zeiter *et al*. (2009)
TGFB1	NM_001166068	CTGAGCCAGAGGCGGACTAC TTGCTGAGGTAGCGCCAGGAATTG	259	Karcher *et al*. (2008)
COL10A1	NM_174634	TGAGCGATACCAAACACCTACAG ACCTTTACCCTTTATGGCATACGG	91	BD
SP7	NM_001102142	CAAAGCAGGCACAAAGAAG GAGGGTAGTCATTGGCATAG	161	BD

RPL13a, ribosomal protein L13a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SOX9, SRY (sex determining region Y)-box 9; COL2A1, collagen type II α1; ACAN, aggrecan; TGFB1, transforming growth factor-β1; COL10A1, collagen type X α1; SP7, Sp7 transcription factor.
*GenBank™ accession number.
**BD, primers designed with Beacon designer software (Premier Biosoft, Palo Alto, CA, USA) and ordered from Sigma.

of the gene of interest is reported as relative to 18S expression ($2^{-\Delta CT}$ method). When gene expression was not detected, the $2^{-\Delta CT}$ value was set to 0 to conduct the statistical analysis. For the 7 days of TGFβ_3 treatment group, beads were also analysed at day 7.

2.9. Statistical analysis

General linear regression models based on ANOVAs were used to examine the effects of seeding (DC and MA), TGFβ treatment (0, 7 and 21 days) and days of culture

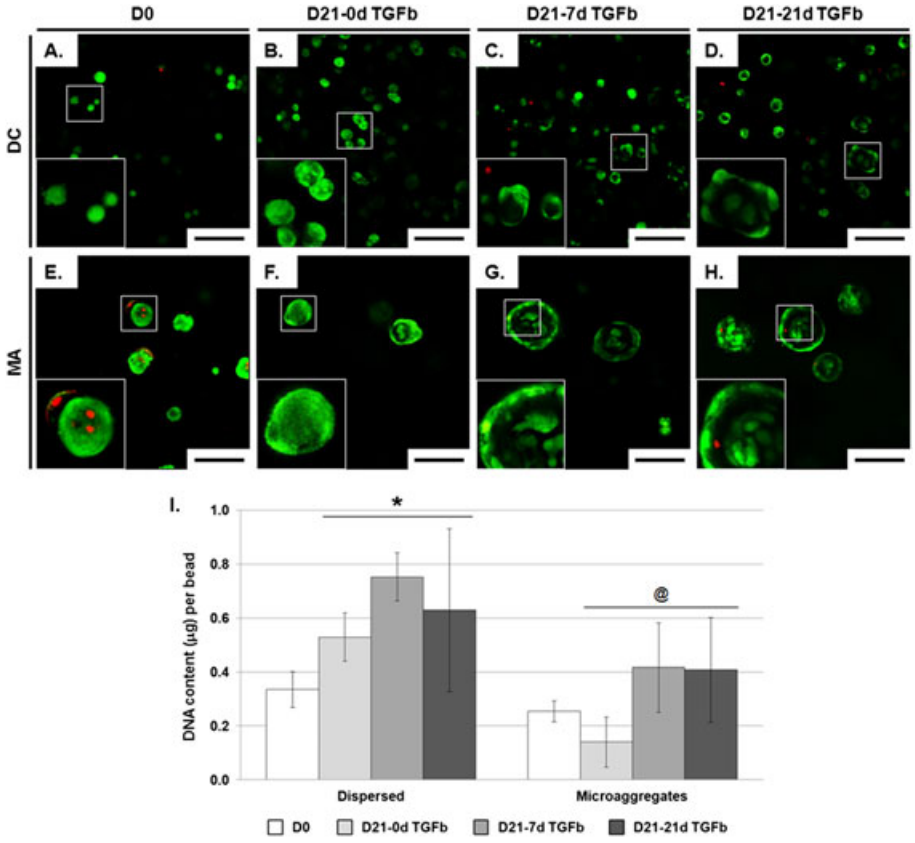

Figure 2. Cell viability and proliferation. (A–H) Bovine BMSCs seeded in alginate beads as dispersed cells (A–D) or as micro-aggregates (E–H) at days 0 (A, E) and 21 after exposure to TGFβ_3 for 0 (B, F), 7 (C, G), and 21 (D, H) days. Cells were stained with calcein AM (green fluorescence) for living cells and propidium iodide (red fluorescence) for dead cells. White frames are ×2.5 digital magnification; representative of three donors/group; scale bar = 100 µm; colour images are available online. (I) DNA content/bead, as determined with Hoechst dye assay; values are mean ± SD; n = 3/group. $^{*}p < 0.05$ vs D0; $^{@}p < 0.05$ vs dispersed cells

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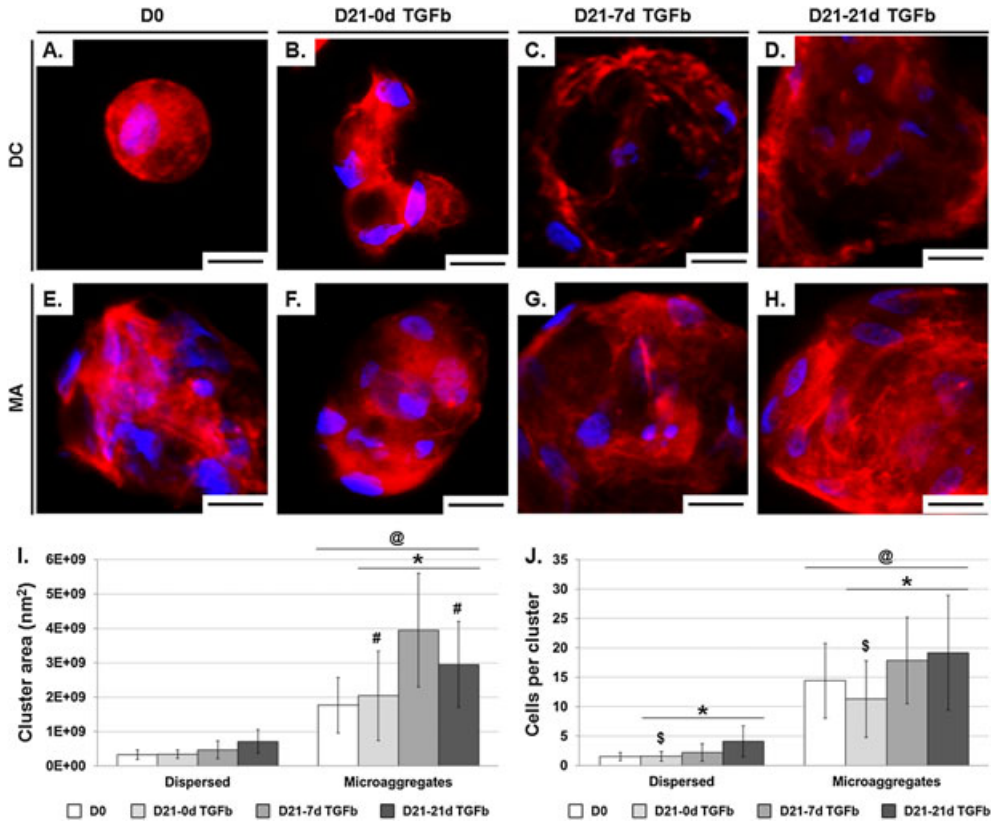

Figure 3. Cell morphology. (A–H) Bovine BMSCs seeded in alginate beads as dispersed cells (A–D) or micro-aggregates (E–H) at day 0 (A, E) and day 21 after exposure to TGFβ_3 for 0 (B, F), 7 (C, G) and 21 (D, H) days. The beads were cryosectioned, fixed and stained with phalloidin (red fluorescence) for F-actin filaments and counterstained with DAPI (blue fluorescence) for cell nuclei; representative of three donors/group; scale bar = 20 μm; colour images available online. (I, J) Morphometric analysis: area covered by cells or clusters (I) and cell number/cluster (J) were determined by image analysis; values are mean ± SD; n = 25 clusters/cells analysed/group. $^{*}p < 0.05$ vs D0; $^{\#}p < 0.05$ vs 7 days of TGFβ_3 treatment; $^{\$}p < 0.05$ vs 21 days of TGFβ_3 treatment; $^{@}p < 0.05$ vs dispersed cells

(days 0, 7 and 21) and their interactions on the variables DNA and GAG/DNA contents, and *sox9*, type II collagen, aggrecan, *TGFβ*, type X collagen and *sp7* gene expression. In all analyses, full factorial models were fitted to the data and then a backwards stepwise procedure was used to remove the non-significant effects. For each significant effect, a Tukey–HSD *post hoc* test was conducted; $p < 0.05$ was considered significant. All data analyses were performed in R v. 2.9.0 (R Development Core Team, 2009).

3. Results

3.1. Cell viability and proliferation

BMSCs showed high cell viability after seeding for all conditions (Figure 2A, E). At day 0, cells appeared as a well-dispersed cell population for DC conditions (Figure 2A) or as dense micro-aggregates for MA conditions (Figure 2E). However, some dead cells could be observed around the micro-aggregates (Figure 2E), most likely due to a higher shear stress exerted on micro-aggregates than dispersed cells when producing the alginate beads. At day 21, cell viability remained high for all conditions (Figure 2B–D, F–H). DNA content confirmed that cells proliferated (Figure 2I). DC conditions, however, led to higher proliferation than MA conditions.

3.2. Cell morphology and adhesion

At day 0, the DC condition led to single dispersed cells (Figure 3A, I), while MA resulted in large clusters (Figure 3E, I) containing, on average, 14 cells (Figure 3J). In DC conditions, after 21 days of culture BMSCs proliferated (Figure 3J) and formed clusters (Figure 3B–D) whose size increased when TGFβ was added, although not significantly (Figure 3I). In MA conditions (Figure 3F–H), micro-aggregates grew during culture, with the bigger clusters observed for 7 days of TGFβ (Figure 3I), although cell proliferation was limited (Figure 3J).

At day 0, cell–cell interactions were more developed in MA than in DC, as shown by the immunostaining of pan-cadherins (Figure 4D and A, respectively), which are glycoproteins involved in cell–cell adhesion. These improved cell–cell interactions, however, disappeared after 3 weeks of culture (Figure 4E). TGFβ treatment had no effects on cadherin expression for either DC or MA (data not shown). Regarding vinculin, a membrane-cytoskeletal protein involved in cell–matrix adhesion, its expression was similar for DC and MA (Figure 4G and J,

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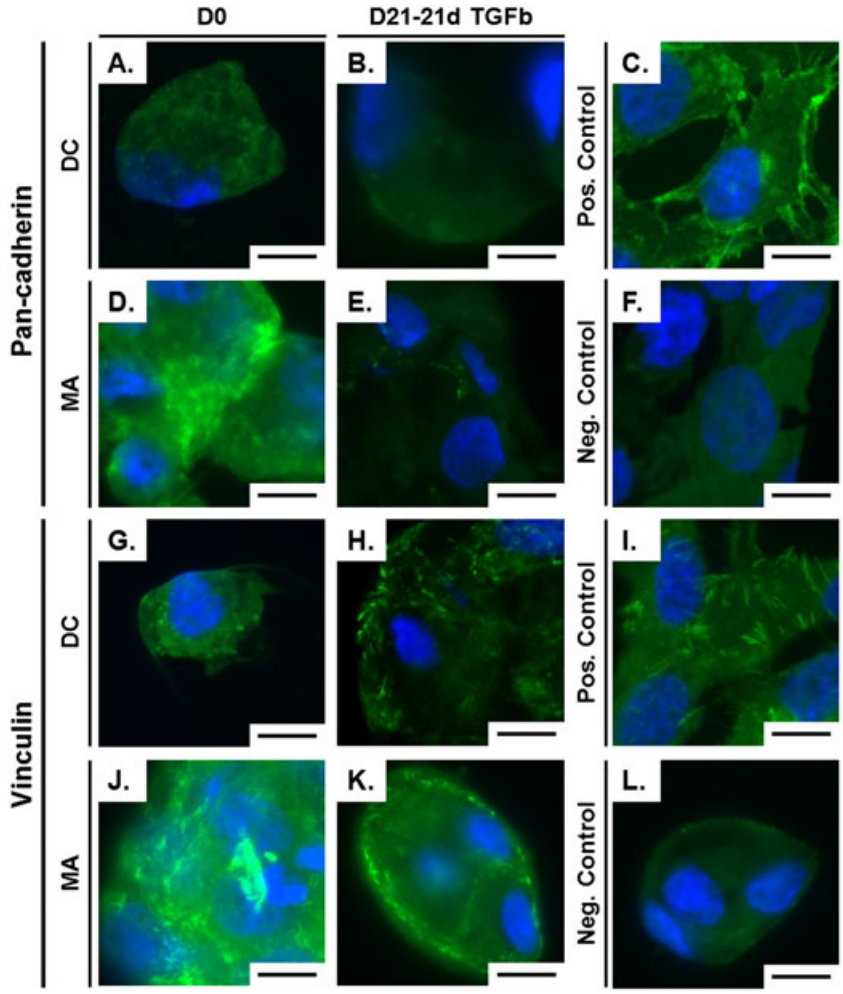

Figure 4. Cell adhesion. (A–F) Bovine BMSCs seeded in alginate beads as dispersed cells (A, B) or as micro-aggregates (D, E) at day 0 (A, D) and after 21 days of exposure to TGFβ_3 (B, E). The beads were cryosectioned, fixed and stained with anti-pan-cadherin (green fluorescence) and counterstained with DAPI (blue fluorescence) for cell nuclei. Human cardiomyocyte progenitor cells were used as positive controls (C) and experimental samples for secondary antibody negative control (F). (G–L) Bovine BMSCs seeded in alginate beads as dispersed cells (G, H) or as micro-aggregates (J, K) at day 0 (G, I) and after 21 days of exposure to TGFβ_3 (H, K). Beads were cryosectioned, fixed and stained with anti-vinculin (green fluorescence) and counterstained with DAPI (blue fluorescence) for cell nuclei. Human cardiomyocyte progenitor cells were used as positive controls (I) and experimental samples for secondary antibody negative control (L); representative of three donors/group; scale bar = 10 μm; colour images available online

respectively), with a dispersed localization of vinculin through the cell surface. At day 21, vinculin condensed in focal adhesions. Distribution seemed similar for DC and MA (Figure 4H and K, respectively), and was not influenced by the different TGFβ stimulation patterns (data not shown).

3.3. Matrix production

In all conditions, proteoglycans (PGs) were deposited (Figure 5A–H), demonstrating successful chondrogenic differentiation of the BMSCs. For both DC and MA conditions, prolonging exposure to TGFβ increased the PG production, as confirmed by a quantitative assay (Figure 5I). However, no differences between DC and MA could be detected. In both conditions, PGs appeared to be concentrated within the clusters (for DC) or the micro-aggregates (for MA), filling the void spaces previously observed.

3.4. Gene expression

Levels of gene expression of chondrogenic markers (*sox9*, a transcription factor involved in early chondrogenesis; type II collagen and aggrecan, both main components of cartilage matrix) increased at day 21 for all conditions (Figure 6A–C). In MA conditions, however, type II collagen and aggrecan mRNA expression were inhibited compared to DC conditions at day 21 (Figure 6B, C). Seven days of TGFβ treatment led to the highest levels of expression of all chondrogenic markers for both MA and DC conditions (Figure 6A–C). At day 0, MA upregulated TGFβ gene expression compared to DC, but this high level of expression disappeared at day 21 under all TGFβ stimulation conditions (Figure 6D).

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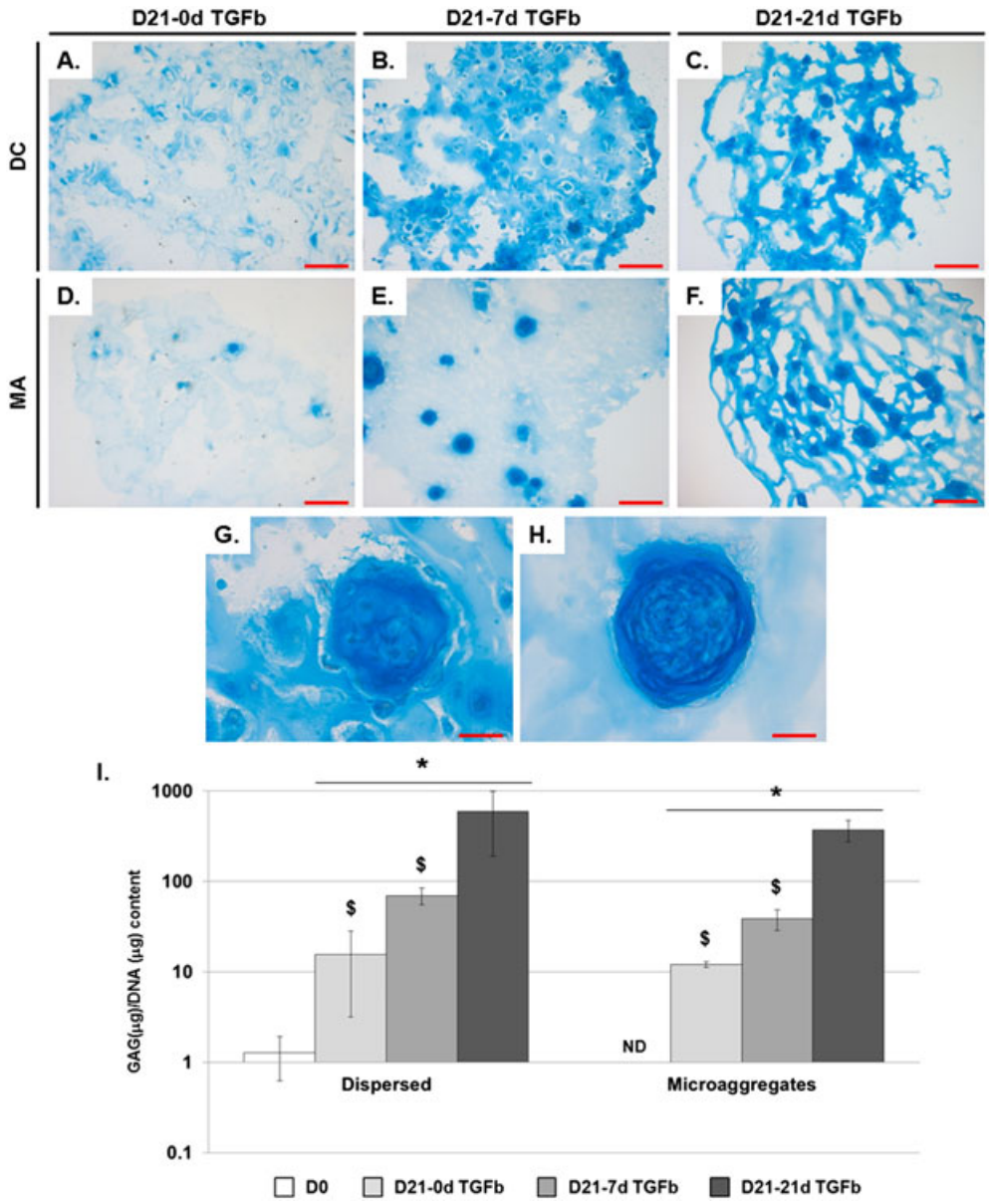

Figure 5. Matrix production. (A–F) Bovine BMSCs seeded in alginate beads as dispersed cells (A-C) or as micro-aggregates (D–F) at day 21 after exposure to TGFβ_3 for 0 (A, D), 7 (B, E) and 21 (C, F) days. Beads were cryosectioned, fixed and stained with Alcian blue for proteoglycans (note that light blue is alginate); representative of three donors/group; scale bar = 200 µm. (G, H) Higher magnifications of (B, E), respectively; scale bar = 50 µm; colour images available online. (I) GAG/DNA content after 21 days of culture, as determined with DMMB and Hoechst dye assays, respectively. Values are mean ± SD (NB: logarithmic *y* axis, and error bars are also logarithmic); n = 3/group; $^{*}p < 0.05$ vs D0; $^{\$}p < 0.05$ vs 21 days of TGFβ_3 treatment; ND, not detected

3.5. Transient TGFβ stimulation

For the 7 days of TGFβ treatment, chondrogenic marker gene expression and matrix production were also evaluated at day 7. The results showed that BMSCs had already started to differentiate at that point. All chondrogenic markers (*sox9*, aggrecan, type II collagen) were already highly upregulated at the gene levels (Figure 7B–D). PGs were also produced at day 7 (Figure 7A), but to a limited amount. PG content and type II collagen expression were significantly higher at day 21 than at day 7, indicating that the cells were still going along the chondrogenic pathway although TGFβ was withdrawn.

3.6. Hypertrophy and osteogenic differentiation

Both type X collagen (Figure 8A), an indicator of chondrocyte hypertrophy, and *sp7* (Figure 8B), a transduction factor involved in early osteogenesis, mRNA expressions increased at day 21 for the DC condition, while only type X collagen expression increased in the MA condition. For both conditions and genes, the highest level of expression at day 21 was for the 7 days of TGFβ treatment. When type X collagen levels of expression were similar for MA and DC conditions, *sp7* mRNA expression was upregulated at day 0 for the MA condition (Figure 8B).

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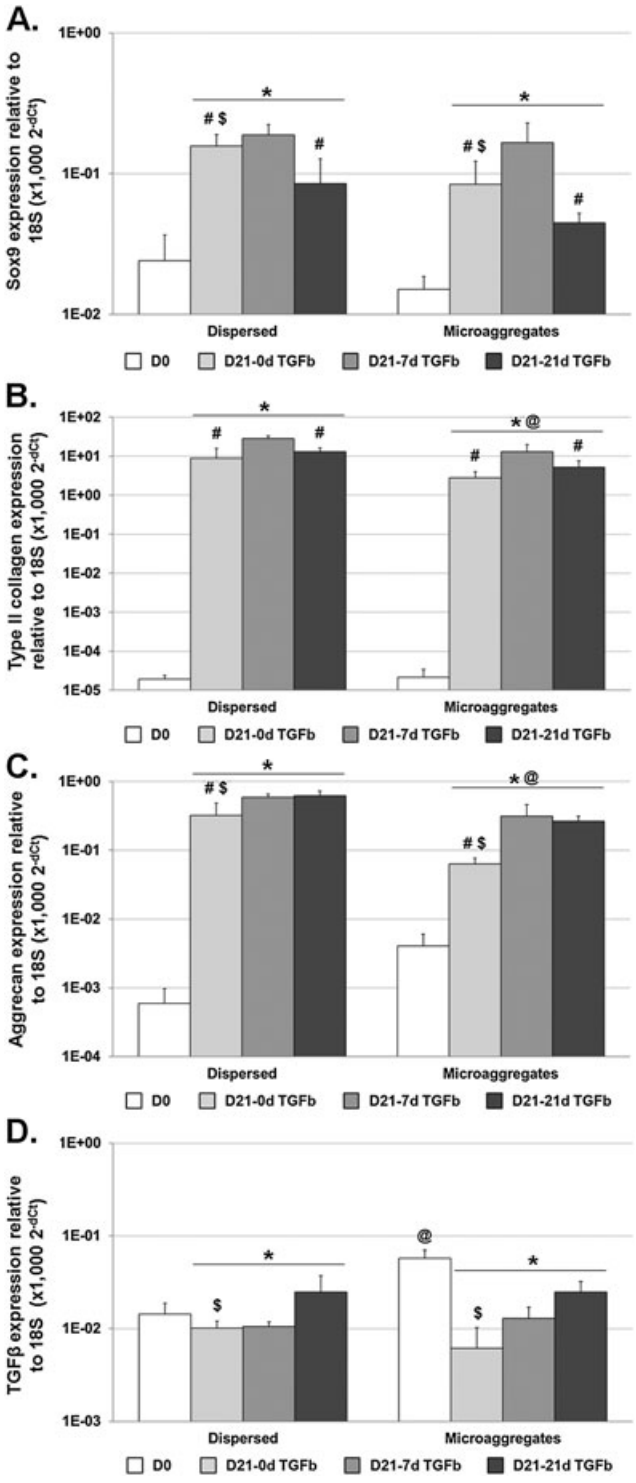

Figure 6. Gene expression – chondrogenesis markers. Gene expression of sox9 (A), type II collagen (B), aggrecan (C) and TGFβ (D), as determined by qRT–PCR. Expression is relative to *18S* reference gene ($2^{-\Delta Ct}$ method). Values are mean + SD (NB: logarithmic *y* axis, and error bars are also logarithmic); $n = 3$/group; $^{*}p < 0.05$ vs D0; $^{\#}p < 0.05$ vs 7 days of TGFβ_3 treatment; $^{\$}p < 0.05$ vs 21 days of TGFβ_3 treatment; $^{@}p < 0.05$ vs dispersed cells

4. Discussion

In summary, these results show that BMSCs underwent (partial) chondrogenic differentiation for all conditions tested (PG production and increased chondrogenic marker expression). MA, however, did not perform better (matrix production) or even as well (gene expression) as DC under all the TGFβ stimulation patterns tested. Nonetheless, these data show that small micro-aggregates can be successfully integrated into hydrogel (alginate). Although cell death was slightly upregulated at day 0 (Figure 2E), BMSCs in MA survived up to 21 days (Figure 2) and differentiated into chondrocytes, with the deposition of a PG-rich matrix within the MA (Figure 5H) and substantial upregulation of *sox9*, type II collagen and aggrecan gene expression (Figure 6A–C).

Increase of *TGFβ* gene expression at day 0 in MA compared to DC (Figure 6D) suggests an early stimulant effect of MA, maybe due to improved juxtacrine signalling (cell–cell contact) rather than paracrine signalling (limited distance between neighbouring cells), as cell–cell contact was improved at day 0 in the MA conditon, as shown by pan-cadherin staining. This upregulation, however, was lost at day 21 and, more importantly, was not translated into enhanced chondrogenic matrix production (Figure 5) or gene expression (Figure 6), even if no exogenous TGFβ was added. One explanation for this absence of effects may be the disappearance, during the 3 weeks of culture, of the cell–cell interactions observed at day 0 (Figure 4). BMSCs in MA most likely lost contact with each other (as shown by pan-cadherin staining) due to extracellular matrix production between the cells, but formed new junctions (as shown by vinculin staining) with this matrix (Figure 5). The lack of effects of *TGFβ* gene expression upregulation may also been explained by a low translation efficiency or by post-transcriptional regulatory mechanisms. Several studies comparing genomic and proteomic analyses report, indeed, moderate correlation between mRNA and protein expression (Chen *et al*., 2002; Huber *et al*., 2004; Oberemm *et al*., 2009; Tian *et al*., 2004). Another explanation for the absence of effects of upregulated *TGFβ* expression might be that BMSCs are not sensitive to the levels of TGFβ they are producing, either because these levels are too low or because BMSCs are less sensitive in MA. Cytoskeleton organization, indeed, has been shown to modulate cell sensitivity to TGFβ. Disorganization of the microfilaments in rabbit articular chondrocytes after treatment with dihydrocytochalasin B enhanced the sensitivity of the cells to TGFβ (increased PG and collagen synthesis) (Benya and Padilla, 1993). In the present study, BMSCs at day 0 displayed more organized microfilaments in MA cells than in the round cells of the DC condition (Figure 3A/E). This difference in cytoskeleton organization may also explain why MA are not upregulating chondrogenic gene expression as well as DC under transient and continuous TGFβ treatment (Figure 6). Although no significant differences were observed at the matrix level (Figure 5), our data support results observed with bovine articular chondrocytes in hydrogel, where small micro-aggregates (5–18 cells) inhibit chondrocyte biosynthesis compared to dispersed cells (Albrecht *et al*., 2006). Distribution of PGs, however, was quite distinct between the two conditions,

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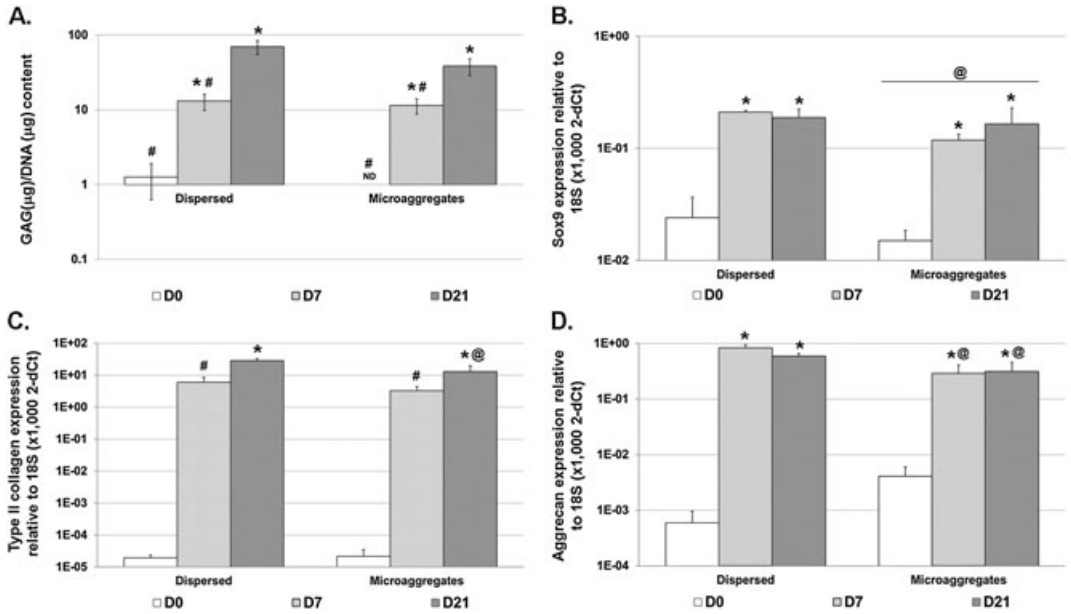

Figure 7. Transient TGFβ stimulation. (A) GAG/DNA content at days 0, 7 and 21, as determined with DMMB and Hoechst dye assays, respectively; values are mean ± SD (NB: logarithmic *y* axis, and error bars are also logarithmic). (B–D) Gene expression of sox9 (B), type II collagen (C) and aggrecan (D), as determined by qRT–PCR; expression is relative to *18S* reference gene ($2^{-\Delta C_T}$ method). Values are mean + SD (NB: logarithmic *y* axis, and error bars are also logarithmic); n = 3/group; $^{*}p < 0.05$ vs D0; $^{\#}p < 0.05$ vs D21; $^{@}p < 0.05$ vs dispersed cells; ND, not detected

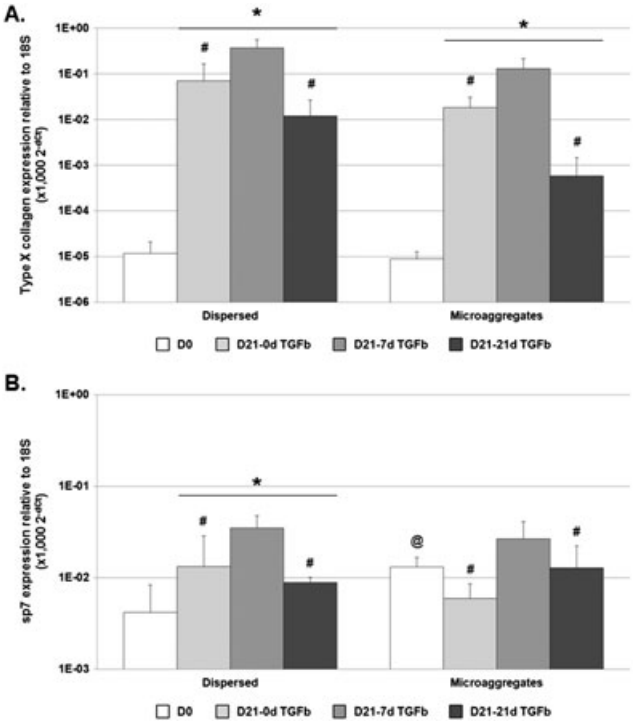

Figure 8. Gene expression – Hypertrophy and osteogenesis markers. Gene expression of type X collagen (A) and sp7 (B), as determined by qRT–PCR; expression is relative to *18S* reference gene ($2^{-\Delta C_T}$ method). Values are mean + SD (NB: logarithmic *y* axis, and error bars are also logarithmic); $^{*}p < 0.05$ vs D0; $^{\#}p < 0.05$ vs 7 days of TGFβ_3 treatment; $^{@}p < 0.05$ vs dispersed cells

with a more evenly distributed matrix for DC (Figure 5). As the cell concentration was similar at day 0 for both conditions, DC resulted in a more dispersed and homogeneous distribution of cells (Figure 2), which could account for a more even distribution of the matrix produced by the BMSCs.

Still, MA might be more potent for the osteogenic differentiation of BMSCs. In fact, MA (not embedded into a hydrogel) have already been shown to promote osteogenic differentiation of human BMSCs compared to 2D culture (increased calcium deposition and osteogenic gene expression) (Kabiri *et al.*, 2012). In the present study, we observed an upregulation of *sp7*, a transcription factor involved in early osteogenic differentiation, in MA at day 0 compared to DC (Figure 8). This suggests a positive influence of the MA on the osteogenic differentiation pathway. The absence of factors required for osteogenic differentiation, such as FBS or β-glycerophosphate, during culture, however, probably nullifies this influence, and additional experiments need to be conducted to assess the potential of MA to promote BMSC osteogenesis.

Contrary to MA, BMSCs in the DC condition proliferated during the 3 weeks of culture. This absence of significant proliferation in MA already containing several cells (Figure 3) may be explained by contact inhibition present in the MA but not in the DC. At day 21, cloned DC spontaneously formed clusters. Although these structures appeared similar to the MA, they were smaller and contained fewer cells (Figure 3). Recreating and amplifying this natural process of cloning and clustering in the MA, however, did not exert any substantial effect on BMSC differentiation, suggesting that cell–cell interactions are not required for initiating chondrogenesis.

These results also confirm that bovine BMSCs can spontaneously differentiate toward the chondrogenic lineage without the presence of TGFβ (PG production and increased *sox9*, type II collagen and aggrecan gene expression; Figures 5–7) when cultured in hydrogel and

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serum-free conditions, as previously reported for bovine BMSCs in micromass culture (Bosnakovski *et al*., 2006). Seven days of TGFβ treatment were enough to enhance the production of cartilaginous matrix, as shown previously with human BMSCs (Buxton *et al*., 2011), but, surprisingly, gave the highest upregulation of chondrogenic marker expression (Figure 6) for both MA and DC. However, the transient TGFβ stimulation also led to higher expression of type X collagen (a marker of chondrocyte hypertrophy). Hence, continuous stimulation with TGFβ resulted in a more stable chondrogenic phenotype; it also led to the highest matrix production (Figure 5).

Conclusions on the (absence of) effects of MA on BMSC chondrogenesis, however, are only valid for the cell concentration and hydrogel tested here. Using a lower concentration may dilute paracrine signalling in the DC condition and, therefore, diminish the chondrogenic differentiation of BMSCs. Buxton *et al*. (2011) have already evaluated the influence of cell concentration on the chondrogenesis of BMSCs seeded into a hydrogel. They reported a maximal PG/collagen synthesis/cell for concentrations in the range 12.5–25 million cells/ml. Lower concentrations led to lower matrix production, indicating the involvement of paracrine signalling in BMSC chondrogenesis. With the concentration used here (7 million cells/ml), paracrine signalling should be diluted in the DC condition and so MA could have a beneficial effect by locally increasing this paracrine signalling. As no positive effect was found for the MA, it seems that cell–cell contact or cytoskeleton organization have a stronger negative effect than paracrine signalling, a positive one for BMSC chondrogenesis. Moreover, the effect of MA on BMSC chondrogenesis has only been tested here in alginate and could, therefore, be an artifact of that system. The previous observation, that micro-aggregates inhibit chondrogenesis of bovine chondrocytes seeded in photo-polymerizable hydrogel (Albrecht *et al*., 2006) when compared to dispersed cells, tends to indicate, however, that the negative effects observed here were not an artifact of alginate.

Another limitation of the study may be the use of exogenous TGFβ if it is the endogenous molecular agent involved in juxtacrine signalling. In this case, adding TGFβ to the culture medium may have overpowered any increase of TGFβ expression present in MA, but not in DC, conditions. Such a beneficial effect, however, should have been observed when the BMSCs were cultured without exogenous TGFβ, when no differences between MA and DC were observed (Figures 5, 6, 0 days TGFβ group). Nonetheless, if TGFβ had been involved in cellular signalling after BMSC differentiation, bone morphogenic protein 2 (BMP2) could have been used to induce BMSC chondrogenic differentiation instead (Schmitt *et al*., 2003).

This study provides important clues about the communication of BMSCs with their environment, where cell–cell interaction seems to have a limited involvement in their (chondrogenic) differentiation. Although DC cloned and spontaneously formed clusters, accelerating and amplifying this process with the MA did not provide beneficial effects. This suggests that influencing cell–matrix, rather than cell–cell, interactions may be a more potent tool to control BMSC differentiation, at least for the chondrogenic pathway.

To conclude, this study shows that micro-aggregates, although potentially promoting cell–cell contacts and improving paracrine signalling, have no beneficial effects on bovine BMSC chondrogenesis in alginate.

Conflict of interest

The authors have no financial interest in the subject matter discussed in this paper.

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