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Identification of candidate regulators of multipotency in human skeletal progenitor cells

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ABSTRACT

Stem cell differentiation is controlled intrinsically by dynamic networks of interacting lineage-specifying and multipotency genes. However, the relationship between internal genetic dynamics and extrinsic regulation of internal dynamics is complex and, in the case of skeletal progenitor cell differentiation, incompletely understood. In this study we elucidate a set of candidate markers of multipotency in human skeletal progenitor cells by systematic study of the relationships between gene expression and environmental stimulus. We used full genome cDNA microarrays to explore gene expression profiles in skeletal progenitor enriched populations derived from adult human bone marrow, minimally cultured in basal, osteogenic, chondrogenic, and adipogenic lineage-specifying culture conditions. We then used a variety of statistical clustering procedures to identify a small subset of genes which are related to these stromal lineages but are specific to none. For a selection of 11 key genes, conclusions of the microarray study were confirmed using quantitative real-time PCR.

Skeletal stem cells (SSCs) constitute a small but significant subpopulation of adherent cells in the adult human marrow [1,2] which are thought to possess the capacity for indefinite self-renewal as well as the ability to differentiate along the principle skeletal lineages, such as bone, cartilage, fat, and muscle [3–5]. Consequently, SSCs play a central role in controlling healthy skeletal tissue turnover and tissue repopulation subsequent to disease or trauma [6,7]. For this reason there is currently significant interest in understanding SSC differentiation for use in clinical settings [3,8,9].

Although pure populations of SSCs cannot currently be isolated, a number of antibodies have been utilized to obtain SSC enriched populations [10–12]. For example, STRO-1 recognizes a trypsin-resistant cell-surface antigen present on a subpopulation of bone marrow cells, including a predominant proportion of the high growth- and differentiation-potential skeletal stem cell and colony forming units-fibroblastic populations [12,13]. Thus, the STRO-1$^+$ fraction constitutes a stem cell enriched, albeit heterogeneous, skeletal progenitor cell population [14].

In light of the difficulty in obtaining a pure population, a comprehensive understanding of the genetic basis of SSC multipotency is currently lacking, although a number of genome-wide studies of skeletal stem- and progenitor cell gene expression have been undertaken. [15–20]. In this report we build upon this literature by comparing the genetic profile of STRO-1$^+$ selected SSC enriched progenitor populations under a variety of lineage-specifying culture conditions.

Materials and methods

Tissue culture reagents and all other biochemical reagents were obtained from Sigma-Aldrich, UK.

Magnetic activated cell sorting. All bone marrow samples were obtained from haematologically normal donors undergoing routine total hip replacement surgery. Samples from four unique donors of both sexes aged between 75 and 84 were used. Throughout, these four samples are denoted S1, S2, S3, and S4. Only tissue that would have been discarded was used, with the approval of the Southampton & south west Hampshire local research ethics committee. STRO-1$^+$ cells were isolated from marrow samples as described in [21]. All studies were conducted using passage 1 STRO-1$^+$ populations.

Cell culture. Freshly isolated STRO-1$^+$ cells were cultured to confluence in a T-75 tissue culture flask under basal conditions (α-mem, supplemented with 10% fetal calf serum, penicillin, and streptomycin) with a media change every 3 days. At confluence, cells were passaged into eight T-75 culture flasks: two T-75s were cultured further in basal conditions for 14 days; two T-75s were cultured in osteogenic media (as described in [22]) for 14 days; two T-75s were cultured in chondrogenic media (described in

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[22]) for 14 days; and two T-75s were cultured in adipogenic conditions for 14 days (described in [22]). In all culture conditions, appropriate media was changed every 3 days. Due to the limited number of cells obtained from STRO-1 selection, S3 cells were not cultured in adipogenic conditions and S4 cells were not cultured in chondrogenic conditions. Thus, we ensured that cells from at least three different donors were cultured in each culture condition.

RNA extraction. Total RNA was extracted from STRO-1$^+$ cells using the TRIzol reagent (Invitrogen). The extracted RNA was subjected to DNAse treatment (DNA-free RNA kit, ZYMO Research) and reverse transcribed using the Super-Script First-strand synthesis system for qPCR (Invitrogen).

Microarray. Microarray processing was conducted by Precision Biomarker Resources (Evanston, Illinois) using the Affymetrix GeneChip Array Station (GCAS) protocol according to manufacturers instructions.

Quantitative real-time PCR analysis. Real-time qPCR was performed using the 7500 Real Time PCR system from Applied Biosystems for analyzing expression of genes. Relative expression levels were normalized to β-actin expression. Primers are given in Supplementary Table 1. The comparative C_T method was employed for quantitation of gene expression. Fold relative expression levels were expressed as mean ± SD ($n = 3$–4 for each treatment group, each sample analysed in duplicate for all genes) for plotting as bar graphs.

Data analysis. All mathematical procedures were conducted using MATLAB using the Bioinformatics and Statistics toolboxes, as well as custom written script. Raw data was background-adjusted using the Robust Multi-array Average (RMA) method and $\log_2$ of the data was taken. All subsequent analysis was conducted on the normalized $\log_2$ dataset.

Statistical analysis. The following analysis was conducted separately on each of the four unique donor datasets. Data was grouped by culture condition (A, adipogenic; B, basal; C, chondrogenic; O, osteogenic), breaking each dataset into 3–4 groups of data (S1A, S1B, S1C, S1O etc). One-way ANOVA was then conducted separately on all 54,675 probesets using these groupings. We identified significant differences in gene expression by setting a Bonferroni-corrected p-value cutoff of $p^* = 0.05$ (corresponding to an absolute ANOVA p-value of $p = 9.14 \times 10^{-7}$) in order to minimize type-1 statistical errors. We followed one-way ANOVA by a multiple-comparison test, identifying as significant pairs which were outside a (1-p) confidence interval of each other. Thus, we obtained six fundamental sets of significant genes, which we denote AB, AC, AO, BC, BO, and CO (so AB represents those genes which were significantly differently expressed by cells cultured in basal conditions by comparison with cells cultured in adipogenic conditions, and so forth). We then used these six fundamental sets to identify genes associated with more than one of the four culture conditions, by finding the following additional sets

$$\text{Adipo-Chondro} = [(\text{BC} \cap \text{CO}) \cap (\text{AB} \cap \text{AO})] \setminus \text{AC}$$
$$\text{Adipo-Osteo} = [(\text{BO} \cap \text{CO}) \cap (\text{AB} \cap \text{AC})] \setminus \text{AO}$$
$$\text{Adipo-Osteo} = [(\text{AO} \cap \text{BO}) \cap (\text{AC} \cap \text{BC})] \setminus \text{CO}$$
$$\text{Adipo-Chondro-Osteo} = (\text{AB} \cap \text{BC} \cap \text{BO}) \setminus (\text{AC} \cap \text{AO} \cap \text{BO})$$

We also calculated the union of these four sets, which we denote the "multipotency" gene set, since it contains those genes differentially regulated by one or more lineage, but specific to none:

$$\text{Multipotency genes} = [\text{Adipo-Chondro}] \cup [\text{Adipo-Osteo}] \cup [\text{Chondro-Osteo}] \cup [\text{Adipo-Chondro-Osteo}]$$

Here ∩ denotes the intersection of sets, ∪ denotes the union of sets and $Y \setminus X$ denotes the relative complement of X in Y (those genes which are members of X but not members of Y). Thus, for example, the Adipo-Chondro set represents those genes which were uniformly up- or down-regulated upon both adipogenic and chondrogenic induction by comparison with basal and osteogenic conditions (and so forth for the other sets). Fig. 1 shows a Venn diagram illustrating how to obtain these sets. Since S3 cells were not cultured in adipogenic conditions we did not obtain Adipo-Chondro, Adipo-Osteo or Adipo-Chondro-Osteo gene sets for this sample; similarly since S4 cells were not cultured in chondrogenic conditions we did not obtain Adipo-Chondro, Chondro-Osteo or Adipo-Chondro-Osteo gene sets for this sample. All other gene sets were obtained by taking intersections of the appropriate available sets.

Sets of genes which had the same pattern of expression in all four donors were obtained by taking the intersection of the identified sets across all four donor samples. In cases where the appropriate S3 and S4 sets were not calculated, we took the intersections across all calculated gene sets. In particular, in the case of the Adipo-Chondro genes and the Adipo-Chondro-Osteo genes this meant that we took intersections of significant gene sets from only 2 donors (in all other cases, intersections from at least 3 of the donor samples were taken). However, the fact that we set such a stringent p-value in our analysis combined with the fact that we examined intersections of sets across independent donor samples, means that the false discovery rate in these cases is still exceptionally low.

Clustering. We examined structure in the data using principle components analysis, multi-dimensional scaling and hierarchical clustering. In order to determine the correct number of clusters we identified natural partitions in the data by finding those points at which introducing another cluster significantly reduced the within-cluster dispersion.

Results

Candidate multipotency genes characterize skeletal progenitor cell state in a donor-independent manner

Using statistical analysis we identified a set of "multipotency" genes: that is, genes which were strongly altered in all four donor samples upon induction of two or more distinct lineages yet are not associated with the third lineage (see Methods). Although the genetic basis of multipotency remains debated, the association of co-expressed genes with the maintenance of skeletal progenitor

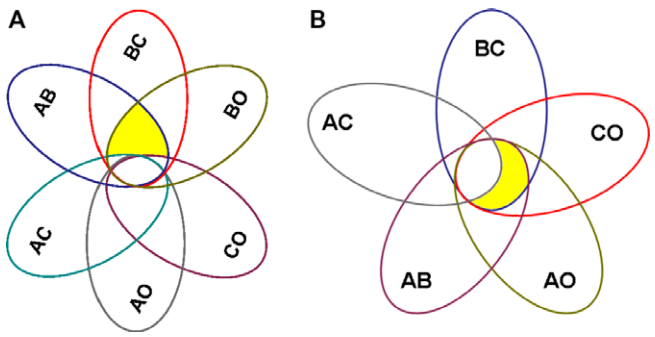

Fig. 1. Venn diagrams illustrating the various gene sets. (a) Adipo-Chondro genes = $[(\text{BC} \cap \text{CO}) \cap (\text{AB} \cap \text{AO})] \setminus \text{AC}$ are in yellow. (b) Adipo-Chondro-Osteo genes = $(\text{AB} \cap \text{BC} \cap \text{BO}) \setminus (\text{AC} \cap \text{AO} \cap \text{CO})$ are in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

multipotency is in accordance with the notion of promiscuous co-expression of key genes during multi-lineage priming [23], and provides a working definition of a multipotency gene which we shall adopt throughout this discussion. In total we identified 38 such multipotency genes which expressed strongly similar expression profiles in all four donor samples. Supplementary Table 2 gives details of these genes.

Principle components analysis shows that these genes characterize the three stromal lineages in a donor-independent manner (see Fig. 2a) and thus may be used as reliable donor-independent markers of skeletal progenitor cell state. Furthermore, we found that the 38 multipotency genes group by expression into four well-defined clusters (see Fig. 2b) corresponding primarily to: (1) those genes up-regulated both upon osteogenic and chondrogenic induction, which we shall call the OC^+ set; (2) those genes down-regulated upon both osteogenic and chondrogenic induction, which we shall call the OC^- set; (3) those genes down-regulated upon both chondrogenic and adipogenic induction, which we shall call the AC^- set; and (4) those genes up-regulated upon both osteogenic and adipogenic induction, which we shall call the OA^+ set.

Candidate markers of Osteo-Chondral bipotency

Since the OC^- cluster contains those genes which are generally down-regulated upon osteogenic and chondrogenic induction (yet remain unaffected by adipogenic induction) we surmise that these genes may play a role in regulating osteo-chondral bipotency. The OC^- cluster contains the following 12 genes: *SLC14A1*, *PTGIS*, *PENK*, *GATA6*, *LDB2*, *GREM1*, *LOC399959*, *QSOX1*, *DCBLD2*, *CLDN11*, *FLG*, and *HAPLN1*. Some of these genes play documented roles in skeletal development, and/or stem cell biology. Others are less well characterized, and therefore constitute novel targets for further research. Based upon their known ontology and potential roles in skeletal biology we selected five of these 12 genes—*GATA6*, *LDB2*, *GREM1*, *QSOX1*, and *CLDN11*—for further investigation by quantitative real-time PCR (qPCR). Expression of these 5 genes as assessed by qPCR is given in Fig. 3. In all cases, expression patterns established by qPCR were in strong agreement with microarray analysis predictions (MCT $p \leqslant 0.012$ in all cases).

The *GATA* family of zinc-finger transcription factors are involved in a variety of developmental and regulatory processes [24–29]. Given that GATA factors are so ubiquitously expressed it is thought unlikely that they act as master regulators of differentiation. Rather, it is more likely that they regulate cellular determination through functional interactions with other tissue-specific transcription factors [24].

LDB2 is a member of the *LIM* domain-binding family which are known to be involved in regulation of cell fate decisions during development [30]. Previous microarray studies have identified the transcriptional cofactor *LDB2* as a marker of pluripotency in human embryonic stem cells [31] and a marker of osteogenesis in human mesodermal precursors, as isolated by extensive subculture [18]. Crucially, *LIM* domain proteins are not thought to interact with DNA directly, but rather to provide a "scaffold" for the formation of multi-protein activator complexes involving additional transcription factors. So, for example, the *LIM* only protein *LMO2* interacts with the *LIM* domain-binding protein *LDB1*, the bHLH transcription factor *TAL1*, the bHLH *E47* protein and *GATA1* to form a multi-protein trans-activating complex which is partly responsible for the maintenance of erythroid precursors in a primitive state [28,29,32].

BMP signaling plays a central role in skeletal tissue maintenance as well as in many developmental process, including osteogenesis and chondrogenesis [33]. *GREM1* is a member of the *DAN* family of antagonists of *BMP* signaling [34], and correspondingly has been shown to play a central role in limb morphogenesis [35,36]. For example, *GREM1* inhibits chondrogenesis during avian limb morphogenesis, and has been implicated regulation of differentiation early mesodermal progenitors to pre-chondrogenic cells in early skeletal development [37]. Furthermore, in complement to its roles during development, *GREM1* is also involved in adult skeletal maintenance. For example, transgenic mice over expressing *GREM1* exhibit widespread skeletal abnormalities including bone modeling defects, spontaneous fractures, disorganized bone structure, reduced trabecular volume and osteopenia [38], indicating that *GREM1* may inhibit osteogenic differentiation of skeletal stem cells by blocking *BMP*-induced differentiation signals [38].

The *GATA6* consensus binding sequence is (*A*/*T*/*C*)*GAT*(*A*/*T*)(*A*) with the sequence *AGATAA* having the greatest binding affinity for *GATA6* [39]. Interestingly, we found that the high affinity consensus sequence *AGATAA* is present in the promoter of the *GREM1* gene [40]. Since *GATA6, LDB2*, and *GREM1* share strongly similar expression patterns as assessed by microarray studies and confirmed by qPCR, together these suggests that *GATA6* may act co-operatively upstream of *GREM1* in control of osteo-chondral bipotency. *GREM1* may then maintain osteo-chondral bipotency by antagonizing *BMP* signaling.

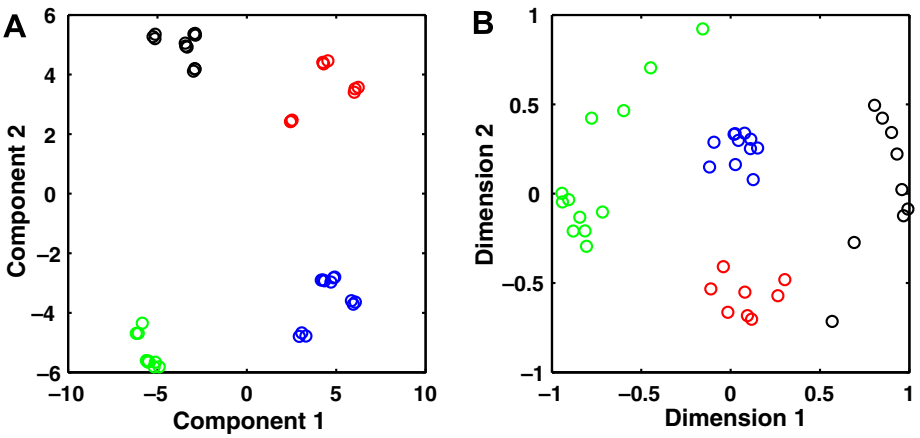

Fig. 2. Cluster analysis of the microarray data. (A) Clustering with donor samples as objects shows that the data cluster primarily by culture condition (black, basal; blue, osteogenic; green, chondrogenic; red, adipogenic). Data is projected onto the first two principle components. (B) Clustering with genes as objects identified 4 distinct clusters (red, blue, black, green). Here the data is projected onto the first two dimensions as identified by multi-dimensional scaling using Pearson correlation distance metric. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

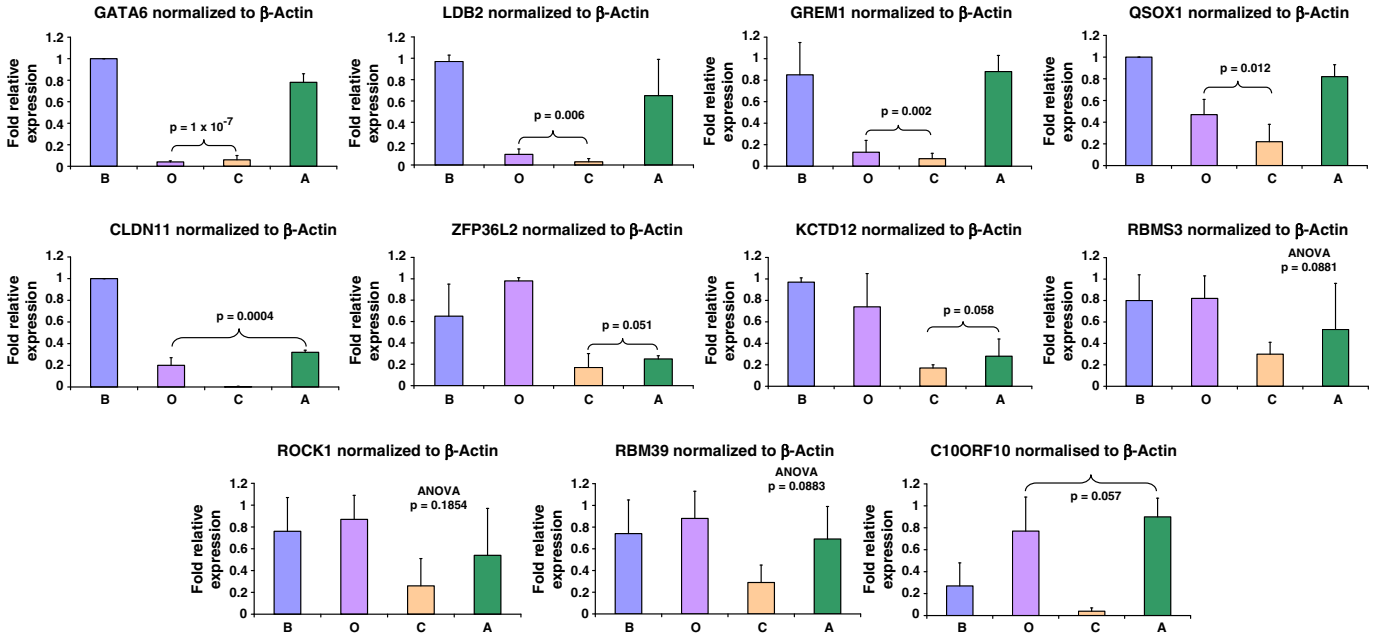

Fig. 3. qPCR expression of 11 skeletal multipotency genes as identified by microarray analysis: unless otherwise stated *p*-values refer to those of the multiple-comparison test.

The OC^+ cluster contains those genes which are generally up-regulated upon osteogenic and chondrogenic induction. Consequently, we surmise that these genes may represent negative markers of osteo-chondrogenic bipotency. The OC^+ cluster contains the following 9 genes:*C22orf16, COL11A1, FKBP5, LOC388610, MT1E, MT1F, MT1X, MT1H/MT1P2*, and *XTLT1*. In addition to containing *COL11A1*, which plays a well-documented role in skeletal biology [41], the OC^+ set also contains various Metallothioneins, which encode for zinc binding proteins, and so may play a role in skeletal progenitor multipotency by affecting the DNA binding affinity of zinc-finger containing transcription factors [42].

Candidate markers of Adipo-Chondral bipotency

Since the AC^- cluster contains those genes which are down-regulated upon adipogenic and chondrogenic induction (yet remain unaffected by osteogenic induction) we surmise that these genes may play a role in genetic regulation of adipo-chondral bipotency. The AC^- cluster contains the following 10 genes: *ZFP36L2*, *KCTD12*, *TIA1*, *RBM39*, *ROCK1*, *Sep-07*, *NOP5/NOP58*, *KIAA1946*, *RBMS3*, *DCLK1*. Based upon their known ontology and potential roles in skeletal biology we investigated expression of five of these 10 genes—*KCTD12*, *RBMS3*, *ZFP36L1*, *RBM39*, and *ROCK1*—by qPCR. Although the same trend was observed in the qPCR data as in the microarray data for each of these genes (see Fig. 3), significance at the $p = 0.05$ level could not be established (although qPCR *KCTD12* and *ZFP36L1* expression confirmed microarray predictions with MCT $p = 0.068$ and MCT $p = 0.061$, respectively). *KCTD12* has been previously identified as a marker of bone marrow stromal cells from a variety of adult human bones by real-time PCR comparison of expression patterns with fibroblasts [43]. The zinc-finger domain containing transcription factor *ZPF36L2* is a molecular target of *LIF* [44], and as such is implicated in maintenance of SSC multipotency through interactions with components of the *LIF*/*STAT3* pathway.

Candidate markers of Osteo-Adipo bipotency

The OA^+ cluster contains those genes which are generally strongly up-regulated upon osteogenic and adipogenic induction. Thus, we surmise that they may represent negative markers of osteo-adipo bipotency. The OA^+ cluster contains the following 7 genes: *LBP*, *CXCL6*, *NPY2R*, *C10orf10*, *PTGFR*, *FAM89A*, and *STC2*. We further investigated expression of *C10orf10* using qPCR. qPCR expression followed microarray predictions with MCT $p = 0.067$ (Fig. 3). *C10orf10* has been shown to activate the transcription factor *ELK1* in a human embryonal kidney cell line [45]. *ELK1*, a target of the *ras-raf-MAPK* signaling cascade, is down-regulated upon differentiation of human embryonic stem cells [46]. Thus, *C10orf10* may be involved in osteo-adipo bipotency via its interaction with *ELK1*.

Discussion

In this study we have compared the genome-wide gene expression patterns of STRO-1^+ human skeletal stem cell enriched populations, minimally cultured in osteogenic, chondrogenic, adipogenic, and basal culture conditions. Using simple analyses, and based upon the notion of promiscuous co-expression during multi-lineage priming [23], we have identified a panel of 38 genes whose expression across multiple donor datasets suggests that they may be associated with maintenance of multipotency in adult human progenitor cells. We found that this set of genes provides a robust donor-independent set of markers of skeletal progenitor cell state, and therefore may be used in a clinical setting. Some of these genes play previously well-documented roles in bone, cartilage and fat biology. Others have previously defined roles in development. Some are less well characterized, and therefore constitute novel targets for further research. Based upon potential roles in skeletal biology we selected 11 of the less well characterized genes for further analysis and confirmed expression patterns using quantitative real-time PCR. In particular, our results suggest a cooperative role for the transcription factor *GATA6*, the transcriptional cofactor *LDB2* and the *BMP* antagonist *GREM1* in co-ordinated regulation of osteo-chondral bipotency. Our current efforts are focussed on further elucidating the nature of this cooperation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.084.

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