Characterisation of the constitutive over-expression of AJ18 in a novel rat stromal bone marrow cell line (D8-SBMC)

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ABSTRACT
Objective: The elucidation of the molecular pathways involved in osteoblast proliferation and differentiation has been greatly enhanced by the availability of cell culture model systems. However, many of the current bone cell culture systems suffer from disadvantages such as the inability to generate mineralised bone-like nodules, a transformed genetic background, cell heterogeneity, and a relatively long time frame from cell seeding to mineralisation, often in the order of several weeks. Here we describe the establishment and characterisation of a novel bone cell line named D8-SBMC. As a first demonstration of their potential value, D8-SBMC was utilised to further support a role for AJ18 during osteogenesis.


Results: D8-SBMC possesses the ability to form robust mineralised bone-like nodules within 8 days proceeding cell confluency. Interestingly, a cement line-like matrix is also generated between the culture dish and a basal monolayer of cells. Constitutive and stable over-expression of AJ18 resulted in an increase in cell proliferation and mineralisation. Expression of bone marker genes, such as bone sialoprotein, osteopontin, osteocalcin, collagen type 1, and osteonectin, was up-regulated by AJ18 over-expression.
1. Introduction

Bone marrow is a complex tissue comprising at least two distinct but interdependent compartments, the haematopoietic system and stroma. The two compartments can be separated largely by the propensity of non-haematopoietic cells to adhere to plastic when bone marrow is plated in culture. The heterogeneous, plastic-adherent cells exhibit many characteristics attributed to bone marrow stromal cells in vivo. Cultures generated from single-cell suspensions of plastic-adherent cells form distinct colonies derived from a single precursor cell and are defined as the colony-forming-unit fibroblast (CFU-F). Historically, these precursor cells are referred to as stromal bone marrow cells (SBMCs). SBMCs are a valuable source of osteoprogenitors for the study of osteoblast differentiation. Multiple in vitro model systems have been developed to study particular aspects of bone biology. Some common cell culture models include primary systems such as foetal rat calvarial cells (FRCCs) and rat bone marrow cells (RBMCs), transformed cell lines such as ROS 17/2.8 or UMR 106, spontaneously immortalised cell lines such as C3H10T1/2, MC3T3-E1, and SBMCs, and cell lines derived from transgenic mice such as MLO-A5. However, there are few if any bone cell line that is non-transformed, clonally derived, and form robust hydroxyapatite (HA) mineralised bone nodules in a relatively short period of time.

Osteoblasts are specialised cells that exclusively form bone in vertebrates. The identification and characterisation of numerous transcription factors and other molecular cues involved in osteogenesis have been facilitated by the availability and utilisation of some of the in vitro systems mentioned above. Thus, the differentiation from multipotential precursor cells to osteoblasts is controlled by at least two key osteogenic transcription factors, Runx2 and Osterix (Osx). Runx2 and Osx are commonly described as master genes for osteogenesis because the ablation of Runx2 or Osterix (Osx) results in the complete absence of bone, although it seems apparent that Osx functions downstream of Runx2, but not of Runx2.

AJ18/Kid3/Znf354c are rat, mouse, and human orthologues, respectively, of a protein comprising a Krüppel-associated box (KRAB) domain and 11 successive C2H2 zinc finger motifs. Rat AJ18 was identified by a differential display screen of foetal rat calvarial cells (FRCCs) as a gene that is up-regulated during osteoblastic differentiation. KRAB/C2H2-containing proteins are a large family of putative transcription repressors whose functions in skeletal tissues are only beginning to be understood. Several lines of evidence have established AJ18 as a regulator of bone formation. Firstly, AJ18 is up-regulated transiently by bone morphogenetic protein-7 (BMP-7) and the temporospatial expression profile of AJ18 is similar to the developmental expression profile of BMP-7. BMP-7 was one of the first BMP family members to be identified and characterised to induce bone formation and stimulate osteoblast proliferation. Secondly, transcription repression by AJ18 was confirmed through the demonstration that AJ18 can compete with Runx2 for the osteoblast-specific element 2 (OSE2). Thirdly, analysis of the AJ18 promoter identified putative DNA recognition sequences specific for factors involved in bone differentiation such as Runx2, Ets, and Smads. Although these observations collectively provide evidence for a role of AJ18 during bone differentiation, the downstream effects of AJ18 such as its influences on the expression of bone-related genes and mineralisation have yet to be investigated.

In this study, the isolation and characterisation of a rat cell line named D8-SBMC is described. D8-SBMC was isolated from a single cell suspension of previously described SBMCs after long-term culture, which in the presence of fibroblast growth factor-2 (FGF-2), dexamethasone (Dex), ascorbic acid (AA), and β-glycerophosphate (β-GP), generate an osteogenic phenotype. D8-SBMC produces robust mineralised, bone-like nodules as early as 8 days after confluence and solely in the presence of AA and β-GP. D8-SBMC expresses Runx2, Osx, and AJ18 during their proliferation and differentiation phases. Subsequently, D8-SBMC was utilised to analyse the role of AJ18 during bone formation. With stable over-expression of AJ18 under the control of the constitutively active cytomegalovirus (CMV) promoter in D8-SBMC, there was a dramatic increase in the formation of bone-like nodules and corresponding expression of bone markers. The effects of CMV-AJ18 over-expression, in part, appear to be due to an increase in the proliferation of D8-SBMC.

2. Materials and methods

2.1. Cell culture and isolation of the D8-SBMC cell line

Long-term cultures of rat SBMCs were received in 25 mm flasks (a generous gift from Dr. S. Pitaru, Tel Aviv University). Two independent rounds of plating in limiting dilutions were performed to isolate individual clonal cell lines. Specifically, SBMCs were plated at 1 cell per well in 96-well dishes with 10% FBS, alpha minimal essential medium (α-MEM), and antibiotics (100 µg/ml penicillin G, 50 µg/ml gentamycin sulphate and 300 µg/ml fungizone), supplemented with dexamethasone (Dex; 10 nM; Sigma Diagnostics Inc.) and rat FGF-2 (3 ng/ml; Sigma Diagnostics Inc.). Cells derived from the first round of single cell populations were then re-plated onto a second 96-well plate at 1 cell per well as described above. These single cell populations were expanded in media supplemented with AA (50 µg/ml; Sigma Diagnostics Inc.) and β-GP (10 mM; Sigma Diagnostics Inc.), and screened for their ability to form
mineralised bone nodules as assessed by von Kossa staining according to Jheon et al.\textsuperscript{24} From the single cell populations isolated, the subpopulation designated D8 showed the most robust bone nodule formation. For all subsequent experiments, D8-SBMC and stably transfected D8-SBMC were plated at 1 $\times$ 10\textsuperscript{4} cells per 35 mm tissue culture dish and grown in 10\% FBS, alpha-MEM, and antibiotics. For mineralisation assays, unless otherwise indicated, AA was added continuously from confluence (defined as 95–100\%; i.e. day 4) and β-GP was added 6 days after confluence (i.e. day 10). In specified experiments, Dex or FGF-2 or β-GP was added continuously at confluence. von Kossa analysis was performed between day 12 and day 18. All cells were grown in a humidified atmosphere of 95\% air and 5\% CO\textsubscript{2} at 37 °C. Medium was changed every 2–3 days. Triplicate dishes were used for analyses at each time point.

2.2. Quantification of von Kossa staining

von Kossa staining was quantified using Adobe Photoshop (version CS2, Adobe Systems, Inc.). Briefly, the amount of positive (black) staining was counted by selecting a single representative area using the “magic wand” tool with the tolerance set to 10. All areas similar to this representative area were then selected using the “similar” function. The total number of pixels was taken as a direct measure of positive von Kossa staining.

2.3. Microscopy

D8-SBMC was grown on theranox plastic coverslips (Thermo Fischer Scientific) until the formation of mineralised bone nodules. Bone nodules were stained by the von Kossa method. Mineralised cultures were fixed in 1\% glutaraldehyde, gently displaced from the cover slips, embedded in LR white acrylic resin (London Resin Company; Berkshire, UK) as previously described,\textsuperscript{32} and sectioned (10 μm). Sections were counterstained with toluidine blue. All sections were visualised under a light microscope (Eclipse 400; Nikon Canada) and photographed using a Coolpix 950 digital camera (Nikon Canada). For transmission electron microscopy (TEM), selected regions were trimmed, and ultrathin sections (80 nm) were placed on polyvinyl formal- and carbon-coated nickel grids. Incubated grids were examined in a JEM 2000FXII transmission electron microscope (JEOL; Tokyo, Japan) operated at 80 kV.\textsuperscript{33}

2.4. Primary rat bone marrow cell preparation

Primary rat bone marrow cells were obtained from young adult (120–130 g) male Wistar rats as previously described.\textsuperscript{31} Briefly, femoral bones were separated from soft tissues under aseptic conditions and washed four times (10 min/wash) in 10\% FBS, α-MEM, and antibiotics. The distal portions of the bone were removed and the marrow cavity flushed into a 7–75 ml flask with a syringe containing 10 ml α-MEM. The cell preparation was dispersed by repeated aspiration through a 20-gauge needle. After 1 week in culture, RBMCs were trypsinised and plated as described for D8-SBMC. Dex (10 nM) was added at confluence for 3 days and total RNA extracted.

2.5. Total RNA isolation and Northern blot hybridisation

Total RNA was isolated from primary RBMCs and D8-SBMC using the RNeasy Mini kit (Qiagen). Northern blots were performed as previously described.\textsuperscript{24}

2.6. qPCR analysis

qPCR primers specific for Runx2, Osx, and AJ18 were prepared using the Primer3 program. All qPCR reactions were performed in triplicates using RNA preparations from 3 separate experiments. Briefly, cDNA was generated using the iScript CDNA synthesis kit (Biorad Laboratories) according to manufacturer’s protocol. qPCR was performed using the iScript SYBR Green RT-PCR kit (Biorad Laboratories) on an iQ5 cyler (Biorad Laboratories). Primer sequences were, Runx2: Forward (F)-GCCGGGAATGATGAGACCA, Reverse (R)-GACCCGTCACCTTGCTACCTT; Osx: F-GAGAGCCAAAGAAGCCATCA, R-GGGAAGGTTGGTGATGTCAT; AJ18: F-CTGCGACCTGTCGACAAGAC, R-TCTTGACGAGCAGTAGGAT; GAPDH: F-AGACAGCCGATCTCTCTTGT, R-CTTGCCTGGGTAAGCTCAT. Levels were normalised to GAPDH. For each gene, the relative levels refer to expression in cells with Dex treatment compared to cells without Dex.

2.7. Preparation of plasmids and stable transfections

Plasmids were constructed using the pEGFP1 vector (Clontech). Full-length GFP was released using restriction enzymes BamHI and NotI to generate an empty vector under the control of the cytomegalovirus5 promoter (pCMV5). The 5' overhangs were filled in with dNTPs and Klenow polymerase (New England Biolabs Inc.) at 37 °C for 15 min and the plasmid religated with T4 ligase (Promega). Full-length AJ18 was PCR-amplified using primers 5'-TAGTTGCTGACACCATGGCTGTG-GATTTGCTGGC-3' and 5'-TAGTTGCTGACACTGATCGAGCAGGATGAAATAGGCT-3' containing engineered SalI sites and cloned into SalI-linearised pCMV5 plasmid to generate vectors containing engineered SalI sites and cloned into SalI-linearised pCMV5 plasmid to generate vectors containing AJ18 in sense (pCMV-AJ18-S) and antisense (pCMV-AJ18-AS) orientations. Restriction enzyme digestions and subsequent sequence analysis were performed to confirm the orientation and sequence of the inserts. D8-SBMC was plated at 100 000 cells per 35 mm dish in 4 ml of 10\% FBS and α-MEM. At 90–95\% confluence, cultures were transfected with the individual plasmids (2 μg each) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Cell selection was initiated by supplementing the media with neomycin (500 μg/ml) after 24–48 h. After 6–7 days of selection, the cells were trypsinised (0.01% trypsin-EDTA) and re-plated to generate pooled populations of selected cells. Stably selected D8-SBMC cell lines were designated as CMV-AJ18 (S), CMV-AJ18 (AS), and CMV (EV).

2.8. Extraction of matrix proteins and Western blot analysis

The matrix proteins were extracted from D8-SBMC at the mineralisation phase using a protocol described by Nagata et al.\textsuperscript{34} and Domenicucci et al.\textsuperscript{35} Briefly, cells were labelled with [35S]-methionine (PerkinElmer) for 24 h 12 days after
confluence. Serial extractions of mineralised bone nodules were performed using 4 M guanidinium chloride (G1 extract), 0.5 M EDTA (E extract), and again with 4 M guanidinium chloride (G2 extract). The extracts were pooled, dialysed, concentrated and stored at -20 °C until analysis. The extracts were separated via PAGE on 10% Tris-glycine minigels. The separated proteins were transferred electrophoretically to a PVDF membrane (Sigma Diagnostics Inc.) in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol). The non-specific sites on the PVDF membrane were blocked by incubation with 5% skim milk in TBS (Tris-buffered saline) - Tween (20 mM Tris, 137 mM NaCl, 0.05%, v/v Tween-20, pH 7.6) for 1 h at RT or overnight at 4 °C. The membrane was washed four times with TBS-Tween and incubated for 1 h at RT with a BSA polyclonal antibody (courtesy of Dr. L.W. Fisher, National Institutes of Health, Bethesda, MD) diluted 1:2000 or mouse anti rat OPN monoclonal antibody (MPIIIB101); Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD) diluted 1:2000 in TBS-Tween/5% skim milk. Following primary incubation, the membrane was washed four times with TBS-Tween, then incubated for 1 h with a 1:25,000 dilution of HRP-conjugated goat anti rabbit IgG (Bio Rad Laboratories) to detect BSP or 1:25,000 HRP-conjugated goat anti-mouse IgG (Bio Rad Laboratories) to detect OPN. The immuno-reactive bands were detected using the Amersham ECL kit (Enhanced Chemiluminescence, GE Healthcare Bio-Sciences Inc.) according to the manufacturer’s protocol, and the membrane exposed to Kodak X-ray film (OMAT-AR).

2.9. Calcium and phosphorus assays

Cells were grown until the mineralisation phase and incubated for 24 h in serum-free medium without AA and β-GP. The conditioned media was removed, centrifuged to remove cell debris, and stored at -20 °C until further analysis. The dishes were washed with PBS and extracellular matrix was extracted with 0.6N HCl for 24 h at 4 °C. The dishes were washed with 0.5 M EDTA (E extract). The extracts were dialysed, concentrated and stored at -20 °C until analysis. The extracts were separated via PAGE on 10% Tris-glycine minigels. The separated proteins were transferred electrophoretically to a PVDF membrane (Sigma Diagnostics Inc.) in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol). The non-specific sites on the PVDF membrane were blocked by incubation with 5% skim milk in TBS (Tris-buffered saline) - Tween (20 mM Tris, 137 mM NaCl, 0.05%, v/v Tween-20, pH 7.6) for 1 h at RT or overnight at 4 °C. The membrane was washed four times with TBS-Tween and incubated for 1 h at RT with a BSA polyclonal antibody (courtesy of Dr. L.W. Fisher, National Institutes of Health, Bethesda, MD) diluted 1:2000 or mouse anti rat OPN monoclonal antibody (MPIIIB101); Developmental Studies Hybridoma Bank, Johns Hopkins University, Baltimore, MD) diluted 1:2000 in TBS-Tween/5% skim milk. Following primary incubation, the membrane was washed four times with TBS-Tween, then incubated for 1 h with a 1:25,000 dilution of HRP-conjugated goat anti rabbit IgG (Bio Rad Laboratories) to detect BSP or 1:25,000 HRP-conjugated goat anti-mouse IgG (Bio Rad Laboratories) to detect OPN. The immuno-reactive bands were detected using the Amersham ECL kit (Enhanced Chemiluminescence, GE Healthcare Bio-Sciences Inc.) according to the manufacturer’s protocol, and the membrane exposed to Kodak X-ray film (OMAT-AR).

2.10. Proliferation assay

Stably transfected and WT cells were plated at various concentrations (10–10 000 cells per well) in triplicate in 96-well dishes and incubated overnight at 37 °C. WST-1 solution (Roche Applied Science) was added directly to the media and incubated for 4 h at 37 °C. Total number of cells was determined by measuring the absorbance at 450 nm in a Titerrek spectrophotometer (Interscience).

2.11. Statistical analysis

Significant effects were assessed using a paired Student’s t test. The degree of significance is indicated by asterisks, where '*' indicates p < 0.05 and '**' indicates p < 0.01. Each experiment presented was performed independently at least three times.

3. Results

3.1. D8-SBMC generates bone-like nodules in vitro

D8-SBMC was isolated as a single cell population from a previously characterised long-term culture of rat SBMCs. From 12 single cell populations examined, D8-SBMC showed robust bone nodule formation as observed by von Kossa staining (Fig. 1A). The extent of mineralisation in D8-SBMC was independent of treatment with Dex and FGF-2 (Fig. 1B) unlike that observed in SBMCs. However, a slight increase in the amount of mineralised nodules was observed with continuous β-GP treatment (i.e. β-GP added at confluence rather than 6 days after; Fig. 1C). von Kossa staining was quantified (Fig. 1D). Light microscopy analysis (Fig. 1E) revealed a continuous monolayer of cuboidal, osteoblast-like cells on top of a continuous sheet of mineralised bone-like matrix. A basal cell layer was also observed underneath the matrix. Transmission electron microscopy of D8-SBMC upon mineralisation (Fig. 1F) showed distinct osteoblasts, foci of mineralisation, and collagenous matrix. In addition, at the interface between cells and the tissue culture polystyrene substratum, a mineralised cement line-like matrix of approximate 0.5 μm thickness was observed (Fig. 1F). The cement line-like matrix and the foci of mineralisation within the collagenous matrix exhibited a fine structure of individual electron-dense lines (white arrows in Fig. 1F) similar to those seen in the cement lines of rat bone marrow cultures. At specific sites, collagen fibres were mineralised and encrusted into the cement line-like matrix (Fig. 1H). It appears that the cement line-like matrix located below the basal cell layer became separated during cell preparation for light microscopy (Fig. 1E). The osteogenic phenotype of D8-SBMC was confirmed by Northern blot hybridisation (Fig. 2). Various genes involved in the proliferation and differentiation of osteoblasts, and the mineralisation of osteoid were analysed over 14 days (cell confluence occurred at day 4). Collagen type 1 (COL1) was up-regulated from day 3, whereas bone sialoprotein (BSP), tissue non-specific alkaline phosphatase (TNAP), and osteonectin (or secreted protein acidic and rich in cysteine, SPARC) were all up-regulated proceeding confluence at day 6. Osteopontin (OPN) was down-regulated soon after confluence. From these expression profiles, in concert with light micrograph observations at various days in culture (data not shown), the phases of proliferation, differentiation, and mineralisation in D8-SBMC were estimated (Fig. 2, bottom).

3.2. Comparison between D8-SBMC and RBMCs

The expression levels of Runx2, Osx, and AJ18 were analysed by qPCR at various times during proliferation and differentiation of D8-SBMC and primary RBMCs. Runx2 and AJ18 showed
Fig. 1 – D8-SBMC form bone-like nodules in vitro. (A–C) von Kossa staining reveals mineralised nodules generated by D8-SBMC (A). von Kossa analysis was performed at day 18 after seeding. D8-SBMC does not appear to be responsive to Dex and FGF-2 (B). Continuous β-GP presence from confluence shows a significant increase in mineralised nodules (C). Bar = 2 mm.

(D) Quantification of mineralised nodules stained by the von Kossa method. Levels are relative to cells in (A).

(E) Light microscopy shows a continuous sheet of bone-like matrix that is mineralised throughout as visualised by von Kossa staining (black) sandwiched between monolayers of cuboidal, osteoblast-like cells (arrow) and basal cells (arrowhead). Sections were counterstained with toluidine blue. Bar = 10 μm.

(F–H) TEM of D8-SBMC at day 14. (F) At the interface to the tissue culture polystyrene substratum, a mineralised cement line (CL)-like matrix, approximately 0.5 μm thick, is seen. Overlying the CL, osteoblasts (Ob) can be seen embedded in the collagenous matrix (cM), which undergoes crystallisation at specific foci of mineralisation (FM). The CL and FM within the collagenous matrix exhibit a fine structure of individual electron-dense lines (arrows). Bar = 1 μm.

(G) At higher magnification, the CL can be seen at the substratum interface, exhibiting a fine structure of electron-dense lines (white arrows).

(H) At specific sites, collagen fibres become mineralised and encrusted into the cement line (arrowhead). Field width: 0.5 μm.
similar biphasic expression profiles during proliferation and differentiation, with up-regulation occurring earlier in D8-SBMC in comparison to RBMCs (Fig. 3). Similar expression profiles of Runx2 and AJ18 have been shown previously in RBMCs. In addition, the expression of Osx is approximately 8-fold higher and occurs earlier in D8-SBMC compared to RBMCs. The early up-regulation of Runx2, Osx, and AJ18 in D8-SBMC correlates with the early formation of bone-like nodules by D8-SBMC compared to RBMCs.

SBMCs require Dex and FGF-2 for bone formation. Dex increases bone formation in RBMCs. In contrast, D8-SBMC reliably generates bone-like nodules independent of Dex and FGF-2 supplementation (Fig. 1A and B). Furthermore, the expression of Runx2, Osx, and AJ18 in D8-SBMC was not influenced by Dex treatment, whereas all three genes were up-regulated by Dex treatment in RBMCs (Fig. 4).

D8-SBMC was transfected with AJ18 in the sense (S) or antisense (AS) orientation under the regulation of a constitutively active CMV-derived promoter. Stably transfected cell lines referred to as S, AS, and EV (empty vector) were established. Total RNA was extracted from the three cell lines 24 h after confluence. Northern blot analyses revealed the expression profiles of endogenous (7 kb in size) and exogenous/transfected (1.8 kb) AJ18. Stably transfected cell lines were grown until the formation of bone nodules and analysed by von Kossa staining (Fig. 5B). Constitutive over-expression of AJ18 (S) resulted in a dramatic increase in mineralisation. Conversely, over-expression of AJ18 in the antisense orientation (AS) showed a decrease in mineralisation. The EV cell line produced levels of bone

Fig. 2 – Northern blot analyses of D8-SBMC. Molecular markers analysed were bone sialoprotein (BSP, 2.0 kb and 1.6 kb), osteopontin (OPN, 1.5 kb), collagen type 1 (COL1, 3.5 kb and 3.0 kb), tissue non-specific alkaline phosphatase (TNAP, 2 kb), and osteonectin or SPARC (secreted protein acidic and rich in cysteine, 2 kb). The amount of total RNA was normalised using 28S rRNA probes (4.7 kb). Proliferation, differentiation, and mineralisation phases are indicated. Confluence occurred at day 4 (C).

Fig. 3 – qPCR analyses of the expression profiles of AJ18, Osx, and Runx2 in D8-SBMC and RBMCs. Initial up-regulation of AJ18, Osx, and Runx2 was observed earlier in D8-SBMC relative to RBMCs. AJ18 and Runx2 show strikingly similar expression profiles particularly in D8-SBMC. Confluence occurred at day 4 (C).

3.3. Constitutive over-expression of AJ18 leads to increased bone nodule formation
formation comparable to WT D8-SBMC (data not shown). von Kossa staining was quantified. The appearance of a continuous sheet of mineralisation (Fig. 5B) compared to bone nodules (Fig. 1A–C) is due to differences in time and area in which D8-SBMC were cultured.

Northern blot hybridisation was performed on total RNA isolated from the stably transfected cell lines along with WT D8-SBMC (Fig. 5C). Total RNA was isolated from proliferation (day 2), differentiation (day 6), and mineralisation (day 12) phases and analysed for the expression of various genes involved in bone formation. All of the bone markers analysed were up-regulated with AJ18 over-expression at each phase compared to the other cell lines tested with the surprising exception of TNAP. AS, EV, and WT cell lines showed similar levels of expression for all transcripts analysed.

3.4. Serial extraction of extracellular matrix proteins demonstrates mineral association of BSP and OPN

Proteins associated with the mineralised tissue matrix were isolated by serial extractions using guanidium thiocyanate, a general protein denaturant, and EDTA, a calcium chelator that dissolves the mineralised HA crystals. By Western blot analysis, the highest amounts BSP and OPN were found with AJ18 over-expression and were largely associated with the mineral component of bone (i.e. present in the E extract; Fig. 6).

BSP was also closely associated with the non-mineralised collagenous matrix as indicated by the presence of BSP in both guanidium thiocyanate extracts (i.e. G1 and G2 extracts). The results show prominent bands of mineral associated proteins in the E extract at ~75 kDa and ~55 kDa corresponding to bone sialoprotein (BSP) and osteopontin (OPN), respectively.10,38

3.5. Calcium and phosphorus in matrix and media

S, AS, and EV cell lines were grown until the formation of bone-like nodules. Concentrations of Ca and P ions, the main components of HA, present in media or incorporated into the extracellular matrix were measured (Fig. 7). Ca and P concentrations associared specifically with matrix correlate with the amount of bone formation. Thus, concentrations were highest in the S cell line (Fig. 7A). The Ca/P ratios in the matrices of S, AS, and EV cells were 1.65, 1.77, and 1.61, respectively, which concur with the stochiometric Ca/P ratio of 1.67 for HA [Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] observed in bone mineral.39 As expected, concentrations of Ca and P in the media were lowest in the S cell line indicative of the depletion of these ions from the media, presumably as a result of their incorporation into the mineralised matrix (Fig. 7B).

3.6. Over-expression of AJ18 results in an increase in cell proliferation.

S, AS, EV, and WT D8-SBMC were plated at various concentrations in 96-well dishes and the number of cells measured after 24 h (Fig. 8). Constitutive over-expression of AJ18 led to an increase in cell proliferation, whereas the AS cell line showed the lowest rate of proliferation. The proliferation rates correlate with an increase or decrease in the number of bone-like nodules in the S or AS cell lines, respectively.

4. Discussion

The isolation of single cell populations from long-term cultures of rat SBMCs1 was undertaken to establish a homogeneous osteoblast cell line. The resulting cell line, named D8-SBMC, shows a robust osteogenic phenotype in a relatively short period of time, generating HA-mineralised, bone-like nodules approximately 8 days following cell confluence. Light microscopy and TEM analyses revealed mineralisation in the presence of a continuous sheet of mineralisation (Fig. 5B) compared to bone nodules (Fig. 1A–C) is due to differences in time and area in which D8-SBMC were cultured.

Northern blot hybridisation was performed on total RNA isolated from the stably transfected cell lines along with WT D8-SBMC (Fig. 5C). Total RNA was isolated from proliferation (day 2), differentiation (day 6), and mineralisation (day 12) phases and analysed for the expression of various genes involved in bone formation. All of the bone markers analysed were up-regulated with AJ18 over-expression at each phase compared to the other cell lines tested with the surprising exception of TNAP. AS, EV, and WT cell lines showed similar levels of expression for all transcripts analysed.

Fig. 4 – Effects of Dex on the expression of AJ18, Osx, and Runx2 in D8-SBMC and RBMCs. (A) qPCR analyses reveal that AJ18, Osx, and Runx2 are not responsive to Dex in D8-SBMC. (B) AJ18, Osx, and Runx2 are responsive to Dex in RBMCs. Relative levels refer to expression of genes in cells with Dex compared to expression in cells without Dex.

Proteins associated with the mineralised tissue matrix were isolated by serial extractions using guanidium thiocyanate, a general protein denaturant, and EDTA, a calcium chelator that dissolves the mineralised HA crystals. By Western blot analysis, the highest amounts BSP and OPN were found with AJ18 over-expression and were largely associated with the mineral component of bone (i.e. present in the E extract; Fig. 6). The mineralised nodules and cement line-like matrix requires definitive analytical proof of hydroxyapatite crystals perhaps via X-ray diffraction and/or energy dispersive spectroscopic analyses. However, our initial characterisation of mineralisation, and in particular the formation of a cement line-like matrix, will make D8-SBMC valuable for biominer-
Fig. 5 – Effects of AJ18 over-expression on mineralisation in D8-SBMC. (A) Northern blot hybridisation was performed with total RNA isolated from stably selected populations of cells over-expressing AJ18 in the sense (S) and antisense (AS) orientations, and empty vector (EV). Similar levels of endogenous AJ18 in S and EV cells were observed. There was a slight decrease in endogenous AJ18 in AS cells. Exogenous AJ18 or AJ18 transgene (AJ18 Tg) is observed. (B) S, AS, and EV cells were assayed for mineralised nodule formation by von Kossa staining showing robust mineralisation in S cells. von Kossa staining was performed 8 days after confluence. Quantification of von Kossa staining is shown below. Levels are relative to EV. Bar = 5 mm. (C) Northern blot analyses of bone markers from total RNA isolated from wild type cells (WT) and stably transfected cell lines. Total RNA was isolated at proliferative (Pro; day 2), differentiation (Diff; day 6), and mineralisation (Min; day 12) phases. Molecular markers analysed were Runx2 (1.8 kb), BSP, OPN, osteocalcin (OC, 0.6 kb), COL1, and ALP. The signal for 28S rRNA indicates similar RNA loading.
alisation studies, especially in the study of interfaces between bone mineral and artificially created surfaces.41

Proliferation, differentiation, and mineralisation phases were determined by a combination of expression profiles of molecular markers and light microscopic observations. The expression profiles of Runx2, Osx, and A18 were up-regulated earlier in D8-SBMC compared to RBMCs. This may, in part, explain the rapid mineralisation of these cultures. Runx2 and A18 both show biphasic expression profiles in D8-SBMC and RBMCs, similar to Northern blot hybridisation analysis of RBMCs by Jheon et al.24 Moreover, the osteogenic phenotype of D8-SBMC is produced independently of Dex and/or FGF-2 unlike SBMCs and RBMCs.1,2,31 D8-SBMC showed a response to earlier β-GP treatment, however, no differences in the molecular profiles of bone markers were detected by Northern blot or qPCR analyses (data not shown). The expression profiles of bone-associated genes in D8-SBMC are unresponsive to Dex and thus far, D8-SBMC has been shown to maintain their bone-forming ability after 30 passages in the absence of Dex and FGF-2 (data not shown).

D8-SBMC possesses characteristics that are highly advantageous for the study of bone formation in vitro compared to...
other previously characterised cell lines. Firstly, D8-SBMC is a homogeneous population derived from two sequential selections of single cell suspensions. This removes complications of analysing heterogeneous populations for the study of osteoblastic differentiation observed with primary foetal rat calvarial cells (FRCCs).\(^9\) RBMCs,\(^10\) or SBMCs.\(^2\) Secondly, D8-SBMC is a non-transformed, spontaneously immortalised, and stable population of cell that possesses the ability to reliably and consistently form bone-like nodules in vitro unlike ROS 17/2.8 or UMR 106.\(^3\)\(^1\)\(^2\) Thirdly, D8-SBMC generates a cement line-like matrix, which will be valuable for the study of cell-surface interactions.\(^3\) Lastly, D8-SBMC produces a large amount of bone-like nodules in the shortest time reported to date in media supplemented with AA and 13-GP alone, without addition of exogenous factors such as Dex and FGF-2. In comparison, D8-SMBCs can generate mineralised nodules as early as 8 days proceeding confluence, whereas greater than 14 days are required for systems such for FRCCs,\(^9\) RBMCs,\(^10\) SBMCs,\(^1\)\(^2\) and MC3T3-E1.\(^14\) MLO-A5 cells have the ability to form bone-like nodules within 6–7 days in the absence of 13-GP, however, these cells were established from a transgenic mouse model overexpressing the large T antigen under the osteocalcin promoter.\(^16\) The combination of the properties, described above, makes D8-SBMC an improved and valuable bone model system for the study of biomineralisation.

D8-SBMC was utilised to study the effects of AJ18 overexpression on osteogenesis. AJ18 is a putative transcriptional repressor that competes with Runx2 for the OSE2,\(^24\) a sequence element present in the promoters of many bone-related genes.\(^21\) Constitutive over-expression of exogenous AJ18 resulted in a significant increase in bone nodule formation along with the corresponding up-regulation of bone-related genes. Conversely, over-expression of antisense AJ18 resulted in a significant decrease in bone nodule formation; however, decreases in bone gene markers were not detected. Interestingly, there was no or little difference in TNEAP expression between the cell lines. This suggests that TNEAP, which is expressed primarily during differentiation and is known to be required for mineralisation,\(^45\) is not a determinant in the amount of mineralisation. However, other isoforms of alkaline phosphatase may be expressed by D8-SBMC and this analysis has yet to be performed.

The increase in bone nodule formation and up-regulation of bone-related markers with over-expression of AJ18 was surprising in light of the competition and repression of OSE2-comprising genes between Runx2 and AJ18.\(^24\) Genes comprising OSE2 such as Runx2, BSP, OC, OPN, and Col1a1 were all up-regulated with AJ18 over-expression. Runx2 has been shown previously to up-regulate these genes.\(^21\)\(^43\) However, over-expression of AJ18 under the control of the 2.4 kb rat Col1a1 promoter showed a decrease in the formation of bone nodules in D8-SBMC with no effects on cell proliferation (work in progress). Therefore, the timing of AJ18 expression, whether constitutively activated under the CMV promoter or temporally activated by the Col1a1 promoter, appears to be a critical determinant of osteogenesis. These results suggest a role for AJ18 during both osteoblast proliferation and differentiation. Recently, Runx2, in addition to its well characterised osteoblast differentiation properties,\(^44\) was shown to play a direct role in the inhibition of cell proliferation by repression of RNA polymerase I-mediated ribosomal RNA (rRNA) synthesis.\(^45\) Therefore, Runx2 is a critical mechanistic link between cell fate, proliferation, and growth. Other “master genes” involved in the formation of muscle (i.e. MyoD, myogenin) and fat (C/EBP-β) also have the ability to repress rRNA synthesis.\(^46\) It would be of great interest to elucidate the role for AJ18, if any, during rRNA synthesis. There is precedent for a role in rRNA synthesis by KRAB/CZH2 proteins. Kid-1, a paralogous gene to AJ18, binds to heteroduplex DNA structures and is localised to the nucleolus, an area of rRNA synthesis by RNA polymerase I.\(^47\) Kid1 induces nucleolar disintegration by presumably reducing rRNA synthesis.

A role for AJ18 during cell proliferation and differentiation is further supported by the strikingly similar expression profiles of AJ18 and Runx2 during both proliferation and differentiation phases of osteogenesis.\(^24\) In addition, AJ18 and Runx2 are both regulated by BMPs.\(^26\)\(^48\) Thus, AJ18 and Runx2, under the influence of BMPs during osteogenesis, may act antagonistically to control the precise number of cells by promoting or inhibiting cell proliferation, respectively. Furthermore, AJ18, which shows similar tissue-specific expression profiles with BMP-7,\(^27\)\(^28\) may be acting as a positive proliferative factor in other tissues. Further experiments must be performed to elucidate the precise physiologic role of AJ18. However, the evidence presented here suggests that AJ18, similar to Runx2, plays an important role in the mechanistic linkage between cell proliferation and growth during osteogenesis.

In summary, we describe the establishment and initial characterisation of a stable, non-transformed, and homogeneous population of osteoblasts called D8-SBMC capable of generating robust and consistent mineralised bone nodules within a short time frame. We predict that the characteristics of these cells, including the ability to form a cement line-like matrix, will make D8-SBMC an invaluable tool for the study of biomineralisation. As a first demonstration of their potential value, D8-SBMC was utilised to further support a role for AJ18 during osteogenesis.

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