Activin/BMP2 chimeric ligands direct adipose-derived stem cells to chondrogenic differentiation

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Abstract Human adipose derived stem cells (hASCs) can be easily isolated and their plasticity has been well characterized. Several TGF-β superfamily ligands can direct hASCs towards chondrocytes. However, these ligands are difficult to purify and expensive. We have developed a library of Activin/BMP2 chimeric ligands (AB2 ligands) by systematically mixing their sequence segments and have tested their chondrogenic potential in hASCs. Cells cultured in monolayer or in a pellet culture system were incubated with a chemically defined medium supplemented with the chimeric ligands for 4 or 6 weeks and showed higher expression levels of type II collagen, aggrecan, and Sox9 mRNAs when compared with control and non-treated cells. Moreover, toluidine blue, alcian blue, and Masson's trichrome staining was markedly increased in treated cells, both in cell pellet and monolayer assays. In addition, immunohistochemical staining for detection of type I collagen, type II collagen, and Sox 9 demonstrated the acquisition of a chondrogenic phenotype in both culture systems. We present here an inexpensive and robust protocol for differentiation of hASCs towards chondrocytes in a reproducible and highly efficient manner. The AB2 ligands employed are easily produced and have properties that may become useful in cell therapy.

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Introduction

Degenerative diseases associated with age represent a major health challenge in developed countries. The intrinsic properties of cartilage tissue, its avascular nature, and deficient self-repair capacity imply that cartilage degeneration or associated injuries may become a chronic problem. To date no effective solution has been found. Moreover, cartilage degeneration causes not only joint pain and dysfunction, but also increases the percentage of people with limited mobility.
Cartilage is a connective tissue formed by only one cell type, the chondrocyte, trapped in the extracellular matrix composed mainly of collagen fibers, aggrecan (a large aggregating proteoglycan) and water. In fact, many efforts are currently being made to create cartilage in laboratories by combining novel biomaterials, growth factors and cells (Moreira-Teixeira et al., 2011).

Clinical treatment for articular cartilage injury includes autologous cell injections of primary chondrocytes expanded in vitro. However, the dedifferentiation of the autologous chondrocytes in culture and the small number of cells that can be obtained limit their clinical applications only to small injuries (Schuerman et al., 2012). Alternative cell sources for cartilage tissue engineering are mainly embryonic stem cells and adult stem cells. Mesenchymal stem cells derived from bone-marrow (MSCs) and adipose tissue (ASCs) have shown significant chondrogenic potential (Diekmann et al., 2010; Estes et al., 2010; Pittenger et al., 1999). Importantly, ASCs can be easily harvested, expanded in vitro and are relatively abundant in comparison with MSC. Therefore, ASCs could be an ideal cell type for generating a large number of functional chondrocytes.

The transforming growth factor-β (TGF-β) superfamily is comprised of almost forty ligands responsible for numerous cellular processes including early embryonic development, tissue patterning and homeostasis, bone formation, wound healing and fibrosis (Attisano et al., 1993; Hogan, 1996a, 1996b). These proteins signal through the simultaneous interaction with one of the 7 type I and one of the 5 type II TGF-β receptor (TGFR-β) kinases. The signaling requires the hetero-dimerization and subsequent activation of both types I and II receptors through the binding of their TGF-β ligands (Derynk and Feng, 1997). Interestingly, different TGF-β ligands, such as Activin (Jiang et al., 1993) as well as several isoforms of bone morphogenetic protein (BMPs) (Denker et al., 1999; Estes et al., 2010; Kramer et al., 2000; Majumdar et al., 2001), TGF-β1 (Awad et al., 2003; Erickson et al., 2002) or TGF-β3 (Hennig et al., 2007), have been shown to promote chondrogenesis. Since Activin A exhibits high affinity for type II receptors and signals through SMAD2/3 transcription factors, while BMP2 possesses higher affinity for type I receptors and signals through SMAD1/5/8 transcription factors, we investigated whether AB2 chimeras created by mixing Activin and BMP2 sequences (Allendorph et al., 2011) would be useful in promoting chondrogenesis. In short, BMP2 and Activin A sequences have been divided into 6 structural segments and these segments have been mixed to create the AB2 library of chimeras with novel functional properties. A systematic swapping strategy of the segments termed Random Assembly of Segmental Chimera and Heteromers (RASCH) was described in detail in Allendorph et al. (2011). The chimeras are fully defined by the code (BXXXXX), where X=A (Activin A) or B (BMP2) depending on which segment is in position X.

Although it has been reported that the use of defined media with specific cytokines induces chondrogenic differentiation in ASCs, the degree of maturation, efficiency and duration of the process still need to be improved. In this study, we have tested the chondrogenic potential of a battery of AB2 chimeras produced in our laboratory and concluded that AB235 could efficiently (i) upregulate the expression of chondrogenic-related genes, (ii) induce collagens and proteoglycans synthesis and (iii) increase chondrogenic cell pellet size using human ASCs.

In conclusion, we present here an inexpensive, robust protocol for differentiation of a large number of human ASCs towards chondrocytes in a reproducible and efficient manner. The scalable number of chondrocytes obtained by the induction with this AB2 ligand may have novel and important implications for cell replacement therapies in cartilage repair clinical protocols.

Material and methods

Generation of TGF-β Chimeras

The AB2 library was generated as previously described (Allendorph et al., 2011). Briefly, the mature domains of both human BMP2 and human Activin A were divided into 6 segments and primers were designed for each segment. An overlapping PCR strategy was used to mix the various segments together to generate full-length PCR fragments of each chimera. The AB235 chimera was created from AB208 (BAAAAA) by mutating IAKDIQN from segment 6 to VVLKNYQD using a Quickchange kit. We introduced this mutation to make AB208 Noggin sensitive. The mutation was designed based on our BMP7/Noggin complex (Groppe et al., 2002). The desired protein sequences were confirmed by DNA sequencing. Proteins were expressed in E. coli as inclusion bodies and purified as previously described (Allendorph et al., 2011).

ASCs cell culture

Human mesenchymal stem cells were derived from the adipose tissue from the subcutaneous abdomen of a 37-year-old Caucasian female (lot number 9061601.12, PromoCell, Heidelberg, Germany). Cells were cultured in “growing medium”: high glucose Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

Induction of monolayer expanded cells towards chondrocytes

hASCs were induced to chondrogenic phenotype as previously described (Estes et al., 2010) with slight variation. Briefly, cells were seeded at 30–40% confluence in a 12 or 24-well plate. Non-treated cells or control cells were grown in “incomplete chondrogenic medium” (CTL): DMEM–high glucose (Invitrogen) supplemented with 10% fetal bovine serum and 1% ITS+Premix (Collaborative Biomedical–Becton Dickinson, Bedford, MA), in the presence of 1% penicillin/streptomycin (Invitrogen). In addition, 50 μg/μL of L-ascorbic acid 2-phosphate (Sigma-Aldrich) was added fresh during each media exchange. To direct chondrogenic differentiation, cells were cultured in “chondrogenic medium”: incomplete chondrogenic medium containing 10 ng/mL of the chimeric ligand. Media was changed every other day. Cells were cultured in chondrogenic media for 2, 4 and 6 weeks depending on the experiment. Complete chondrogenic medium with 10 ng/mL of BMP2, known to be capable of chondrogenesis, was used as positive control. In addition,
we compared the chondrogenesis potential of AB2 chimera ligands and TGF beta3 at two different concentrations: 10 ng/ml and 50 ng/ml.

**Chondrogenic differentiation in cell pellet culture**

Two different methods were used to induce the formation of a cell pellet culture. In the first method, a suspension of 250,000 cells, in either growing media, incomplete chondrogenic medium or chondrogenic medium, was added to 15-ml conical tubes and centrifuged at 300 g at 21 °C for 5 min to form a pellet at the bottom of each tube. The pellets appeared as a round-shaped mass at the bottom of the tube. In the second method, cells were grown in 12-well plates in chondrogenic medium and after confluence, the monolayer spontaneously detached from the plastic and took the form of a crumpled paper ball. Control cells grown in incomplete chondrogenic medium did not detach spontaneously from the plastic and, therefore, the monolayer was manually separated using a sterile tip. The emerging pellets were carefully transferred to 15-ml conical tubes. This was done after two weeks from seeding cells in the 12-well plates. Tubes were incubated with loosened tops at 37 °C and 5% CO2. Medium was exchanged every other day for the duration of the experiment, and tubes were gently shaken to avoid the adherence of the pellet to the plastic walls.

**RNA isolation and real time-PCR analysis**

Real-time PCR was performed to study the differentiation profile of the hASCs in order to analyse the expression of key cartilage markers. Total cellular RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer’s recommendations. 2 μg of DNase1 (Invitrogen) treated total RNA was used for cDNA synthesis using the SuperScript II Reverse Transcriptase kit for RT-PCR (Invitrogen). Real-time PCR was used for cDNA synthesis using the SuperScript II Reverse Transcrptase kit for RT-PCR (Invitrogen). Real-time PCR was performed using the SYBR-Green PCR Master mix (Applied Biosystems). Sequences of primers were 5′ CACAGTCCATGCC3′ (COL2A1); 5′ TGACCTGGCCGTG3′ (COL1A1); 5′ GGCTGAGGGTTAAAGGCAGTG3′ (SOX9); 5′ GGCGGATGCTCTCAATCTGGT3′ for type II collagen (COL2A1); 5′ TGCGTAAAAGACCTACCCCTC3′ for aggrecan (ACN); 5′ GAGACAGCATGACGCGGAG3′ and 5′ GGGAATCCTGCTCTAATCTGG3′ for type II collagen (COL2A1); 5′ ATGGATGAGGAAACTGGCAACT3′ and 5′ GCCATCGAACGAAACATGTAAGT3′ for type I collagen (COL1A1); 5′ ACTCCGAGACGTGGCACATC3′ and 5′ TGTAGGTGACCTGGCTGG3′ for Sox9 (SOX9); 5′ AGAACCTCGAACAGACATGTC3′ and 5′ GGCTGAGGGTTAAAGGCAGTG3′ for Runx1. The gene expression levels were normalized to corresponding GAPDH values and are shown as fold change relative to the value of the control sample. All the samples were done in triplicate for each gene.

**Cell monolayer and cell pellet processing**

For histological and immunocytochemistry analyses, monolayer expanded cells and culture pellets were processed as follow. Culture pellets were fixed with 4% paraformaldehyde for 20 min at room temperature and embedded in 2.3 M sucrose for 1 h. Cell pellets were embedded in Tissue Freezing Medium, Blue (Electron Microscopy Sciences) and frozen on dry ice. Sections of 2–6 μm in thickness were cut with a microtome and placed in the centre of a coated slide. Sections were washed with PBS in a humid chamber, until the excess sucrose washed away. Monolayer expanded cells were washed thrice in PBS and fixed with 4% paraformaldehyde for 20 min at room temperature (RT).

**Histochemical determinations**

Toluidine blue staining: 0.1 g of toluidine blue (Sigma) was dissolved in 100 ml of dH2O. Fixed cells and pellet sections were stained in toluidine blue solution for 1–5 min at RT, and rinsed with dH2O until the excess stain washed away. Alcian Blue Staining: 1 g of Alcian blue (Sigma) was diluted in 3% acetic acid solution, and pH was adjusted to 2.5 with acetic acid. Fixed cells and pellet sections were stained in Alcian blue solution for 20 min at RT, and washed with dH2O until the dye was gone. Masson’s Trichrome Staining: we used Masson’s trichrome kit (Sigma) for the detection of total collagen content. The collagen fibers will be stained blue, the nuclei will be stained dark brown/purple and the cytoplasm is stained red/pink.

**Fluorescence microscopy**

Briefly, after fixation, monolayer expanded cells and pellet sections were blocked and permeabilized for 1 h at 37 °C with 5% BSA/5% appropriate serum/1X PBS with 0.1% Triton X100. Subsequently, cells and sections were incubated with the indicated primary antibody overnight at 4 °C. Monolayer expanded cells and pellet sections were next washed thrice with 1X PBS and incubated for 2 h at 37 °C with the respective secondary antibodies and washed thrice with 1X PBS. DAPI (0.5 μg/ml in PBS) was used to visualize the nuclei. Cells were mounted with aqueous mounting media before analysis.

Primary antibodies used were anti-type I collagen antibody (rabbit polyclonal antibody (ab292), Abcam, Cambridge, MA), anti-type II collagen antibody (mouse monoclonal antibody (ab3092), Abcam, Cambridge, MA), type X collagen (Neomarkers, Fremont, CA) or anti-Sox9 antibody (rabbit polyclonal antibody (AB5335), Chemicon). Secondary antibodies were Alexa Fluor 568 or 488 (Neomarkers). The pictures were taken with either a Leica TCS SP2 A0BS confocal or a Nikon E-800 microscope.

**Results**

Upregulated expression of chondrogenic genes after induction with chimeric ligands in ASCs

To select chimeric ligands with higher ability to induce chondrogenesis, we performed real time PCR analyses of cells grown in monolayer for two weeks in chondrogenic induction media. We observed enhanced gene expression of the principal cartilage extracellular matrix components, type II collagen (Col II) and aggrecan in all of the chimeras tested when compared to control cells, suggesting the enormous potential of these constructs (Fig. 1). Among all the chimeras, AB235 showed the most prominent chondrogenic effect since it markedly upregulated Col II mRNA to an average of 13-fold...
compared with the control. This chimera also increased aggrecan mRNA expression 14-fold and Sox 9 mRNA expression 9-fold. In addition, AB204 and AB215 also showed increased expression of these chondrogenic markers (Fig. 1).

Based on the real time PCR results we concluded that the chimeric ligands that induced major increase in chondrogenic markers expression were, in order from most to least, AB235, AB204, and AB215. Therefore these ligands were chosen to perform further experiments to fully assess their chondrogenic induction capacity.

**Formation of extracellular proteoglycan matrix**

The degree of maturation after chondrogenic differentiation was further assessed by toluidine and alcian blue staining of monolayer cultured cells. Accumulation of glycosaminoglycans in the extracellular environment indicates the formation of a cartilage matrix, and can be positively stained by toluidine and alcian blue. We observed a noticeable increase in the intensity of alcian blue staining in cells treated with AB235 for two weeks when compared with control cells (CTL) or cells treated with different chimeras, such as AB204 and AB215. Furthermore, the intensity of the alcian blue staining was comparable between AB235- and BMP2-treated cells (positive control cells) (Fig. 2A–K). Fig. 2G–K shows, in more detail, the striated distribution of the cartilage extracellular matrix and a strong blue staining for AB325- and BMP2-treated cells is apparent (Fig. 2G and I, respectively). We also tested toluidine blue staining in cells treated with two different concentrations of AB235 (Fig. 2L–M) and TGF-β3 (Fig. 2N–O). We observed that AB235- and TGF-β3-treated cells showed a similar dose-dependent increase in the intensity of toluidine blue staining. However, the intensity of staining in control cells was low (Fig. 2P). These results revealed the presence of acidic proteoglycans characteristic of the cartilaginous matrix in cells treated with chimeric ligands.

**Formation of cartilage-like tissue**

We used a cell pellet culture model which requires a high cell density to create compact cell-cell contacts mimicking the cellular condensation process occurring in normal limb
development (Johnstone et al., 1998). As far as we know, the method to induce pellet formation as a detached monolayer with "crumpled paper ball" form has not been used before to induce chondrogenesis (described in mat. and met. section). This method was development based in the concept of prechondrogenic mesenchymal condensations. In fact, mesenchymal cells seem to acquire a cohesive cell behavior, termed compaction, which is a critical cellular event required for cartilage formation (Barna and Niswander, 2007). Fig. 3F–J shows that the cell pellets achieved a cartilage-like appearance with a white shiny look. In addition, we clearly observed that both pellets prepared with the traditional centrifugation method (Fig. 3A–E) and with the new methodology (Fig. 3F–J) differed in size depending on the ligands included in the medium. In both methods, we observed a significant increase in cell pellet size when AB235 (Fig. 3B and G) or BMP2 (Fig. 3D and I) was added to the medium in comparison with cell pellets grown in incomplete chondrogenic medium with no ligands (Fig. 3E and J), or with AB215 (Fig. 3A and F), or AB204 (Fig. 3C and H). This increment in size was slightly larger when the pellets were obtained by previous monolayer culture. Furthermore, we were not able to form a pellet when cells were fed only with growing medium (data not shown). We further investigated the formation of cartilage-like tissue using toluidine blue staining in cell pellet sections. Active matrix production was detected in AB2 chimeras- and BMP2-supplemented pellet cultures (Fig. 3K–N). Note that the control cells (Fig. 3O) did not display tissue-like organization and rather present the appearance of a cell aggregation with no clear organization. A non-uniform distribution pattern of toluidine blue staining in treated cells is shown in more detail in Fig. 3P–S. These dense regions of a characteristic positive metachromatic staining are evidence of glycosaminoglycans synthesis.

Furthermore, the Masson's trichrome staining of pellet cultures grown with AB235 supplementation (Fig. 4A–C) revealed the typical appearance of cartilage tissue with blue staining for collagen fiber deposits that form a dense matrix containing the differentiated chondrocytes, stained in dark brown, within lacunae (Fig. 4C). These results reinforce the observed high potential of AB235 in promoting chondrogenesis.

To quantify the chondrogenic potential of the hASC cultured in a pellet system, we performed real-time PCR analysis of genes related to cartilage differentiation 6 weeks after treatment with chimeric ligands (Fig. 4D). Upon AB235 treatment, we observed increased expression of a number of chondrogenic markers including type II collagen (455-fold), Sox9 (9-fold) and Aggrecan (67-fold) as compared with the control, whereas the expression of the fibrous tissue marker type I collagen (Col I) decreased. These results suggested that ASCs were successfully induced towards an articular chondrogenic lineage by the chimeric ligand AB235.

Figure 2 Photographs of monolayer expanded cells cultured for 14 days with 10 ng/ml of different AB2 ligands and BMP2, showing a different increment of proteoglycans deposition (revealed by alcian blue labelling) depending on the ligands added to the medium (A). Higher resolution images of the same cells as in A are shown in B–K. L–O: Chondrogenic induction of the ligands was dose-dependent as demonstrated with the accumulation of toluidine blue positive material. Cells were cultured in chondrogenic medium with 10 ng/ml (L and N) or 50 ng/ml (M and O) of: AB235 (L–M) and TGF beta3 (N–O) and compared with control cells (P). Scale bars B–F and L–P: 6 mm; Original magnification for G–K was 10×.
Chondrogenic protein expression

Next, to further evaluate the chondrogenic potential of the chimeric ligands, we performed immunocytochemistry assays on cells grown in monolayer for 4 weeks (Fig. 5) or a pellet system differentiated after 6 weeks (Fig. 6). Monolayer cultured cells treated with AB204 (Fig. 5A–C) and AB235 (Fig. 5G–I) clearly demonstrated an increase in the expression of type II collagen which develops a dense filamentous matrix network deposited over the cells (Fig. 5B–C, H–I respectively). When cells were treated with AB208 (Fig. 5D–F) Col II expression was slightly lower, showing a similar pattern as BMP2 treated cells (Fig. 5J–L).

Furthermore, immunostaining of Col II and Col I in AB235-treated cells cultured in a pellet system (Fig. 6) also showed a substantial number of Col II positive cells (Fig. 6E, F, H and I), which formed a dense filamentous network of collagen II connecting the cells. Interestingly, we did not detect co-localization of collagen I (Fig. 6D and G) and II as Col I-positive cells were located surrounding Col II-positive cells, which strongly resemble a stratified structure in the tissue (Fig. 6F and I). Control cells (Fig. 6A–C) did not show tissue-like appearance or a strong, positive label of the chondrocyte markers tested.

In addition, expression of the chondrogenic transcription factor Sox9 was highly enriched in AB253-treated cells (Fig. 6L) when compared with control cells (Fig. 6J) in pellet sections. Sox9 protein was found localized in both nucleus and cytoplasm. In agreement with our results, others authors have also found Sox9 protein localization within the cytoplasm of chondrocytes (Aigner et al., 2003).

Finally, we stained the pellet section with type X collagen, a marker of hypertrophic chondrocytes committed to the osteogenic lineage, and did not observe significant positive staining in pellet section cultured with AB235 (Fig. 7H–I), while BMP2 treated cells showed a slight but significant expression of Col X (Fig. 7E–F). Altogether, these data suggested that ASCs acquired a mature chondrocyte-like phenotype induced by the activity of the chimeric ligand AB235.

Time points of chondrogenesis induction

To analyze the timing of the chondrogenic differentiation process during the exposition of the hASC to chimeric ligands, we collected samples at different time points (2, 4 and 6 weeks) and studied the expression of some early (Sox9 and Runx1) and late (Col II and I) markers of the chondrogenic pathway. Gene expression at the different time points was compared to cells that had been maintained in the same conditions as the treated cells, and collected at the same time points, but grown in media without any chimeric ligand or BMP2.

Fig. 8A shows that Col II gene expression increased over the period of differentiation and reached a 39-fold increment...
6 weeks after the treatment with AB253. Col I expression was significantly increased in both treatment groups (AB235 and BMP2) as compared to control cells (Fig. 8B) reaching maximum expression after four weeks of treatment. Moreover, after 14 days of treatment with chondrogenic medium we observed a modest and transient enhancement of the Runx1 mRNA level, which was more evident in AB235 treated cells. The Runx1 expression level decreased in later time-points for all treatments (Fig. 8C). Chondrocyte differentiation is initiated by expression of the transcription factor Sox9. After 2 weeks of culture in media supplemented with BMP2, Sox9 expression enhanced 21-fold but dropped to 9-fold after 4 weeks. The addition of AB235 to the media also increased Sox9 gene expression 9-fold after four weeks of culture. After 6 weeks of culture the expression of this transcription factor decreased for both treatments (Fig. 8D).

Discussion

Differentiated chondrocytes are capable of proliferating and secreting numerous growth factors and cytokines to form the extracellular matrix in mature cartilage (Fischer et al., 2010; Keller et al., 2011). During chondrogenesis, these events are precisely regulated by different growth factors and soluble factors released from cartilage elements as well as from the perichondrium (Hwang et al., 2011). Previous studies have shown that chondrocyte-derived factors may influence the fate of mesenchymal cells via paracrine, juxtacrine or gap-junction signalling pathways (Cheng et al., 2009; Gelse et al., 2009), suggesting that these factors can stimulate the formation of extracellular matrix in precursor cells and could be essential for regenerative medicine applications (Hwang et al., 2008). These factors are, however, difficult to produce in sufficient quantity. The development of new molecules with increased activity in driving MSCs and ASCs towards chondrogenic differentiation is, therefore, a priority in regenerative medicine and pharmaceutics.

On the other hand, there is accumulating evidence that co-culture multipotent stem cells, such as ASC and MSC, with patient derived chondrocytes can be used to induce chondrogenesis without the need for exogenous cytokines and growth factors (Wu et al., 2012a; Leijten et al., 2012). In addition, it has been shown that trophic factors secreted by the MSCs/ASCs induce cartilage formation by stimulating chondrocyte proliferation and matrix deposition by chondrocytes, rather than MSCs actively undergoing chondrogenic differentiation (Wu et al., 2011; 2012b). Although these findings are interesting and could

Figure 4 Masson’s Trichrome staining of AB235 treated cells in pellet cultures (A–C) showed marked collagen fibbers with blue label. A high magnification view of (A) is shown in (B). A stained section from a different pellet is shown in (C), where the formation of collagen matrix lacunae can be seen. Expression of chondrogenic markers in a pellet culture (D). Pellets were cultured with AB235 or BMP2 for 6 weeks. RT–PCR analysis of chondrogenic gene markers (Col II, Col I, Sox9 and Aggrecan) normalized to GAPDH and shown as relative gene expression to cells cultured in incomplete chondrogenic medium. Data are shown as average ± 95%CI (n= 3) from at least 2 independent experiments. Original magnification: 5× for A; 10× for B and 40× for C.
represent a suitable alternative for cartilage repair, we present here a different approach that is not based on the use of autologous chondrocytes. In this respect, we previously established a procedure that allowed us to generate a large quantity of BMP2/BMP6 heterodimer (BMP-2/6) by a chemical refolding method, and demonstrated that BMP-2/6 is a better inducer of differentiation of human embryonic stem cells (hESCs) than its homodimeric counterparts (Valera et al., 2010). In addition, using a segment-swapping RASCH strategy with BMP2 and Activin-A sequences, we have demonstrated that one such AB2 library chimera, AB208, exhibits the refolding characteristics of BMP2 while possessing Activin-like signaling attributes (Allendorph et al., 2011). Since BMP2 has been proven to enhance gene expression of cartilage-specific genes and proteoglycans in hMSCs (Sekiya et al., 2001, 2005), these chimeras can be good candidates for stem cell differentiation/guidance.

In the present study we analyzed the chondrogenic potential of A/B2 chimera ligands on hASC. Firstly, we assayed their ability to up-regulate the expression of genes involved in chondrogenesis. AB2-ligands increased collagen II, aggrecan or Sox9 expression, which demonstrated a potential in inducing chondrogenic differentiation (Li et al., 2011; Yoo et al., 1998). The level of induction of chondrogenic genes was highly dependent on the chimeric ligands used, with AB235 showing the strongest chondrogenic potential both in monolayer and pellet culture systems. Time point evaluations confirmed that chondrogenic differentiation induced by AB235 exhibited a similar pattern to that of the cartilage maturation process with an initial increase of Col I and Sox9 and a decline at later times, a time-dependent increase in expression of Coll II, and a marked decrease of Runx1 levels. Sox9 acts in early stages of chondrocyte differentiation, which directly induces type II collagen (Lefebvre et al., 1997) and is expressed in mesenchymal condensations (Lefebvre and de Crombrugghe, 1998). Decreased Col I gene expression suggested that AB2 ligands induced articular chondrocyte differentiation, as type-I collagen is either present in very small amounts or absent in hyaline cartilage, but abundant in fibrocartilage (Eyre and Muir, 1977; Roberts et al., 2009). Runx1 is a transcription factor highly expressed during early chondrogenesis and is downregulated in late stages of chondrocyte differentiation. Moreover, it has been demonstrated that Runx1 is capable of accelerating induction of MSC differentiation towards chondrogenesis by increasing the expression of early chondrocyte maturation markers (Wang et al., 2005). Thus, our results indicate that AB235 is superior in inducing chondrogenesis from early to late stages of maturation.

Members of the TGF-β family, TGF-β1 or TGF-β3, have been shown to induce chondrogenic differentiation (Johnstone et al., 1998; Mackay et al., 1998; Tanaka et al., 2004). In this respect, Lorda-Diez et al. (2011) have performed an elegant in vivo study to identify the temporal hierarchy of the genes which orchestrate chondrogenesis. The experimental model consisted in the induction by TGF-β of an ectopic digit in the developing embryo and concluded with the postulation of three different periods in the transition from mesoderm towards cartilage: i) the pre-condensation stage, characterized mainly by upregulation of Sox9; ii) condensation stage, with the activation, among others, of Activin bA and BMP receptor genes; and iii) pre-cartilage period, characterized by the intensification of the expression of Sox9 which precedes the upregulation of Aggrecan and type II Collagen genes.

The TGF-β family is divided into two subfamilies, the TGF-β/Activin/Nodal and the BMP/GDF, which members play...
a key role in regulating development, differentiation, and tissue repair and display complementary effects (Chng et al., 2011). TGF-β signaling via a heteromeric cell surface serine/threonine kinase receptor complex consisting of a dimeric ligand and a pair of both type I and type II receptors (Attisano and Wrana, 2002; Heldin et al., 1997). The type II receptors

Figure 6  AB2 ligands-treated cell pellets formed a "cartilage like" tissue organization. hASC cell pellet cultures were grown in medium supplemented with AB235 (D–I) and incomplete chondrogenic medium (A–C) for 6 weeks, fixed and immunostained with Col I and Col II antibodies. Protein expression was detected in red for Col I and green for Col II. Cell nuclei were labelled with DAPI (blue channel). Tissue organization in stratified layers showed Col I stained cells enveloping Col II producing cells. Expression of chondrogenic transcription factor Sox9 in culture pellet sections. Immunofluorescence of Sox9 was analysed after 6 weeks of treatment with AB235 (K–L) and control cells (J). Proteins were indirectly labelled with secondary antibodies (green channel) and cell nuclei labelled with DAPI (blue channel). (K) shows nuclear staining of the same field as (L). In (L), note nuclear localization of Sox9 (arrows). Original magnification 20x for: A–F and J; 40× for: G, H, I, K; and L.
kinases are activators of the type I receptor known as activin receptor-like kinases (ALKs). TGF-β ligands assemble a receptor complex of one of five type II TGF-β receptor kinases and subsequent cross-phosphorylation of one of seven type I receptors which triggers phosphorylation of the cytoplasmic receptor-regulated SMAD molecules. Indeed we show here that the major difference between AB235 and BMP2 treatment was visible in the upregulation of Sox9 expression. This observation could indicate that Sox9 upregulation is linked to both SMAD2/3 and SMAD 1/5/8 activation, whereas the increase in Col II is mainly regulated by BMP2 presumably through SMAD 1/5/8. In fact, it has been recently shown that Smad 4 silencing (blocks both SMAD 1/5/8 and SMAD 2/3 intracellular signaling) targeted to limb mesoderm causes a dramatic inhibition of chondrogenesis of digit progenitors (Bénazet et al., 2012). Moreover, both Sox9 and Col II proteins were synthesized in great amount by the hASCs supplemented with AB235 for 6 weeks. It has been previously demonstrated that Sox9 in concert with L-Sox5 and Sox6, regulates cartilage formation and maintains the chondrocyte phenotype in the mature cartilage by activating expression of several cartilage-specific genes, including Col2a1, Col9a1 and Col11a1, Acan and Comp (Han and Lefebvre, 2008).

BMPs have a synergistic effect with TGF-β on promoting MSCs into hyaline-like cartilage tissue (Toh et al., 2005) and also signal through activin type II receptors (ActrII) or ALKs. In addition, signaling through the BMP receptors is required for the maintenance of the articular cartilage in post-natal organisms (Rountree et al., 2004).

Finally, we showed here that while AB235-treated cells almost did not express Col X, this hypertrophic marker slightly increased after BMP2 treatment. In fact, BMP2 also mediates the replacement of chondrogenesis by endochondral ossification comprising chondrocyte maturation, hypertrophy, and transition from type II to type X collagen with subsequent chondrocyte apoptosis. Thus, chondrogenic cultures induced by BMP2 display high expression of genes associated with chondrocyte hypertrophy (Steinert et al., 2009). In addition, in vivo studies have demonstrated that BMP2 expressing MSCs resulted

Figure 7  Hypertrophic marker type X collagen expression in pellet section grown in incomplete chondrogenic medium (A–C) and in grown in medium supplemented with BMP2 (D–F) or AB235 (G–I). Col X was labelled in red and cell nuclei were visualized by DAPI staining (blue). Original magnification 20× for: A, B, C, G, H and I; 40× for: D, E and F.
in tissue hypertrophy and the formation of osteophytes, when transplanted orthotopically to osteochondral defects (Gelse et al., 2003).

The results presented here confirm that AB235 enhanced hASCs chondrogenic differentiation in comparison with individual treatment with BMP2, suggesting that this chimeric ligand could signal through both type I and type II receptors more efficiently, thereby increasing cartilage maturation. In fact, both BMP2 and Activin can bind ActrII (activin with very high affinity and BMP with lower affinity) (Chen et al., 2002).

In addition, differences in the level of endogenously secreted growth factors and/or in the expression of their specific receptors might modulate the resulting phenotype of the target cell and could be responsible for the success of chondrogenic differentiation in MSC (Hennig et al., 2007).

A main strategy of regenerative medicine is the construction of a biocompatible scaffold that, in combination with living cells and/or bioactive molecules, replaces, regenerates or repairs damaged cells or tissue (Perán et al., 2012; López-Ruiz et al., 2012). Growth factors are commonly used to enhance the scaffold’s bioactivity and facilitate tissue growth and regeneration. The rationale of incorporating growth factors within a construct, is to allow the surrounding cells to attach to the scaffold via surface receptor binding, activate their signaling pathways and dictate them to migrate, proliferate, differentiate and synthesize proteins during tissue regeneration (Lutolf and Hubbell, 2005). Thus further studies to evaluate the effect of the incorporation of AB235 to bio-compatible scaffolds could have important clinical application in the near future.

Conclusions
Here we present an optimized, reproducible and inexpensive protocol to direct the differentiation of adipose stem cells into the chondrogenic lineage. Our chimeric ligand AB235 has proven to be highly efficient to induce chondrogenesis from hASC. This novel differentiation culture system may be extended to other cell and tissue types and thus could have broad clinical applications.

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