JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2013; **7**: 371–382.
Published online 4 June 2012 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.532

RESEARCH ARTICLE

Effect of basic fibroblast growth factor in mouse embryonic stem cell culture and osteogenic differentiation

Laura C. Rose[1], Ross Fitzsimmons[1], Poh Lee[2], Roman Krawetz[2], Derrick E. Rancourt[2,3] and Hasan Uludağ[1,4,5]*

[1]*Department of Biomedical Engineering, University of Alberta, Edmonton, Canada*
[2]*Department of Oncology, University of Calgary, Canada*
[3]*Department of Biochemistry and Molecular Biology, University of Calgary, Canada*
[4]*Faculty of Pharmacy and Pharmaceutical Sciences, University of Calgary, Canada*
[5]*Department of Chemical & Materials Engineering, University of Alberta, Edmonton, Canada*

Abstract

Embryonic stem cells are actively explored as a cell source in tissue engineering and regenerative medicine involving bone repair. Basic fibroblast growth factor (bFGF) has been a valuable growth factor to support the culture of human stem cells as well as their osteogenic differentiation, but the influence of bFGF on mouse embryonic stem (mES) cells is not known. Towards this goal, D3 cells were treated with bFGF during maintenance conditions and during spontaneous and osteogenic differentiation. In feeder-free monolayers, up to 40 ng/ml of exogenous bFGF did not support self-renewal of mES without LIF during cell expansion. During spontaneous differentiation in high-density cultures, bFGF stimulated cell proliferation under certain conditions but did not influence differentiation, as judged by stage-specific embryonic antigen-1 expression. The addition of bFGF reduced the alkaline phosphatase (ALP) activity associated with osteoblast activity during differentiation induced by osteogenic supplements, although the extent of mineralization was unaffected by bFGF. The bFGF increased the mesenchymal stem cell marker Sca-1 in an mES cell population and led to an enhanced increase in osteocalcin and runx2 expression in combination with BMP-2. These results suggest that bFGF could be utilized to expand the cell population in high-density cultures in addition to enriching the BMP-2 responsiveness of mES cells.

Received 14 March 2011; Revised 8 July 2011; Accepted 26 September 2011

Supporting information may be found in the online version of this article.

Keywords basic fibroblast growth factor; mouse embryonic stem cells; osteogenic differentiation; self-renewal; pluripotency

1. Background

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst (Evans and Kaufman, 1981). These cells are identified by their pluripotency and unlimited self-renewal properties. These characteristics make ES cells promising as a cell source for tissue engineering and regenerative medicine, as adult stem cells are restricted with respect to lineage commitment and expansion potential. The pluripotency of ES allows differentiation of the cells into any tissue in the body under the appropriate culture conditions. Their self-renewal capabilities make it possible to attain large cell numbers in culture necessary for clinical transplantation. The ES cells are being particularly investigated as a source of osteoblasts for bone regeneration, after being treated with osteogenic supplements in culture (Jukes *et al.*, 2010). Among the osteogenic supplements, basic fibroblast growth factor (bFGF) is well known to play a central role in the culture of adult stem cell populations, since it facilitates *ex vivo* bone marrow stromal cell (BMSC) expansion (Varkey *et al.*, 2006). The BMSCs contain a

* *Correspondence to: H. Uludağ, 2–020 Research Transition Facility, Department of Chemical and Materials Engineering, University of Alberta, Edmonton, Alberta T6G 2E1, Canada. E-mail: hasan.uludag@ualberta.ca*

population of mesenchymal stem cells (MSCs; multipotent stem cells), which bridge the transition from pluripotent ES cells to fully differentiated osteoblasts. Exposure to bFGF positively selects for mesenchymal cell progenitors in adult cell populations (Bianchi *et al.*, 2003; Maegawa *et al.*, 2007) and stimulates bone formation and repair *in vivo* when the protein is administered in animal models (Nagai *et al.*, 1995; Martin *et al.*, 1997). Therefore, bFGF could be an important supplement for the cultivation of ES cells as well when they are expanded for osteogenic tissue repair.

In initial studies, mouse ES (mES) cells were cultured on a feeder layer to prevent spontaneous differentiation. Leukemia inhibitory factor (LIF) produced by the feeder cells was found to be critical for maintaining pluripotency (Williams *et al.*, 1988; Smith *et al.*, 1988). In mES cells, the activity of LIF is mediated by activation of the Janus kinase–signal transducer and activator (JAK/STAT) pathway via the gp130 receptor. In contrast, treatment with LIF was not sufficient for human ES (hES) cell pluripotency or self-renewal. Unlike mES cells, hES cells did not express the receptor gp130 to allow LIF binding and STAT3 activation (Thomson *et al.*, 1998; Humphrey *et al.*, 2004). The mitogen bFGF was then explored in hES cell culture and this growth factor appeared to significantly influence the self-renewal of hES cells (Amit *et al.*, 2000). Elevated bFGF concentrations (~100 ng/ml) were able to maintain cell numbers and surface markers when undifferentiated hES cells were maintained on Matrigel™, with similar efficiency to that of feeder cell-conditioned medium (Levenstein *et al.*, 2006; Xu *et al.*, 2005a). Some non-human primate (Furuya *et al.*, 2003) and rabbit ES cells (Honda *et al.*, 2003) also benefited from bFGF supplementation during their culture; however, it is not clear whether the observed bFGF influences are universal, given the interspecies differences in the signalling pathways responsible for self-renewal and pluripotency (Martins-Taylor and Xu, 2010). There have been no systematic investigations of the effect of bFGF on mES cells, despite the fact that FGF receptors 1–4 (FGFR1–4) were shown to be upregulated during osteogenic differentiation of mES cells and that blocking the signalling activity of the FGFRs reduced the proliferation of mES cells (Ng *et al.*, 2007).

This study was conducted to explore the role of bFGF supplementation during mES cell expansion and differentiation toward the osteogenic lineage. The mES cells present fewer technical difficulties during cultivation than hES cells, and they are an important model for studying embryonic development, given the difficulties in employing human tissues for this purpose. The availability of numerous mouse models of bone diseases makes it possible to utilize mES cells in animal models of bone repair. Hence, in this study, we set out to: (a) explore the influence of bFGF on maintenance cultures of mES cells; and (b) determine the potential of bFGF in directing mES cells toward an osteogenic lineage during spontaneous and BMP-induced differentiation.

2. Methods

2.1. Materials

D3 mES cells were obtained from American Type Culture Collection (Rockville, MD, USA). (ESGRO) was obtained from Millipore (Temecula, CA, USA), trypan blue solution from MP Biomedicals (Solon, OH, USA) and CyQuant DNA kit from Molecular Probes (Eugene, OR, USA). Mitomycin-C, HCl and sulphuric acid were obtained from Fisher (Fairlawn, NJ, USA). bFGF was obtained from R&D Systems (Minneapolis, MN, USA); its bioactivity is confirmed as we routinely use it for culture and expansion of human adult bone marrow stromal cells. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) high-glucose medium with L-glutamine, penicillin/streptomycin and β-mercaptoethanol were all from Invitrogen (Grand Island, NY, USA). Gelatin, *p*-nitrophenol phosphate (*p*-NPP), ascorbic acid, β-glycerol phosphate, calcium standards, *o*-cresolphthalein, 2-amino-2-methyl-propan-1-ol and 8-hydroxyquinoline were all purchased from Sigma (St Louis, MO, USA). Phycoerythrin (PE)-conjugated mouse anti-human/mouse stage-specific embryonic antigen-1 (SSEA-1) was purchased from Stemgent (San Diego, CA, USA). The RNeasy kit was purchased from Qiagen (Missassauga, ON, USA) and the RNA 6000 Nano Chip kit was from Agilent (Santa Clara, CA, USA). M-MLV reverse transcriptase and random primers were from Invitrogen (Carlsbad, CA, USA). Oligos (dT_{18}) and real-time PCR primers were purchased from Fermentas (Burlington, ON, Canada) and IDT (San Diego, CA, USA), respectively. The *Escherichia coli*-derived BMP-2 used in this study was kindly provided by Dr W. Sebald (University of Würzburg).

2.2. Cell culture

The stock cultures of D3 embryonic stem cells were co-cultured with Mitomycin C-arrested human foreskin fibroblasts (HFFs) or mouse embryonic fibroblast (MEF) feeder cells on gelatin-coated plates to maintain pluripotency and a normal karyoptype (Meng *et al.*, 2008). Cells were grown in basal medium with 1000 U/ml ESGRO, which contains LIF. Basal medium was composed of high-glucose DMEM with L-glutamine and 15% ES-qualified FBS, 0.1 mM non-essential amino acids, 50 U/ml each of penicillin and streptomycin and 100 μM β-mercaptoethanol. In order to investigate the effect of growth factors on mES cells, basal medium was supplemented with exogenous bFGF or BMP-2 in the specified groups. The medium was changed daily and the cells were passaged every 2–3 days. Feeder cells were removed just prior to experiments during a 30 min incubation on gelatin to selectively plate feeders.

2.3. Effect of exogenous bFGF on pluripotent mES cells

Maintenance mES cells were grown on MEF feeders for one passage prior to experiments. Feeder cells were

DOI: 10.1002/term

removed from the cultures and mES cells were seeded in 24-well gelatin-coated plates in basal medium with combinations of bFGF (0–40 ng/ml) and LIF (1000 U/ml). Medium was changed daily and cultures were subcultured every 3 days, as dictated by the density of the 0 ng/ml bFGF with LIF group. At each passage, the remaining cells were taken for live cell counts and for alkaline phosphatase (ALP) activity, as an indicator of pluripotency. Trypan blue was used as an exclusion agent for live cell counts in order to identify dead cells. Alkaline phosphatase (ALP) activity was determined as previously described (Clements *et al*., 2009). In brief, cell cultures assessed for ALP activity were washed with HBSS and incubated in ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol, 0.1% v/v Triton-X, pH 10.5) at room temperature for 2 h to lyse cells. Phosphatase substrate *p*-NPP was added to the lysate in 96-well plates to give a concentration of 1 mg/ml. Kinetic ALP activity was attained from the maximum slope of eight absorbance readings (at 405 nm), 90 s apart. ALP activity was normalized to DNA content in each sample.

2.4. Embryoid body formation and differentiation

Embryoid body (EB) formation and differentiation was induced via hanging drop culture, as described previously (Zur Nieden *et al*., 2003). After the removal of feeder cells, mES cells were counted, resuspended in medium and hanging drops (750 cells/drop) were made on the inner sides of the lids of tissue culture plates containing 3 ml sterile PBS. On day 3, aggregates were transferred to bacteriological Petri dishes in 10 ml medium. On day 5, EBs were transferred to 48-well tissue culture plates with medium containing 0 or 1000 U/ml LIF and bFGF concentrations of 0, 2, 10 and 40 ng/ml. The medium was changed as needed, typically at 2–3 day internals.

2.5. Effect of exogenous bFGF during early differentiation

To determine the effect of bFGF during EB formation, ES cells were exposed to bFGF (0–40 ng/ml), with and without LIF, in basal medium from day 0 to day 15. At days 5, 10 and 15, EBs cultures were harvested to assay for DNA content, ALP activity and SSEA-1 staining. Cultures for DNA content and ALP activity were washed with HBSS and incubated at 37 °C in ALP buffer for 2 h. Cultures for SSEA-1 staining were trypsinized, resuspended in 50 μL HBSS and incubated for 1 h on ice with 10 μL PE-anti-mouse/human SSEA-1. After washing with HBSS, cells were analysed by flow cytometry at the FL2 channel (Quanta; Beckman Coulter), with unstained cells set to 1% as a background control.

2.6. Osteogenic differentiation of mES cells

Osteogenic differentiation was induced by treatment with osteogenic factors at day 5, after plating EBs in 48-well plates. EBs were grown in basal medium with 50 μg/ml ascorbic acid and combinations of 10 mM β-glycerol phosphate, 1 μM dexamethasone, 10 ng/ml bFGF and 500 ng/ml BMP-2. At days 15 and 25, cultures were harvested to assay for DNA content, ALP activity and extracellular calcium content. For alizarin red staining, the EBs were transferred to glass coverslips and incubated with medium (in six-well plates) containing the indicated supplements (see Figure 8B). Alizarin red staining was performed according to Zur Nieden *et al*. (2003) on days 15 and 25.

2.7. DNA content, calcium deposition and ALP activity assays

The cultures were washed with HBSS and incubated with ALP buffer. After incubation, the plates were washed with HBSS and the extracellular matrix was dissolved in 0.5 N HCl. A 500 μl solution of 2-amino-2-methyl-propan-1-ol (1.5% v/v) and *o*-cresolphthalein (37 mM) was mixed with 100 μl 8-hydroxyquinoline (28 mM) and sulphuric acid (0.5% v/v) in 48-well plates containing of 20 μl of sample. The absorbance was determined at 570 nm and compared against calcium standards. Kinetic ALP activity was measured as described above and DNA content was quantified using a CyQUANT assay kit.

2.8. Real-time polymerase chain reaction (PCR)

RNA was harvested from osteogenic cultures on day 14, after treatment with medium containing ascorbic acid (50 μg/ml), bFGF (0 or 10 ng/ml) and BMP-2 (0 or 500 ng/ml) in 24-well plates. Cultures were washed with HBSS and then RNA was extracted with an RNeasy Kit, according to the manufacturer's instructions. The obtained RNA concentration was measured with a GE NanoVue spectrophotometer; RNA quality was determined with an RNA 6000 Nano Chip Kit. All but one RNA sample (6.1) had RNA integrity number (RIN) above 7.2. From each sample, 150 ng RNA was reverse-transcribed using M-MLV reverse transcriptase, following the manufacturer's instructions, in a reaction volume of 20 μL. In addition to random primers, oligos (dT_{18}) were used to synthesize cDNA template. Primer sets (Table 1) were validated by ensuring equal PCR efficiencies between endogenous control *GAPDH* and the gene of interest over a five-fold change in template cDNA concentration (see Supporting information, Figure S1). The stability of the *GAPDH* endogenous control was confirmed by ensuring similar cycle threshold (C_T) values from different biological replicates of all four treatment groups. SYBR green dye, which binds double-stranded DNA, was used to monitor real-time reaction products on a 7500 Fast Real-Time PCR System (Applied Biosystems). From the 20 μL reverse-transcription reactions, 1 out of 10 dilutions were made for template in real-time PCR. The 10 μL real-time reaction mixture consisted of 2.5 μL cDNA template, 2.5 μL 3.2 μM primers and 5 μL 2× master mix containing SYBR green dye, dNTPs and salts. Reaction mixtures were heated to 95 °C for 2 min before going through 40 cycles of a denaturation step (15 s at 95 °C)

DOI: 10.1002/term

Table 1. Real-time PCR primers

Primer set	Marker	Direction	Primer Sequence (5′ to 3′)
1	*ALP*	Forward	GGCCAGCAGGTTTCTCTCTTG
		Reverse	GCAGGGTCTGGAGAATATATTTGG
2	*Cbfa1/*	Forward	GCCGGGAATGATGAGAACTACT
	Runx2	Reverse	AGATCGTTGAACCTGGCTACTTG
3	*CD29*	Forward	CCAGGGCTGGTTATACAGAATCA
		Reverse	CCACATACATCACTGGGAATTCC
4	*CD105*	Forward	CCTCCCAGTGGAGACTTCAGAT
		Reverse	AGTGCCGTGTCTTTCTGTAATCC
5	*Col-1a1*	Forward	CCCTGCCTGCTTCGTGTAAA
		Reverse	TTGGGTTGTTCGTCTGTTTCC
6	*FGFR1*	Forward	TGAGCTTGGCTTCCTATAGTTTTTC
		Reverse	GCAGAATTGAGTTGCCAAGTTG
7	*GAPDH*	Forward	ATGTGTCCGTCGTGGATCTGA
		Reverse	CCTGCTTCACCACCTTCTTGA
8	*OCN*	Forward	CGGCCCTGAGTCTGACAAAG
		Reverse	AGGTAGCGCCGGAGTCTGTT
9	*OP*	Forward	AGGCATTCTCGGAGGAAACC
		Reverse	CAAACAGGCAAAAGCAAATCAC
10	Sca-*1*	Forward	CAAGGTGGGAGTAGTGTGTGAAAT
		Reverse	GCCCTAGAGAGGATTAGAGCACCTA
11	*SSEA-1*	Forward	AGCTGTGACTAACATTGCCTCATT
		Reverse	GAAACCCTGTCTGAAAAACCAAA

and an annealing/elongation step (60 s at 60 °C), during which SYBR green fluorescence data were collected. A $^{\Delta\Delta}C_T$ analysis was used to determine differences in gene expression, as compared to no treatment controls. *GAPDH* was employed as an endogenous control, to which the C_T of the gene of interest was normalized. Changes in gene expression were determined by normalizing the differences in cycle threshold ($^{\Delta}C_T$) to spontaneous differentiation (no treatment) controls. RNA from three biological replicates was pooled and run in triplicate to screen for changes in gene expression. Any changes from the no treatment control were verified in three independent biological replicates, with each real-time reaction performed in triplicate.

2.9. Statistical analysis

For all results, except EB formation for osteogenic differentiation, error bars represent the standard deviation (SD) of results in triplicate. Error bars in osteogenic differentiation experiments (ALP activity, DNA and calcium content) represent SD for groups with five or six replicates. To determine significant changes due to bFGF treatment, one-way analysis of variance (ANOVA) was employed, followed by Dunnett's test, which compares all treatment groups to a single control.

3. Results

3.1. Effect of exogenous bFGF on pluripotent mES cell cultures

To investigate the bFGF response of mES cells, the cells were cultured with 0, 2, 10 and 40 ng/ml bFGF with (1000 U/ml) and without LIF. Cell numbers and ALP activity were used as measures of self-renewal and pluripotency. ALP is a membrane-bound enzyme that exhibits biphasic behaviour. It is expressed on the surface of pluripotent undifferentiated ES cells and disappears as cells begin to differentiate. If cells are directed toward an osteogenic lineage, its expression is upregulated as cells mature into osteoblasts. For clarity, activity associated with undifferentiated ES cells is here denoted as ALP_{Embryo}, while activity associated with osteogenic differentiation is denoted as ALP_{Osteo}. The 0 ng/ml bFGF with 1000 U/ml LIF group served as the control, as this condition mimicked the maintenance cultures of mES cells, and the visual cell density of this group was used as an indicator of the timing of cell passaging. mES cells at each passage were counted and cell numbers in treatment groups were expressed relative to the control group.

In the absence of LIF, mES cells showed a rapid decrease in cell numbers with cell passage and cell viability was essentially lost at the second passage under the feeder-free conditions (Figure 1). In the presence of LIF, bFGF resulted in a slow decrease in cell numbers over the five passages, as compared to the maintenance conditions. There was no evidence of a dose–response relationship in relative cell numbers within the employed bFGF concentrations. The control group itself also showed a decrease in cell numbers over the study period (Figure 1, insert).

Similar to the changes in cell numbers, the ALP_{Embryo} activity was decreased in the absence of LIF but remained constant with LIF (Figure 2A). However, specific ALP_{Embryo} activity (ALP_{Embryo} activity/cell) remained relatively constant relative to the maintenance condition (Figure 2B). There was no effect of bFGF on the ALP_{Embryo} activity under the conditions of this experiment.

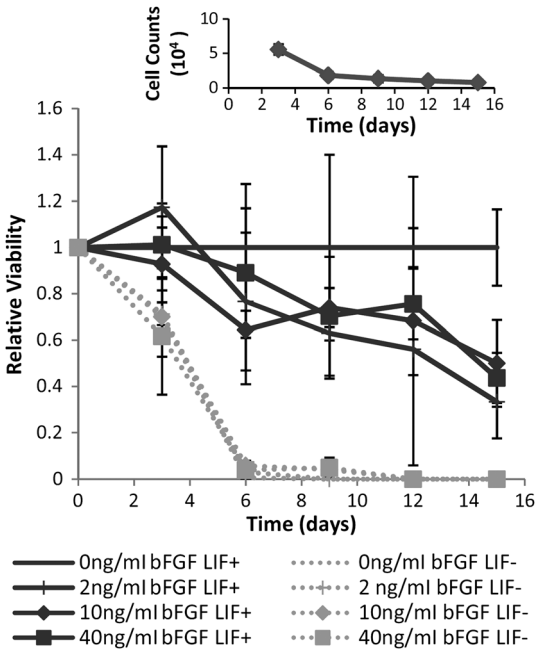

Figure 1. Effect of bFGF on self-renewal during maintenance conditions. Cell counts of mES cells treated with 0, 2, 10 and 40 ng/ml bFGF in the absence and presence (1000 U/ml) of LIF. Regular maintenance conditions (i.e. 0 ng/ml bFGF and 1000 U/ml LIF) were used to normalize cells counts at each time point. Note that the regular maintenance group to which treatment groups were normalized did show a gradual decline. (Insert) Cell numbers of cultures grown under maintenance conditions (0 ng/ml bFGF and 1000 U/ml LIF)

DOI: 10.1002/term

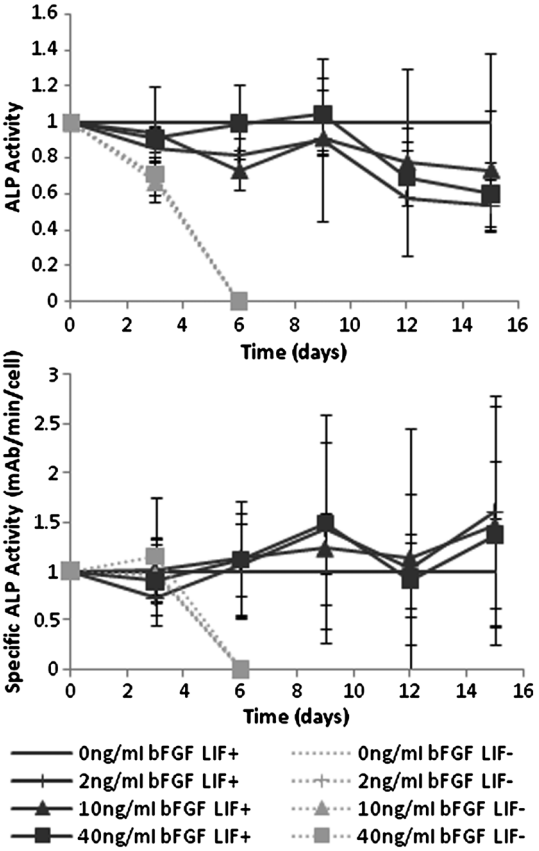

Figure 2. Effect of bFGF on ALP activity during maintenance conditions. ALP activity for mES cells treated with 0, 2, 10 and 40 ng/ml bFGF in the absence and presence (1000 U/ml) of LIF. ALP activity (2A) was normalized to the maintenance conditions of 0 ng/ml bFGF with LIF. Specific ALP (2B) activity (mAb/min/μg DNA) was normalized to the maintenance conditions of 0 ng/ml bFGF with LIF

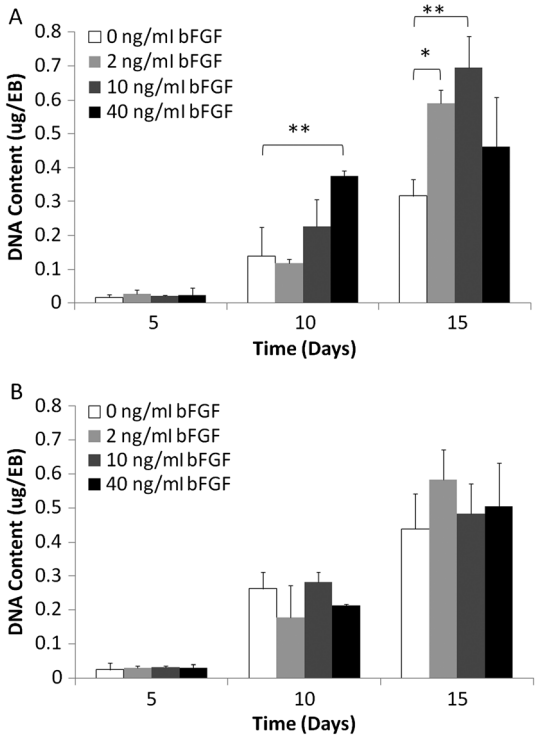

Figure 3. DNA content of EBs during early phase of differentiation. DNA content of EBs harvested on day 5, 10 or 15. Cultures were grown in basal medium containing 0, 2, 10 or 40 ng/ml bFGF with (1000 U/ml; B) and without LIF (A) from hanging drop formation at day 0. Treatment groups were compared to the negative control of 0 ng/ml bFGF at each time point; *$p < 0.05$, **$p < 0.01$

3.2. Effect of bFGF during EB formation and early phase of differentiation

To investigate the effects of bFGF during spontaneous differentiation in high-density cultures, EBs were grown in basal medium containing 0–40 ng/ml bFGF with (1000 U/ml) and without LIF from hanging drop formation. The EBs in 48-well plates were harvested on days 5, 10 and 15 to assay for DNA content, ALP_{Embryo} activity and SSEA-1 expression. DNA content was used as an indicator of cell number, since it was not possible to obtain accurate cell counts, due to technical difficulties in dissociating EBs into uniform cell suspensions. At day 5, there were no significant differences in DNA content among the study groups without or with LIF (Figure 3A and B, respectively). Proliferation in subsequent days was evident in EBs even in the absence of LIF (Figure 3A), which was unlike the monolayer cultures (see Figure 1). At day 10 in the absence of LIF, significant increase in DNA content was obtained when mES cells were cultured with 40 ng/ml bFGF ($p < 0.01$ vs 0 ng/ml bFGF). Similarly, at day 15 significant increase in DNA content was obtained at 2 and 10 ng/ml bFGF ($p < 0.05$ and $p < 0.01$ vs 0 ng/ml bFGF). In the presence of LIF (Figure 3B), there were no significant differences in DNA content among the study group.

The specific ALP_{Embryo} activity did not vary significantly among the study groups (Figure 4). A drop in the specific ALP_{Embryo} activity was evident for some of the mESs cultured without LIF (Figure 4A), but bFGF did not have a stimulatory or inhibitory effect on this activity at different time points. In the presence of LIF (Figure 4B), an upward trend in the specific ALP_{Embryo} activity was apparent, but bFGF did not influence the specific ALP_{Embryo} activity under these conditions either.

Flow cytometry was used to detect SSEA-1 positive cells as a function of time. Sufficient cells were not collected on day 5 to obtain an accurate assessment of SSEA-1, but there were no apparent differences among the study groups, as judged by the average fluorescence of the sample (data not shown). On day 10 (Figure 5), all four groups treated with LIF showed significant SSEA-1-positive cells ($p < 0.01$ for 0, 2 and 40 ng/ml bFGF and $p < 0.05$ for 10 ng/ml bFGF vs without LIF). On day 15, the level of SSEA-1 expression was significantly reduced as compared to the day 10 levels, and only the cells treated with 10 ng/ml bFGF and LIF showed significant ($p < 0.01$) variation in SSEA-1-positive cells compared to LIF treatment alone (0 ng/ml bFGF). Addition of bFGF did not prevent the reduction in the levels of SSEA-1-positive cells from day 10 to day 15.

3.3. Effect of bFGF during osteogenic differentiation of mES cell cultures

To investigate the effect of bFGF during osteogenic differentiation, EBs were treated with different combinations of osteogenic supplements (β-GP, DEX and BMP-2) in basal

DOI: 10.1002/term

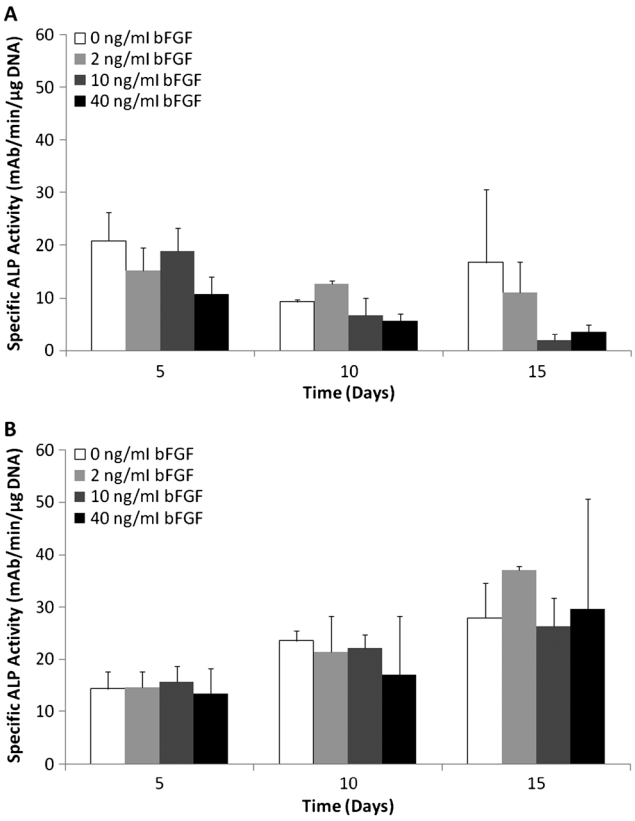

Figure 4. ALP activity of EBs during early phase of differentiation. ALP activity (mAb/min) of EBs normalized to the amount of DNA in each sample. Cultures were grown in basal medium containing 0, 2, 10 or 40 ng/ml bFGF with (1000 U/ml; B) and without LIF (A) from hanging drop formation at day 0. Treatment groups were compared to the negative control of 0 ng/ml bFGF each time point

medium containing ascorbic acid but without LIF. The EB grown in each combination was exposed to either 0 or 10 ng/ml bFGF. This concentration of bFGF was chosen based on the increases in DNA content found when mES cells were cultured without LIF during early differentiation. The EBs in 48-well dishes were harvested on days 15 and 25 to assay for the DNA content, ALP_{Osteo} activity and calcification. The addition of exogenous bFGF did not result in significant increase in DNA content at day 15 (Figure 6). EBs treated with BMP-2 alone showed a significant increase ($p < 0.05$) in DNA content, along with the EBs treated with β-GP/bFGF and β-GP/DEX/bFGF ($p < 0.01$). At day 25, only the DEX/BMP-2/bFGF group showed a significant ($p < 0.05$) increase over control without bFGF, indicating no clear effect of bFGF supplementation on the DNA content under osteogenic stimulation.

None of the specific ALP_{Osteo} values were significantly different among the study groups on day 15 (Figure 7). This was the case in the absence or presence of bFGF. However, on day 25, EBs treated with BMP-2 ($p < 0.05$), DEX/BMP-2 ($p < 0.01$) and β-GP/DEX/BMP-2 ($p < 0.05$) showed significant increases in specific ALP_{Osteo} activity as compared to the control EBs (EBs treated with no osteogenic supplements and no bFGF). In the presence of bFGF, these groups did not lead to a significant increase in specific ALP_{Osteo} activity, indicating the ability of bFGF to downregulate induced ALP activity. Accordingly, there were no differences in the specific ALP_{Osteo} activity among the treatment groups.

The EBs treated with β-GP/BMP-2 and β-GP/DEX/BMP-2/bFGF (Figure 8A) showed significant ($p < 0.01$) calcium deposition on day 15, but the calcium deposition with EBs treated with β-GP/DEX was not significant. The latter group again did not give significant calcium accumulation on day 25 compared to the control EBs (EBs treated with no osteogenic supplements and no bFGF). However, the EB cells treated with β-GP/BMP-2, β-GP/BMP-2/bFGF, β-GP/DEX/BMP-2 and β-GP/DEX/BMP-2/bFGF combinations all showed significant calcium deposition ($p < 0.01$). The presence of the bFGF in these groups did not alter the level of calcium deposition. Consistent with the biochemical calcium assay, alizarin red staining indicated significant mineralization in EB cultures, which generally increased from day 15 to day 25 (Figure 8B).

3.4. Effect of bFGF on gene expression during osteogenic differentiation

Changes in gene expression with exposure to bFGF during osteogenic differentiation were investigated using real-time

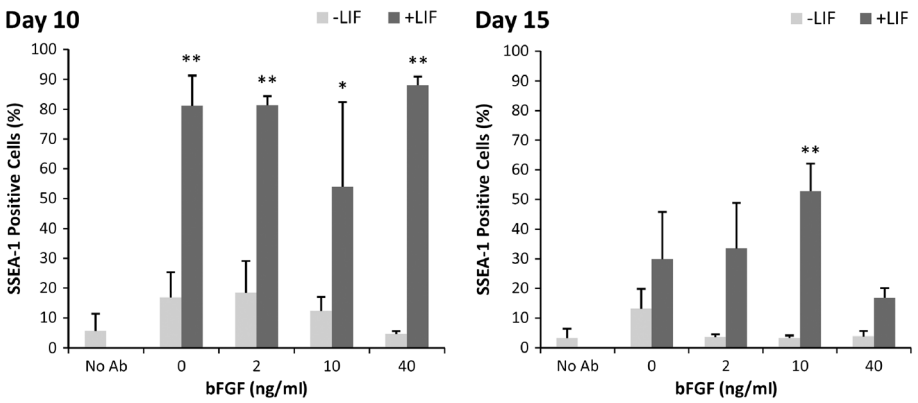

Figure 5. SSEA-1 staining of EBs during early phase of differentiation. Flow cytometry analysis of SSEA-1 expression on cells during the early phase of differentiation. Cells stained with phycoerythrin-labelled SSEA-1 antibodies, with unstained cells, were set a 1% positive. Cultures were grown in basal medium containing 0, 2, 10 or 40 ng/ml bFGF with (1000 U/ml) and without LIF from hanging drop formation at day 0 until harvest at day 10 and 15. Treatment groups were compared to the negative control of 0 ng/ml bFGF without LIF at each time point; *$p < 0.05$, **$p < 0.01$

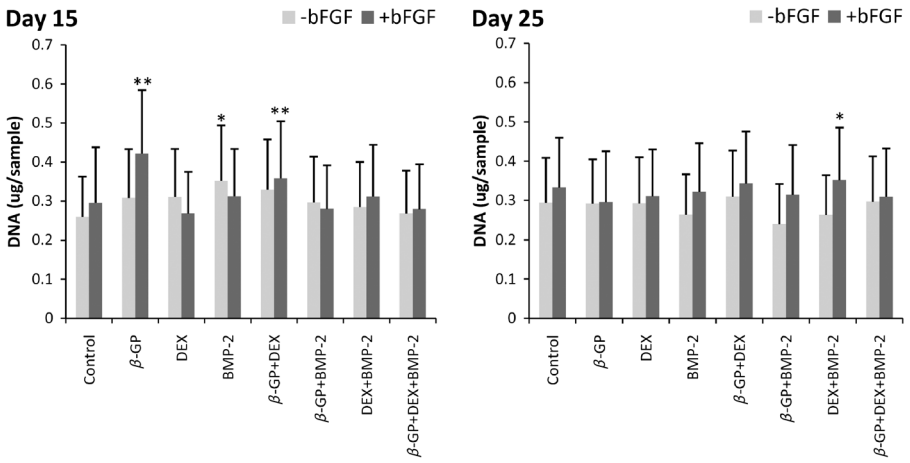

Figure 6. DNA content of EBs during osteogenic differentiation; DNA content of EBs following exposure to osteogenic supplements. β-GP, DEX, BMP-2 and bFGF were added to basal medium, which contained ascorbic acid. Basal medium was included as a control. EBs were grown in media containing different combinations of osteogenic supplements and were harvested on days 15 and 25 after hanging drop formation to assess DNA content. Significantly different groups compared to control medium without bFGF are indicated (*$p < 0.05$, **$p < 0.01$)

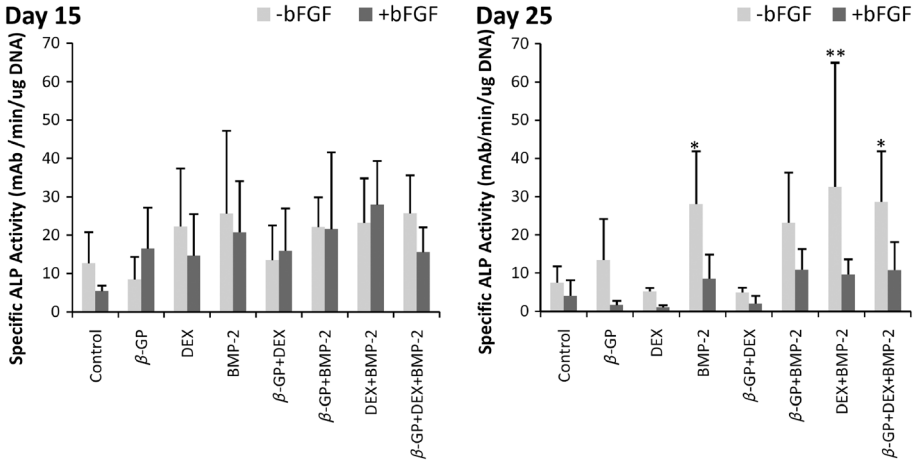

Figure 7. ALP activity of EBs during osteogenic differentiation. ALP activity of EBs exposed to different osteogenic supplements. Different combinations of β-GP, DEX, BMP-2 and bFGF were added to basal medium, which contained ascorbic acid. Basal medium alone served as a control. EBs were grown in medium containing different combinations of osteogenic supplements. ALP was measured on days 15 and 25, and was normalized to DNA content. Significant differences from control medium without bFGF are indicated; $p < 0.05$, **$p < 0.01$

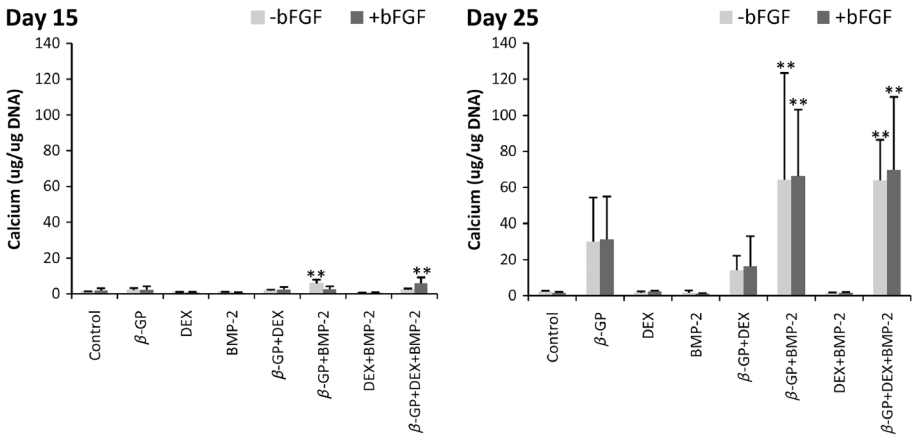

Figure 8. Extracellular matrix calcification of EBs during osteogenic differentiation. (A) Calcium content of EBs following exposure to osteogenic supplements. EBs were grown in basal medium, which contained ascorbic acid, with combinations of β-GP, DEX, BMP-2 and bFGF. Basal medium alone served as a control. Cultures were harvested at day 15 or 25 and assessed for calcification. Calcium content of the cultures was normalized to DNA content. Significant differences from control medium without bFGF are indicated; $p < 0.05$, **$p < 0.01$. (B) Representative alizarin red-stained EBs, which were cultured in the presence of the indicated supplements. EB cultures stained after day 15 (left) and day 25 (right) are shown. Note that the pictures are intended as a qualitative measure of calcification in EB cultures and should not be used for quantitative purposes to compare different groups

DOI: 10.1002/term

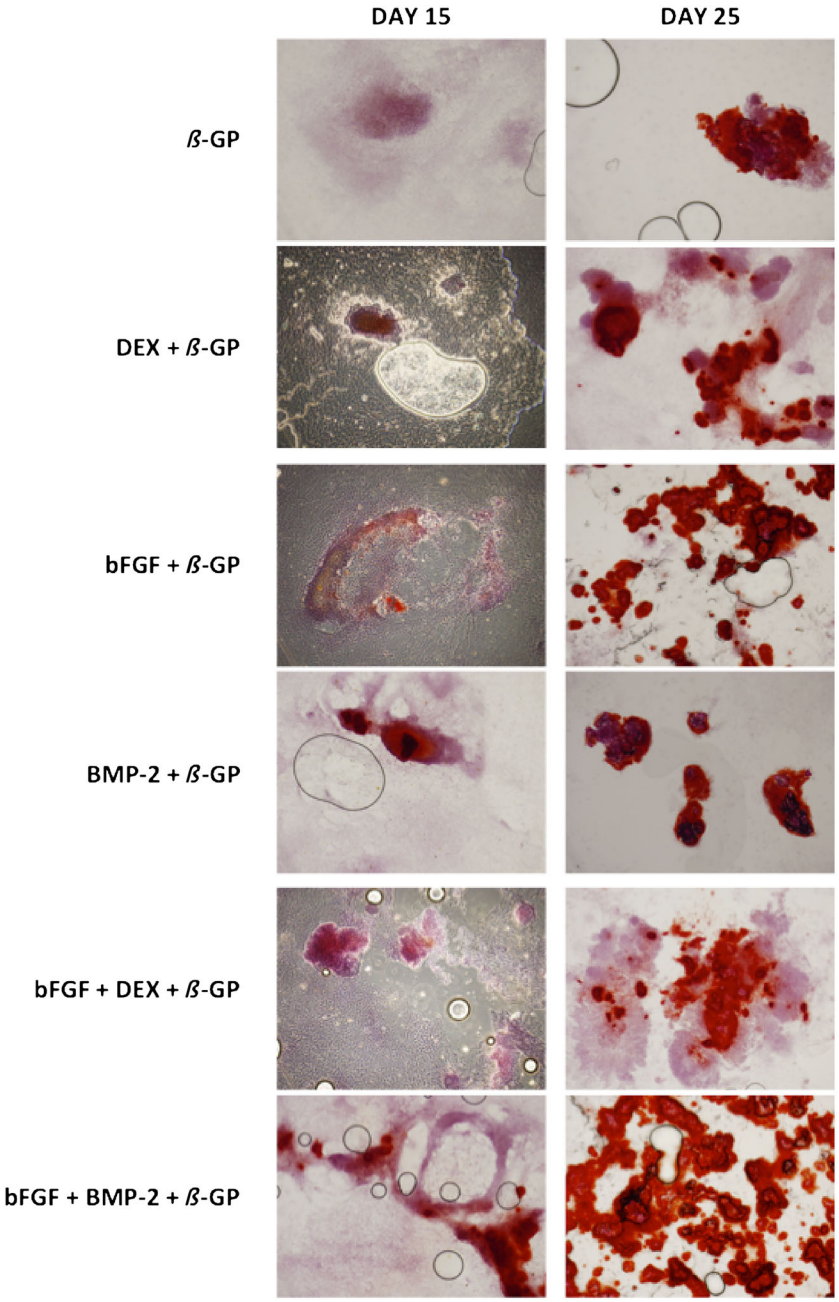

Figure 8. Continued

PCR. The EBs were treated with bFGF (10 ng/ml), BMP-2 (500 ng/ml) and a combination of bFGF and BMP-2 (10 and 500 ng/ml, respectively), and RNA was harvested from cultures on day 14 or 21 for cDNA templates. Untreated mEB served as a control in the PCR analysis. Osteogenesis- associated genes analysed included runt-related transcription factor 2 (*Runx2*), osteocalcin (*OCN*), osteopontin, collagen and *ALP*. Markers associated with ES cell pluripotency (*SSEA-1*), mesenchymal stem cells (*CD105*, *CD29*, *Sca1*) and haematopoetic stem cells (*Sca1*) were also included.

The *ALP* mRNA was found to increase 1.8–2.2-fold at days 14 and 21 after BMP-2 treatment of EBs (Figure 9). However, the combination of bFGF and BMP-2 led to a decrease in ALP expression at day 21, similar to the results from the enzyme activity assay, where the addition of bFGF during osteogenic differentiation led to a decrease in ALP activity.

The bFGF treatment resulted in a ~2.5-fold increase in Sca1 (a marker present on both mesenchymal and haematopoetic stem cells) expression on day 14 as compared to no treatment. In combination with BMP-2, a ~5.2-fold increase in Sca1 expression was observed. A small increase in Sca-1 expression (~1.5-fold) was evident at day 21 with bFGF treatment.

On day 14, an increase in OCN expression was observed following exposure to BMP-2 (2.2-fold); however the combination of BMP-2 and bFGF led to an even higher (6.7-fold) OCN levels over BMP-2 alone (Figure 9). A similar trend was observed on day 21.

At day 14, a significant (1.9-fold) increase in Runx2 over no treatment was observed when EBs were exposed to a

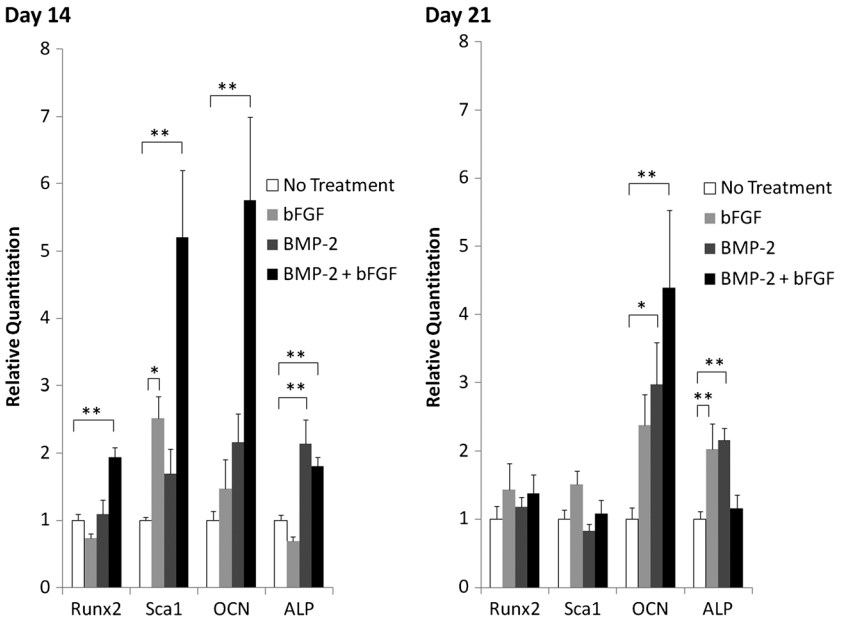

Figure 9. Changes in gene expression during osteogenic differentiation. Quantitative PCR results showing gene expression of *ALP*, *Sca-1* and *OCN* (osteocalcin) during BMP-2-induced osteogenic differentiation. Cells were exposed to either BMP-2 alone or a combination of BMP-2 and bFGF. Basal medium without BMP-2 or bFGF served as a control. Cells were harvested at days 14 and 21 and a $^{\triangle\triangle}C_T$ analysis was used to evaluate changes in expression of target genes, using *GADPH* as an endogenous control. Expression in the bFGF, BMP-2 and BMP-2 + bFGF treatment groups were normalized to a control group without BMP-2 or bFGF; $*p < 0.05$, $**p < 0.01$

combination of BMP-2 and bFGF, but not BMP-2 or bFGF alone. No changes in Runx2 were seen at day 21 for all groups.

PCR analysis for SSEA-1 expression indicated no increase in expression following the addition of bFGF (not shown) on day 14 or 21, confirming the flow cytometry results.

The other molecular markers (CD29, CD105, Cpl-1a1, FGFR1 and OP) were not significantly changed (not shown) from the no treatment group under the investigated conditions.

4. Discussion

The mechanism of bFGF-mediated feeder-free culture has not been uncovered, but bFGF is proposed to interfere with bone morphogenetic protein (BMP) signalling, associated with unconditioned medium, that causes differentiation of hES cells (Xu *et al.*, 2005b). Although bFGF is routinely used in the culture of pluripotent hES cells, the effects of bFGF on mES cell cultures is not known. Based on the experience with hES cells and human/mouse BMSCs (Jung *et al.*, 2010; Wang *et al.*, 2010), we expected bFGF to enhance survival and proliferation of the cells and, therefore, facilitate the expansion of these cells. In addition, we were interested to determine whether bFGF could enhance BMP-2 mediated osteogenic differentiation. An understanding of how mES cells respond to these commonly used growth factors would greatly enhance our understanding of stem cells, which would further facilitate the use of stem cells that are vitally needed for regenerative medicine approaches. We conducted the current study to explore the beneficial effect, if any, of bFGF mES cell expansion and osteogenic differentiation. The effects of exogenous bFGF on D3 mES cells were investigated in three different environments: during maintenance conditions, at an early stage of spontaneous differentiation (in high-density EB formation) and during osteogenic differentiation. The feeder-independent nature of the D3 line allowed feeder-free conditions (i.e. on gelatin-coated plates) during experiments, so that only the effects of exogenous growth factor were observed. This prevented any synergistic effects from combinations of bFGF and other growth factors produced by the feeder layers.

Under the maintenance conditions with regular passaging, bFGF did not sustain self-renewal capabilities of mES cells in the absence of LIF and it was no substitute for LIF in prolonging self-renewal. In combination with LIF, there was a slight decrease in the number of cells after five passages with bFGF compared to basal conditions (with LIF) in the absence of bFGF. However, the specific ALP_{Embryo} activity of bFGF-treated mES cells was unaffected, indicating that the addition of bFGF did not appear to result in differentiation and maintained pluripotency.

Since self-renewal was not supported in cultures without LIF, the effect of bFGF alone on pluripotency could not be assessed under maintenance conditions. To investigate the effect of bFGF on mES differentiation, EBs were formed via hanging drops in medium, since the cells were previously cultivated without LIF under these conditions (Zur Nieden *et al.*, 2003). This was shown to be the case here as well. Under these conditions and in the absence of LIF, bFGF treatment did stimulate proliferation in EBs under certain concentrations, suggesting that a careful optimization might be necessary to obtain this beneficial effect of bFGF on EBs. When mES cells are cultured in

DOI: 10.1002/term

tissue-engineering scaffolds it is likely that EB formation will occur more readily, due to the confined spaces of the scaffolds, and our results indicate that bFGF may help in mEB cell expansion under these conditions. The bFGF did not stimulate cell proliferation in the presence of LIF, since the cells displayed a more robust proliferation under this condition.

Based on the chances in ALP_{Embryo} of the EBs treated with LIF, the cytokine was found to be beneficial to maintaining the pluripotency of the mES cells, even in EBs. A similar conclusion was reached based on SSEA-1 expression, which is present in undifferentiated cells and disappears as ES cells undergo differentiation. Although not statistically significant, the addition of bFGF appeared to cause a larger decrease in ALP_{Embryo} activity compared in the absence of LIF. Taking these factors together, bFGF did not appear to adversely affect the pluripotency of mES in EBs. Although different signalling pathways are employed to maintain pluripotency in mouse and human ES cells, some of these differences may be attributed to ES cells derived from different temporal origins (Brons *et al*., 2007). Regardless, bFGF pluripotency and self-renewal pathways do not appear to be present in mES cells, given the unresponsiveness of mES cells to the exogenous bFGF.

The effects of bFGF during osteogenic differentiation were subsequently investigated. The EBs were exposed to all combinations of β-GP, DEX and BMP-2 in order to induce osteogenic differentiation, and cultured with and without bFGF to investigate the changes in osteogenic differentiation. The success of these treatments in inducing osteoblast characteristics was assessed by alizarin red staining (Buttery *et al*., 2001), ALP_{Osteo} levels (Toumadjie *et al*., 2003), formation of discrete mineralized nodules (Duplomb *et al*., 2007; Buttery *et al*., 2001) and expression of osteogenic lineage-specific genes (Zur Nieden *et al*., 2003). In our differentiation experiments, it is possible that the ALP_{Osteo} activity at day 15 was not entirely due to induction of the osteogenic pathway, but ALP_{Osteo} activity at day 25 was most likely due to the presence of osteoblast-like cells. Some markers of pluripotency, such as Oct3/4, remain strong for at least 7 days after the removal of LIF, but markers associated with the primitive endoderm have been found to increase at day 6 (Toumadjie *et al*., 2003), suggesting that the molecular pathways involved in differentiation are activated relatively early.

The presence of bFGF did not consistently lead to an increase in DNA content during the imposed osteogenic differentiation (days 15–25). However, bFGF did appear to have negative effects on specific ALP_{Osteo} activity. EBs grown with BMP-2, DEX/BMP-2 or β-GP/DEX/BMP-2 all showed significantly higher ALP_{Osteo} activity but the addition of bFGF to these combinations resulted in significant decreases in specific ALP_{Osteo} activity. Although we were not able to show significant ALP_{Osteo} activity with our AA/β-GP/DEX positive control, a similar bFGF-dependent inhibition of ALP_{Osteo} activity (Varkey *et al*., 2006; Clements *et al*., 2009) was seen in adult BMSCs grown in osteogenic medium containing AA/β-GP/DEX. Others reported conflicting results on the effect of bFGF on osteogensis of human BMSC, including ALP activity (reviewed in Clements *et al*., 2009), this despite the fact that bFGF administration in rat and mouse models resulted in a stimulation of bone formation (Nagai *et al*., 1995; Dunstan *et al*., 1999). The decreased ALP_{Osteo} activity observed with bFGF did not necessarily correlate with decreased calcium deposition, as the combinations of β-GP/BMP-2 and β-GP/BMP-2/DEX that gave significant calcification showed no change upon addition of bFGF. β-GP/BMP-2 and β-GP/BMP-2/DEX/bFGF appeared to have accelerated calcification, and these two groups were the only ones that had significant calcium deposition on day 15. Nevertheless, all BMP-2-containing groups gave increased calcification by mES cells in EBs, consistent with the expected activity of this well-established osteogenic protein (Lecanda *et al*., 1997).

Addition of β-GP was essential for calcification; the combination of DEX/BMP-2 led to significantly higher specific ALP_{Osteo} activity in EBs, but required the addition of β-GP for the formation of any significant calcification (also seen in alizarin red-stained EBs). It must be noted, however, that although not statistically significant, the addition of β-GP alone resulted in some calcification. It is possible that DEX and BMP-2 are required to induce osteo-specific markers, whereas β-GP may result in spontaneous calcium precipitation on its own (Roach, 1992).

In order to determine the effects of bFGF on osteogenic lineage specific gene expression, quantitative PCR was used to analyse changes in gene expression following EBs formation and treatment with BMP-2 and bFGF. Changes in *ALP* gene expression correlated well with the changes in enzymatic (colorimetric) ALP activity. Osteocalcin was upregulated when the cells were treated with BMP-2 (consistent with a previous report on mES (Ohba *et al*., 2007), and was even further increased with a combination of bFGF and BMP-2 treatments, along with *Runx2*. This confirmed that the cells did undergo osteogenic differentiation with BMP-2 and, based on this marker alone, bFGF did stimulate osteogenic differentiation. Further evidence to suggest that bFGF may alter the differentiation process was the expression profile of *Sca-1* at day 14, a marker present in both mesenchymal and haematopoetic stem cells (Zhu *et al*., 2010). The addition of bFGF to the culture medium caused an increase in Sca-1 at day 14, which indicated expansion of the mesenchymal/haematopoetic stem cells that was consistent with the loss of pluripotency marker *SSEA-1* expression. The disappearance of Sca-1 at day 21 was most likely due to further differentiation, and decrease in mesenchymal stem cells in the obtained cell population. This Sca-1 upregulation indicated that bFGF increased the population of mesenchymal-like cells, in agreement with previous results obtained from BMSCs (Bianchi *et al*., 2003; Maegawa *et al*., 2007). To fully assess osteogenic differentiation in mES cells, more characterization will be needed for changes in the markers of pluripotency (e.g. *Oct4*, *SOX2*, etc.) to better realize the potential of teratoma formation, if the osteogenic

DOI: 10.1002/term

differentiation mES cells are used for transplantation. The chances of teratoma formation are likely to be diminished if the pluripotency markers are decreased as a result of osteogenic differentiation. Future studies should elucidate such changes.

5. Conclusion

We conclude that, despite the known mitogenicity of bFGF, we have not observed any consistent increase in cell numbers or DNA content of mES cells during regular maintenance conditions in monolayers. LIF was indispensable under these conditions. The bFGF on its own (i.e. without LIF) did stimulate cell proliferation without causing osteogenic differentiation in high-density EB cultures. These results pointed to significant differences between human and mouse bFGF-dependent pluripotency and self-renewal pathways. During differentiation under the influence of osteogenic supplements, the addition of bFGF prevented the increase in ALP_{Osteo} activity of EBs exposed to osteogenic factors, which was reminiscent of the behaviour of adult BMSCs exposed to bFGF. Upregulation of *Sca-1* and osteogenesis-related genes suggested that bFGF may enhance the population of mesenchymal stem cells and lead to osteogenic differentiation under these conditions. We conclude that, while LIF was indispensable for long-term survival, bFGF could be useful in expansion of stem cells in EBs cultures and can further support BMP-2-induced osteogenesis at the gene level.

Acknowledgements

The authors are grateful for real-time PCR guidance from Troy Locke at the Molecular Biology Facility, University of Alberta, Canada. Financial support for this project was provided by a CIHR Team Grant on Skeletal and Regenerative Medicine (to P.I. and D.E.R.), an Alberta Advanced Education and Technology (AET) grant, the Alberta Heritage Foundation for Medical Research (AHFMR) for equipment and a CIHR Operating Grant (to P.I. and H.U.); L.R. was partly supported by a Queen Elizabeth II Scholarship from the University of Alberta. We are grateful for Professor Walter Sebald (Biozentrum, University of Würzburg, Germany) for providing us with recombinant BMP-2 for this study.

Supporting information on the internet

The following supporting information may be found in the online version of this article.

Figure S1. Priming efficiency of primers for real-time PCR. The difference in cycle threshold (ΔC_T) compared to *GAPDH* endogenous control was plotted against the log of template DNA (cDNA) concentration for each of the primer sets to show primer efficiency. No significant change in priming efficiency was observed over a five-fold change in template DNA concentration

Author contributions

L.C.R. designed experiments, collected and analysed data and drafted the manuscript; R.F. and P.L. contributed to real-time PCR study design and experiments; R.K., D.E. R. and H.U. conceived of the study, contributed to experimental design and data interpretation and critically revised the manuscript. All authors read and approved the final manuscript.

References

Amit M, Carpenter M, Inokuma M *et al.* 2000; Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* **227**: 271–278.

Bianchi G, Banfi A, Mastrogiacomo M *et al.* 2003; *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* **287**: 98–105.

Brons IGM, Smithers LE, Trotter M *et al.* 2007; Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nat Lett* **448**: 191–195.

Buttery LDK, Bourne S, Xynos JD *et al.* 2001; Differentiation of osteoblasts and *in vitro* bone formation from murine embryonic stem cells. *Tissue Eng* **7**: 89–99.

Clements BA, Hsu C, Kucharski C *et al.* 2009; Non-viral delivery of basic fibroblast growth factor gene to bone marrow stromal cells. *Clin Orthop Relat Res* **467**: 3129–3137.

Duplomb L, Dagouassat M, Jourdon P *et al.* 2007; Differentiation of osteoblasts from mouse embryonic stem cells without generation of embryoid body. *In Vitro Cell Dev Biol Anim* **43**: 21–24.

Dunstan CR, Boyce R, Boyce BF *et al.* 1999; Systemic administration of acidic fibroblast growth factor (FGF-1) prevents bone loss and increases new bone formation in ovariectomized rats. *J Bone Min Res* **14**: 953–959.

Evans M, Kaufman M. 1981; Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**: 154–156.

Furuya M, Yasuchika K, Mizutani K *et al.* 2003; Electroporation of *Cynomolgus* monkey embryonic stem cells. *Genesis* **3**: 180–187.

Honda A, Hirose M, Ogura A. 2003; Basic FGF and Activin/Nodal but not LIF signalling sustain undifferentiated status of rabbit embryonic stem cells. *Exp Cell Res* **315**: 2033–2042.

Humphrey R, Beattie G, Lopez A *et al.* 2004; Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* **22**: 522–530.

Jukes JM, van Blitterswijk CA, de Boer J. 2010; Skeletal tissue engineering using embryonic stem cells. *J Tissue Eng Regen Med* **4**: 165–180.

Jung S, Sen A, Rosenberg L *et al.* 2010; Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells. *Cytotherapy* **12**: 637–657.

Lecanda F, Avioli LV, Cheng SL. 1997; Regulation of bone matrix protein expression and induction of differentiation of human osteoblasts and human bone marrow stromal cells by bone morphogenetic protein-2. *J Cell Biochem* **67**: 386–396.

Levenstein M, Ludwig T, Xu RH *et al.* 2006; Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* **24**: 568–574.

Maegawa N, Kawamura K, Hirose M *et al.* 2007; Enhancement of ostoblastic differentiation of mesenchymal stromal cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and

fibroblast growth factor-2 (FGF-2). *J Tissue Eng Regen Med* **1**: 306–313.
Martin I, Muraglia A, Campanile G *et al.* 1997; Fibroblast growth factor-2 supports *ex vivo* expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology* **138**: 4456–4462.
Martins-Taylor K, Xu RH. 2010; Determinants of pluripotency: from avian, rodents, to primates. *Cell Biochem* **109**: 16–25.
Meng GL, Zur Nieden N, Liu SY *et al.* 2008; Properties of murine embryonic stem cells maintained on human foreskin fibroblasts without LIF. *Mol Reprod Dev* **75**: 614–622.
Nagai H, Tsukuda R, Mayahara H. 1995; Effects of basic fibroblast growth factor (bFGF) on bone formation in growing rats. *Bone* **16**: 367–373.
Ng KW, Speicher T, Dombrowski C *et al.* 2007; Osteogenic differentiation of murine embryonic stem cells is mediated by fibroblast growth factor receptors. *Stem Cells Dev* **16**: 305–318.
Ohba S, Nakajima K, Komiyama Y *et al.* 2007; A novel osteogenic helioxanthin derivative acts in a BMP-dependent manner. *Biochem Biophys Res Commun* **357**: 854–860.
Roach HI. 1992; Induction of normal and dystrophic mineralization by glycerophosphates in long-term bone organ culture. *Calcif Tissue Int* **50**: 553–563.
Smith A, Heath J, Donaldson D *et al.* 1988; Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**: 688–690.
Thomson JA, Itskovits-Eldor J, Shapiro S *et al.* 1998; Embryonic stem cell lines derived from human blastocysts. *Science* **282**: 1145–1147.
Toumadjie A, Kusumoto KI, Parton A *et al.* 2003; Pluripotent differentiation *in vitro* of murine ES-D3 embyronic stem cells. *In Vitro Cell Dev Biol Anim* **39**: 449–453.
Varkey M, Kucharski C, Haque T *et al.* 2006; *In vitro* osteogenic response of rat bone marrow stromal cells to bFGF and BMP-2 treatments. *Clin Orthop Relat Res* **442**: 113–123.
Wang L, Huang Y, Pan K *et al.* 2010; Osteogenic responses to different concentrations/ratios of BMP-2 and bFGF in bone formation. *Ann Biomed Eng* **38**: 77–87.
Williams RL, Hilton D, Pease S *et al.* 1988; Myeloid leukaemia inhibitory factor maintains the development potential of embryonic stem cells. *Nature* **336**: 684–687.
Xu C, Rosler E, Jiang J *et al.* 2005a; Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells* **23**: 315–323.
Xu RH Peck RM, Li DS *et al.* 2005b; Basic FGF and suppression of BMP signalling sustain undifferentiated proliferation of human ES cells. *Nat Methods* **2**: 185–190.
Zhu H, Guo ZK, Jiang XX *et al.* 2010; A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. *Nat Protoc* **5**: 550–560.
Zur Nieden N, Kempka G, Ahr HJ. 2003; *In vitro* differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation* **71**: 18–27.

DOI: 10.1002/term