Chondrogenic potential of human mesenchymal stem cells and expression of Slug transcription factor

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Chondrogenic potential of human mesenchymal stem cells and expression of Slug transcription factor

Short title: Slug content allows the identification of hMSCs capable of chondrogenesis

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Abstract

The scientific literature rarely reports experimental failures or inconsistent outcomes in the induction of cell differentiation; however researchers commonly experience poor or unsuccessful responses to differentiating agents when culturing stem cells. One way of investigating the underlying reasons for such responses is to look at the basal expression levels of specific genes in multipotent stem cells prior to differentiation induction. In addition to shedding light on the complex properties of stem cells and the molecular modulation of differentiation pathways, this strategy can also lead to the development of important time- and money-saving tools that aid the efficient selection of cellular specimens; in this case, stem cells that are more prone to differentiate towards specific lineages and are thereby more suitable for cell-based therapeutic protocols in regenerative medicine. To address this latter aspect, we focused on understanding the reasons why some human mesenchymal stem cell (hMSC) samples are less efficient at differentiating towards chondrogenesis. We show that analysis of the basal expression levels of Slug, a negative regulator of chondrogenesis in hMSC, provides a rapid and simple tool for distinguishing stem cell samples with the potential to form a cartilage-like matrix, and that are therefore suitable for cartilage tissue engineering. Indeed, we show that high basal levels of Slug prevent the chondrogenic differentiation of hMSCs, even in the presence of TGFβ and elevated levels of Sox9.

Keywords hMSCs; chondrogenic differentiation; Slug transcription factor; gene expression

Despite the availability of established protocols, researchers working with stem cells are often faced with difficulties in stimulating cells to differentiate towards a certain lineage. The failure to achieve successful differentiation is usually attributed to the heterogeneity of cell populations or to slight variations in culture conditions. Thus, most investigations directed at improving differentiation success are focused on identifying the best sources of progenitor cells and the ideal 'cocktail' of differentiation inducers.

Much evidence now exists showing gene expression profile differences in differentiated stem cells, but the evaluation of basal levels of specific genes expressed in pre-differentiation condition is often lacking. In this context, the major challenge is to recognise informative differences in the basal expression levels of a particular gene between stem cell samples and to correlate them with differences in the differentiation potential of the cells. Such analyses could provide clues as to why '*in vitro*' cell differentiation induction fails in some cells, whilst providing a tool to help select the stem cell specimens more prone to differentiating towards specific lineages.

Pre-differentiation gene expression analysis is not only able to help elucidate the complex properties of stem cells and the molecular mechanisms that regulate specific differentiation intracellular pathways, but it may also lead to the development of improved cell-based therapeutic protocols.

In the present study, we focused on investigating why it is difficult to induce some human mesenchymal stem cells (hMSCs) samples to differentiate towards chondrogenesis *in vitro*. hMSCs provide an attractive cell source for cartilage tissue engineering applications as they are readily expandable and capable of differentiating into chondrocytes (Quintana *et al*., 2009). However, hMSCs are heterogeneous cell populations that exhibit varying chondrogenic potential (Delorme *et al*., 2009), and this may limit their clinical relevance. By means of *post hoc* analysis, we investigated whether the basal expression levels of Slug (Snail2) transcription factor (Nieto, 2002), a negative regulator of chondrocyte differentiation (Goldring *et al*., 2006; Kim *et al.,* 2010; Savagner *et al.,* 1998), could be exploited to predict the outcome of chondrogenesis. Our previous

findings demonstrated that Slug knockdown negatively affects the maturation process of osteoblasts, and that it has a pro-chondrogenic effect in hMSCs (Lambertini *et al*., 2009; Torreggiani *et al*., 2012). Since many studies have shown that the correct levels of transcription factors are crucial for the successful differentiation of stem cells towards a desired lineage (Barzilay *et al*., 2009; Chang *et al*., 2008), we investigated whether Slug expression levels could be critical in determining the susceptibility of skeletal progenitor cells to exogenous differentiating agents.

The present study investigated hMSCs isolated from subcutaneous adipose tissue (hASCs) and bone marrow (hBMSCs). Cells were immunophenotypically characterised, as previously described (de Girolamo *et al*., 2009; Torreggiani *et al*., 2012), by flow cytometric analysis (data not shown). In the subsequent experiments, only the samples expressing high levels of the main MSC surface markers (such as, CD13, CD29, CD44, CD54, CD90 and CD105 antigens) and that lacked hematopoietic markers (CD14, CD31, CD45 and CD133) were used. hMSCs were cultured in standard media supplemented with specific differentiating agents to induce either osteogenesis or chondrogenesis. Here we focused on a group of well characterised hMSC samples that, despite efficiently differentiating into cells of the osteogenic lineage, did not acquire the chondrogenic phenotype. When cultured in osteogenic medium, the cells expressed typical osteogenic markers, including alkaline phosphatase (ALP), Runx2 and collagen, and were able to deposit mineral matrix (Figure 1). However, when the hMSCs were maintained for 21 days in pellet cultures in the presence of chondrogenic differentiating agent TGFβ1, we observed a different modulation of the chondrocyte phenotype markers. In particular, quantitative RT-PCR revealed that the expression of Sox9, a critical transcription factor necessary for MSC commitment towards chondrogenesis, remained expressed (although fluctuating) during TGFβ1 exposure (Figure 2A). The expression of aggrecan, the main proteoglycan found in cartilage, significantly increased in nine samples out of ten, but the total production of glycosaminoglycans (GAGs), measured by dimethylmethylene blue assay, was upregulated in just four samples out of ten (Figure 2A). When the chondrogenic phenotype was assessed as the production of collagen type 2 (Col2A1), the predominant collagen

type in cartilage, all samples were negative, both at the mRNA and protein level, in comparison with our positive control sample 1 (Figure 2B). Indeed, unsuccessful chondrogenic differentiation of MSC pellets is widely defined as the absence of Col2A1 expression, as detected by immunocytochemical analysis.

The expression levels of Slug transcription factor were also analysed during hMSC differentiation towards both osteogenesis and chondrogenesis. In all osteo-differentiated hMSCs, Slug expression significantly increased both at the mRNA and protein level, as revealed by qRT-PCR and Western blot analysis, respectively, confirming the positive role of this transcription factor in osteogenesis (Lambertini *et al*., 2009) (Figure 2C). However, Slug expression would be expected to be switched off during the chondrogenesis of hMSCs cultured in pellet or micromass in the presence of TGFβ1, since it is known to be a negative regulator of this kind of differentiation (Goldring *et al*., 2006). Nevertheless, Slug expression (at both the mRNA and protein level) was only found to be significantly reduced just in five samples out of ten (samples 1, 2, 3, 4, and 5), whereas it was unaffected or even increased in the others (samples 6, 7, 8, 9, and 10) (Figure 2D). This last group included sample number 10, which was the hardest to induce towards chondrogenesis, being the only aggrecan-negative population following TGFβ1 exposure (indicated as: AGG-negative sample). It should also be noted that this expression profile did not correlate to the source from which the hMSCs were isolated.

These data prompted us to investigate whether the chondrogenic potential of hMSCs could be recognised in advance in the uninduced condition, focusing on Slug expression as a marker of poor chondrogenic differentiation. The experiment was performed using the same ten samples and four additional control samples, which all tested positive for Col2A1 following TGFβ1 treatment (similar to control sample 1). Prior to differentiation induction, samples were cultured for 24 hours in suitable conditions and then analysed for basal Slug content. As shown in Figure 2E, Slug expression levels were high in standard 2D culture conditions in all the samples analysed. Conversely, Slug expression was significantly lower in cells growing in pellet cultures (3D

condition). Interestingly, Slug levels also resulted as being quite low in the five samples able to differentiate towards chondrogenesis (group A), but it was unmodified, respect to the 2D condition, in the AGG-negative sample (sample 10). Slug levels in groups B (samples 2-5) and C (samples 6-9) were significantly higher than those found in group A, although they were lower than that of sample 10, and this may explain their TGFβ unresponsiveness.

Our findings shed light onto the role of Slug in chondrogenic context and suggest that a physical stimulus, such as the pellet three-dimension culturing technique, may in itself negatively affect Slug expression. However, we also show that the 3D environment is necessary but not sufficient for decreasing Slug expression levels. A number of studies have been focused on understanding the correlation between extrinsic factors, microenvironmental signals and related changes in gene expression that may affect the differentiation potential of hMSCs. In our experimental model, the microenvironment is represented by a 3D system that is uniquely supported by the extracellular matrix (ECM) produced by the cell pellet and, as we show here, it may be insufficient. This is consistent with recent data demonstrating that, when MSCs proliferate in an inappropriate ECM, proper differentiation is not accomplished due to an aberrant expression of several transcription factors (Hoshiba *et al*., 2012).

Together, our data suggest that induction medium is only effective in promoting chondrocyte differentiation of hMSCs when transcriptional regulatory networks that predominantly operate in cartilage development are activated in the correct way. In other words, in the multistep pathway of chondrogenesis, high basal levels of Slug expression may represent a “restriction signal” that, together with other factors, prevents hMSCs from differentiating into chondrogenic-like cells, even in the presence of TGFβ and elevated Sox9 levels. This could partially explain the varying conclusions that many studies have arrived at about TGFβ signalling during chondrogenesis and homeostasis, reflecting the complex context-dependent regulation of TGFβ on both the tissue and cellular level (Keller *et al.*, 2011; Patil *et al*., 2011; Quintana *et al*., 2009).

Another important issue to be considered is the relationship between Sox9 and Slug transcription factors. Sox9 has always been considered as the master regulator of chondrogenesis that exerts its effect at an early stage, in spite of its fluctuating expression levels after TGFβ treatment, as observed here. However, the role that we suggest to assign to Slug is consistent with the hypothesis that increased levels of transcription factors with critical roles in initiating the chondrogenic process, such as Sox9, may be secondary to other events in hMSC chondrogenic differentiation (Hollander *et al*., 2010; Ghone *et al*., 2012; Oldershaw *et al*., 2008). We suggest that low levels of Slug should be considered as one of the important priming signals (in addition to FGF-2 pretreatment) necessary for the correct modulation of the hMSC molecular machinery, such that they may respond more efficiently to chondrogenic cues, thus enhancing differentiation (Handorf *et al*., 2011).

In conclusion, without needing to invest time in identifying the 'best' sources of MSCs with a strong chondrogenic potential and without the need to set up long differentiation procedures, the results of the present study provide a quick and easy way of distinguishing which hMSCs possess the greatest chondrogenic potential and represent the best candidates for cartilage tissue engineering.

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Statements

A statement explicitly describing the ethical background of the research performed is not applicable to the present study.

The authors declare to have no conflict of interests.

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Figure Legends

Figure 1. Osteogenic potential of hMSCs. Osteogenic differentiation was evaluated by analysing (A) intracellular alkaline phosphatase (ALP) activity, (B) Runx2 mRNA expression by quantitative RT-PCR, (C) collagen production by Sirius Red F3BA and (D) extracellular calcified matrix deposition by Alizarin Red-S.

hMSCs from subcutaneous adipose tissue and bone marrow were cultured on polystyrene up to 21 days in control (Ctrl) and osteogenic medium (Osteo) containing 10 nM dexamethasone, 10 mM glycerol-2-phosphate, 150 μM L-ascorbic acid-2-phosphate and 10 nM cholecalciferol (Sigma-Aldrich). hASCs were obtained from fat tissue of healthy donors undergoing plastic surgery. hBMSCs were obtained from bone-marrow aspirates of the iliac crest from patients undergoing ankle surgery. In all cases, informed consent was obtained by the Ethical committee of Galeazzi Orthopaedic Institute of Milan or the Rizzoli Orthopaedic Institute of Bologna.

Alkaline phosphatase (ALP) enzymatic activity, secreted collagen and ECM calcification were evaluated as previously described (de Girolamo *et al*., 2009, Sacerdote et al., 2013). Briefly, for collagen production, hMSCs were fixed for 1 hour with Bouin's solution (Sigma-Aldrich) and then stained for 1 hour with 0.1% (w/v) Sirius Red F3BA in saturated picric acid (Sigma-Aldrich). Stained samples were extracted using 0.1 M NaOH for 5 min and then read at 550 nm. A standard curve of known concentrations of calf skin type I collagen (Sigma-Aldrich) was created to assay the concentration of secreted collagen. Extracellular matrix calcification was determined using fixed hMSCs stained with 40 mM Alizarin Red-S (AR-S, pH 4.1; Fluka). Mineral deposition was quantified by incubating the stained sample with 10% w/v cetylpyridinium chloride (CPC; Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) for 15 min to extract AR-S. Absorbance was read at 550 nm with a Wallac Victor II plate reader. Representative images of hMSCs stained with Sirius Red and AR-S are reported in inserts (C) and (D), respectively.

The threshold cycle (Ct) of Runx2 tested by quantitative RT-PCR was normalised against the house-keeping gene, GAPDH. Data were standardised with respect to the control, set at 1. Data are expressed as the mean of ten samples analysed. Error bars indicate standard error of the mean (SEM). *P ≤ 0.05. **P ≤ 0.01.

Figure 2. Chondrogenic potential of hMSCs. Chondrogenic differentiation was evaluated in samples cultured in the presence of chondrogenic differentiating agents (DMEM supplemented with 1% FBS, 100 nM dexamethasone, 110 mg/L sodium pyruvate, 150 µM L-ascorbic acid-2-phosphate, 1X ITS and 10 ng/mL TGF-β1) for 21 days, or in their absence (Ctrl). (A) Sox9 and aggrecan expression was analysed by quantitative RT-PCR, and GAG production was assessed by dimethylmethylene blue staining after normalisation against DNA content.

qRT-PCR data were calculated using the Ct method (normalised to GAPDH house-keeping gene levels), determined in triplicate and expressed as the fold difference value from the calibrator (day 0) for each sample. Error bars indicate SEM. Statistical analysis was performed for data relating to day 21 in the absence of differentiating agents (Ctrl) versus day 21 in the presence of differentiating agents (+TGFβ). *P ≤ 0.05.

(B) Col2A1 expression was analysed by immunocytochemistry (NB600-844 COLLAGEN TYPE II, Novus Biological). Representative images of pellets cultured for 21 days either in Ctrl or chondrogenic medium (+TGFβ) are reported.

(C) Slug gene expression in osteo-differentiated hMSCs. Slug mRNA expression levels were assessed by qRT-PCR and the results calculated using the Ct method. Expression levels were normalised to GAPDH levels from the same sample. Data represent the average ± SEM of ten samples (1-10). Statistical analysis was performed for data relating to day 21 in the absence of differentiating agents (Ctrl) versus day 21 in the presence of differentiating agents (Osteo). *P ≤ 0.05.

(D) Slug gene expression in chondro-differentiated hMSCs. Slug mRNA expression levels were

assessed by qRT-PCR. qRT-PCR data and statistical analysis were processed as shown in panel A. *P ≤ 0.05.

(E) Slug gene expression in uninduced hMSCs. qRT-PCR analysis for the detection of Slug mRNA levels was performed on hMSCs at day 0, before the addition of differentiating agents, following the culturing of cells for 24 hours in adhesion 2D or pellet 3D systems. In the 2D condition, the average values of all samples studied are calculated (data represent the average ± SEM of fourteen samples). In the 3D condition, three groups (A, sample 1 and another four samples capable of differentiating towards chondrogenesis; B, samples 2-5; C, samples 6-9) were considered and compared to sample 10.

Total RNA was extracted from one aggregate per sample (for cells cultured in pellet) or from the cells cultured in adhesion in 6-well plates using an RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instruction and as previously described (Lambertini *et al.*, 2009).

RT-PCR results were calculated using the Ct method. The statistical significance between groups is marked with symbols: *2D versus groups A, B, C and sample 10; ** sample 10 versus groups A, B, and C. P ≤ 0.05.

All qRT-PCRs were carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). TaqMan technology, the Assays-On-Demand kit (Applied Biosystems, Foster City, CA, USA) for human Slug (Hs00950344_m1), Runx2 (Hs00231692_m1), Sox9 (Hs00165814_m1) and aggrecan (Hs00153936_m1) were used.

Western blot analysis for Slug protein is reported in the inserts in panels C and D. Ten μg of whole cell lysates were assayed on a 12% SDS-PAGE. Proteins were then transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). After blocking with PBS-0.05% Tween 20 and 5% dried milk, the membranes were probed with Slug antibody (L40C6) from Cells Signaling Technology (Denvers, CA, USA). Anti-IP3K (06-195) from Upstate Biotechnology (Lake Placid,

NY, USA) was used to confirm equal protein loading. The proteins were visualised using Supersignal Femto Substrate (Pierce). Size markers are reported (kD).

Figure 1

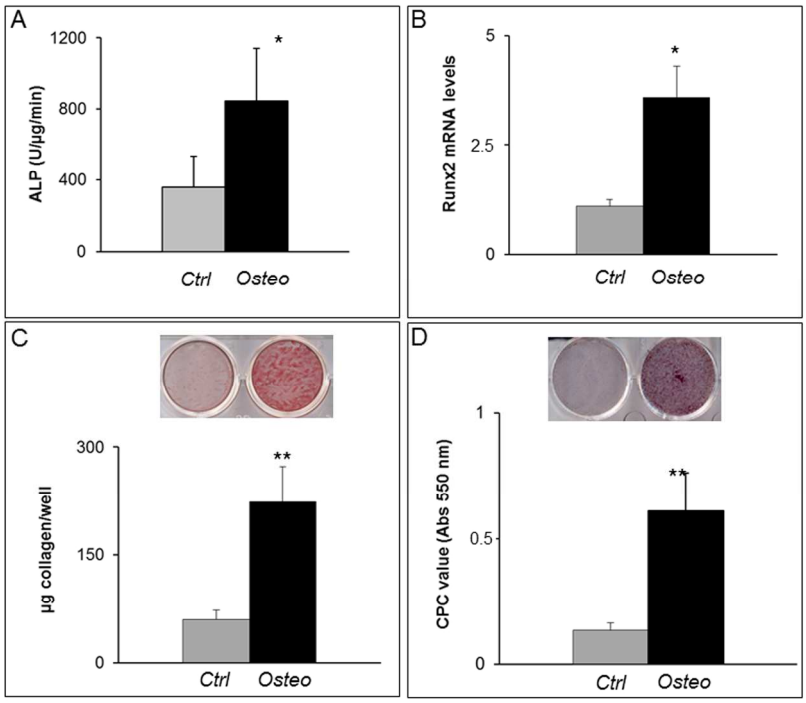

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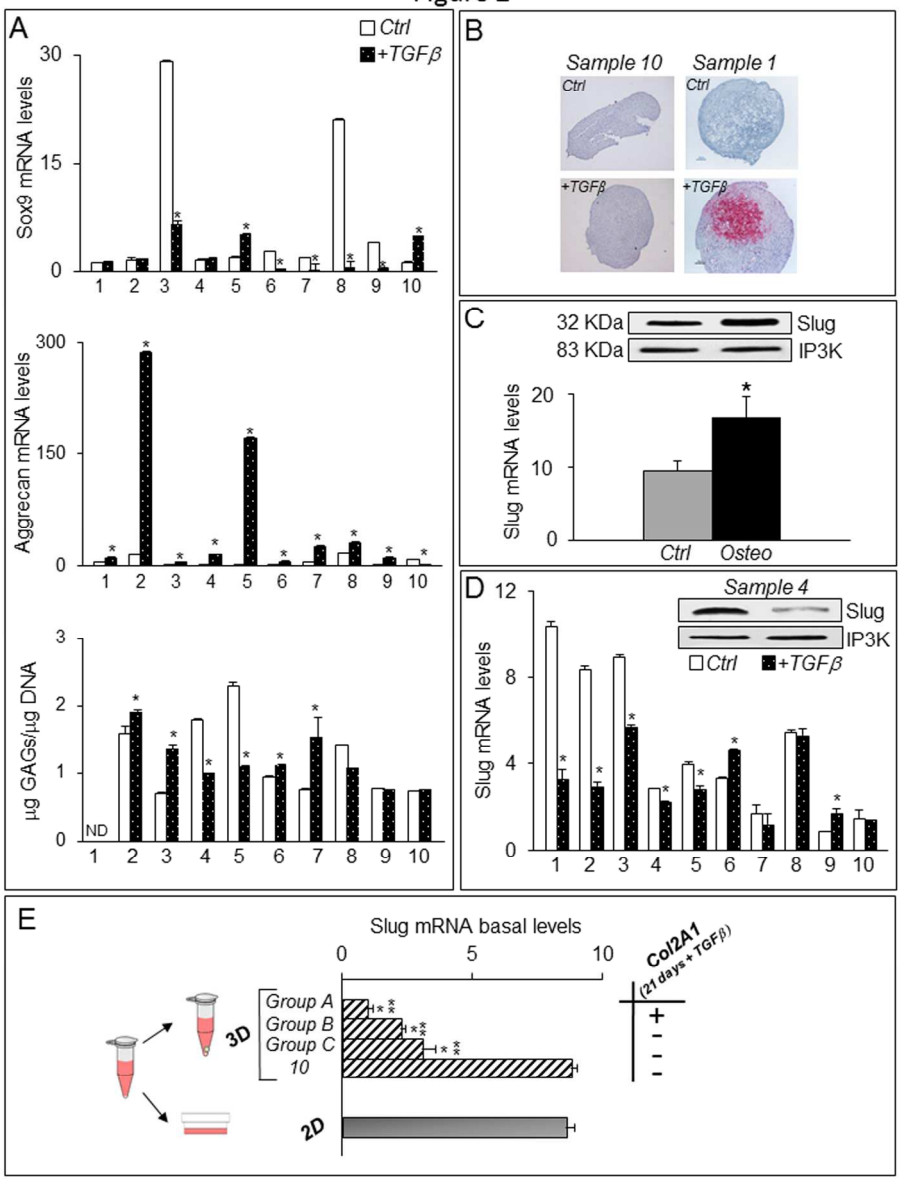
Figure 2



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