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Chondrogenic potential of stem cells derived from adipose tissue: A powerful pharmacological tool

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ABSTRACT

Chondrogenesis has been widely investigated *in vitro* using bone marrow-derived mesenchymal stromal cells (BM-MSCs) or primary chondrocytes. However, their use raises some issues partially circumvented by the availability of Adipose tissue-derived MSCs. Herein; we characterized the chondrogenic potential of human Multipotent Adipose-Derived Stem (hMADS) cells, and their potential use as pharmacological tool. hMADS cells are able to synthesize matrix proteins including COMP, Aggrecan and type II Collagen. Furthermore, hMADS cells express BMP receptors in a similar manner to BM-MSC, and BMP6 treatment of differentiated cells prevents expression of the hypertrophic marker type X Collagen. We tested whether IL-1β and nicotine could impact chondrocyte differentiation. As expected, IL-1β induced ADAMTS-4 gene expression and modulated negatively chondrogenesis while these effects were reverted in the presence of the IL-1 receptor antagonist. Nicotine, at concentrations similar to those observed in blood of smokers, exhibited a dose dependent increase of Aggrecan expression, suggesting an unexpected protective effect of the drug under these conditions. Therefore, hMADS cells represent a valuable tool for the analysis of *in vitro* chondrocyte differentiation and to screen for potentially interesting pharmacological drugs.

1. Introduction

Increase of life span is accompanied by an increase of age-related diseases including osteoarthritis. It is the most common cause of rheumatic complaints and is characterized by extensive remodeling of subchondral bone and permanent destruction of articular cartilage leading to joint pain [1]. Development of cellular models is of great interest to decipher differentiation mechanisms and to screen for drugs. Human mesenchymal stem cells (hMSCs), originally isolated from bone marrow (BM-MSC), are multipotent adult stem cells. They are able to differentiate into various cell types, such as osteoblasts, adipocytes and chondrocytes, with the ability to undergo extensive self-renewal *in vitro* [2].

Even though the plasticity of adult stem cells is frequently debated [3], spontaneous *in vitro* chondrogenic differentiation [4] or differentiation in the presence of growth factors such as transforming growth factor-β (TGFβ), bone morphogenetic proteins (BMPs) or insulin-like growth factor-1 (IGF-1) has been reported [5]. Unfortunately, the use of BM-MSCs has some limitations associated with patient discomfort and the pain of bone marrow harvesting. Furthermore, the self-renewal capacity of BM-MSCs is low and can only be passaged *in vitro* for a limited number of times before senescence and growth arrest [6]. Alternatively, chondrocytes obtained arthroscopically from healthy articular cartilage lose rapidly their morphological and biochemical characteristics [7]. Therefore, finding other sources of easily accessible cells which exhibit stable chondrogenic differentiation capacities represent an important goal.

It has been shown that adipose tissue-derived stem cells (ATSC) obtained from either lipoaspirate or lipectomy can differentiate into various cells types, including chondrocytes [8]. The potential

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of using these cells is attractive as subcutaneous fat is abundant and the procedure to obtain samples is minimally invasive for the patients [9]. However, the limited life span (2-3 weeks), the limited amount of cells obtained after isolation from individuals, and the inability of the cells to withstand freezing/thawing, represent important drawbacks for a routine use. In addition, while BM-MSCs produced BMP-2, 4 and 6 endogenously, ATSC showed rarely expression of these BMPs and required the presence of exogenous BMPs for their differentiation [10].

Recently, human Multipotent Adipose-Derived Stem (hMADS) cells have been isolated and characterized from adipose tissue of young donors and can be maintained *ex vivo* using an original procedure [11–13]. hMADS cells display extensive self-renewal capacity *in vitro*, exhibit a normal diploid karyotype and maintain the capacity to undergo differentiation into many mesenchymal cell types, including chondrocytes [12–14]. hMADS cells are able to freezing/thawing procedure with no loss of multipotency properties [12–13].

In this study, we have characterized the chondrogenic potential of hMADS cells compared to that of BM-MSCs based on the expression of various markers, i.e. Aggrecan, COMP, type I and X Collagens (Col I, Col X) as well as specific staining of the extracellular matrix. Furthermore, as hMADS cells were responsive to drugs known to affect cartilage degradation, i.e. IL-1β and nicotine, they represent a valuable pharmacological tool for drug screening.

2. Materials and methods

2.1. Reagents

Cell culture media, serum, buffers, and trypsin were purchased from Lonza (Verviers, Belgium), other cell culture reagents were from Sigma–Aldrich Chimie (Saint-Quentin Fallavier, France). BMP6, IL-1β and TGF-β3 were purchased from Peprotech (France).

2.2. Cell culture

The establishment and characterization of the multipotency of hMADS cells have been extensively described [13–14]. Briefly, these cells, isolated from white adipose tissue removed from surgical scraps of infants undergoing surgery, did not enter senescence while exhibiting a diploid karyotype, were non-transformed though expressing significant telomerase activity, did not show any chromosomal abnormalities after 140 population doublings, and maintained their differentiation properties after 160–200 population doublings [13–14]. hMADS cells were able to withstand freeze/thaw procedures and their differentiation could be directed under different culture conditions into various lineages [12–13]. In the experiments reported herein, hMADS cells established from male infant were used between passages 15 and 30 corresponding to 35–80 population doublings. hBM-MSC were purchased from Cambrex (Paris, France) and used as recommended by the manufacturer.

Cultures were performed either as mono-layer (2D) or as three dimensional (3D) pellets. For 2D analysis, hBM-MSC or hMADS cells were seeded, in 12 multi-well plates, at a density of 25×10^3 - cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM)/low glucose (1.5 g/l), supplemented with 10% FCS, 15 mM HEPES, 2.5 ng/ml hFGF-2, 60 μg/ml penicillin, and 50 μg/ml streptomycin defined as growth medium. hFGF-2 was removed when cell reached confluence. At day 2 post-confluence (designated day 0), cells were induced to differentiate in the presence of DMEM/high glucose (4.5 g/l) supplemented with 500 μM ascorbic acid, 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium, 1 mM sodium pyruvate, 40 μg/ml L-proline, 100 nM dexamethasone and 10 ng/ml TGF-β3 (defined as differentiation medium). When indicated, BMP6 was added at 10 ng/ml.

For 3D analysis, hMADS cells were seeded at 5×10^5 cells per 15-ml polypropylene tube and centrifuged 5 min at 1000 rpm. The resulting cell pellet was maintained in growth medium for 3 days and chondrogenesis was induced in the differentiation medium described above. The tube's lid was left open to allow gas exchange. The cells were then the treated or not with BMP6.

IL-1β, anti-IL1βR and nicotine were added at the indicated doses for the indicated periods. The media were changed every 3 days and analyzed at the indicated days.

2.3. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

These procedures follow MIQE recommendations [15]. Total RNA was extracted using TRI-Reagent kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. Quality control for purity and integrity of RNA were tested by UV spectrophotometry on a NanoDrop 1000 device and by SYBR Gold-stained agarose analysis (Invitrogen). Reverse transcription-polymerase chain reaction (RT) was conducted as described previously [16–17]. Primer sequences, designed using Primer Express software (Applied Biosystems, Courtaboeuf, France), are listed in Supplementary Table 1 and were tested for their specificity, efficiency, reproducibility and dynamic range. For quantitative PCR (QPCR), final reaction volume was 10 μl using SYBR qPCR premix Ex TaqII from Takara (Ozyme) and assays were run on a StepOne Plus ABI real-time PCR machine (PerkinElmer Life and Analytical Sciences, Boston). The expression of selected genes was normalized to that of TATA-box binding protein (TBP) and 36B4 housekeeping genes and quantified using the comparative-ΔC_t method.

2.4. Histological analysis and immunohistochemistry

Cell pellets were fixed in phosphate buffered formaldehyde, then dehydrated through a series of ethanol concentrations, cleared with xylene, embedded in paraffin and 5 μm sections were prepared. For histological examination, the sections were stained using Haematoxylin–Erythrosine–Safran (HES) for morphology analysis and alcyan blue for proteoglycan detection. Indirect immunostaining assay was performed as described previously [18] using rabbit anti-type IIα Collagen antibodies (Millipore, France).

2.5. Statistical analysis

Data are expressed as mean values ± standard error of the mean (sem) and are analyzed using the 2-tailed student's *t* test. Differences were considered statistically significant at $P \leqslant 0.05$.

3. Results

3.1. Analysis of hBM-MSCs and hMADS cells chondrogenesis

Chondrogenic differentiation of hBM-MSCs and hMADS was evaluated by qRT-PCR based on the expression of chondrogenic markers including Aggrecan (ACAN), Cartilage oligomeric matrix protein (COMP), alpha chain of type I Collagen (Col Ia1) and type X Collagen (Col X).

Cell culture in mono-layer (2D) was performed in the presence of growth factors that are known to induce the chondrogenic differentiation such as TGF-β3 and BMP6. hMADS cells, as well as hBM-MSC, expressed BMP and TGFβ receptors, BMPR1A, BMPR2, TGFβR1, 2 and 3 (data not shown). Chronic treatment between

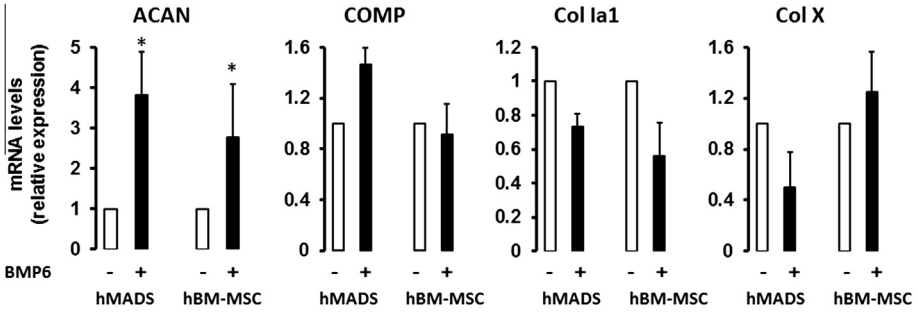

Fig. 1. Expression of molecular markers in differentiated hMADS and hBM-MSC cells: effect of BMP6. hMADS cells and hBM-MSC were seeded in mono layer (2D) culture in multiwell plates and induced to differentiate into chondrocytes in the differentiation medium in the absence or presence of 10 ng/ml BMP6 for 21 days. RNA was analysed for the expression of aggrecan (ACAN), Cartilage oligomeric matrix protein (COMP), type Ia1 Collagen (Col a1) and type X Collagen (Col X) by quantitative RT-PCR. Results are representative of three independent experiments performed on different series of cells. $^{*}P < 0.05$ versus day non-treated cells.

days 0 and 14 with 10 ng/ml TGF-β3 in the absence or the presence of 10 ng/ml BMP6, was performed in order to analyze its effect on gene expression. The data showed no differences between hMADS cells and hBM-MSC (Fig. 1). Indeed, TGF-β3 differentiated hMADS and hBM-MSC cells expressed chondrogenic markers, and BMP6 treatment induced a significant increase of ACAN and COMP mRNA levels in hMADS cells and to a lower extent in hBM-MSC (Fig. 1). However, Col Ia1 and Col X mRNA levels were down regulated (Fig. 1).

3.2. hMADS cells chondrogenesis in pellets (3D)

We subsequently analyzed whether hMADS cells were able to differentiate into chondrocytes in a three-dimension assay. For that purpose, hMADS cells were maintained in pellets as described in the Material and Methods section for 21 days in the differentiation medium supplemented with 10 ng/ml TGF-β3 and in the absence or the presence of 10 ng/ml BMP6. As expected, differentiated cells expressed chondrogenic markers (Fig. 2). Furthermore, BPM6 treatment induced a significant increase of ACAN and COMP mRNA levels and a decrease in those of Col Ia1 and X (Fig. 2A).

To confirm the differentiation of hMADS cells to cartilage cell morphology in response to TGF-β3 and BMP6, samples were evaluated using alcyan blue staining. Control pellets without TGF-β3 and BMP6 appeared very small in size and rather loose (Fig. 2Ba). Treatment of hMADS cells with TGF-β3 alone (not shown) or TGF-β3 and BMP6 resulted in mostly compact high density cultures (Fig. 2Bb).hMADS cells cultured in the presence of TGF-β3 and BMP6 differentiated into cartilage cells that exhibited distinct deep blue and orange staining representative of polysaccharides and proteoglycans reflecting an improved quantity of synthesized extracellular matrix. The presence of vacuoles confirmed the formation of cartilage-like structures. Moreover, differentiated hMADS cells did express type II Collagen as shown by specific immunostaining (Fig. 2Bc).

In order to further characterize hMADS-derived chondrocytes, we investigated whether these cells were responsive to well-known effectors of chondrogenesis, such as IL-1β and nicotine.

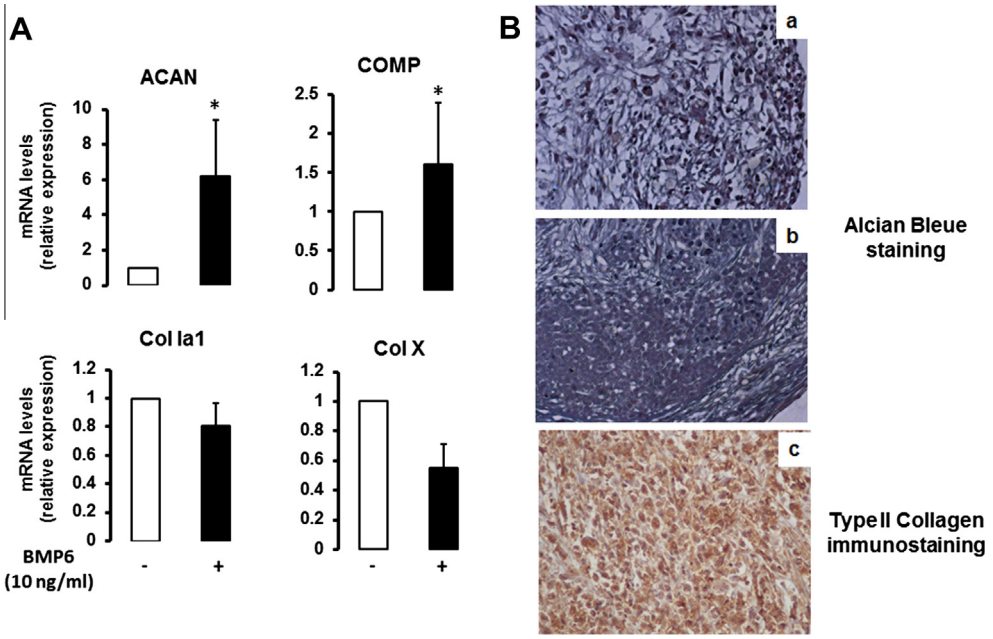

Fig. 2. Analysis of the chondrogenic potential of hMADS cells in a 3D assay. (A) Chondrogenic differentiation of hMADS cells was performed in pellet (3D) culture as described in Materials and Methods section in the absence or presence of 10 ng/ml BMP6. At day 21, RNA was analysed for the expression of ACAN, COMP, Col a1 and Col X by quantitative RT-PCR. Results are representative of three independent experiments performed on different series of cells. $^{*}P < 0.05$ versus day non-treated cells. (B) Histological examination of the pellet sections with Haematoxylin–Erythrosine–Safran for morphology, Alcyan blue and safranine for proteoglycans (Ba). Indirect immunostaining assay was performed using rabbit anti-type IIα Collagen antibodies (Bc).

3.3. Effect of nicotine on chondrogenesis

A recent report has described a positive effect of nicotine on the chondrogenic differentiation [19]. hMADS cells were induced to differentiate into chondrocytes in 12 multi-well plates in the presence of 10 ng/ml TGF-β3 and 10 ng/ml BMP6 during 14 days. Cells were treated with various amounts of nicotine during the whole differentiation process and RNA was extracted and analyzed at day 14. ACAN mRNA levels significantly increased in a dose dependent manner to nicotine (Fig. 4) and a tendency to an increase in those of COMP was observed. In parallel, Col Ia1 and Col X mRNA levels were not affected or showed a slight trend to decrease at 25 ng/ml nicotine.

3.4. Effect of IL-1β on chondrogenesis gene expression

hMADS cells were induced to differentiate into chondrocytes in 12 multi-well plates in differentiation medium containing 10 ng/ml TGF-β3 and 10 ng/ml BMP6 during 14 days. Cells were exposed for the last two days (day 12 to day 14) to 10 ng/ml IL-1β. ACAN and COMP mRNA levels were down regulated in presence of IL-1β and this effect was reversed by the interleukin-1 receptor antagonist, anakinra (Fig. 3). Col Ia1 and X mRNA levels were regulated in a similar way but to a lower extent.

Interestingly, IL-1β treatment led to an up regulation of ADAMTS-4 mRNA levels which was reversed by the IL-1 receptor antagonist. An inverse situation was observed for ADAMTS-5 mRNA levels, for which we observed a down regulation in presence of IL-1β reversed by IL-1 receptor antagonist.

4. Discussion

The results reported herein clearly show that established hMADS cells are able to differentiate into chondrocytes under appropriate serum-free medium and in two and three dimension conditions. Moreover, these cells possess properties similar to those of commonly used human bone-marrow derived chondrocytes. Differentiated hMADS cells expressed cartilaginous matrix containing collagenous and proteoglycans as demonstrated by gene expression and histological and immune-staining analysis.

In vitro chondrocyte formation using hBM-MSCs has been described; intensive investigations showed that the combination of TGFβ and BMP was the most appropriate condition for chondrogenesis including MSCs from adipose tissues [10,20–22]. In our study, the combination of TGF-β3 and BMP6 regulated the expression of chondrogenic genes in a similar way both in hMSCs and hMADS.

Several reports have indicated that chondrocytes differentiated from adipose-derived MSCs, embryonic stem cells and BM-MSC show a higher capacity to express *in vitro* chondrogenic markers including Aggrecan (ACAN) than natural articular chondrocytes [23–24]. In our study, ACAN expression appeared higher in hMADS cells than in BM-MSCs.

A number of studies have clearly shown that MSCs undergoing chondrogenic differentiation become hypertrophic chondrocytes [25–27]. It has been shown that BMPs including BMP4 plays an important role in maintaining a chondrogenic phenotype, both by enhancing matrix production and suppressing the production of type X Collagen (Col X) [28]. By stimulating matrix production, BMPs impairs chondrocyte dedifferentiation and thus prevents chondrocyte hypertrophy. In the present study, hypertrophic markers such as Col X were down regulated in the presence of BMP6 and TGF-β3 in both hMADS cells and hBM-MSCs. The down regulation of Col X was more pronounced in hMADS cells than BM-MSC in agreement with previous studies using MSCs derived from adipose tissues [29]. Thus, with regard to the expression of chondrocytes markers, hMADS cells appear as a suitable model of chondrogenesis.

IL-1 is a major pro-inflammatory cytokine involved in cartilage and bone loss during the pathogenesis of arthritis. IL-1 inhibits the cartilage-specific, type II Collagen production and induces cartilage ACAN degeneration by aggrecanases (ADAMTS). In this way, the induction of ADAMTS-4 (or aggrecanase 1) gene expression and the inhibition of ACAN and COMP expression by IL-1β observed in hMADS cells are in agreement with previous studies [30], indicating that differentiated hMADS is also a model of cartilage degeneration. Of interest, in hMADS cells, ADAMTS-5 (aggrecanase 2) expression is regulated in an opposite way in response to IL1. This result is in agreement with previous studies demonstrating that, by contrast to the *in vivo* situation, ADAMST-5 is not regulated *in vitro* by catabolic cytokines, including IL1, but seems to be constitutively expressed [31].

Inhibition of the IL-1β pathway by a specific IL-1 receptor antagonist is present endogenously in cartilage in physiological condition, and is an already used strategy in clinic to prevent cartilage degradation during osteoarthritis pathogenesis [32–33]. One of the classically used molecules in therapy is the recombinant anakinra, an analog of the physiological human IL-1Ra [34]. Reversion by anakinra of IL-1β effect in differentiated hMADS cells, demonstrated clearly that these cells can be used in pharmacological screening in order to found new anti-arthrosic compounds.

A more controversial situation is the association between smoking and osteoarthritis, as some reports have suggested that smoking protected against osteoarthristis [35] whereas others have shown deleterious effects of smoking [36]. For instance a significant positive effect of nicotine has been reported on the proliferation of human articular chondrocytes with an up-regulation of Collagen synthesis *in vitro* [19,37]. However, a toxic effect at higher doses has also been reported [38]. Nicotine treatment of differentiating hMADS cells led to a further induction of ACAN mRNA expression with no effect on the other markers indicating that this

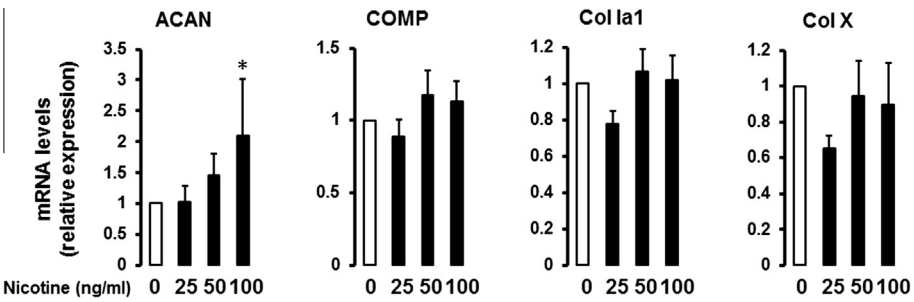

Fig. 3. Effect of nicotine on gene expression in hMADS-derived chondrocytes. hMADS cells chondrogenic differentiation in mono layer culture in the differentiation medium supplemented with 10 ng/ml BMP6 during 14 days in the presence of various amounts of nicotine. RNA was analysed for the expression of ACAN, COMP, Col a1 and Col X by quantitative RT-PCR. Results are representative of two independent experiments performed on different series of cells. $^{*}P < 0.05$ versus day non-treated cells.

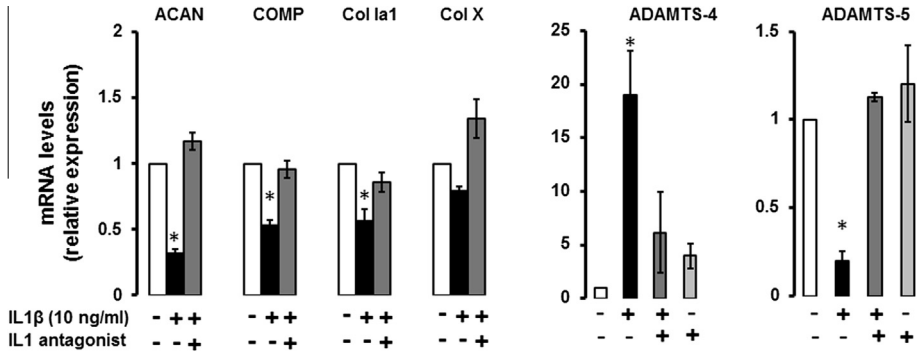

Fig. 4. Effect of IL-1β on gene expression in hMADS-derived chondrocytes. hMADS cells were induced to differentiate in differentiation medium containing 10 ng/ml BMP6 during 14 days. Cells were exposed for the last two days to 10 ng/ml IL-1β in the absence or presence of interleukin-1 receptor antagonist anakinra. RNA was analysed for the expression of ACAN, COMP, Col a1, Col X, ADAMTS-4 and 5 by quantitative RT-PCR. Results are representative of two independent experiments performed on different series of cells. $^{*}P < 0.05$ versus day non-treated cells.

drug may promote a specific effect without affecting the whole process of chondrogenesis. Even if a slight chondrogenic effect is displayed by nicotine treated hMADS cells, these results are only partially in agreement with the study of Ying et al [19]. Indeed, in their study a positive effect was found on type II Collagen but not on ACAN expression. Moreover, this nicotine characteristic is in discordance with the fact that nicotine is a well-known inductor of pro-inflammatory IL-1 and PGE2 pathway, 2 cartilage degradation pathways. It is possible that *in vitro*, a positive effect of nicotine was found due to the activation of pro-chondrogenic TGFβ pathway, as it is demonstrated in other models that nicotine is able to induce further of these pathways [39].

We showed in this study that hMADS cells are able to differentiate into chondrocytes in a two and three-dimension assay and to express typical chondrocyte markers including cartilage specific Collagen type II. Furthermore, hMADS cells respond to different pharmacological stimuli affecting chondrocyte properties. Therefore, due to their remarkable properties, i.e. displaying extensive self-renewal capacity, exhibiting a normal diploid karyotype, maintaining the capacity to undergo differentiation even after extensive expansion and resisting to freezing/thawing procedure with no loss of multipotent properties, hMADS cells represent a valuable and reproducible tool for the analysis of *in vitro* chondrocyte differentiation and for the screening of potentially interesting drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.012.

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