Derivation of a novel undifferentiated human foetal phenotype in serum-free cultures with BMP-2

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Abstract

Skeletal stem and progenitor populations provide a platform for cell-based tissue regeneration strategies. Optimized conditions for ex vivo expansion will be critical and use of serum-free culture may allow enhanced modelling of differentiation potential. Maintenance of human foetal femur-derived cells in a chemically defined medium (CDM) with activin A and fibroblast growth factor-2 generated a unique undifferentiated cell population in comparison to basal cultures, with significantly reduced amino acid depletion, appearance and turnover, reduced alkaline phosphatase (ALP) activity and loss of type I and II collagen expression demonstrated by fluorescence immunocytochemistry. Microarray analysis demonstrated up-regulation ofCLU, OSR2, POSTN and RABGAP1 and down-regulation of differentiation-associated genes CRYAB, CSRP1, EPAS1, GREM1, MT1X and SRGN as validated by quantitative real-time polymerase chain reaction. Application of osteogenic conditions to CDM cultures demonstrated partial rescue of ALP activity. In contrast, the addition of bone morphogenetic protein-2 (BMP-2) resulted in reduced ALP levels, increased amino acid metabolism and, strikingly, a marked shift to a cobblestone-like cellular morphology, with expression of SOX-2 and SOX-9 but not STRO-1 as shown by immunocytochemistry, and significantly altered expression of metabolic genes (GFPT2, SC4MOL and SQLE), genes involved in morphogenesis (SOX15 and WIF1) and differentiation potential (C1orf19, CHSY-2, DUSP6, HMGCS1 and PPL). These studies demonstrate the use of an intermediary foetal cellular model for differentiation studies in chemically defined conditions and indicate the in vitro reconstruction of the mesenchymal condensation phenotype in the presence of BMP-2, with implications therein for rescue studies, screening assays and skeletal regeneration research.

Keywords: foetal • osteoprogenitor • BMP-2 • serum-free medium • differentiation • tissue regeneration

Introduction

The application of stem/progenitor cells to injury or disease management will require sufficient cell number, thus necessitating derivation of ex vivo expansion protocols as the native stem cell resource is limited by their availability, typically less than one in every 40,000 cells [1, 2]. Expansion of cultures in vitro must be tempered with the ability of the cells to maintain a proliferative capacity and stem/progenitor phenotype prior to targeted lineage differentiation. Most tissue culture techniques utilize foetal calf serum (FCS), a complex undefined mixture of factors, although batch variability has been shown to have significant effects on expansion kinetics of human bone marrow stromal cells (hBMSCs) [3]. The use of human-derived serum has been shown to support hBMSC and chondrocyte cell growth; however, these studies are limited by issues of cost and availability [4, 5]. Use of a serum-free growth medium may permit better modelling of differentiation potential [6–8], and a serum-free chemically defined medium (CDM) developed by Johansson and Wiles [6] was later optimized for undifferentiated human embryonic stem cells (hESC) culture by Vallier and colleagues with the addition of
either activin A or Nodal as inhibition of activin/Nodal signalling, though not Nodal alone, resulted in increased differentiation [9]. Following supplementation with fibroblast growth factor-2 (FGF-2), hESCs maintained pluripotency in long-term cultures suggesting FGF-2 as a competence factor in the activin/Nodal pathway [9].

Many tissue regeneration strategies rely on the application of stimulatory agents to induce differentiation following expansion [10, 11] and various factors have been applied to mesenchymal cell populations [12–14]. Ascorbic acid 2-phosphate (ascorbate), acts as an enzyme co-factor for collagen synthesis and has been shown to enhance the osteogenic response of human osteoblasts in vitro [15] as has dexamethasone, a glucocorticoid hormone which promotes the catabolism of carbohydrates, fats and proteins and antagonizes insulin signalling [12, 16–18]. The bone morphogenetic proteins (BMPs) are potent mitogens for the formation of new skeletal tissue, and have been extensively studied for their effects on osteogenic and chondrogenic differentiation [19–21] and the proliferative ability of cells [19, 20, 22–25]. First described as components of demineralized bone matrix by Urist and coworkers [26], BMPs are key in the signal transduction of SMAD proteins 1, 5 and 8 which up-regulate runt-related transcription factor 2 (RUNX2, also referred to as CBFA1 or core-binding factor 1) and result in downstream up-regulation of bone matrix proteins type I collagen, osteocalcin and osteopontin, necessary for osteoblast maturation [27]. Various studies have reported on the osteogenic properties of human foetal bone cells. Harris and coworkers demonstrated increased levels of alkaline phosphatase (ALP) and osteocalcin in an immortalized human foetal osteoblastic cell line in the presence of vitamin D₃ [28] although studies with primary bone cells from foetuses at 11–14 weeks after conception (WPC) by Campagloni et al. [29] or 13–16 WPC by Montjovent et al. [30], indicated the proliferation of foetal bone cells was increased in the presence of dexamethasone and showed that following treatment with vitamin D₃, ascorbic acid and β-glycerophosphate, foetal populations displayed enriched ALP activity, with up-regulation of type I collagen, ALP, osteocalcin and RUNX2 gene expression [30]. In addition, we have demonstrated an osteoprogenitor phenotype of human foetal femur-derived cells at 7.5–11 WPC with expression of type I collagen, ALP activity and BMP receptor 1A in basal conditions, and up-regulation of non-collagenous bone proteins osteopontin and osteocalcin in the presence of ascorbate and dexamethasone [31].

Human foetal femur-derived cells provide an important intermediary cellular model as a pre-natal but non-embryonic source, between hESC and adult cell populations. Delineation of their osteogenic potential for application to modelling studies will be critical. The current studies, therefore, set out to model the effects of potent osteogenic growth factors ascorbate/dexamethasone and BMP-2 on human foetal femur-derived cells in a serum-free CDM, to provide greater understanding therein for ex vivo expansion, application to growth factor screening and skeletal tissue engineering.

Materials and methods

Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) including α-MEM (minimum essential medium, α-modification, M0644), bovine serum albumin (BSA, A1470), monothioglycerol (M6145), PBS (phosphate buffered saline, P4417), trypsin-EDTA (ethylenediamine tetra-acetic acid, T4174), Tris-EDTA (Tris-hydrochloric acid EDTA, T9285), TRITON® X-100 (X100), alkaline buffer solution (A9226), ALP assay kit with Sigma 104® phosphatase substrate (104–0) and AP standard (104–1), IGEPAL® CA-630 (I3021) and primers for real-time quantitative RT-PCR (qRT-PCR), FCS (10106169), Iscove’s modified Dulbecco’s medium (IMDM, 21980–032), F-12 (Ham’s) nutrient mixture (31765–027), Lipid 100× mix (11905–031), TIRzol reagent (15596–018), Super-Script First-strand synthesis system for PCR (11904–018), Cell Tracker Green™ CMFDA (5-chloromethyl-fluorescein diacetate, C7025), ethidium homodimer-1 (E1169) and DAPI (4’,6-diamidino-2-phenylindole, D3571) were purchased from Invitrogen (Paisley, UK), Collagenase B (1088807), insulin (1378497) and transferrin (652202) were procured from Roche (Manheim, Germany), Activin A (120–14) and FGF-2 (100–18B) were purchased from PeproTech EC (London, UK). DNA-free RNA Kit (R1013) was obtained from Zymo Research Corporation (Orange, CA, USA). BMP-2 protein was obtained from Prof. Walter Sebald, University of Würzburg, Germany. Undiluted culture supernatant was derived from the STRO-1 hybridoma (IgM) provided by Dr. J. Beresford, University of Bath. Type I collagen polyclonal rabbit antibody was a gift from Dr. Larry Fisher, of the National Institutes of Health (NIH). Type II collagen polyclonal rabbit antibody (234187) was purchased from Calbiochem (Nottingham, UK). SOX-2 (AB5603) and SOX-9 (AB5535) polyclonal rabbit antibodies were obtained from Chemicon International, Inc. (Temecula, CA, USA). Goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugate (115–096–075) and goat anti-rabbit IgG TRITC (terramethyl rhodamine isothiocyanate)-conjugate (111–026–047) secondary antibodies were purchased from Jackson ImmunoResearch Labs., Inc. (West Grove, PA, USA and Stratech Scientific Ltd., Soham, Cambridgehire, UK). Power SYBR® Green PCR Master Mix (4367859) was purchased from Applied Biosystems (Foster City, CA, USA). All images were captured on a Carl Zeiss Axiovert 200 microscope with Axiovision software (version 4.5) via an AxioCam MRc digital camera for phase images or via an AxioCam MRc with appropriate filters for fluorescence microscopy (Carl Zeiss Ltd., Welwyn Garden City, UK).

Cell culture

Human foetal tissue was obtained with informed and written consent following termination of pregnancy, according to guidelines issued by the Polkinghorne Report [32] and ethical approval from the Southampton & South West Hampshire Local Research Ethics Committee for use of tissue at 7.5–11 WPC. Tissue was obtained from both sexes (as obtained from placental identification), and foetal age was determined by measuring foot length, with a total of 15 human foetal samples utilized (mean 8.8 weeks, with a total of 15 human foetal samples utilized (mean 8.8 weeks).
**Chemically defined medium**

CDM comprising 50% IMDM/50% F-12, supplemented with 5 mg/ml BSA, Lipid 100× at 1% concentration, 450 μM monothioglycerol, 7 μg/ml insulin and 15 μg/ml transferrin was used with the addition of activin A and FGF-2 where appropriate at 10 ng/ml and 12 ng/ml, respectively [9]. Primary cultures were established as above then seeded to well plates. After 24 hrs, cells were washed twice with PBS then transferred to either CDM with activin A/FGF-2 or back to basal medium conditions with media changes every 48 hrs until harvest at 7 days for metabolic or biochemical analysis.

**Osteogenic modulatory factors with CDM**

Primary foetal cells were seeded across well plates for biochemical, histological and metabolic analysis or tissue culture flasks for microarray studies. After 24 hrs, cells were washed twice with PBS then given either CDM with activin A/FGF-2 (CDM + A/F) or basal medium to establish culture conditions. Media changes were performed on respective cultures every 48 hrs, and after 5 days CDM + A/F cultures were refreshed with either CDM alone (still labelled as CDM + A/F), CDM with 100 μM ascorbate/10 nM dexamethasone, CDM with 150 ng/ml BMP-2 or basal medium with basal medium changes on control wells. Concentrations of modulatory factors were used as established [12, 20]. Cultures were maintained for a further 5 days with media changes every 48 hrs prior to harvest after a total of 10 days in CDM or control conditions (culture regimen is presented diagrammatically in Fig. S1).

**Biochemical analysis**

Following fixation in 95% ethanol, cell lysate in 0.05% TRITON®-X100 was utilized for colorimetric turnover of para-Nitrophenylphosphate (pNPP), measured at 410 nm to quantify ALP activity as nmol pNPP/hr or assayed using the PicoGreen® double-stranded DNA quantification reagent (480 nm excitation, 520 nm emission) to calculate DNA content, given as ng/mL. Specific ALP activity results were expressed as nmol pNPP/ng DNA/hr. Absorbance and fluorescence were measured on an ELX-800 Universal Microplate Reader and FLX-800 Microplate Fluorescence Reader, respectively, using Bio-Tek KC4 Kinetical for Windows software (version 3.01, revision 7) (Bio-Tek Instruments, Inc., Winooski, VT, USA, http://www.biotek.com). Values are expressed as mean ± standard deviation (S.D.). Studies were run in triplicate with multiple culture wells (n = 6). Statistical significance compared to basal control cultures was calculated using ANOVA with Tukey’s Multiple Comparisons post-test. All statistical analyses were performed with GraphPad Instant Software (GraphPad Software Inc, San Diego, CA, USA, http://www.graphpad.com).

**Metabolic activity**

Following washes in PBS, samples were incubated in Earle’s balanced salt solution supplemented with 0.5% (v/v) human serum albumin, 1 mM glucose, 5 mM lactate, 0.47 mM pyruvate and a complete mixture of amino acids, for 2 hrs at 37°C prior to fixation. Reverse phase high performance liquid chromatography was performed as previously described [33]. Following correction for blank wells (media in the absence of cells), amino acid data were expressed as either appearance or depletion of amino acids from the culture medium, or the sum of the two given as ‘turnover’, expressed as pmol/ng DNA/h, mean ± standard error of the mean (S.E.M.). Amino acid data were analysed to determine whether they were normally distributed using the Ryan-Joiner normality test. Differences between basal conditions and CDM with activin A and FGF-2 were analysed using either a Student’s t-test or a Mann-Whitney U-test. Differences between CDM with activin A/FGF-2, CDM with ascorbate/dexamethasone and CDM with BMP-2 were analysed using one-way ANOVA followed by a Fisher’s test.

**Cell viability**

Cells were labelled with Cell Tracker Green™ CMFDA and ethidium homodimer-1 for viable or necrotic cells, respectively, as per manufacturer’s instructions. Following fixation in 95% ethanol, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:100 in PBS) for 5 min.

**Fluorescence immunocytochemistry**

Cultures were fixed in 4% paraformaldehyde and stored in PBS at 4°C prior to immunostaining. Following blocking (1% BSA in PBS) and permeabilization where necessary (0.01% TRITON®-X100) primary antibodies were incubated at 4°C overnight with respective dilutions in 1% BSA in PBS; type I collagen (LF67), 1:300; type II collagen, 1:1000; STRO-1, 1:2; Sox-2, 1:200 or Sox-9, 1:150. Following PBS washes appropriate secondary antibodies were applied for 1 hr at room temperature and counterstained with DAPI. Negative controls lacked the primary antibody, wherein no staining was observed (Fig. S2).

### Table 1 Foetal samples used and sample age, weeks after conception (WPC)

| Foetal sample | Foot length | Age (WPC) |
|---------------|-------------|-----------|
| H549          | 10.0        | 11.0      |
| H589          | 6.5         | 8.5       |
| H815          | 6.5         | 8.5       |
| H827          | 7.5         | 9.0       |
| H858          | 6.0         | 8.5       |
| H860          | 6.5         | 8.5       |
| H948          | 7.0         | 9.0       |
| H988          | 6.0         | 8.5       |
| H993          | 6.5         | 8.5       |
| H1052         | 5.0         | 7.5       |
| H1054         | 7.0         | 9.0       |
| H1119         | 7.0         | 9.0       |
| H1124         | 7.0         | 9.0       |
| H1126         | 6.5         | 8.5       |
| H1148         | 6.0         | 8.5       |
**RNA extraction and cDNA synthesis**

Total RNA was extracted from cell cultures using the TRIzol reagent as per manufacturer’s instructions and subjected to DNase treatment then either sent for microarray analysis or reverse transcribed using the super-script first-strand synthesis system for PCR.

**Microarray analysis**

Microarray processing was conducted by Precision Biomarker Resources, Inc., (Evanston, IL, USA, http://www.precisionbiomarker.com). Following confirmation of sample quality on a nanodrop ND-1000 (for optical densities at 230, 260 and 280) and a Bioanalyzer Nanochip (Agilent’s protocol), 1.0 µg of labelled cRNA from each sample was hybridized to Affymetrix U133 plus 2.0 chips in triplicate (giving three technical replicates per sample, with three biological replicates) and processed using the Affymetrix GeneChip Array Station (GCAS) protocol according to manufacturer’s instructions. To determine significantly altered gene expression for basal medium versus CDM with activin A/FGF-2, a pairwise Student’s t-test with a Bonferroni correction (P < 0.05/length or array, or 54,675 probe sets) was performed from log2 normalized expression data with MATLAB® software (version 7.3, The Mathworks Inc., Natick, MA, USA, http://www.mathworks.com) (Fig. S3A). A three-way ANOVA was performed to compare basal, CDM with activin A/FGF-2 and CDM plus BMP-2 conditions by Dr. Eric Bremer of Precision Biomarker Resources, Inc. using Partek® Genomics Suite software (version 6.08, Partek Inc., St. Louis, MO, USA, http://www.partek.com). Significantly altered gene expression (P < 0.05) above a twofold threshold was determined by the G mean ratio of log2 normalized expression data (Fig. S3B). Appropriate genes of interest for qPCR validation were determined following literature review. Microarray data were deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and Array Express (http://www.ebi.ac.uk/arrayexpress/) in accordance with MIAME guidelines [34].

**Quantitative PCR**

Real-time qRT-PCR was performed with the Applied Biosystems 7500 Real Time PCR System for primers as illustrated in Table 2. Values were calculated using the comparative threshold cycle (Ct) method and normalized to β-ACTIN expression. Results of combined experiments have been presented (three donor samples) and expressed as mean ± S.D. Statistical analyses were performed with Student’s t-test for CDM and activin A/FGF-2 cultures in comparison to basal conditions or one-way ANOVA with Tukey’s multiple comparisons post-test for basal, CDM with activin A/FGF-2 and CDM with BMP-2 comparisons.

**Results**

**Human foetal femur cells grow in a serum-free chemically defined medium**

To investigate the efficacy of the CDM, human foetal femur-derived cells were cultured in CDM with activin A/FGF-2 (Fig. 1). After 7 days, growth was observed to be equivalent to basal controls maintained in serum (Fig. 1A); however, the addition of activin A/FGF-2 to α-MEM alone resulted in a significantly reduced cell numbers as assessed by DNA content, (Fig. 1A) and negligible ALP activity (Fig. 1B). Cultures containing serum displayed a baseline level of ALP activity which was 10-fold greater than cultures with CDM and activin A/FGF-2 (Fig. 1B). Following genome-wide expression array analysis, 46 genes were identified as significantly different in three different foetal samples (Table S1). Down-regulated genes included matrix metalloproteinases (MMPs) 1, 3 and 13, the chemokine (C-X-C motif) ligands 1 and 6, interleukins 6 and 8 and metallothionein variants. Validated expression by qPCR demonstrated up-regulation of clusterin, odd-skipped related 2, periostin and RAB GTPase 1 (Fig. 1C) and a loss of expression for crystallin αB, cysteine and glycine rich protein 1, endothelial PAS protein 1, gremlin 1, metallothionein 1X and serglycin (Fig. 1D). Metabolic analysis demonstrated significantly reduced amino acid depletion, appearance and turnover in cultures of CDM with activin A and FGF-2 compared to basal controls (Fig. 1E).

**Use of CDM to model osteogenic modulatory factors in human foetal cell cultures**

The addition of 100 µM ascorbate and 10 nM dexamethasone to basal medium enhanced ALP activity in comparison to basal controls cultures (Fig. S4). No increase of ALP was observed with 150 ng/ml BMP-2, and, furthermore, supplementation of BMP-2 to ascorbate/dexamethasone-treated cultures attenuated their ALP activity (Fig. S4). We therefore modelled the effect of osteogenic modulatory factors on cultures in CDM (Fig. 2). In comparison to basal cultures, cells cultured in CDM supplemented with either activin A/FGF-2, ascorbate/dexamethasone or BMP-2 displayed reduced cell number, according to DNA content, which was restored to control levels when transferred back to basal culture conditions (Fig. 2A). The addition of ascorbate/dexamethasone resulted in only partial recovery of ALP activity in comparison to basal cultures (Fig. 2B). Moreover, the lowest specific ALP activity was observed in cultures grown in CDM plus BMP-2 (Fig. 2B). Morphologically, negligible difference was observed between basal conditions and CDM cultures containing either activin A/FGF-2, ascorbate/dexamethasone or medium with serum (Fig. 2C–F). In contrast, a marked change was observed in cultures supplemented with BMP-2 with appearance of a cobblestone-like cellular morphology, surrounded by cells with typical fibroblast morphology (Fig. 2G compared with Fig. 2C–F). Nuclear counter-staining demonstrated a shift in nuclear location to the cell edge and a kidney-shaped morphology (Fig. 2H and inset). To determine culture viability, CDM plus BMP-2 conditions were maintained for 21 days with negligible evidence of cell death (Fig. 2I and J). Metabolic studies showed significantly increased amino acid depletion, appearance and turnover in CDM with ascorbate/dexamethasone and CDM with BMP-2 cultures in comparison to CDM and activin A/FGF-2 (Fig. 2K).
### Table 2 Primer sequences of human genes used for quantitative PCR validation

| Gene abbr. | Full name                                      | Accession number | Primer sequences | Amplicon |
|------------|------------------------------------------------|------------------|------------------|----------|
| β -Actin   | β-actin                                        | NM_001101        | F: 5’ ggc atc ctc acc ctg aag ta 3’  | 81 bp    |
|            |                                                |                  | R: 5’ agg tgt ggt gcc aga ttt tc 3’  |          |
| ALP        | Alkaline phosphatase                           | NM_000478        | F: 5’ gga act cct gag cct tga cc3’ | 85 bp    |
|            |                                                |                  | R: 5’ tcc tgt tca gct cgt act gc 3’ |          |
| COL1A1     | Collagen type I, α1                            | NM_000088        | F: 5’ aac agc cgc ttc acc tac ag 3’  | 99 bp    |
|            |                                                |                  | R: 5’ ggg agg tct tgg tgg ttt tg 3’ |          |
| OCN        | Bone γ-carboxyglutamate protein                | NM_199173        | F: 5’ ggc agc gag gta gtg aag ag 3’  | 101 bp   |
|            | (osteocalcin)                                  |                  | R: 5’ ctc cac ctc ctc cct g 3’       |          |
| RUNX2      | Runt-related transcription factor 2            | NM_001015051     | F: 5’ tct tca cca atc tcc ccc 3’     | 230 bp   |
|            |                                                |                  | R: 5’ tgg att aaa agg act tgg tg 3’   |          |
| CLU        | Clusterin                                      | NM_001831        | F: 5’ cca gac ggt ctc aga cca tg 3’  | 95 bp    |
|            |                                                |                  | R: 5’ gtg ccc ccc cgt tga cag 3’     |          |
| CRYAB      | Crystallin, αb                                 | NM_001885        | F: 5’ tgg gat tga tgg agg tg 3’      | 81 bp    |
|            |                                                |                  | R: 5’ tcc tgt gga act tgg tg 3’      |          |
| CSRP1      | Cysteine and glycine-rich protein 1            | NM_004078        | F: 5’ gct ggg tat caa gca cga g 3’   | 91 bp    |
|            |                                                |                  | R: 5’ ctc gga gcc acc aat ctt ct 3’  |          |
| EPAS1      | Endothelial PAS domain protein 1               | NM_001430        | F: 5’ aac ctc aag tca gcc acc tc 3’  | 75 bp    |
|            |                                                |                  | R: 5’ tgg agg agg gca gtt gt 3’      |          |
| GREM1      | Gremlin 1                                      | NM_013372        | F: 5’ cac act caa ctg ccc tga a 3’   | 73 bp    |
|            |                                                |                  | R: 5’ gca acg aca ctg ctt cac 3’     |          |
| MT1X       | Metallothionein 1X                             | NM_005952        | F: 5’ caa ctg ctc ctg ccg 3’         | 104 bp   |
|            |                                                |                  | R: 5’ ggc acg agg agc agc gcc 3’     |          |
| OSR2       | Odd-skipped related 2                          | NM_053001        | F: 5’ tgt aca tct gcc aca agg 3’      | 104 bp   |
|            |                                                |                  | R: 5’ tcc ttt ccc aca ctc ctg 3’     |          |
| POSTN      | Periostin, osteoblast specific factor          | NM_006475        | F: 5’ gca cgg cgt aat gag gct tg 3’  | 61 bp    |
|            |                                                |                  | R: 5’ gca cgg cgt aat gag gct tg 3’  |          |
| RABGAP1    | RAB GTPase activating protein 1                | NM_012197        | F: 5’ tgc tct cca aac ctc tac gc 3’  | 83 bp    |
|            |                                                |                  | R: 5’ ggg ctc cca aca cca gag 3’     |          |
|            |                                                |                  | R: 5’ gcc act cta gtg aca aca 3’     |          |
| SRGN       | Serglycin                                      | NM_002727        | F: 5’ ccc tct gag ctc taa ct 3’      | 93 bp    |
|            |                                                |                  | R: 5’ ccc gag ctc taa cca gag 3’     |          |
| C1orf19    | Chromosome 1 open reading frame 19             | NM_052965        | F: 5’ tgt tac ctc gac ctc atg g 3’   | 86 bp    |
|            |                                                |                  | R: 5’ cca aca agg cag atg agc 3’     |          |

Continued
Phenotypic characterization demonstrated a loss of type I collagen expression in both CDM with BMP-2 and CDM plus activin A/FGF-2 conditions in comparison to basal cultures (Fig. 3A versus Fig. 3B and C), with minimal expression of type II collagen (Fig. 3D versus Fig. 3E and F). STRO-1 was seen in selected cells in three conditions (Fig. 3G–I) although not in the cells with the altered nuclear positioning (Fig. 3I). SOX-9 expression was observed in basal (Fig. 3J) and CDM with activin A/FGF-2 cultures (Fig. 3K) but limited to the cobblestone-like cells in CDM plus BMP-2 (Fig. 3L). In addition, expression of SOX-2 was observed in the CDM plus BMP-2 cultures only and was restricted to the cobblestone-like cells (Fig. 3M and N versus Fig. 3O). Expression of OCT4 and TRA-1–60 were not seen in any of the treatments (Fig. S5).

Microarray analysis of CDM plus BMP-2 cultures

To better define the mechanisms behind the altered phenotype in the CDM plus BMP-2 cultures, genome-wide expression array analysis was used to identify differential gene expression compared to basal and CDM with activin A/FGF-2 conditions (Fig. 4). Principal component analysis of genome-wide expression arrays showed a distinct shift in the dimensionality of gene clustering, indicating a markedly altered gene expression profile in cells cultured in CDM with BMP-2 compared to basal or CDM plus activin A/FGF-2 conditions (Fig. 4A). Results of a three-way ANOVA comparing the culture conditions demonstrated significantly altered expression of 58 genes and hierarchical cluster analysis showed basal conditions as more distinct to either CDM treatment group as shown by the length of the vertical dendrogram arms, with little variation between replicates or samples (Fig. 4B). Ten genes were identified as representative from the six cluster groups formed at the fourth level of horizontal dendrogram arms and validated with qPCR (Fig. 5). Expression levels for chromosome 1 open reading frame 19 increased in CDM with activin A/FGF-2 cultures over basal and was higher still in CDM plus BMP-2 conditions (Fig. 5A), a pattern repeated for 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (Fig. 5B), sterol-C4-methyl oxidase-like (SC4MOL) (Fig. 5C), Wnt inhibitory factor 1 (WIF1) (Fig. 5D), and several others (Table 2).

Table 2
Continued

| Gene abbr. | Full name | Accession number | Primer sequences | Amplicon |
|------------|-----------|------------------|------------------|----------|
| CHSY-2     | Chondroitin synthase-2 | NM_175856 | F: 5' cgc cga cga cga tgt cta c 3' | 85 bp |
| DUSP6      | Dual specificity phosphatase 6 | NM_022652 | F: 5' ggg caa gaa ctg tgt ct 3' | 59 bp |
| GFPT2      | Glutamine-fructose-6-phosphate transaminase 2 | NM_005110 | F: 5' cgg ctg gag tac aga ggc ta 3' | 99 bp |
| HMGCS1     | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | NM_001098272 | F: 5' cgt ccc act cca aat gat g 3' | 94 bp |
| PPL        | Periplakin | NM_002705 | F: 5' gga ggc act ctg tga ctt ctg 3' | 84 bp |
| SC4MOL     | Sterol-C4-methyl oxidase-like | NM_001017369 | F: 5' cat ggg tga cca cta ctt ctg 3' | 146 bp |
| SOX15      | SRY (Sex determining region Y)-box 15 | NM_006942 | F: 5' aac tgc tgc cca cct ata cc 3' | 65 bp |
| SQLE       | Squalene epoxidase | NM_003129 | F: 5' gat ggg agt tca cga gag 3' | 68 bp |
| WIF1       | Wnt inhibitory factor 1 | NM_007191 | F: 5' cgg ctt acc cgg att cta ctt 3' | 59 bp |

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oxidase-like (Fig. 5C), and Wnt inhibitory factor 1 (Fig. 5D). The highest expression for dual specificity phosphatase 6, periplakin and SOX15 were observed in CDM with activin A/FGF-2 conditions, compared to basal and CDM plus BMP-2 cultures (Fig. 5E–G, respectively). In CDM plus BMP-2 conditions, expression of chondroitin synthase-2 was comparable to basal medium (Fig. 5H) but had the highest expression of glutamine-fructose-6-phosphate transaminase 2 (Fig. 5I) and squalene epoxidase (Fig. 5J) in comparison to the other two treatment groups.

Discussion

We have utilized a serum-free CDM for human foetal femur cell expansion and demonstrated the derivation of an undifferentiated population compared to cultures maintained in serum. Furthermore, the addition of BMP-2 provides a novel cellular model for differentiation studies.

These studies highlight several key findings; firstly, culture in CDM with activin A and fibroblast growth factor-2 [6, 9] resulted in an undifferentiated phenotype as indicated by reduced amino acid turnover [35], negligible expression of ALP activity and type I and type II collagens. Expression of STRO-1 and SOX-9, as markers of mesenchymal progenitors, was maintained in selected cells [36, 37]. Microarray studies confirmed this phenotype, with up-regulation of clusterin, involved in anti-apoptosis [38, 39], odd-skipped related 2 and peristin, as key regulators of pre-osteoblast proliferation [40, 41] and RAB GTase 1, indicated in cell cycle maintenance [42]. In addition, we observed down-regulation of crystallin αB, suggested to have a role in osteogenic differentiation [43], gremlin 1, a BMP antagonist [44] and cysteine and
glycine-rich protein 1, involved in the regulatory processes of development and cellular differentiation [45] and implicated in the non-canonical Wnt signalling pathway [46]. Down-regulated metallothioneins correlate to studies which showed a positive regulatory role of metallothionein isoforms in osteogenesis [47] and reduced serglycin expression with CDM and activin A/FGF-2 suggests a role in the proliferative capacity of these cells [48]. Further genes down-regulated include interleukin 6 (IL-6), necessary for osteoblast signalling in osteoclastogenesis [49] and chemokine C-X-C motif ligands 1 and 6, seen in early neural progenitors during development of the human foetal brain [50] and in mesenchymal cells [51], respectively. Down-regulation of MMPs 1, 3 and 13, confirmed the de-differentiation effects of serum-free CDM with activin A and FGF-2, with the latter isoform seen in hypertrophic chondrocytes of human foetal bone tissue [52], similar in locus and morphology to the foetal femur tissue utilized in the current studies [31].

Additionally, although the application of ascorbate/dexamethasone to adult and foetal mesenchymal populations has been previously...
reported [12, 53] and BMP-2 has been shown to positively affect hBMSC proliferation [20, 22, 54] the current study demonstrated the addition of BMP-2 resulted in a significant reduction of ALP activity and foetal cell number. Moreover, culture in CDM with BMP-2 resulted in a marked change to colonies of cobblestone-like cells supported by typical fibroblastic cells, and although cobblestone-like cells displayed an altered nuclear location and shape; the cells maintained viability in extended culture. Reduced expression of the cell adhesion protein periplakin [55], in comparison to cultures with activin A/FGF-2, may be linked to the cobblestone-like morphology. Although STRO-1+ cells were observed with BMP-2, these were not the cobblestone-like cells. Conversely, SOX-9 expression was restricted to the cobblestone-like cells only, as was SOX-2, a recognized stem cell marker [56], although the extranuclear expression may be linked to the altered nuclear shape. In contrast to the study of foetal mesenchymal cells by Guillot and coworkers [57], expression of other hESC markers OCT4 and TRA-1–60 [7, 58, 59] were not seen in the human foetal femur-derived cells. These cultures were further characterized by an increased metabolic activity in comparison to CDM and activin A/FGF-2 conditions, however, suggestive of a differentiating population [33, 60], as supported by a marked increase in the expression of metabolic gene glutamine-fructose-6-phosphate transaminase 2 [61] and two markers of sterol biosynthesis – sterol-C4-methyl oxidase-like [62] and squalene epoxidase [63]. Up-regulation of the tRNA splicing endonuclease 15 homolog chromosome 1 open reading frame 19 suggests these populations may also utilise extra mechanisms to maintain cell growth [64]. Down-regulation of differentiation regulatory genes was confirmed with SOX15, involved in skeletal muscle development [65], and DUSP6, a regulator of the MAP kinase pathway involved osteogenic signal transduction [66]. In addition, increased expression of Wnt inhibitory factor 1, a major antagonist of BMP-signalling [67], was seen in CDM cultures with BMP-2, as was the synthesizing
3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, part of the statins family previously indicated in osteogenesis [68].

FGF-2, a developmental mitogen, has been shown to preferentially target primary and precursor cell populations for the expansion of chondro- and osteoprogenitors [69–74]. Interestingly, Martin and coworkers suggested FGF-2 may result in a de-differentiation of committed mesenchymal progenitors prior to the addition of BMP-2 to promote lineage differentiation [75], in contrast however to the current studies. FGF-2 signalling may be mediated by chondroitin sulphate glycosaminoglycans (CS-GAGs) [76] via a recently proposed mechanism whereupon release from a CS-GAG-protected extracellular matrix environment exposes a newly divided daughter cell to exogenous growth factors [77]. We observed increased levels of chondroitin synthase-2 (CHSY-2); dual specificity phosphatase 6 (DUSP6); glutamine-fructose-6-phosphate transaminase 2 (GFTT2); 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1); periplakin (PPL); sterol-C4-methyl oxidase-like (SC4MOL); SRY (sex determining region Y)-box 15 (SOX15); squalene epoxidase (SQLE) and Wnt inhibitory factor 1 (WIF1). Ten genes of interest, shown in bold, were identified from the six cluster groups formed at the fourth level of horizontal dendrogram arms: Chromosome 1 open reading frame 19 (C1orf19); chondroitin synthase-2 (CHSY-2); dual specificity phosphatase 6 (DUSP6); glutamine-fructose-6-phosphate transaminase 2 (GFTT2); 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1); periplakin (PPL); sterol-C4-methyl oxidase-like (SC4MOL); SRY (sex determining region Y)-box 15 (SOX15); squalene epoxidase (SQLE) and Wnt inhibitory factor 1 (WIF1). n = 3 per treatment group as shown by key. Abbreviations: BMP-2, bone morphogenetic protein-2; FGF-2, fibroblast growth factor 2.
BMPs promote transient SOX gene expression influential in the terminal differentiation of chondrocytes [21] and in serum-free CDM conditions may therefore promote a chondrogenic response in the human foetal cells through the selection of a sub-population of cells or early progenitors. This is supported by the study of Schmitt and coworkers who observed differentiation along the chondrogenic, but not osteogenic or adipogenic lineages, in hBMSCs in a serum-free environment with BMP-2 [78]. That the cobblestone-like cells alone expressed SOX-9 and SOX-2, but not STRO-1 or type I and II collagens suggested a differential response of sub-populations to BMP-2. These findings indicate a novel in vitro model for the recapitulation of the condensation phenotype, in the presence of CDM and ongoing work in our laboratories is focused on functionality of cobblestone-like cell preparations.

Finally, a key consideration for further serum-free CDM studies is the potential for osteogenic rescue. However, the use of serum-free CDM remains an important tool for the ex vivo expansion of human mesenchymal cells [84]. Moreover, CDM conditions may prove more appropriate for mesenchymal expansion as the latent osteogenic activity seen in the human foetal femur-derived cells in standard basal culture conditions demonstrates competency factors within serum, with implications for in vitro expansion and osteogenic rescue.

**Fig. 5** Quantitative PCR validation of significantly altered gene expression between basal and serum-free treatment groups. Foetal cell cultures were maintained in either basal medium with serum (Basal), CDM with 10 ng/ml activin A and 12 ng/ml FGF-2 (CDM + A/F) or CDM plus 150 ng/ml BMP-2 (CDM + BMP-2) and gene expression measured for: (A) Chromosome 1 open reading frame 19 (C1orf19), (B) 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1); (C) Sterol-C4-methyl oxidase-like (SC4MOL); (D) Wnt inhibitory factor 1 (WIF1); (E) Dual specificity phosphatase 6 (DUSP6), (F) Periplakin (PPL), (G) SRY (sex determining region Y)-box 15 (SOX15), (H) Chondroitin synthase-2 (CHSY-2), (I) Glutamine-fructose-6-phosphate transaminase 2 (GFPT2) and (J) Squalene epoxidase (SQLE). Transcript levels were determined using the comparative threshold cycle (Ct) method and normalized to β-ACTIN expression with basal conditions set as 1. Results expressed as mean ± S.D. with n = 3; *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: BMP-2, bone morphogenetic protein-2; FGF-2, fibroblast growth factor 2, PCR, polymerase chain reaction.
osteogenic studies, namely (i) additional factors may be neces-
sary for osteogenic differentiation, as; (ii) osteogenesis may not
be the result of added modulatory factors alone and (iii) peripheral
differentiation pathways may therefore be overlooked.

In conclusion, the current studies confirm the potential of
CDM-mediated ex vivo expansion of human foetal femur-derived
cells and the derivation of a unique undifferentiated phenotype in
the presence of activin A and FGF-2. The primitive foetal popula-
gations generated with BMP-2 offer a new model to investigate the
underlying molecular mechanisms in skeletal stem and progenitor
cell biology with implications for tissue regeneration strategies.

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Supporting Information

Additional Supporting Information may be found in the online ver-
sion of this article:

Fig. S1 Diagrammatic representation of chemically defined medium
(CDM) experiments culture regimen, showing isolation in basal
medium (α-MEM) prior to maintenance in either basal or CDM with
10 ng/ml activin A and 12 ng/ml FGF-2 conditions for 5 days, fol-
lowed by culture in either CDM alone, CDM with 10 ng/ml activin A
and 12 ng/ml FGF-2, CDM with 150 ng/ml BMP-2, CDM with 100 μM
ascorbate and 10 nM dexamethasone or back to basal conditions.

Fig. S2 Control immunostaining for phenotypic marker expres-
sion. In respective cultures of either basal medium, chemically de-
efined medium (CDM) supplemented with 10 ng/ml activin A and
12 ng/ml FGF-2 or CDM with 150 ng/ml BMP-2, no staining was
observed in control wells lacking primary antibody for (A–C) Type
I collagen, (D–E) Type II collagen, (G–I) STRA-1, (J–L) SOX-9 or
(M–O) SOX-2. (P) Human embryonic stem cells (hESC) as posi-
tive control for SOX-2 staining, and (Q) Negative hESC control
lacking primary SOX-2 antibody. hESC cultures (HUES 7 cell lines)
were cultured on mouse embryonic feeder (MEF) layers in hES
knockout medium and were fixed at passage 11 of culture. Bars =
50 μm. Abbreviations: BMP-2, bone morphogenetic protein-2;
FGF-2, fibroblast growth factor 2.

Fig. S3 Diagrammatic representation of results from microarray
analysis. (A) Comparison of three donor samples of human foetal
femur-derived cells maintained in either basal medium containing
10% serum or a serum-free chemically defined medium (CDM)
supplemented with 10 ng/ml activin A and 12 ng/ml FGF-2, result-
ing in 51 matches or 46 genes excluding duplications. H988,
H1052 and H1054 denote the three foetal samples utilized. (B)
Results of two-way ANOVA analysis, shown in diagrammatic form,
for human foetal femur-derived populations cultured in either
basal medium or CDM with activin A and FGF-2 or CDM with
BMP-2 to determine significantly altered expression of genes
giving 67 matches or 58 genes excluding duplications, for three
donor foetal samples. Abbreviations: BMP-2, bone morphogenetic
protein-2; FGF-2, fibroblast growth factor 2.

Fig. S4 Effects of osteogenic stimulatory factors Ascorbate/
Dexamethasone and BMP-2 on human foetal femur derived cells.
Primary cells were passaged and seeded to well plates. After 24
hrs basal medium was refreshed in control wells or supplemented
with either 100 μM ascorbate and 10 nM dexamethasone, 150
ng/ml BMP-2 or a combination of the two conditions and
refreshed every 48 hrs. Cultures were stopped at day 5 for bio-
chemical analysis. (A) Cell number as measured by PicoGreen®
double-stranded DNA assay. (B) Specific alkaline phosphatase
(ALP) activity comparing the four culture treatments. Results
expressed are as mean ± S.D. with n = 6; bars with the same
superscript are significantly different; a, e, P < 0.05; b, c, P <
0.01; d, f, P < 0.001. Abbreviations: α-MEM, α-modification min-
imum essential medium; BMP-2, bone morphogenetic protein-2.

Fig. S5 Fluorescence immunochemistry for human embry-
onic stem cell (hESC) markers. (A–C) Respective cultures of either
basal medium, chemically defined medium (CDM) supplemented
with 10 ng/ml activin A and 12 ng/ml FGF-2 or CDM with
150 ng/ml BMP-2 were stained for OCT4, as were; (D) hESC pos-
itive controls and (E) negative control wells lacking primary OCT4
antibody. (F) Staining for TRA-1–60 in basal; (G) CDM with activin
A/FGF-2; (H) CDM plus BMP-2 and (I) hESC cultures. (J) Negative
hESC control lacking primary TRA-1–60 antibody. Immunoocy-
tochemistry was performed as per methods section for OCT4
(1:100 dilution) and TRA-1–60 (1:100 dilution). hESC cultures (HUES
7 cell lines) were cultured on mouse embryonic feeder (MEF)
layers in hES knockout medium and were fixed at passage 11 of
culture. Bars = 50 μm. Abbreviations: BMP-2, bone morpho-
genetic protein-2; FGF-2, fibroblast growth factor 2.

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