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High levels of ephrinB2 over-expression increases the osteogenic differentiation of human mesenchymal stem cells and promotes enhanced cell mediated mineralisation in a polyethyleneimine-ephrinB2 gene-activated matrix

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Abstract

Gene therapy can be combined with tissue engineering constructs to produce gene-activated matrices (GAMs) with enhanced capacity for repair. Polyethyleneimine (PEI), a non-viral vector, has previously been optimised for high efficiency gene transfer in rat mesenchymal stem cells (rMSCs). The use of PEI to transfect human MSCs (hMSCs) with ephrinB2 is assessed here. Recently a role for the ephrinB2 ligand and EphB4 receptor duo has been proposed in bone remodelling. Herein, over-expression of the ephrinB2 ligand resulted in increased osteogenic differentiation in hMSCs. As ephrinB2 is a cell surface anchored ligand which only interacts with cells expressing the cognate EphB4 receptor through direct contact, we have shown that direct cell-cell contact between two neighbouring cells is responsible for enhanced osteogenesis. In an effort to begin to elucidate the molecular mechanisms at play downstream of ephrinB2 over-expression, RT-PCR was performed on the GAMs which revealed no significant changes in *runx2* or *BMP2* expression but an upregulation of *osterix* (*Osx*) and *Dlx5* expression prompting the belief that the mode of osteogenesis is independent of the *BMP2* pathway. This select interaction, coupled with the transient gene expression profile of PEI, makes the PEI-ephrinB2 GAM an ideal candidate matrix for a bone targeted GAM.

Introduction

In the field of tissue engineering, a triad of a biomaterial scaffold, an appropriate cell source and cell cues such as mechanical signals or growth factors, are the foundation for the development of novel therapeutics. Recently, there has been a surge in research to produce gene-activated matrices (GAMs) which involves combining gene therapy with scaffold-based templates to enhance the capacity for repair. GAMs offer several key advantages in the derivation of therapeutics for bone repair in that sustained, controllable, localised release and expression of the transgene of interest can be achieved in these systems minimising unwarranted offsite side effects [1-3]. Unlike many other applications of gene therapy, a transient expression profile is ideal in the design of GAMs for tissue regeneration applications as expression is only desirable for a timeframe relative to tissue healing. Non-viral vectors can provide this profile whilst also offering some other key advantages such as decreased immunogenicity, cytotoxicity and safety concerns. Polyethyleneimine (PEI) is a

non-viral vector for gene transfer [4] which has recently shown promise as a transfection tool in rat mesenchymal stem cells (rMSCs) both in monolayer culture and in GAMs [5]. PEI transfection has been reported in human bone marrow derived MSCs and human adipose derived MSCs but with low transfection efficiencies of approximately 4 to 19 % [6-8]. Several groups have reported the success of GAMs for bone regeneration which incorporate genes encoding growth factors such as bone morphogenetic protein 2 (BMP2) [9] or vascular endothelial growth factor (VEGF) [10]. Herein, the use of the ephrinB2 ligand in a PEI-ephrinB2 GAM is proposed as an ideal candidate gene for a highly specific bone-targeted construct.

Ephrin ligands and their cognate receptors are the largest subset of receptor tyrosine kinases. A ligand and receptor duo within this family, ephrinB2 and EphB4, have recently been identified as having an important functional role in bone turnover and remodelling [11, 12]. Bidirectional signalling between an ephrinB2-expressing osteoclast and an EphB4-expressing osteoblast results in enhanced osteoblast differentiation and decreased osteoclastogenesis and when this mechanism is obstructed it leads to a reduction in osteoblast activity and an increase in osteoclast differentiation [11-13]. There is also evidence supporting a role aside from bone remodelling. Preliminary findings by Takyar et al. [14] have shown that ephrinB2 is required for the complete differentiation of osteoclasts and osteoblasts *in vivo*. Xing et al. [15] have also shown that ephrinB2 is upregulated following mechanical loading to the tibia in a murine model. Furthermore, Zhao et al. [11] showed that the over-expression of ephrinB2 in a murine osteoblast cell line causes an increase in osteogenesis *in vitro*.

The ephrinB2 ligand and the EphB4 receptor have previously been shown to be expressed on the surface of MSCs [16]. MSCs are non-haemotopoietic stem cells capable of the differentiation and therefore the regeneration of tissues of mesenchymal origin such as bone [17, 18]. This quality, coupled with relative ease of harvest, isolation, immunomodulatory properties and their ability to home to a site of injury [19] has assured their prominent role in the development and assessment of constructs in regenerative medicine. Various growth factors and molecular signals can prompt MSCs to differentiate down an osteoblastic lineage with the growth factor BMP2 having the most profound effects on cell differentiation and bone formation [20]. However, the transition from initial BMP2 assessment in animal models to human treatments has not proved smooth with differential responses between species reported [21]. Moreover, the high doses often necessary to avoid diffusion and the short-half life of the protein with BMP2 usage can result in soft tissue inflammation, ectopic bone formation and potential carcinogenic implications [22-24]. In bidirectional ephrin signalling, forward signalling occurs in the receptor-expressing cells and reverse signalling occurs in the ligand-expressing cell [25] and both ligand and receptor are cell surface anchored [26]. Therefore it is believed that signalling is dependent upon an *in trans* interaction occurring between adjacent cells which requires intimate cell contact [27]. This is a very significant advantage in the development of a GAM for bone repair. Growth factors such as BMP2 are released from the cell into the surrounding vicinity potentially causing detrimental off-site effects. As ephrinB2 mediated signalling occurs on the surface of the transfected cell, this gives rise to a very select GAM where only EphB4-expressing cells are targeted, leading to a local therapeutic effect.

The development of GAMs also requires a suitable scaffold platform to act as the template for tissue repair. Previous research from our laboratory has documented the potential of collagen-nanohydroxyapatite (nHa) scaffolds [28] to serve as a basis for GAMs whereby the collagen-nHa component was incorporated to act as an osteoinductive trigger and also facilitated enhanced transgene expression of cells seeded on the GAMs [5, 29]. The development of a PEI collagen-nHa GAM which was tailored for optimised rat mesenchymal

stem cell transfection was recently reported using reporter genes [5]. The ability of this GAM to transfect human bone marrow derived MSCs and the substitution of therapeutic genes which promote bone repair in place of the reporter genes remains to be investigated. As we begin to better understand ephrin/Eph interactions it is also becoming plausible to configure bioengineering applications involving the ephrinB2 ligand.

Therefore, the chief goal of this study was to investigate the potential of a PEI-ephrinB2 GAM as a therapeutic for bone repair. The initial specific objectives were to optimise PEI for monolayer hMSC transfection and to subsequently examine the effects of ephrinB2 over-expression on their osteogenic capabilities. Following this, the potential of engineering a novel PEI-ephrinB2 GAM for bone repair was evaluated by seeding it with hMSCs and analysing the subsequent transfection and ensuing osteogenesis.

Material and Methods

Cell culture

hMSCs were derived from bone marrow aspirates obtained from human volunteers, with informed consent at the Regenerative Medicine Institute, the National University of Ireland, Galway. All procedures were performed with ethical consent from the Clinical Research Ethical Committee at University College Hospital, Galway. The hMSCs were isolated using standard protocols and stringent analysis of cell phenotype (tri-lineage differentiation and a full panel of cell surface markers), as published in Duffy et al. [30]. Cells were isolated from the iliac crest of healthy donors and expanded in culture by direct plating as previously described [31]. Cells were cultured in standard T-175 growth flasks in hMSC growth medium, i.e. low-glucose DMEM (Sigma-Aldrich, Ireland) supplemented with 10 % HyClone FBS (Fisher Scientific, Ireland), 1 % penicillin/streptomycin (Sigma-Aldrich, Ireland) and maintained at 37°C and 5 % CO₂ in a humidified incubator. Upon reaching 90 % confluency, cells were detached with 0.25 % trypsin-EDTA (Sigma-Aldrich, Ireland) and re-plated at 5.7×10^3 cells/cm³. Cells were used at passage 5 in all experiments within.

Nucleofection

Nucleofection is used within as a comparative method of gene transfer in contrast to PEI transfection. 5×10^5 cells were harvested and resuspended in 100 µl of pre-warmed nucleofection solution (Amaxa, Lonza Cologne AG, Germany). 2 µg of ephrinB2-DNA in TE buffer was added to the cell suspension. The sample was transferred to an Amaxa cuvette (Amaxa, Lonza Cologne AG, Germany) and placed into the holder of the Nucleofector (Amaxa, Lonza Cologne AG, Germany) and subjected to the high transfection efficiency hMSC-specific program. The cuvette was removed immediately and 500 µl of pre-warmed growth medium was added to the cell suspension. 300 µl of the resulting solution was added to each of 2 wells of an adherent 6 well plate and the cells were then cultured as normal before harvesting for RT-PCR analysis.

Optimisation of PEI as a non-viral vector in monolayer human MSCs

Branched 25 kDa PEI (Sigma-Aldrich, Ireland) was condensed with Green Fluorescent Protein (GFP) plasmid DNA, (Amaxa, Lonza Cologne AG, Germany) to form PEI-DNA polyplexes. Briefly, cells were cultured until 90 % confluent then seeded at a density of 5×10^4 cells per well in adherent 6 well plates 24 h prior to transfection. The medium was removed 1 h before transfection and a 1.5 ml phosphate buffered saline (PBS; Sigma-Aldrich, Ireland) rinse was performed. 1.5 ml of OptiMEM serum-free medium (Gibco, Ireland) was then added to each well and incubated at 37°C for 1 h. PEI-GFP polyplexes were produced in OptiMEM at varying N/P ratios (1, 5, 7, 10) and doses (1 µg, 2 µg, 4 µg) of DNA and incubated at room temperature for 30 min. (The N/P ratio can be

defined as moles of amines in PEI(N)/moles of phosphate in DNA(P) and the term dose refers to the quantity of DNA contained within the polyplex. Furthermore, polyplexes of increasing DNA dose therefore contain increased levels of PEI to preserve a consistent N/P ratio.) The OptiMEM was removed from the wells and the polyplex solutions were added to the monolayer in 500 µl suspensions and incubated at 37°C for 15 min. After 15 min a further 1 ml of OptiMEM was added and the cells were incubated for approximately 4 h. Following this transfection period the medium was removed and a PBS rinse was performed. Complete culture medium was then added and the cells were incubated at 37°C to allow expression of the transgene. To demonstrate transfection efficiency, flow cytometry analysis was performed 72 h post transfection using the FACS Canto 11 DIVA software. Transfection efficiency (%) was calculated by expressing GFP fluorescing cells versus non-fluorescing cells as a percentage as described previously [5]. An evaluation of cell viability 24 h and 72 h post transfection using a range of polyplex N/P ratios and DNA doses was performed with an MTT Cell Growth Assay (Millipore™, Ireland) according to the manufacturer's instructions. Cell viability (%) was estimated according to the following equation: $\text{abs}_{[\text{transfected}]} / \text{abs}_{[\text{control}]} \times 100$. All experiments were carried out in triplicate and non-transfected cells served as a 100 % viability control. PEI polyplexes were made in a similar fashion using an ephrinB2 plasmid and a luciferase plasmid (pGaussia-luciferase, New England Biolabs, Massachusetts, USA) in subsequent experiments. The ephrinB2 plasmid was cloned into a mammalian expression vector as previously described [32]. All plasmids were propagated using a plasmid purification kit (Qiagen, Ireland).

Osteogenesis assay

Cells were plated at a density of 2.5×10^4 cells per well in a 12 well plate and cultured for 24 h prior to the addition of osteogenic medium. In the case of transfected cells, normal medium was removed and they were cultured in osteogenic medium 24 h post transfection. Osteogenic medium comprised of low glucose DMEM supplemented with 10 % FBS, 1 % penicillin/streptomycin, 10 mM β-glycerophosphate (Sigma-Aldrich, Ireland), 50 µM ascorbic acid 2-phosphate (Sigma-Aldrich, Ireland) and 100 nM dexamethasone (Sigma-Aldrich, Ireland). The medium was replaced every 3 days for the duration of the assay and the plates were assayed for calcium deposition using a Stanbio calcium assay (Calcium CPC Liquicolour, Stanbio Inc., USA) 14 days post transfection. Briefly, the medium was removed and cells were rinsed with 1 ml of PBS. 500 µl of 0.5 M HCl (Sigma-Aldrich, Ireland) was added to each well and the contents were scraped into eppendorfs and left to shake overnight at 4°C. Calcium levels were then quantified using the assay according to manufacturer's instructions. In the case of the osteogenic differentiation analysis of GAMs, hMSCs were seeded onto PEI-ephrinB2 GAMs and after 24 h the GAMs were transferred to new wells containing 2 ml of osteogenic medium. On the day of harvest, the GAM was removed and placed in 1 ml of 0.5 M HCl, homogenised using a hand held rotor-stator homogeniser (Omni Int., Germany) and left shaking overnight at 4°C. Thereafter, the Stanbio calcium assay was carried out as normal.

EphrinB2/Fc and EphB4/Fc-chimera protein binding assay

The presence of the ephrinB2 ligand and the EphB4 receptor on the surface of hMSCs was demonstrated by assessing the binding of receptor and ligand chimera proteins respectively. Cells were starved for 2 h in serum free medium and treated with 2 µg/ml IgG/Fc, ephrinB2/Fc or EphB4/Fc chimeras (R&D Systems, UK) as described previously [33]. Fc-chimera proteins were pre-clustered with goat anti-human IgG/Fc antibody (Jackson Laboratories, USA) for 30 min at 37°C (0.2 µg anti-Fc per µg of Fc-chimera protein). Cells were detached from the flask using a cell dissociation agent (MP Bio) washed in D-PBS (-)/5

% FBS twice and incubated with 2 µg/ml Fc-chimera proteins (ephrinB2, EphB4 or CD6/Fc) for 30 min on ice. After a further two washes cells were incubated with FITC-conjugated goat anti-human IgG1 antibody for 30 min at room temperature. After a further two washes stained cells were analysed using the GUAVA EasyCyte or the BD LSR. Histograms of cell number versus fluorescence intensity were recorded for 10,000 cells per sample and analysed using FCS Express 2 (DeNovo Software).

Phospho-Akt flow cytometry

It was suspected that stimulation of hMSCs with chimera proteins would result in Akt phosphorylation. hMSCs were grown to 60 % confluency and then serum starved for 2 h in OptiMEM serum-free medium (Gibco, Ireland) prior to stimulation with Fc-chimera proteins. Cells were treated with 2 µg/ml of pre-clustered ephrinB2/Fc, EphB4/Fc or IgG/Fc alone for 30 min. Normal medium containing 10 % serum acted as a positive control. Cells were trypsinised and fixed immediately in BD PhosFlow Perm Buffer II (BD Biosciences, Ireland) to maintain phosphorylation state, and incubated at 37°C for 10 min to insure permeabilisation. Cells were pelleted and washed in BD PhosFlow Perm/Wash Buffer I (BD Biosciences, Ireland). Cells were then resuspended in wash buffer and stained with rabbit anti-phospho Akt Alexa Fluor 647 (BD Biosciences, Ireland) at a 1:100 dilution and incubated for 1 h at room temperature. Histograms of cell number versus APC fluorescence intensity were recorded for 10,000 cells per sample at each time point with the BD FACS Canto and analysed using FCS Express 2 (DeNovo Software). Unstained cells and cells in serum free medium acted as negative controls.

Blocking peptide assay

To ascertain whether ephrinB2-EphB4 signalling was responsible for osteogenesis in PEI-ephrinB2 hMSCs, a blocking peptide assay was performed. Briefly, transfections were carried out as normal and osteogenic medium containing blocking or control peptides (JPT Peptide Technologies, Germany) [12, 16] was added to the cells 24 h post transfection. The blocking peptide (TNYLFSPNGPIARAW) blocks the ephrinB2 binding site of the EphB4 receptor whereas the control peptide (RTVAHHGGLYHTNAEVK) has no effect on the cells. Cells remained in culture for 14 days where the peptide-supplemented medium was changed twice weekly.

Scaffold fabrication

Highly porous homogeneous collagen-nHa scaffolds were fabricated by a lyophilisation technique using a procedure developed by O'Brien et al. [34]. Collagen slurries were made by adding 3.6 g of collagen (Integra Life Sciences, USA) to 720 ml of 0.05 M glacial acetic acid (HOAc) and blending at 15,000 rpm for 90 min using an overhead blender maintained at 4°C. (Ultra Turrax T18 Overhead Blender, IKA Works Inc., USA). nHa was synthesised using a novel dispersant-aided precipitation technique [35] which was incorporated into collagen slurries using a suspension method [28]. Scaffolds were cut to 9.5 mm diameter using circular biopsy punches post-lyophilisation. All scaffolds were rehydrated in PBS, dehydrothermally crosslinked and chemically crosslinked with a mixture of 14 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC; Sigma-Aldrich, Ireland) and 5.5 mM N-Hydroxysuccinimide (NHS; Sigma-Aldrich, Ireland) [36] to both sterilise and structurally reinforce the scaffold.

Gene-activated matrix production

GAMs were fabricated as formerly described [5]. PEI-DNA (ephrinB2/GFP/luc) polyplexes were prepared at a ratio of N/P 10 as detailed above using 2 µg of DNA in 50 µl

solutions. (A range of DNA doses from 2 to 20 μg was produced for PEI-luc GAMs.) 25 μl of the polyplex solution was added per side of the scaffold by direct loading then incubated for 15 min at room temperature after loading. Subsequently, 2.5×10^5 hMSCs were seeded per side of the scaffold in a 25 μl suspension with 15 min incubation between seeding each side. 2 ml of OptiMEM was then added and the plates were incubated at 37°C for 4 h after which the medium was removed and replaced with 2 ml of normal hMSC medium. 24 h post transfection the GAM was transferred to a new well and cultured in 2 ml of normal/osteogenic medium which was changed twice weekly for the duration of the experiment. PEI-GFP GAMs were imaged at intervals using a Leica DMIL microscope (Leica Microsystems, Switzerland). A LumiFlex GLuc assay (New England Biolabs, Isis Ltd, Ireland) was used to quantify the expression of luciferase from hMSCs seeded on the GAMs as described previously [5] according to manufacturer's instructions.

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was harvested from monolayer cells by adding 300 μl of Qiazol reagent (Qiagen, Ireland) per well and the RNA was extracted using the RNeasy Kit (Qiagen, Ireland). RNA quality and quantity was determined using a Nanodrop (Applied Biosystems, Ireland) with the absorbance set at 260 nm. A DNase clean up step was performed by incubating the RNA in 7 μl of DNase (Qiagen, Ireland) for 15 min to eliminate any potential genomic/plasmid DNA present in the RNA prior to PCR. Quantitative RT-PCR was then implemented with a one-step SYBR Green RT-PCR kit (Qiagen, Ireland) using 15 ng of RNA on a 7500 Real Time polymerase chain reaction system (Applied Biosystems, Ireland) utilising the QuantiTect primer for ephrinB2 (Hs_EFNB2_1_SG), and the 18S primer (Hs_RRN18S_1_SG) as a housekeeping gene. A one-step kit was used due to low RNA yields in this instance. In the case of scaffolds/GAMs, they were placed in 1 ml of Qiazol reagent (Qiagen, Ireland) and homogenised using a hand held rotor-stator homogeniser (Omni Int., Germany). 200 μl of chloroform was added to separate the solution into protein, DNA and RNA phases. RNA was extracted from the aqueous RNA phase using the RNeasy kit and a DNase step was also completed as outlined above. Genomic DNA was removed by heating the RNA to 42°C for 2 min using a genomic DNA wipeout buffer (Qiagen, Ireland) before transcribing the RNA to cDNA using a reverse transcriptase enzyme (Qiagen, Ireland) according to manufacturer's instructions. RT-PCR was then performed on cDNA using the following primers; Hs_EFNB2_1_SG, Hs_EPHB4_1_SG, Hs_DLX5_1_SG, Hs_SP7_1_SG, Hs_BMP2_1_SG, Hs_RUNX2_1_SG for ephrinB2, EphB4, Dlx5, Osx, BMP2 and runx2 respectively. Relative expression was then calculated using the $\Delta\Delta\text{CT}$ method [37] using averages of 3-4 samples for each group utilising 18S as a housekeeping gene.

Statistics

Results are expressed as mean \pm standard deviation and each experiment was carried out in triplicate. A One-way ANOVA followed by a Holm-Sidak post-hoc pairwise multiple comparison test was carried out on all data sets where $p < 0.05$ was considered to indicate statistical significance. A Two-way ANOVA was conducted in Fig. 1 and Fig. 5, again followed by a Holm-Sidak post-hoc pairwise multiple comparison test.

Results

Optimisation of PEI for hMSC transfection

To optimise the non-viral vector PEI for hMSC transfection, a range of polyplexes from N/P 1 to N/P 10, containing 2 μg of GFP plasmid DNA, were made and applied to hMSCs in monolayer culture. Flow cytometry conducted 72 h post transfection revealed N/P 7 and N/P 10 polyplexes to possess the highest % transfection efficiency at approximately 25

% and were statistically significant to all other groups ($p < 0.05$; **Fig. 1A**). An MTT assay was subsequently carried out in order to determine whether these polyplexes hampered hMSC viability (**Fig. 1B**) which showed that N/P 10 polyplexes had a detrimental effect on the hMSCs relative to all other groups with only 28.5 ± 5.6 % remaining viable 72 h post transfection ($p < 0.05$; **Fig. 1B**). The effect of increasing polyplex dose was also investigated with the N/P ratio remaining fixed at N/P 7 and using DNA doses of 1 μ g, 2 μ g and 4 μ g (**Fig. 1C**). Transfection using polyplexes containing 4 μ g of DNA had a profound effect on cell viability and resulted in only 17.3 ± 3.9 % of cells remaining metabolically active after 72 h which was statistically significant to all other groups ($p < 0.05$). In summary, aside from 2 μ g N/P 10 polyplexes and 4 μ g N/P 7 polyplexes, all other polyplexes analysed did not affect cell viability severely. The high transfection efficiency achieved using 2 μ g N/P 7 polyplexes without compromising cell viability indicates that these polyplexes possess the optimal N/P ratio and dose for transfecting hMSCs in monolayer culture to maximise transfection efficiency whilst minimising cytotoxicity.

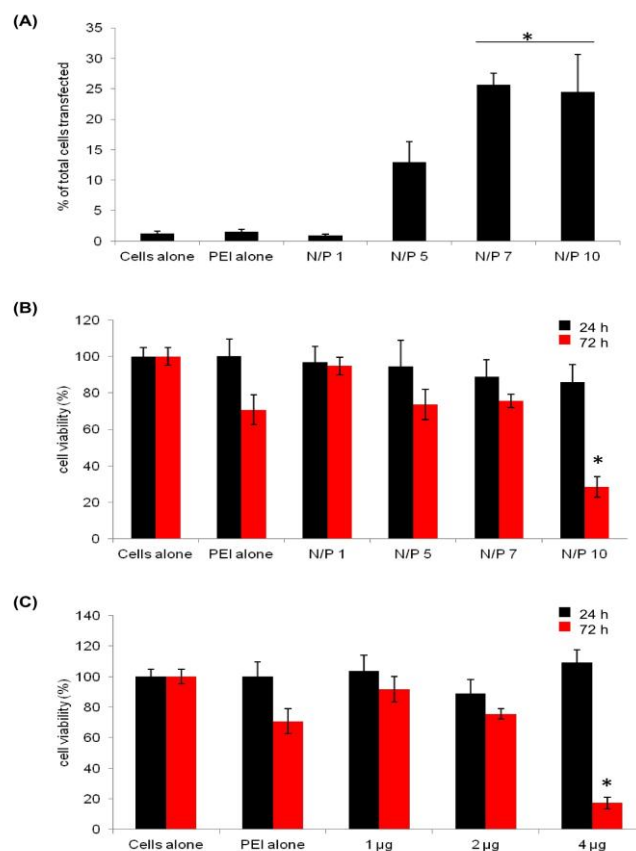


Fig. 1 PEI transfection and cell viability in human MSCs. Flow cytometry was performed on hMSCs 3 days post transfection with PEI-GFP polyplexes to determine transfection efficiency (A). The viability of hMSCs transfected with a range of 2 μ g N/P ratios (B) and various N/P 7 polyplex doses (C) was also assessed. * indicates statistical significance relative to all other groups ($p < 0.05$).

EphrinB2 over-expression results in enhanced osteogenesis of hMSCs

Relative gene expression analysis using RT-PCR confirmed both successful nucleofection (**Fig. 2A**) and PEI transfection (**Fig. 2B**) of ephrinB2 in the hMSCs. Nucleofection of hMSCs with ephrinB2 resulted in an 85-fold increase in ephrinB2 expression after 48 h ($p < 0.05$; **Fig. 2A**) in comparison to a 1241-fold increase with PEI transfection ($p < 0.05$) (**Fig. 2B**). hMSCs cultured in osteogenic medium had increased calcium deposition after 14 days in culture and hMSCs transfected with PEI-ephrinB2 polyplexes exhibited a further increase in calcium deposition ($p < 0.05$; **Fig. 2C**).

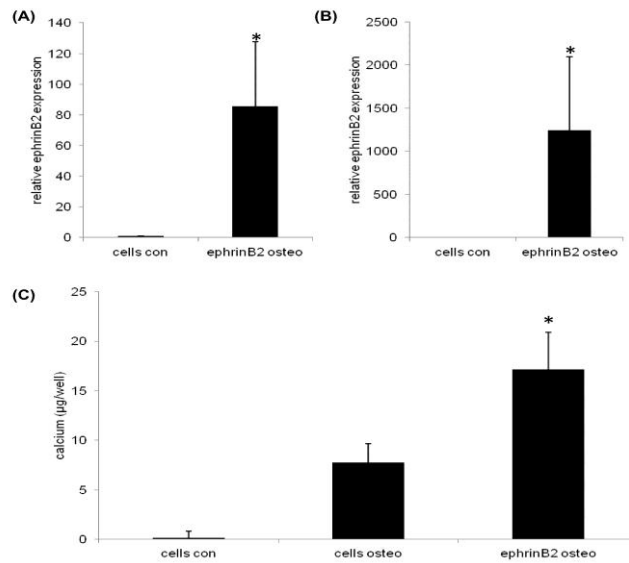


Fig. 2 EphrinB2 over-expression in hMSCs and the ensuing effect on osteogenesis. hMSCs were nucleofected (A) or transfected using PEI (B) with the ephrinB2 plasmid whereby transfection was measured by RT-PCR 48 h later. ‘Cells con’, ‘cell osteo’ and ‘ephrinB2 osteo’ refers to cells cultured in growth media, cells in osteogenic media and ephrinB2-transfected cells in osteogenic media respectively. PEI-ephrinB2 hMSCs cultured in osteogenic medium demonstrated significantly more calcium deposition after 14 days in monolayer culture (C). * indicates statistical significance relative to all other groups ($p < 0.05$).

hMSCs innately express the EphB4 receptor which causes Akt phosphorylation upon stimulation

We sought to demonstrate the presence of ephrinB2 and EphB4 on the surface of MSCs by assessing the binding of EphB4/Fc and ephrinB2/Fc chimeras respectively. Cells were incubated with 2 µg/ml of EphB4/Fc or ephrinB2/Fc for 30 min and bound protein was analysed using flow cytometry. It was found that EphB4/Fc and ephrinB2/Fc bound to the surface of MSCs (**Fig 3A**) which indicated the co-expression of the ligand and the receptor on the cells. Exposure to ephrinB2/Fc and EphB4/Fc for 3 h and 30 min also resulted in a marked increase in Akt phosphorylation (**Fig. 3Bi and ii**) whereas IgG/Fc alone did not have the same stimulatory effect (**Fig. 3Bi**). After 3 h in culture a drop in Akt phosphorylation was seen in both Fc-chimera treatment groups whereas there was no drop in cultures in medium containing 10 % FBS (**Fig. 3Biii**). These results demonstrate that ephrinB2 and EphB4 are present on hMSCs and can be activated with stimulation increasing Akt phosphorylation which has known downstream effects on cell proliferation and migration [38].

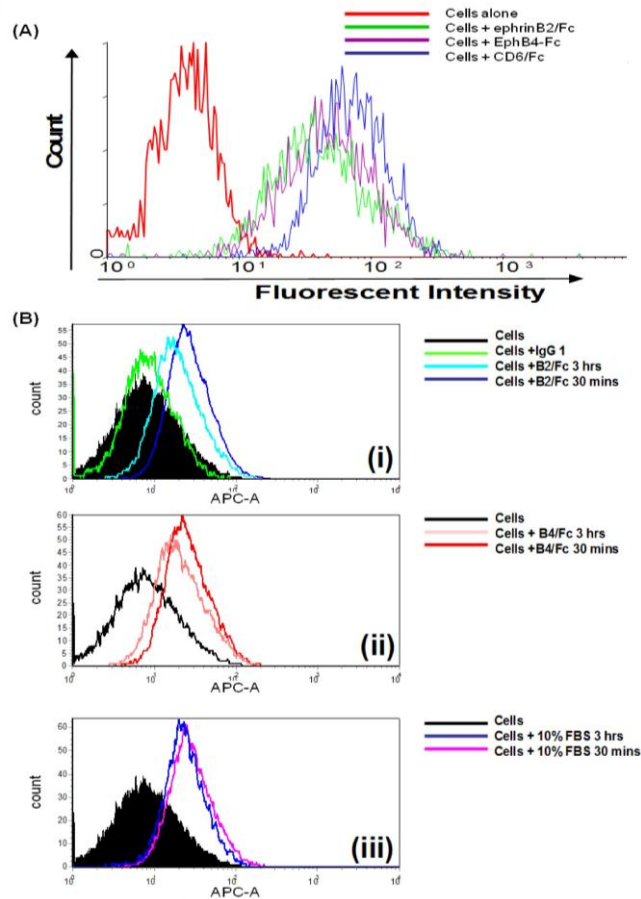


Fig. 3 hMSCs innately express the EphB4 receptor which causes Akt phosphorylation upon stimulation. Co-expression of both the EphB4 receptor and the ephrinB2 ligand was confirmed by EphB4/Fc and ephrinB2/Fc chimera proteins binding to the cell surface of the hMSCs using CD-6/Fc as a positive control (A). EphrinB2/Fc and EphB4/Fc exposure resulted in an increase in Akt phosphorylation (Bi and ii). IgG/Fc alone did not have the same stimulatory effect (i) and cultures in medium containing 10 % FBS (Biii) exhibited no decrease in Akt phosphorylation.

Signalling between ephrinB2 and EphB4 is crucial to osteogenesis

As ephrinB2-EphB4 interactions have been shown to have a prominent role in bone remodelling [11], we sought to determine whether the cognate EphB4 receptor was associated with the observed osteogenic effects of over-expressing ephrinB2. We examined that potential interaction by blocking the ephrinB2 binding site on EphB4 with a TNYLFSPNGPIARAW peptide (Fig. 4). hMSCs were transfected with PEI-ephrinB2 in monolayer culture in osteogenic medium containing the blocking peptide or a control peptide. Following 14 days of culture, PEI-ephrinB2 cells in normal osteogenic medium or medium containing the control peptide displayed significantly higher levels of calcium deposition than PEI-ephrinB2 cells cultured with the blocking peptide ($p < 0.05$). This denotes that the enhanced osteogenesis seen with ephrinB2 over-expression is induced by a signalling cascade initiated through interaction with the EphB4 receptor on neighbouring cells.

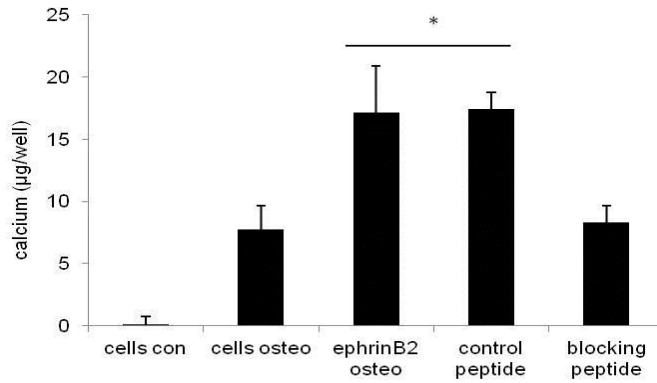


Fig. 4 Signalling between ephrinB2 and EphB4 is crucial to enhanced osteogenesis. 'cells con', 'cell osteo', 'ephrinB2 osteo', 'blocking peptide' and 'control peptide' refers to cells cultured in growth media, cells in osteogenic media and ephrinB2-transfected cells cultured in osteogenic media alone, or osteogenic media containing an EphB4 blocking peptide or a control peptide respectively. Mineralisation was significantly inhibited upon addition of the EphB4 blocking peptide to PEI-ephrinB2 hMSCs indicating that enhanced osteogenesis is associated with EphB4/ephrinB2 signalling. * indicates statistical significance relative to all other groups ($p < 0.05$).

The development of PEI-GAMs targeted for transient transfection of hMSCs

In order to investigate whether PEI-GAMs could successfully induce hMSC transfection as shown in monolayer culture, a series of GAMs were fabricated containing either PEI-GFP or PEI-luc polyplexes (**Fig. 5**). Initially N/P 7 polyplexes were used as optimised for monolayer transfection (**Fig. 1**) but the levels of transfection seen in the 3D GAM environment were not as high (data not shown) so it was decided to revert to N/P 10 polyplexes for use within the GAM as published previously with rat MSCs [5]. Fluorescent images of PEI-GFP GAMs (**Fig 5A-C**) produced using 2 µg N/P 10 polyplex doses and seeded with hMSCs proved the GAMs capable of retaining and transiently transfecting hMSCs over a 14 day period. In order to quantitatively measure this transfection and to ascertain the effects of increasing dose in the GAM, PEI-luc GAMs were fabricated using a range of N/P 10 polyplex doses and seeded with hMSCs. A luciferase assay carried out on medium samples from the matrices corroborates the transient expression profile in the GAM whilst also showing that 2 µg doses are preferential in this environment (**Fig 5D**). Luciferase expression from GAMs containing 2 µg PEI-luc polyplexes had statistically higher expression at day 3 relative to all other groups ($p < 0.05$).

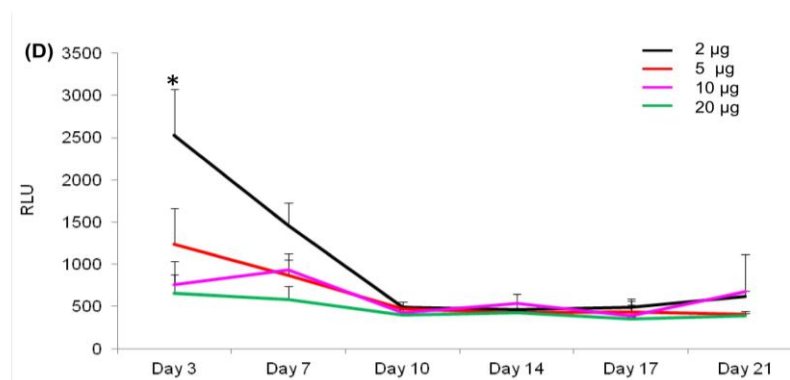
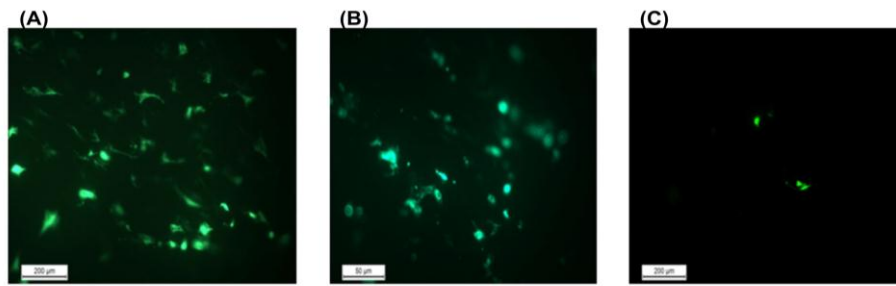


Fig. 5 GAM development. Images depicting hMSC transfection in 2 µg N/P 10 PEI-GFP GAMs at 3 (A), 7 (B) and 14 (C) days post cell seeding. Relative luciferase expression was determined by assaying medium samples from a range of N/P 10 PEI-luc GAMs with different polyplex doses over a 21 day culture period (D). * indicates statistical significance relative to all other groups ($p < 0.05$) at the day 3 timepoint.

The osteogenic assessment of a novel PEI-ephrinB2 GAM

PEI-ephrinB2 GAMs were synthesised and their transfection potential evaluated by seeding them with hMSCs and analysing their response. Firstly, RNA isolates from PEI-ephrinB2 GAMs were analysed for ephrinB2 transgene expression using RT-PCR after 7 and 14 days of culture (**Fig. 6A, B**) which verified high levels of transient ligand expression in hMSCs on the GAM over this timeframe. RT-PCR on RNA isolates from hMSCs seeded on PEI-ephrinB2 GAMs demonstrated an approximate 5000-fold increase in ephrinB2 relative RNA expression after 7 days ($p < 0.05$; **Fig. 6A**) which declined to approximately 800-fold increase after 14 days ($p < 0.05$; **Fig. 6B**). This decline in gene expression after 14 days supports the transient transfection profile of PEI as seen previously [5]. Next, relative expression of the EphB4 ligand was examined to determine whether over-expression of its cognate ligand triggered any change in the expression of the receptor (**Fig. 6C, D**). There was no significant fluctuation in expression after 7 days but there was significantly less EphB4 expression relative to the osteogenic control after 14 days ($p < 0.05$). Calcium deposition was measured in PEI-ephrinB2 GAMs after 14 (**Fig. 6E**) and 35 (**Fig. 6F**) days of culture. There was a statistically significant rise in calcium deposition relative to controls at the 14 day timepoint ($p < 0.05$; **Fig. 6E**). After 35 days in culture no significant difference in the ephrinB2 GAMs relative to the controls was seen (**Fig. 6F**) which could be attributed to a saturation effect in the matrix over an extended culture period.

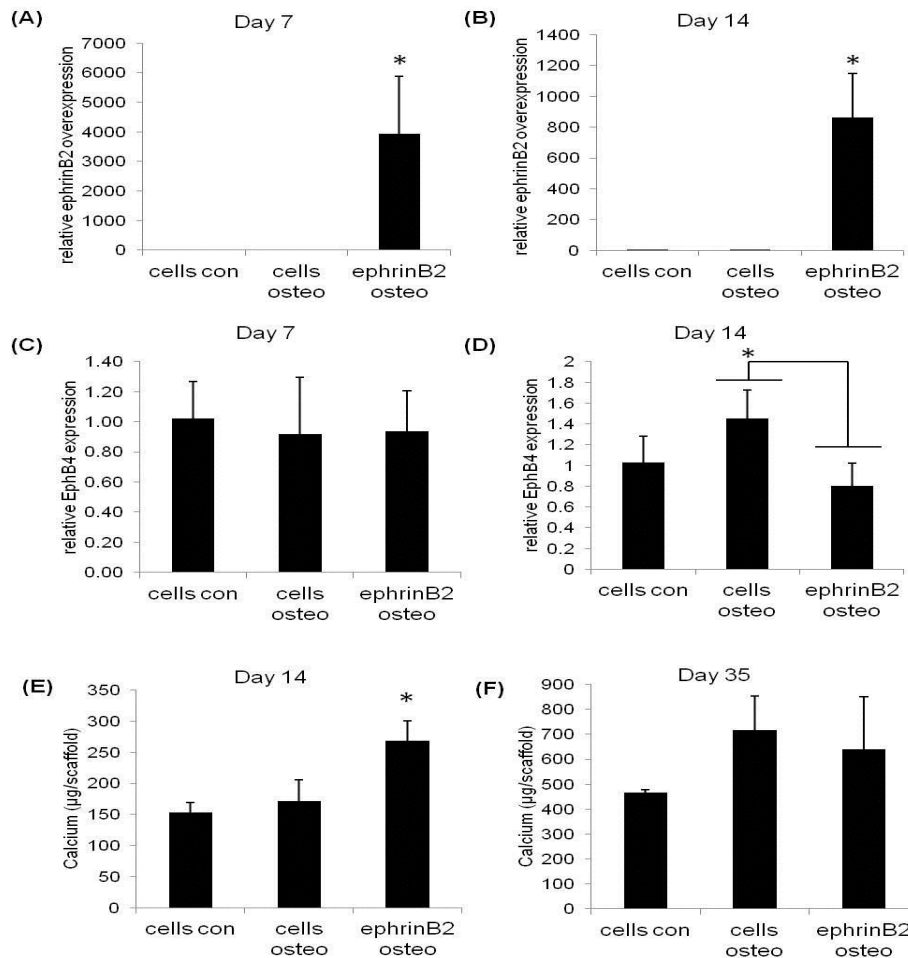


Fig. 6 High levels of transient ephrinB2 over-expression in a PEI-ephrinB2 GAM induces enhanced osteogenesis. High levels of ephrinB2 over-expression are seen in PEI-ephrinB2 GAMs seeded with hMSCs after 7 days (A) which has begun to subside after 14 days (B) in culture. ‘Cells con’, ‘cell osteo’ and ‘ephrinB2 osteo’ refers to cells cultured in growth media, cells in osteogenic media and ephrinB2-transfected cells in osteogenic media respectively. There are no fluctuations in EphB4 expression after 7 days in culture (C), however after 14 days there is a decline in EphB4 expression relative to the cells in osteogenic medium control (D). hMSCs transfected on a PEI-ephrinB2 GAM deposit significantly more calcium after 14 days of culture in comparison to both cells in control and osteogenic medium (E). However, this effect is not seen when culture periods are prolonged to 35 days (F) suggesting that enhanced osteogenesis is dependent on the level of ephrinB2 expression. * indicates statistical significance ($p < 0.05$).

EphrinB2 osteogenesis is independent of the BMP2 pathway

Gene expression analyses carried out on PEI-ephrinB2 GAMs seeded with hMSCs using RT-PCR suggests that the mode of osteogenesis is independent of the BMP2 pathway, the foremost pathway involved in osteogenesis [20]. There was no upregulation in relative RNA expression of either BMP2 or runx2 in PEI-ephrinB2 GAMs relative to controls at either 7 days or 14 days in culture suggesting that they are not involved in the signalling cascade leading to osteogenesis via ephrinB2 over-expression (Fig. 7A-D). BMP2 and runx2 expression was significantly upregulated in the osteogenic control relative to cells in standard medium at the day 7 timepoint ($p < 0.05$). EphrinB2 hMSCs even displayed a reduction in BMP2 expression in comparison to hMSCs in osteogenic medium at the day 14 timepoint. Interestingly, two key transcription factors critical for osteogenesis were significantly upregulated on PEI-ephrinB2 GAMs, namely Dlx5 and Osx ($p < 0.05$; Fig. 7E-H). Where cells cultured on coll-nHa scaffolds in control medium and osteogenic medium served as controls, Dlx5 expression was significantly enhanced on PEI-ephrinB2 GAMs at the day 7 timepoint ($p < 0.05$) but there was no significant difference in expression when compared to the osteogenic control after 14 days (Fig. 7E, F). However, levels of relative Osx expression were upregulated at both timepoints ($p < 0.05$; Fig 7G, H) which may suggest that Dlx5 is

upstream of *Osx* in this signalling system. In brief, these two transcription factors are implicated downstream of *ephrinB2* over-expression which is acting to increase mineralisation and the committal of the cells to an osteoblastic lineage.

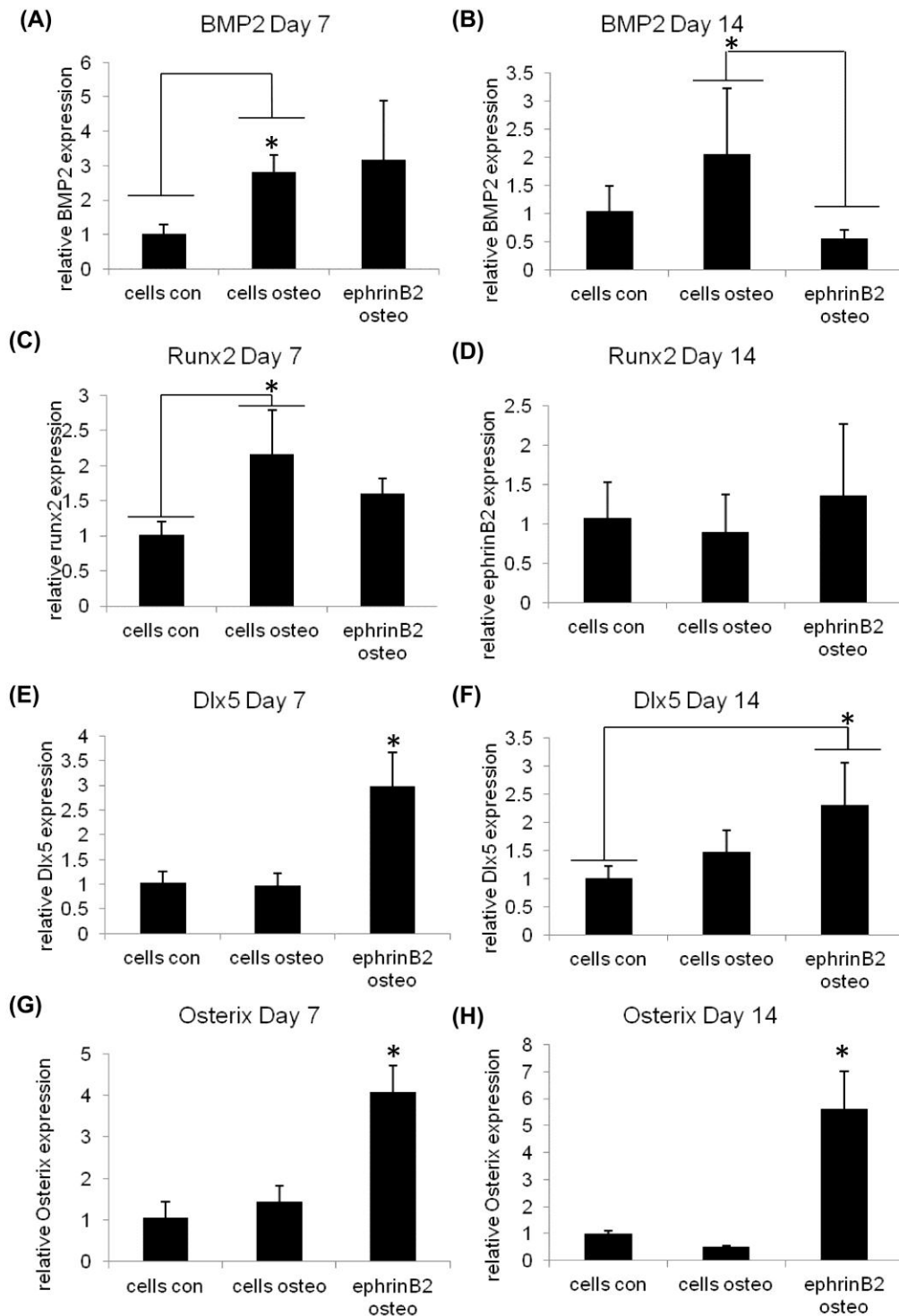


Fig. 7 EphrinB2 osteogenesis is independent of the BMP2 pathway. RNA isolates were analysed by qRT-PCR for BMP2 (A, B), *runx2* (C, D), *Dlx5* (E, F) and *Osx* (G, H) expression 7 and 14 days post addition of cells to the GAMs. 'Cells con', 'cell osteo' and 'ephrinB2 osteo' refers to cells cultured in growth media, cells in osteogenic media and ephrinB2-transfected cells in osteogenic media respectively. * indicates statistical significance ($p < 0.05$).

Discussion

The goal of this work was to develop an ephrinB2 gene-activated matrix (GAM) for application in bone tissue repair and regeneration. Specifically, this involved optimising PEI as a transient non-viral vector for (hMSC) MSC transfection to achieve transfection efficiencies higher than those previously reported [6-8] and combining PEI-ephrinB2 polyplexes with a collagen-nHa scaffold previously engineered for bone repair. The results within have demonstrated that high levels of ephrinB2 over-expression in hMSCs cultured in osteogenic medium are causal to enhanced osteogenesis. The development of a novel PEI-ephrinB2 GAM was achieved and upon seeding with hMSCs its potential to drive these cells to an osteoblastic lineage was proven. When hMSCs were cultured on this GAM, an increase in calcium deposition in the matrix was observed as early as 14 days in culture. Furthermore, gene expression analysis on the cell seeded GAMs also provided some insights on the molecular mechanisms underlying increased hMSC osteogenesis. No increases were seen in BMP2 or runx2 expression, prompting the belief that the mode of osteogenesis is independent of the BMP2 pathway. Interestingly, RT-PCR analysis also revealed upregulation of Dlx5 and incremental increases in Osx gene expression, two key transcription factors involved in osteogenesis which have been shown to be capable of acting separately to the BMP2 pathway. Collectively, these results classify ephrinB2 as a potent inducer of osteogenesis in hMSCs and may also signify a role for the ligand in the differentiation of progenitor MSCs to the osteoblast phenotype. Of most relevance, this cell surface ligand has been shown to be an ideal candidate gene for inclusion in a GAM specifically targeted for bone repair applications which was validated by the osteogenic differentiation of hMSCs seeded on a PEI-ephrinB2 GAM.

The results from this study demonstrate that ephrinB2 over-expression results in enhanced osteogenesis in hMSCs, an osteoprogenitor cell type of direct relevance to bone healing in humans. Arthur et al. [16] have proposed that the Eph/ephrins are involved in osteochondral differentiation of MSCs and ephrinB2 over-expression has been previously reported in murine osteoblasts [11] and in this study ephrinB2 over-expression has been shown to enhance osteogenesis in human MSCs. The implications of this are manifold. Firstly, the augmentation in differentiation capability when ephrinB2 is over-expressed would imply that ephrinB2 hMSCs could be a valuable enhancement to MSCs routinely explanted and isolated for stem cells therapies directed at bone repair. Secondly, as PEI is a transient non-viral vector in which the level of expression will gradually deteriorate to basal levels in MSCs in a manner of weeks [5], the ligand would not be expressed long term, avoiding unwanted side effects such as ectopic bone formation, cytotoxicity or safety concerns.

The binding of clustered ephrinB2/Fc and EphB4/Fc respectively to the hMSCs as verified by flow cytometry attests that both the ligand and the receptor are innately present. Exposure to both of these clustered molecules also stimulates the MSCs as shown by an increase in Akt phosphorylation demonstrating that each can be activated by their receptor/ligand cohorts. These data point to a role for Eph/ephrin signalling within the MSC population itself exclusive of interaction with other cell types as seen in bone homeostasis.

This Eph/ephrin signalling was further investigated by using a forward signalling blocking peptide which specifically binds to the ephrinB2 binding domain of the EphB4 receptor. When ephrinB2 hMSCs were cultured in osteogenic medium containing the peptide, the previously seen increases in osteogenesis did not occur indicating that the mode of action is completely reliant on EphB4/ephrinB2 signalling. Indeed, when the EphB4 receptor was blocked the levels of osteogenesis dropped to approximately the same level as cells in osteogenic medium. This also indicates that the EphB4 receptor may not be involved in the differentiation process attributed to culture in osteogenic induction medium. Diminished mineralisation has been previously seen when the EphB4 blocking peptide was added to

mouse K4b10 cells and MSCs in culture where ephrinB2 was not over-expressed [16, 39]. In bone remodelling and bone formation it is established that osteoblasts expressing ephrinB2 and osteoclasts expressing EphB4 are in concert to induce osteoblast differentiation through forward signalling (via EphB4) and to inhibit osteoclastogenesis through reverse signalling (via ephrinB2) [11, 40, 41]. Here, signalling between ephrinB2 and EphB4 is shown in a singular cell population, hMSCs, with a definitive effect on osteogenesis. As the same cell type can express both the receptor and the ligand [25, 41-43] it is proposed that ephrinB2/EphB4 *in trans* signalling [26, 44] is at play here between two adjacent cells.

EphrinB2 over-expression does not stimulate over-expression of the EphB4 receptor. Perhaps the over-expression of the ligand or the receptor causes increased Eph/ephrin interactions with proximal cells simply due to the sheer volume of the ligand present. The extremely high levels of ephrinB2 over-expression induced are suspected to be the key contributor to the osteogenic effects seen. Previously reported findings showed transfection efficiencies of approximately 46 % with nucleofection [32] and herein we show a transfection efficiency of approximately 26 % in hMSCs with PEI. However, when these varying modes of non-viral transfection were analysed for ephrinB2 transgene expression by RT-PCR, an 85.6-fold increase was seen in ephrinB2 nucleofected cells whereas a 1241.4-fold increase was seen in PEI transfected hMSCs. This may be interpreted as, although the transfection efficiencies are lower, multiple copies of the ligand are produced and ubiquitously expressed on the surface of the PEI transfected cells which increases the likelihood of an interaction with the EphB4 receptor. A prior paper published by this group has reported that ephrinB2 over-expression by nucleofection acts as an angiogenic stimulus in hMSCs whereby decreased osteogenesis was also observed [32]. Based on the data in this study and our previous work we now hypothesise that the level of ligand over-expression dictates the fate of hMSCs. Further support for this theory was recently demonstrated whereby variability in receptor levels and signalling has been shown to result in opposing effects in cancer cells. High levels of ephrin-dependent EphA2 and EphB4 forward signalling can suppress tumorigenesis whereas low levels can promote tumorigenicity [13].

Following the optimisation of PEI as a high efficiency, low cytotoxicity vector in hMSCs and confirmation of the pro-osteogenic effects of ephrinB2 conducted through the EphB4 ligand, this research has led to the development of a PEI-ephrinB2 GAM which can induce a terminal osteoblastic nature and high levels of mineral deposition in hMSCs within a 14 day timeframe. hMSCs seeded on the GAMs successfully attached and migrated throughout the GAM becoming transfected to transiently over-express the ephrinB2 transgene endowing increased potential for osteogenesis in a cell type and scaffold already accomplished for a role in bone repair. The osteogenic enhancements witnessed in monolayer culture were mirrored in the PEI-ephrinB2 GAM and with significantly elevated levels of osteogenesis seen within 14 days it provides great expectations for *in vivo* translation. Only a 2 µg polyplex dose is contained in the GAM which is significantly lower to doses used in previous GAMs [45] minimising potential cytotoxicity. This GAM offers a key advantage to growth factor-containing [46, 47] or growth factor-encoding [48, 49] matrices. The matrix acts as a depot for the polyplexes whereby only cell types which home to a site of injury such as MSCs are transfected and only cells which possess the EphB4 receptor are affected by transgene expression.

Ephrin bidirectional signalling is quite unique in that both the receptor and the ligand are capable of sending and transducing signals, and therefore it is not a straightforward system which adds to the complexity of the potential signalling cascades [43]. Hence, the upstream and downstream signals associated with this bidirectional signalling remain somewhat elusive [50]. The PEI-ephrinB2 GAM allowed us to investigate cell signalling downstream of ephrinB2 over-expression in an environment more akin to the *in vivo* niche.

Activating the BMP2 pathway has the most pronounced effect on osteogenesis and bone formation [20] so BMP2 and the most essential master transcription factor of the BMP2 pathway, runx2 [51], were investigated for involvement downstream of ephrinB2 over-expression. There were no elevations in the levels of BMP2 or runx2 expression in PEI-ephrinB2 GAMs at the message level at either day 7 or 14 which would insinuate that the molecules do not have a role in ephrinB2-induced osteogenesis. There was however, a significantly higher level of runx2 and BMP2 expression in the cells in the osteogenic medium relative to the cells in control medium at day 7 so the cells were responding accordingly to osteogenic induction medium. Dlx5 is another transcription factor involved in the terminal differentiation of osteoblasts which has been implied as an upstream regulator of Osx expression [51]. Similarly, the transcription factor Osx is a multifunctional player in the regulation of bone homeostasis and bone growth [52] and is required in the progenitor cell's final commitment to a pre-osteoblastic lineage [51, 53]. As both of these factors can act to increase osteogenesis independent of the BMP2 pathway, they were analysed as possible contenders acting downstream of ephrinB2 over-expression. Dlx5 was upregulated at the day 7 timepoint whereas Osx expression remained upregulated at both the 7 day and 14 day timepoint. Together these results would indicate that Dlx5 and Osx do have a role here downstream of ephrinB2/EphB4 activation and that perhaps Dlx5 is acting upstream of Osx expression. Likewise, the upregulation of both Dlx5 and Osx has previously been seen in a murine osteoblast cell line cultured in the presence of ephrinB2/Fc [11] which corroborates our findings. The addition of ephrinB2 chimera proteins being causal to this upregulation also supports the theory that forward signalling through the EphB4 receptor is behind osteogenic induction. Based on these results, a schematic of the believed mechanism of a BMP2-independent action is hypothesised in Fig. 8. To summarise, PEI-mediated over-expression of ephrinB2 induces an osteogenic effect in hMSCs in monolayer culture. This effect is stipulated to be BMP2 pathway independent as there were no fluctuations in the levels of runx2 or BMP2 observed. Levels of Dlx5 expression were upregulated at the 7 day timepoint and levels of Osx expression remained elevated at both 7 and 14 days. It has also been shown that ephrinB2-EphB4 signalling is intrinsic to the osteogenic effect as enhanced osteogenesis was quenched when the EphB4 receptor was blocked. Therefore, it is proposed that upon extremely elevated levels of ephrinB2 expression and subsequent binding to the EphB4 receptor an unknown signal is relayed to Dlx5 which acts on Osx to commit the cells to an osteoblastic lineage and increases calcium production in the cell (Fig. 8).

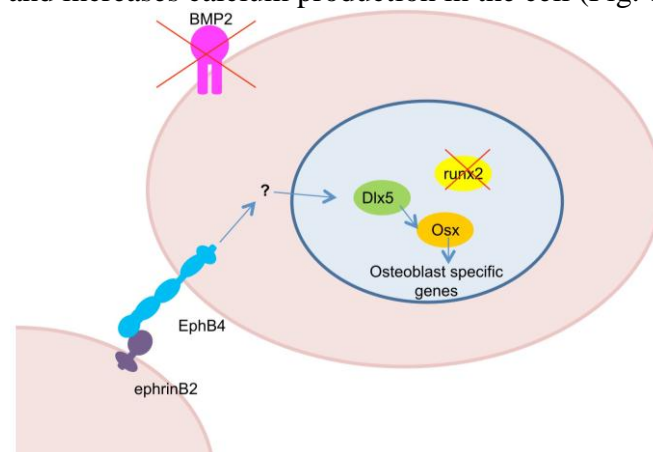


Fig. 8 Insights into the molecular mechanisms involved in ephrinB2-associated osteogenesis. To summarise the findings within, PEI over-expression of ephrinB2 induces an enhanced osteogenic effect in hMSCs in monolayer culture (**Fig. 2C**). This effect is stipulated to be BMP2 pathway independent as the levels of runx2 or BMP2 were not observed to be upregulated relative to controls at either timepoint (**Fig. 7A-D**). Levels of Dlx5 expression were upregulated at the 7 day timepoint (**Fig. 7E**) and levels of Osx expression remained elevated at both 7 and 14 days (**Fig. 7G, H**). It has also been shown that ephrinB2-EphB4 signalling (**Fig. 4C**) is intrinsic to the osteogenic effect as enhanced osteogenesis was quenched when the EphB4 receptor was blocked. Therefore, it is hypothesised that upon extremely elevated levels of ephrinB2 expression and subsequent binding to the EphB4 receptor an unknown signal is relayed to Dlx5 which acts on Osx to commit the cells to an osteoblastic lineage and increases calcium production in the cell.

In summation, this GAM offers several key advantages in that it is composed of a natural collagen-nHa base which is biocompatible, biodegradable and has already demonstrated a capacity to promote osteogenesis. The incorporation of a non-viral vector also offsets the negative connotations associated with viral vectors in gene therapy [54]. Indeed, using gene delivery also resolves the issues of short half-life, diffusion, poor distribution and expense associated with protein delivery [4]. Additionally, ephrinB2 is only expressed in the matrix for a period of weeks, and therefore has an expression profile relative to that required for bone healing. Due to the localised expression of the ligand on cells recruited and transfected in the GAM, the low levels of exogenous PEI polymer used and the fact that the ligand can only affect EphB4 expressing cells, we hypothesise that there is also less scope for off target effects such as ectopic bone formation.

Conclusion

It has been demonstrated that high levels of ephrinB2 over-expression induces the committal of MSCs to an osteoblastic lineage. The increases in osteogenesis indisputably rely on the interaction of ephrinB2 with its cognate receptor, EphB4. Furthermore, we have demonstrated that the transcription factors *Dlx5* and *Osx* respectively are implied in the downstream signalling events post ephrinB2-EphB4 interaction. hMSCs seeded on PEI-ephrinB2 GAMs were shown to be of pre-osteoblastic nature within 14 days based on increased calcium deposition in the matrix which eludes to the possibility of such matrices being applied to bone defects. It is believed that these PEI-ephrinB2 GAMs offer a novel, selective and alternative approach to bone repair applications whereby the next step will be to evaluate their performance in an *in vivo* model.

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