Is Osteogenic Differentiation of Human Nucleus Pulposus Cells a Possibility for Biological Spinal Fusion?

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Abstract

Objective. The purpose of this study was to investigate whether a simple, biologically robust method for inducing calcification of degenerate intervertebral discs (IVD) could be developed to provide an alternative treatment for patients requiring spinal fusion. *Design.* Nucleus pulposus (NP) cells isolated from 14 human IVDs were cultured in monolayer and exposed to osteogenic medium, 1,25-dihydroxyvitamin D_3 (VitD$_3$), parathyroid hormone (PTH), and bone morphogenic proteins (BMPs) 2/7 to determine if they could become osteogenic. Similarly explant cultures of IVDs from 11 patients were cultured in osteogenic media with and without prior exposure to VitD$_3$ and BMP-2. Osteogenic differentiation was assessed by alkaline phosphatase activity and areas of calcification identified by alizarin red or von Kossa staining. Expression of osteogenic genes during monolayer culture was determined using polymerase chain reaction and explant tissues assessed for BMP inhibitors. Human bone marrow–derived mesenchymal stromal cells (MSCs) were used for comparison. *Results.* Standard osteogenic media was optimum for promoting mineralization by human NP cells in monolayer. Some osteogenic differentiation was observed with 10 nM VitD$_3$, but none following application of PTH or BMPs. Regions of calcification were detected in 2 of the eleven IVD tissue explants, one cultured in osteogenic media and one with the addition of VitD$_3$ and BMP-2. *Conclusions.* Human NP cells can become osteogenic in monolayer and calcification of the extracellular matrix can also occur, although not consistently. Inhibitory factors within either the cells or the extracellular matrix may hinder osteogenesis, indicating that a robust biological fusion at this time requires further optimization.

Keywords

intervertebral disc, calcification, nucleus pulposus, spinal fusion, *in vitro*

Introduction

Degeneration of the intervertebral disc (IVD) is a contributory factor to the back pain experienced by some patients[1] and although conservative treatment will alleviate the symptoms for many, for some spinal fusion becomes their only option. Spinal fusion has been shown to be effective in approximately two-thirds of patients treated,[2,3] and has been referred to as the "gold standard" for treating back pain.[4,5] It involves the degenerate IVD being fused to its adjacent vertebrae, thus immobilizing the intervertebral space, reducing movement and alleviating the patient's pain. The technique is both invasive and expensive and usually requires removal of disc material and replacement with autologous, allogeneic, xenogeneic, or synthetic bone and/or metal "cages" to replace the IVD,[6] sometimes in combination with bone morphogenic proteins (BMPs) to stimulate new bone growth. Unfortunately, the procedure can still fail with nonfusion rates of up to 30% reported.[7]

Normally, the human IVD does not mineralize, but under certain conditions, calcification of the disc can occur, particularly in the thoracic spine of males, with increasing age and where there is loss of disc height.[8] Calcification in the disc has long been associated with several pathological conditions such as myositis ossificans progressive, juvenile rheumatoid arthritis, and ankylosing spondylitis[9] where spinal fusion occurs first via bony spurs between vertebrae, followed by calcification of the IVD. Calcification, which is reported to occur more commonly in the outer annulus fibrosus (AF) than the central nucleus pulposus (NP),[10] has also been reported to occur following trauma; for example, 30% of children with calcification in their cervical discs had experienced previous severe trauma.[11] In scoliotic patients,

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Table 1. Patient Demographics.

	NP		MSCs	
No. of samples (male:female)	25 (14:11)		10 (3:7)	
	Location	No.	Location	No.
Sample source	L2/3:	1	Iliac crest:	8
	L3/4:	1		
	L4/5:	17	Tibial bone:	2
	L5/S1:	6		
Disc degeneration grade	II	1		
	III	9		
	IV	11		
	V	4		

MSC = mesenchymal stromal cells; NP = nucleus pulposus.
Details of patients from whom intervertebral discs and bone marrow–derived MSCs were obtained for this study including a summary of the degenerative grade of the donated discs as assessed via T2-weighted magnetic resonance images and the Pfirrmann et al.[21] grading algorithm.

calcification has been identified in discs adjacent to calcified cartilage end-plates[12] particularly in regions subjected to altered loading; calcium deposits have also been found in degenerate disc tissue.[13]

In previous work from our laboratory and others, the presence of local mesenchymal progenitor cells in IVD tissues has been highlighted within both the central NP and the outer AF of degenerate and nondegenerate human discs.[14-18] They have shown that these cells can be driven down an osteogenic lineage by culturing in appropriate media. Nosikova et al.[19] have also shown that bovine cells isolated from the AF are capable of mineralization while Haschtmann et al.[20] induced ossification of the AF in lapine discs with BMP-2 and transforming growth factor–β.

For many years, research has focused on the biological repair or regeneration of the degenerate IVD, but little attention has been paid to facilitating a cell therapy to lead to spinal fusion for immobilizing the spine and reducing pain. The aim of this study was to determine the most successful way of inducing osteogenic differentiation of IVD cells, with the long-term aim of developing a minimally invasive, biological spinal fusion. Hence, we have investigated the potential of known osteogenic-promoting factors, including 1,25 dihydroxyvitamin D_3 (VitD$_3$), parathyroid hormone (PTH) and BMPs, on directing degenerate human NP cells, some of which have been shown to have stem cell-like properties,[14] down an osteogenic pathway.

Methods

Sample Source

Following ethical approval from Shropshire Research Ethics Committee (04/02/RJH) and Isle of Wight, Portsmouth and South East Hampshire Research Ethics Committee (09/H0501/95) and written informed consent, degenerate human IVD samples (graded II-V on the Pfirrmann MRI score[21]) were obtained from 25 patients (14 males, 11 females, aged 41.0 ± 10.1 years) undergoing routine surgery for back and leg pain (16 with herniations and 9 with degenerative disc disease [DDD]; **Table 1**). Bone marrow from iliac crest and tibial bone chips was obtained from 10 patients (3 males, 7 females, aged 48.5 ± 13.9 years) undergoing knee arthroplasty to provide mesenchymal stromal cells (MSCs) for positive controls of cells with the capacity to go down an osteogenic lineage.

Cell Isolation and Maintenance

Nucleus pulposus was identified macroscopically and dissected from IVDs obtained from 14 patients (aged 37 ± 10 years), minced and digested for 16 hours at 37°C in 250 IU/mg collagenase (Type 2, Worthington Biochemical Corporation, Lakewood, NJ, USA).[22] Cells were filtered, washed, and seeded at 5000 cells/cm^2 and maintained in monolayer with "standard" culture medium (DMEM/F12 [Life Technologies, Paisley, UK] containing 10% [v/v] fetal bovine serum [FBS; Life Technologies], 0.5% [v/v] ascorbic acid [10 mg/mL solution in phosphate buffered saline, PBS; Sigma-Aldrich, St Louis, MO, USA], 0.5% [v/v] gentamicin [Life Technologies], 1% [v/v] penicillin and streptomycin [Life Technologies] and 0.05% [v/v] fungizone [Life Technologies]) at 5% CO_2 in a humidified atmosphere. MSCs were also cultured in monolayer as previously described.[14] All cells were passaged as standard by trypsinization on reaching 80% confluence.

Osteogenic Differentiation in Monolayer (2-Dimensional Culture)

Monolayer cultures of NP cells (at passages 1-4) and 100% confluency were treated with factors to induce osteogenic differentiation. For each experiment, cells from 3 different patients were used, and cells were seeded in triplicate in either 24- or 96-well plates for each patient and condition. All factors were compared with standard osteogenic medium, which contained 100 nM dexamethasone (Sigma Aldrich), 50 μM L-ascorbic acid-2-phosphate (Sigma Aldrich), and 10 mM β-glycerophosphate (Sigma Aldrich).[23] Medium was replaced every 2 to 3 days for up to 21 days. We established both the effect of the individual components (and different combinations) of the osteogenic medium and also the following purported osteogenic inducers at concentrations previously found to promote osteogenesis in *in vitro* studies: (a) VitD$_3$ (Sigma Aldrich) at 0.1, 1, or 10 nM[24-26]; (b) the 1-34 fragment of human PTH (Sigma Aldrich) at 1, 10, or 100 nM, either continuously or intermittently for 1 hour every 48 hours[27-29] and

(c) BMPs 2, 7, and 2/7 heterodimer (R&D systems, Minneapolis, MN, USA) at 10 or 100 ng/mL.[30,31]

Osteogenic Differentiation In Situ (3-Dimensional Culture)

Disc tissue, with the macroscopic appearance of NP, from 11 patients aged 44 ± 12 years was dissected into similar sized pieces (approximately 5 mm $\times$ 5 mm) and placed inside dialysis tubing (molecular weight cut off of 3.5kDa) and maintained in explant culture in standard or osteogenic culture medium for a minimum of 21 days, whereby swelling of the tissue was controlled by the inclusion of 10% polyethylene glycol (PEG, molecular weight 20kDa).[32] Three samples were cultured for 8 weeks and maintained in osteogenic medium plus 50 ng/mL BMP-2 with each change of medium having an initial 15-minute transient treatment with 10 nM VitD$_3$ to activate vitamin D receptors, as previously described by Chen *et al.*[33]

Assessment of Osteogenic Differentiation

In cultured cells, alkaline phosphatase activity was determined either histologically or via a colorimetric assay.[34] For histological staining, cells were fixed for 10 minutes with 10% formalin, washed with PBS (Life Technologies) then incubated for 1 hour at room temperature (RT) with the stain solution: 25 mg naphthol AS-BI phosphate in 0.5 mL dimethylformamide and 50 mg Fast Red TR in 50 mL Tris-HCl buffer (pH 9; all Sigma Aldrich). Cells were washed with PBS and the percentage of cells stained were counted and the staining intensity semi-quantitatively assessed with scores ranging from 1 to 6, representing light to strong staining intensity. (All 3 wells for 1 patient's cells per condition were viewed in their totality and ascribed an average score for both the number of cells [as a percentage of the total cells] and intensity in their staining.) Where appropriate, alkaline phosphatase activity was also determined via colorimetric assay in a 96-well format. For this, cells were lysed with 0.1% Triton X in PBS and 50 μL of lysate incubated for 1 hour at 37°C with 0.7% (v/v) 0.5 M MgCl$_2$ and 1.24% (w/v) *p*-nitrophenol phosphate in 50 mM sodium carbonate and 50 mM Tris buffer (pH 9.5). Color change was read at 405 nm and background absorbance corrected with readings at 650 nm.

Three-dimensional explant cultures were assessed for calcification via von Kossa and alizarin red staining. Explants were snap-frozen in liquid nitrogen–cooled hexane and 7-μm thick cryosections were collected onto poly-L-lysine–coated slides and stored at −20°C. Tissue sections were fixed in 10% formalin for 10 minutes at RT then either alizarin red solution or 5% solution of silver nitrate (von Kossa) was added with these latter sections needing ultraviolet light to develop the stain. Adjacent sections were stained with Mayer's hematoxylin and eosin.

Immunohistochemical Staining for BMP Inhibitors

Cryosections of explant cultures were also assessed for the presence of BMP inhibitors via immunohistochemistry (IHC). Slides were brought to RT and fixed in 4% paraformaldehyde for 15 minutes. All immunostaining steps were carried out at RT and PBS used for each wash unless stated otherwise. Sections were blocked for 1 hour with 10% normal horse serum, and then the following rabbit polyclonal antibodies applied overnight at 4°C: chordin (K-25, sc-130720, 2 μg/mL); gremlin (FL-184, sc-28873, 4 μg/ml); noggin (FL-232, sc-25656, 1 μg/Ml) all from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA. Negative control sections were incubated with either (a) rabbit normal serum or rabbit IgG (sc-2027, Santa Cruz) at the appropriate concentration or (b) PBS. Sections were then incubated with secondary biotinylated goat anti-rabbit IgG (Vectastain Elite ABC, Vector Laboratories Ltd, Peterborough, UK) for 30 minutes before blocking for endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 30 minutes. Immunolabeling was visualized using the streptavidin-biotin detection system with diaminobenzidine tetrachloride (Sigma Aldrich) as substrate. Sections were washed with distilled water and dehydrated using serial isopropyl alcohol solutions (70%-100%) before being cleared in xylene and mounted in Pertex (CellPath, Newtown, UK).

Osteogenic Gene Expression

To determine individual osteogenic genes, which could be altered following treatment of monolayer cells for osteogenic differentiation, RNA was extracted using the RNeasy Mini Kit (Qiagen, Manchester, UK). cDNA was synthesized from extracted RNA using the RT^2 First strand kit (Qiagen) and RT-Q PCR performed using an ABI 7500 RT-PCR system (Applied Biosystems) for *Runt-related transcription factor 2* (*RUNX2*), *osteocalcin*, and *osteopontin* with *GAPDH* as the reference gene (Qiagen). Fold changes greater than ±2 were noted and the reference gene expression checked to ensure it was constant throughout.

Statistical Analyses

Statistical analyses were performed using Microscoft Excel and Analyse-It (Analyse-it Software Ltd, Leeds, UK). Distribution was determined using the Anderson-Darling test for normality and data sets compared using Student *t*-test or 1-way analysis of variance with a Bonferroni post hoc test. Data are presented as mean ± standard deviation and significance set at $P \leq 0.05$.
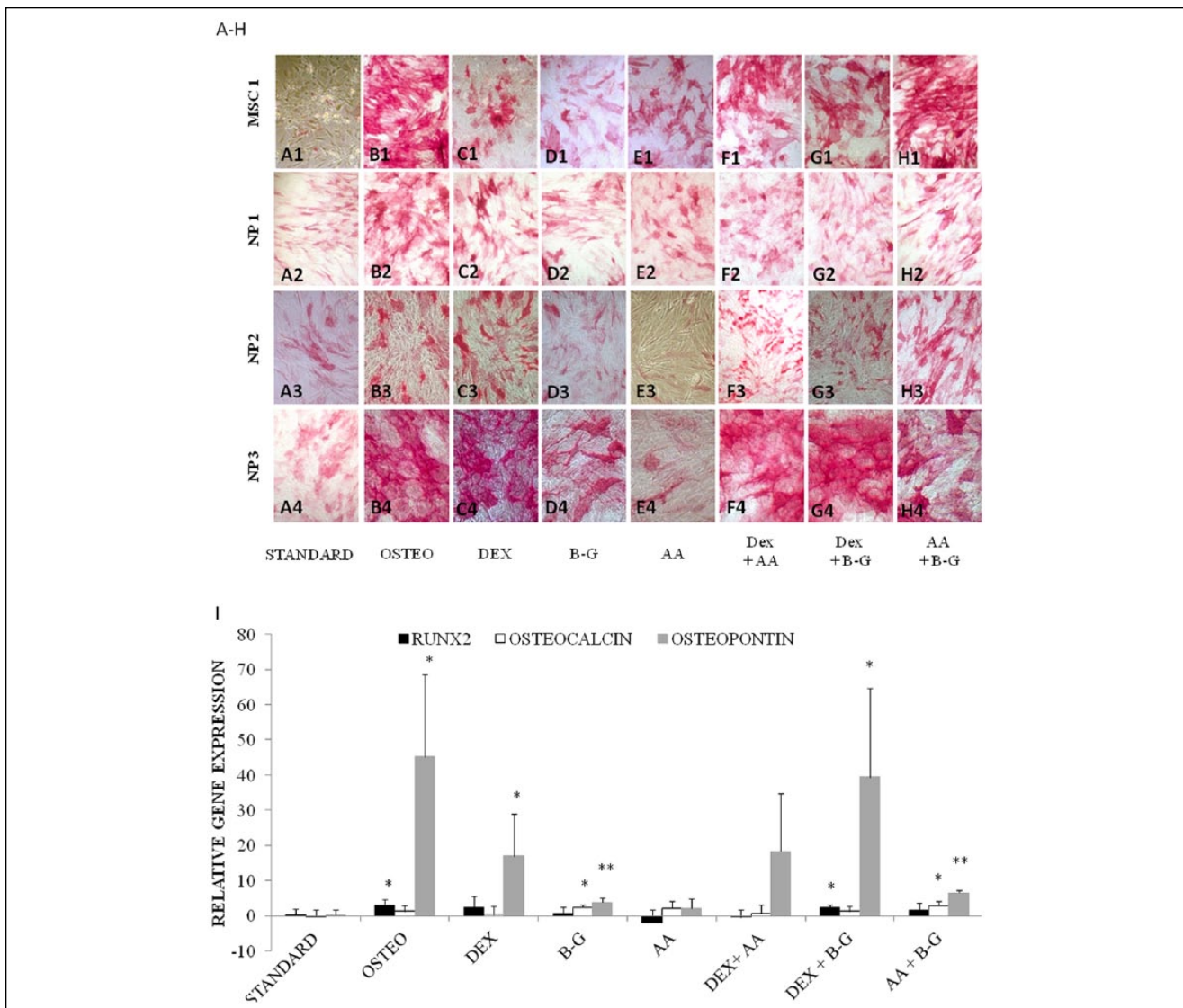
Figure 1. (**A-H**) Alkaline phosphatase staining of cultured mesenchymal stromal cells (MSCs) and human nucleus pulposus (NP) cells maintained in monolayer for 21 days with osteogenic medium (osteo) (**B1-B4**) and its components, dexamethasone (DEX) (**C1-C4**), β-glycerophosphate (B-G) (**D1-D4**), L-ascorbic acid-2-phosphate (AA) (**E1-E4**) and in combination (**F1-H4**), in comparison with the control of "standard" medium (**A1-A4**). (**I**) Expression of osteocalcin, osteopontin, and RUNX2 genes (relative to GAPDH) for each corresponding culture condition at 21 days. Results show the mean values of NP cell cultures from 3 different patients and the bar represents the standard deviation. Significant differences are denoted by *$P < 0.05$ and **$P < 0.005$.

Results

Optimal Media Conditions for Osteogenic Differentiation of NP Cells

Positive staining for alkaline phosphatase activity was seen in all monolayer NP cells treated with osteogenic medium for 21 days, although the extent of staining varied considerably, between patients, both in number (from 10% to 80% of cells) and intensity (**Figs. 1B2-B4**, **2B2-B4**, and **2H1-H3**). The effect of each component of the osteogenic medium (dexamethasone, β-glycerophosphate, and L-ascorbic acid-2-phosphate) was also assessed either singularly (**Fig. 1C1-C4**,

1D1-D4, and **1E1-E4**, respectively) or in different combinations (**Fig. 1F1-F4**, **1G1-G4**, and **1H1-H4**). Maximal alkaline phosphatase activity was observed in cells treated with complete osteogenic media, with 57% ± 23% of cells having strong positive staining. In particular, dexamethasone and β-glycerophosphate, either individually or in combination increased alkaline phosphatase activity. No relationship between degenerative grade and intensity and percentage cells stained for alkaline phosphatase was observed in this study. As expected, MSCs treated with osteogenic induction medium (**Fig. 1B1**) stained positively for alkaline phosphatase (75% ± 7%) though surprisingly, in NP cells maintained in standard

culture medium, a small amount of alkaline phosphatase staining was also observed (**Fig. 1A2-A4**). PCR analysis demonstrated an increase in osteopontin expression, with cells cultured in osteogenic medium to be greatest (45-fold; **Fig. 1I**) compared with cells maintained in standard culture medium ($P < 0.01$), followed by dexamethasone either in combination with β-glycerophosphate (39-fold, $P < 0.05$), or on its own (17-fold, $P < 0.05$). Osteopontin expression also increased in response to β-glycerophosphate in combination with L-ascorbic acid-2-phosphate (6.5-fold, $P < 0.0005$) and on its own (3.9-fold, $P < 0.005$). At 21 days, 2-to 3-fold significant increase in RUNX2 expression was observed when NP cells were cultured with osteogenic or dexamethasone in combination with β-glycerophosphate and also in osteocalcin expression when cultured with β-glycerophosphate alone or in combination with L-ascorbic acid-2-phosphate (all $P < 0.05$). In case any change in *RUNX2* expression had been missed by 21 days, the experiment was repeated with an earlier endpoint of 7 days. A 5-fold increase in RUNX2 expression confirmed our suspicions that this transcription factor is expressed earlier than 21 days in response to osteogenic medium.

Vit D$_3$ Induces Osteogenic Differentiation

Little or no osteogenic differentiation was seen at 21 days in cells treated with 0.1 nM VitD$_3$ for 21 days (**Fig. 2C2-C4**) with faint alkaline phosphatase staining being present in only approximately 4% of cells. With 1 or 10 nM VitD$_3$, however, some alkaline phosphatase staining was seen in both the NP cells (10%) and MSCs (25%-40%; **Fig. 2D1-D4** and **2E1-E4**). Cells treated with complete osteogenic medium were run as a positive control and showed the greatest staining (~45% in NP cells and 80% in MSCs) for alkaline phosphatase (**Fig. 2B1-B4**). When 10 nM VitD$_3$ was added to standard or osteogenic media it did not augment the alkaline phosphatase activity (**Fig. 2G1-G3** and **I1-I3**, respectively). Interestingly, PCR analysis detected significantly decreased osteocalcin and osteopontin expression in NP cells maintained in osteogenic medium with 10 nM VitD$_3$ compared to osteogenic medium without VitD$_3$ (**Fig. 2J**).

Lack of Osteogenic Differentiation Following Application of pTH or BMPs

Neither continuous nor intermittent doses of PTH over the concentration range of 1 to 100 nM increased alkaline phosphatase (assessed both via histological staining or activity assay) in the NP cells or MSCs (data not shown). In addition, there was no observed increase in alkaline phosphatase as assessed by activity assay in NP cells or MSCs treated with BMP-2, -7, or -2/7 heterodimer at concentrations between 10 and 100 ng/mL (data not shown). In fact the application of BMPs, in particular BMP-2/7,

seemed to reduce alkaline phosphatase activity in human NP cells compared with those maintained in standard culture.

Three-Dimensional Culture of NP Tissue and Presence of BMP Inhibitors

Calcification was identified in two of the herniated human NP tissues maintained in explant culture (**Fig. 3A** and **B**). Calcification was detected via alizarin red staining in disc tissue isolated from a 29-year-old female (L4/5) and maintained in osteogenic media for 21 days and also in disc tissue from a 44-year-old male (L5/S1) maintained in osteogenic media with the addition of VitD$_3$ followed by BMP2 for 8 weeks. All other discs grown as explant cultures showed no induction of mineralization regardless of culture conditions or time in culture. As in monolayer, degenerate grade did not appear to be associated with calcification of the explant tissues.

All 5 of the IVD explants assessed for the presence of the BMP inhibiting molecules chordin, gremlin, and noggin within the cells and extracellular matrix were found to be positive for gremlin (**Fig. 4D-G**), but the number of cells and intensity of staining varied from few and pale to numerous and dark. Cells were positive for chordin in 3 of 5 samples while noggin was detected in 4 of the samples. Neither chordin nor noggin were clearly detected in any the extracellular matrix of the explants. No clear relationship between the presence of these BMP inhibitors and calcification of the extracellular matrix was observed.

Discussion

We have shown that human degenerate IVD cells are capable of becoming osteogenic in both monolayer culture and *in situ* within NP tissue. In monolayer culture, cells from human degenerate NPs, maintained in osteogenic media, were consistently capable of osteogenic differentiation. Some NP cells stained positive for alkaline phosphatase even under standard conditions, indicating a "natural" predilection for, or tendency to becoming osteogenic. This may reflect the presence of progenitor cells that have previously been identified in disc tissue.[14-17,35] We have shown that 11% to 20% of cells within degenerate discs have positive staining for the putative stem cell markers, Notch-1 and cytokeratins 8 and 19, although there is a huge range between individual patients' discs (e.g., from 0% to 96% for Notch-1).[14] Recent work by Tekari *et al.*[36] identified approximately 9% of bovine NP cells as being positive for the angiopoietin-1 receptor (Tie2). Cells positive for Tie2 have been shown to have progenitor-like multipotency and in human discs their incidence decreases with both age and degeneration.[35]
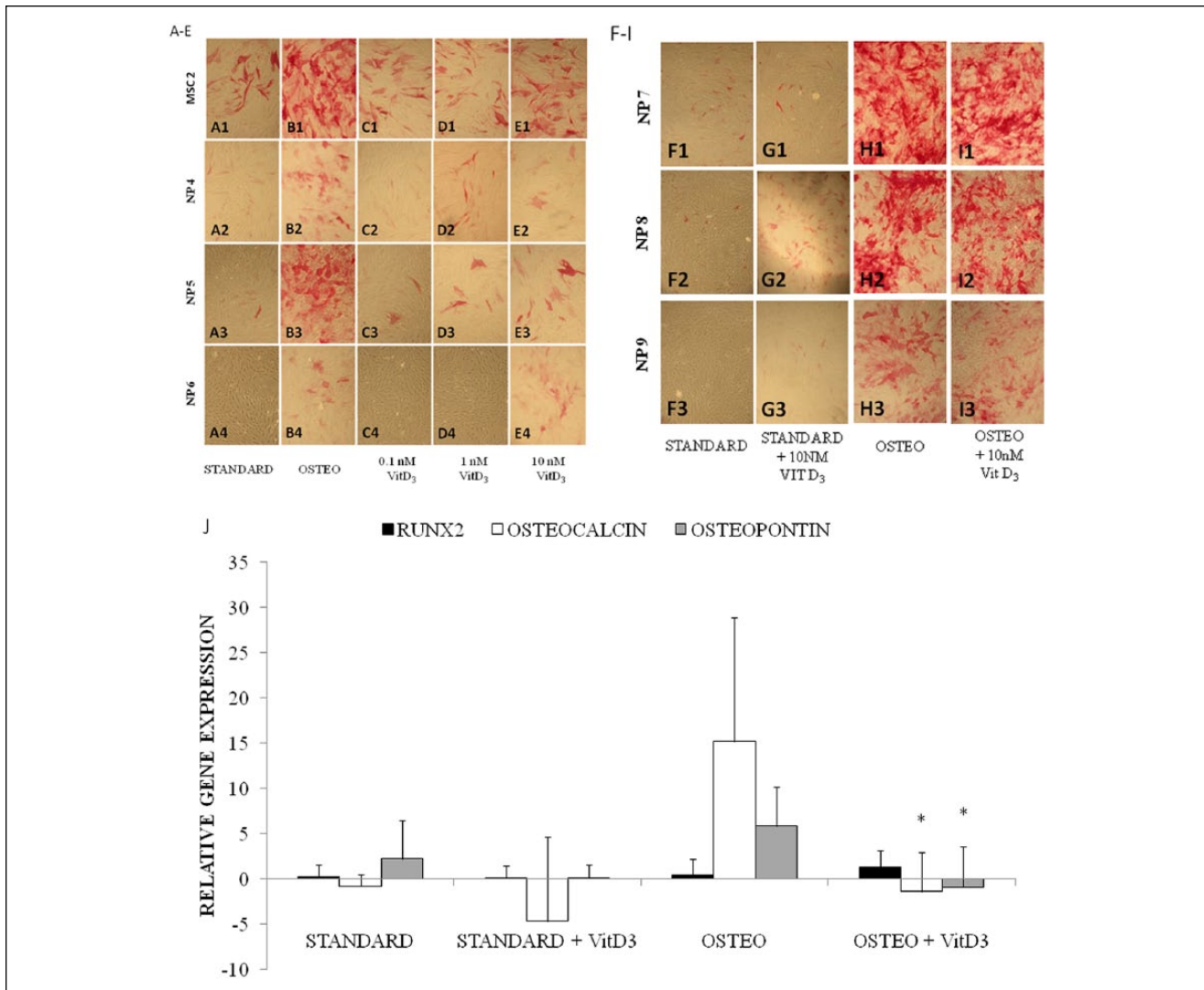
Figure 2. (**A-E**) Alkaline phosphatase staining of monolayer cultured human nucleus pulposus (NP) cells from 3 patients and one population of mesenchymal stromal cells (MSCs) maintained with osteogenic medium (**B1-B4**) and 3 doses of VitD$_3$ (0.1, 1, and 10 nM; **C1-4**, **D1-4**, and **E1-4**, respectively) in comparison to standard medium (**A1-A4**) and (**F-I**) cultured human NP cells from 3 patients exposed to standard and osteogenic medium ± 10 nM VitD$_3$, all at 21 days. (**J**) Expression of osteocalcin, osteopontin, and RUNX2 genes (relative to GAPDH) for each culture condition in (**F-I**). Results show the mean values of NP cells cultured from $n = 3$ patients with bars representing the standard deviation. Significant differences between osteogenic media with and without VitD$_3$ are denoted by *$P < 0.01$.

Cells express different markers at different stages of osteogenesis. RUNX2 is one of the initial markers prior to alkaline phosphatase production, followed by osteopontin and then osteocalcin.[37] RUNX2 is a key transcription factor essential for osteoblastic differentiation and is usually expressed early by committed osteoprogenitor cells or immature osteoblasts. The expression of RUNX2 in our experiments was found to be increased at 7 days but by 21 days this upregulation had ceased, typical of its early production in osteogenesis.[19] The later markers, osteocalcin and osteopontin, are both secreted by mature osteoblasts, but osteopontin is also produced in

other tissues. Both these molecules are involved in the regulation of hydroxyapatite formation with osteopontin, in particular, having a role in inhibiting crystal growth.[38] At 21 days, osteocalcin was significantly upregulated (up to 3-fold) when β-glycerophosphate, either alone or with L-ascorbic acid-2-phosphate, was added to the monolayer cultures. Osteopontin was significantly expressed at high levels during culture with osteogenic media and all culture conditions with dexamethasone except when VitD$_3$ was added.

Interestingly, factors which are commonly used as osteogenic agents in the clinic (VitD$_3$, PTH, and BMPs) had less
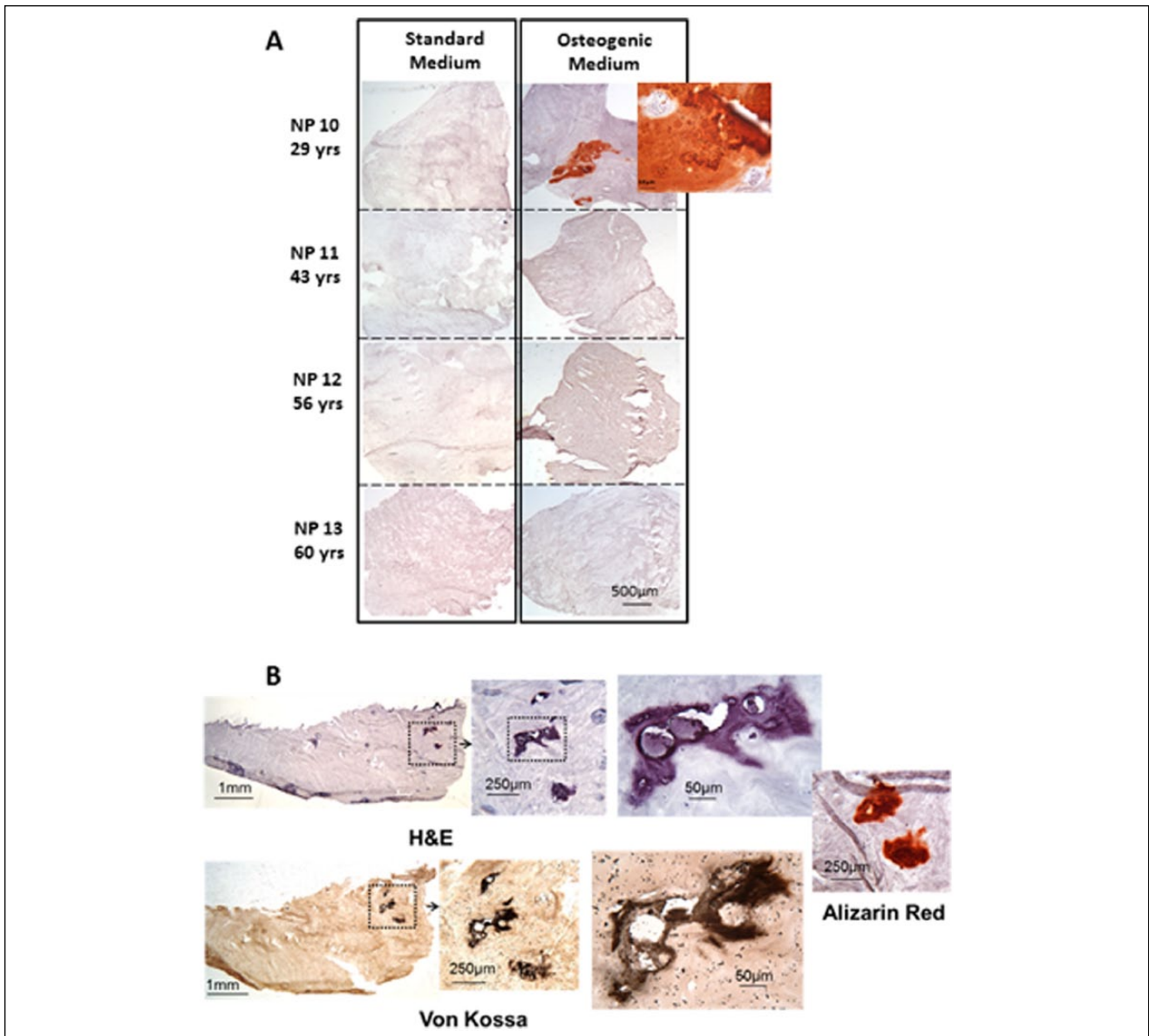
Figure 3. (**A**) Four representative disc explants cultured in standard and osteogenic culture medium for 21 days and stained with alizarin red. Only the explant from the youngest patient (NP10) showed calcification. (**B**) Explant cultures of disc from a 44 year old male cultured in Vit D_3 for 15 minutes prior to bone morphogenic protein (BMP) osteogenic medium for 8 weeks, showing ossification in sections stained with hematoxylin and eosin, alizarin red, and von Kossa.

effect on disc cells and MSCs than osteogenic culture medium traditionally used in *in vitro* systems. *In vivo*, the active form of vitamin D, VitD$_3$, maintains the homeostasis of calcium and phosphate ions and reduces osteoporotic risk.[39] In osteoblasts, 2 receptors, 1 nuclear (vitamin D receptor [VDR])[40] and 1 in the plasma membrane (protein disulfide isomerase family A member 3 [Pdia3]),[41,42] have been identified for VitD$_3$. We found that the lower concentration (0.1 nM) of VitD$_3$ did not stimulate upregulation of alkaline phosphatase in human degenerate disc cells, but at higher concentrations, such as 1 or 10 nM, alkaline phosphatase activity was increased. A similar response to 10

nM VitD$_3$ has been observed in other studies using MSCs obtained from vertebral bodies whereby this concentration promoted osteogenesis.[24,25] At a concentration of 10 nM, VitD$_3$ increased alkaline phosphatase staining in NP cells and MSCs, but did not augment the effect of osteogenic medium, perhaps due to dexamethasone interfering, as has been shown to occur in human osteoblasts.[43] In contrast, in response to the addition of VitD$_3$ to osteogenic medium, both osteocalcin and osteopontin expression were significantly decreased in NP cells (compared with osteogenic medium alone) and yet alkaline phosphatase staining remained the same.
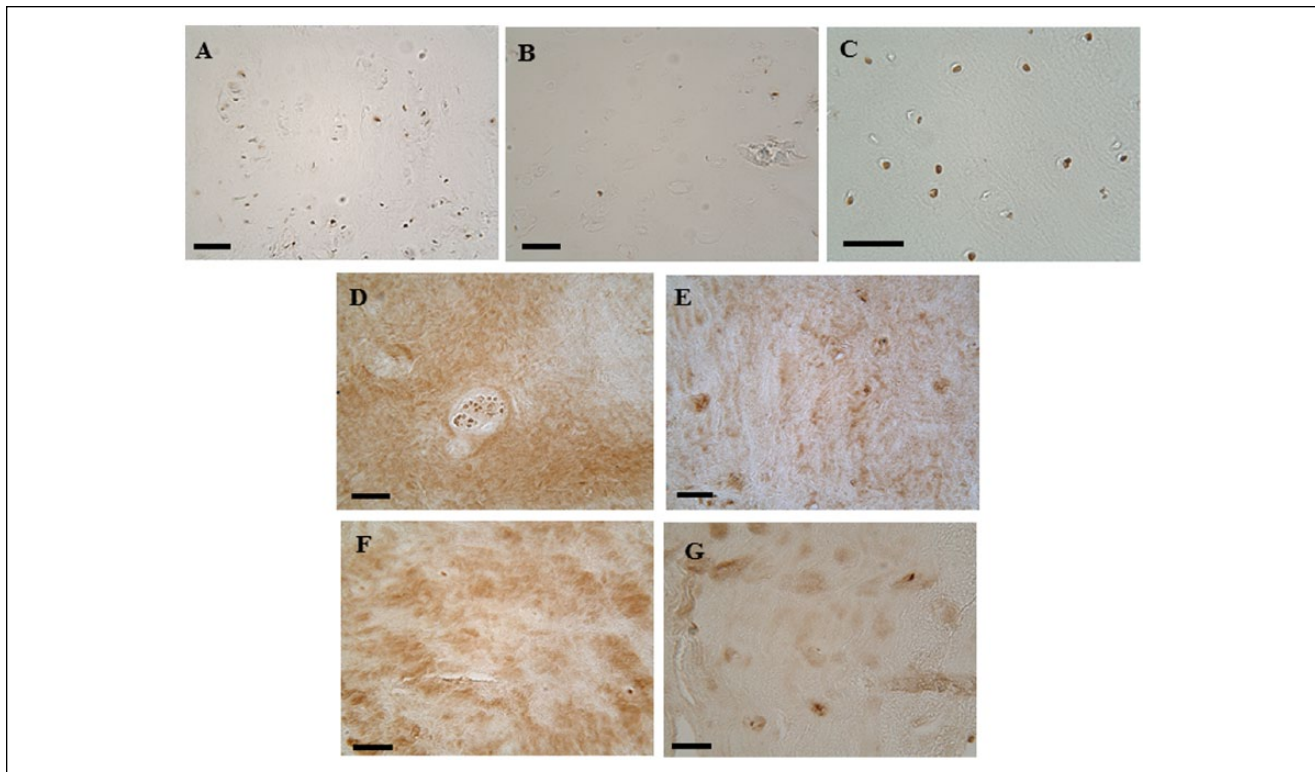
Figure 4. Immunohistochemical of staining for the bone morphogenic protein (BMP) inhibitors chordin, gremlin, and noggin. Human osteoarthritic cartilage was used as a positive control for chordin and gremlin (**A** and **B**, respectively) and bovine cartilage for noggin (**C**). Images **D-G** show positive gremlin staining in human degenerative explant tissues cultured in standard medium (**D**), osteogenic medium (**E** and **F**) and osteogenic with VitD$_3$ and BMP2 (**G**). (**D** and **E**) and (**F** and **G**) are from the same intervertebral disc (IVD) explants with the sample in (**E**) being positive for calcification (NP10 from **Fig. 3A**). Scale bar = 50 micron.

In vivo, PTH acts to increase the presence of calcium ions by binding to osteoblasts and increasing RANKL expression but inhibiting osteoprotegerin (OPG) production. RANKL is then free to bind to RANK instead of OPG, thus stimulating osteoclast precursors to fuse and form new osteoclasts which break down bone and release calcium ions from the bone matrix. However, the presence of both intermittent[28] and continuous[27] application of 1-45 PTH has been shown to induce alkaline phosphatase in rodent MSCs, although Yang *et al.*[27] found that intermittent application reduced alkaline phosphatase levels in their rat MSCs to lower than controls and Fujita *et al.*[44] found continuous PTH decreased alkaline phosphatase in mice osteoblasts. Neither intermittent nor continuous application of PTH in this study was found to affect the presence of alkaline phosphatase in human degenerate NP cells. Disc cells may behave differently from MSCs, since Madiraju *et al.*[45] applied PTH to degenerate discs cells and found that it reduced alkaline phosphatase activity in human NP cells (but not AF cells).

BMP-2 and -7 have long been known to be involved in inducing bone and cartilage formation and in osteoblast differentiation. They have also been reported to have an anabolic effect on IVD cells and tissues, by increasing proteoglycan and matrix production. Kim *et al.*[46] have shown that human NP cells produce alkaline phosphatase and at a higher level by cells from less degenerate discs than more degenerate ones. Injections of BMP-7 into canine discs have been shown to stimulate the production of peridiscal bone.[47] However, the dose of BMP injected into these discs was more than 1000-fold of that used in the present study where we found no response. It is also known that disc cells are capable of producing inhibitors to osteogenic factors such as BMPs; these include noggin, gremlin, and chordin. Chan *et al.*[48] have shown that human disc cells express the genes for these antagonists and co-culture of disc cells reduced osteogenesis in MSCs. In addition, expression of GREM1 was higher in NP cells than AF cells. The same group have shown reversal of the disc's inhibitory effect on MSCs with the addition of a BMP2 variant (L51P).[49] Immunohistochemical staining for the BMP inhibitors in our study demonstrated that they were present in some disc samples and to varying degrees.

There are conflicting reports in the literature about the propensity of NP cells and tissues to mineralize, both *in vitro* and *in vivo*. For example, Nosikova *et al.*[19] actually used bovine NP cells as a negative control in their *in vitro*

study on mineralization of AF cells (and our laboratory has found a similar resistance of bovine NP cells to become osteogenic (unpublished results)). There are many pathological syndromes, however, where ectopic calcification of discs occurs naturally in canines, sheep,[50] and humans. The etiology of the dystrophic calcification is not known and indeed the calcification process itself is complex such that there are numerous factors that can influence the many pathways involved. The samples used for explant culture were from both degenerate (n = 5) and herniated discs (n = 6) but only 2 herniated discs demonstrated calcification following culture under osteogenic conditions. These findings concur with others that found herniated discs more prone to calcification than normal discs.[51] Other reasons for the variation of human degenerate IVDs to calcify are that perhaps some discs may have a higher number of progenitor cells (and so be more likely to calcify). This may explain the calcification observed in the NP of the 29-year-old in **Figure 3A** while other samples, from older patients, may have lower numbers of progenitor cells. Alternatively, some discs may have higher levels of molecules that are known to inhibit mineralization, such as gremlin, noggin, and chordin, which have been investigated here. Other factors that are known to influence the likelihood of calcification are pH and levels of phosphate, calcium ions, alkaline phosphatase activity, and pro-inflammatory cytokines, but these have not been examined in the current study. Variability between individual patients in all these factors could explain the lack of a clear pattern of calcification in our samples as well, perhaps, as explain why some patients are resistant to BMP-2 treatment for spinal fusion.

Not studying all the factors known to influence calcification may be considered as a limitation of this study. A further limitation of the study is the lack of adequate cells available from the limited surgical waste material and being unable to provide enough at an early passage as was preferred for all the replicate tests performed; hence cells were assessed in monolayer at passages varying between P1 and P4. Although the explant culture model enables maintenance of an environment much more akin to *in vivo* than monolayer cultures, it is still not perfect. For example, whilst it restricts swelling to some extent, it may not completely mimic the physical restriction nor reflect the limited nutrient supply that the disc is exposed to *in vivo*.

In conclusion, this work has shown that cells from degenerate human IVDs have the capability to be driven down an osteogenic pathway, such that they can produce calcium and phosphate salts when grown in monolayer and in some disc tissue itself. Unfortunately, this study has not shown that factors commonly used in the clinic for inducing bone formation and mineralization will reliably do this within discs, perhaps due to the complexity of the factors that influence the calcification process. Further developing our understanding of these control points, and being better able to manipulate them, could lead to a relatively simple and minimally invasive approach for fusing the spine in back pain patients in the future.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical approval for this study was obtained from the Shropshire Research Ethics Committee (04/02/RJH) and Isle of Wight, Portsmouth, and South East Hampshire Research Ethics Committee (09/H0501/95).

Informed Consent

Written informed consent was obtained from all subjects before the study.

Trial Registration

Not applicable.

References

1. Brinjikji W, Diehn FE, Jarvik JG, Carr CM, Kallmes DF, Murad MH, *et al*. MRI findings of disc degeneration are more prevalent in adults with low back pain than in asymptomatic controls: a systematic review and meta-analysis. AJNR Am J Neuroradiol. 2015;36:2394-9.
2. Turner JA, Ersek M, Herron L, Haselkorn J, Kent D, Ciol MA, *et al*. Patient outcomes after lumbar spinal fusions. JAMA. 1992;268:907-11.
3. Stromqvist B, Fritzell P, Hagg O, Jonsson B. The Swedish Spine Register: development, design and utility. Eur Spine J. 2009;18(suppl 3):294-304.
4. Fritzell P, Hagg O, Wessberg P, Nordwall A. 2001 Volvo Award Winner in Clinical Studies. Lumbar fusion versus nonsurgical treatment for chronic low back pain: a multicenter randomized controlled trial from the Swedish Lumbar Spine Study Group. Spine (Phila Pa 1976). 2001;26:2521-32.
5. Carreon LY, Glassman SD, Howard J. Fusion and nonsurgical treatment for symptomatic lumbar degenerative disease: a systematic review of Oswestry Disability Index and MOS Short Form-36 outcomes. Spine J. 2008;8:747-55.
6. Babu MA, Coumans JV, Carter BS, Taylor WR, Kasper EM, Roitberg BZ, *et al*. A review of lumbar spinal instrumentation:

evidence and controversy. J Neurol Neurosurg Psychiatry. 2011;82:948-51.

7. Watkins R, Watkins R III, Hanna R. Non-union rate with stand-alone lateral lumbar interbody fusion. Medicine (Baltimore). 2014;93:e275.

8. Chanchairujira K, Chung CB, Kim JY, Papakonstantinou O, Lee MH, Clopton P, et al. Intervertebral disk calcification of the spine in an elderly population: radiographic prevalence, location, and distribution and correlation with spinal degeneration. Radiology. 2004;230:499-503.

9. Dussault RG, Kaye JJ. Intervertebral disk classification associated with spine fusion. Radiology. 1977;125:57-61.

10. Kerns S, Pope TL Jr, de Lange EE, Fechner RE, Keats TE, Cimmino C. Annulus fibrosus calcification in the cervical spine: radiologic-pathologic correlation. Skeletal Radiol. 1986;15:605-9.

11. Sonnabend DH, Taylor TKF, Chapman GK. Intervertebral disc calcification syndromes in children. J Bone Jt Surg. 1982;64-B:25-31.

12. Roberts S, Menage J, Eisenstein SM. The cartilage end-plate and intervertebral disc in scoliosis: calcification and other sequelae. J Orthop Res. 1993;11:747-57.

13. Hristova GI, Jarzem P, Ouellet JA, Roughley PJ, Epure LM, Antoniou J, et al. Calcification in human intervertebral disc degeneration and scoliosis. J Orthop Res. 2011;29:1888-95.

14. Turner S, Balain B, Caterson B, Morgan C, Roberts S. Viability, growth kinetics and stem cell markers of single and clustered cells in human intervertebral discs: implications for regenerative therapies. Eur Spine J. 2014;23:2462-72.

15. Henriksson H, Thornemo M, Karlsson C, Hagg O, Junevik K, Lindahl A, et al. Identification of cell proliferation zones, progenitor cells and a potential stem cell niche in the intervertebral disc region: a study in four species. Spine (Phila Pa 1976). 2009;34:2278-87.

16. Feng G, Yang X, Shang H, Marks IW, Shen FH, Katz A, et al. Multipotential differentiation of human anulus fibrosus cells: an in vitro study. J Bone Joint Surg Am. 2010;92:675-85.

17. Brisby H, Papadimitriou N, Brantsing C, Bergh P, Lindahl A, Barreto HH. The presence of local mesenchymal progenitor cells in human degenerated intervertebral discs and possibilities to influence these in vitro: a descriptive study in humans. Stem Cells Dev. 2013;22:804-14.

18. Risbud MV, Schoepflin ZR, Mwale F, Kandel RA, Grad S, Iatridis JC, et al. Defining the phenotype of young healthy nucleus pulposus cells: recommendations of the Spine Research Interest Group at the 2014 Annual ORS meeting. J Orthop Res. 2015;33:283-93.

19. Nosikova Y, Santerre JP, Grynpas MD, Kandel RA. Annulus fibrosus cells can induce mineralization: an in vitro study. Spine J. 2013;13:443-53.

20. Haschtmann D, Ferguson SJ, Stoyanov JV. BMP-2 and TGF-β3 do not prevent spontaneous degeneration in rabbit disc explants but induce ossification of the annulus fibrosus. Eur Spine J. 2012;21:1724-33.

21. Pfirrmann CW, Metzdorf A, Zanetti M, Hodler J, Boos N. Magnetic resonance classification of lumbar intervertebral disc degeneration. Spine. 2001;26:1873-8.

22. Garcia J, Mennan C, McCarthy HS, Roberts S, Richardson JB, Wright KT. Chondrogenic potency analyses of donor-matched chondrocytes and mesenchymal stem cells derived from bone marrow, infrapatellar fat pad, and subcutaneous fat. Stem Cells Int. 2016;2016:6969726.

23. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem. 1997;64:295-312.

24. D'Ippolito G, Schiller PC, Perez-stable C, Balkan W, Roos BA, Howard GA. Cooperative actions of hepatocyte growth factor and 1,25-dihydroxyvitamin D_3 in osteoblastic differentiation of human vertebral bone marrow stromal cells. Bone. 2002;31:269-75.

25. Chen K, Aenlle KK, Curtis KM, Roos BA, Howard GA. Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. Bone. 2012;51:69-77.

26. De Kok IJ, Hicok KC, Padilla RJ, Young RG, Cooper LF. Effect of vitamin D pretreatment of human mesenchymal stem cells on ectopic bone formation. J Oral Implantol. 2006;32:103-9.

27. Yang C, Frei H, Burt HM, Rossi F. Effects of continuous and pulsatile PTH treatments on rat bone marrow stromal cells. Biochem Biophys Res Commun. 2009;380:791-6.

28. Rey A, Manen D, Rizzoli R, Ferrari SL, Caverzasio J. Evidences for a role of p38 MAP kinase in the stimulation of alkaline phosphatase and matrix mineralization induced by parathyroid hormone in osteoblastic cells. Bone. 2007;41:59-67.

29. Rickard DJ, Wang FL, Rodriguez-Rojas AM, Wu Z, Trice WJ, Hoffman SJ, et al. Intermittent treatment with parathyroid hormone (PTH) as well as a non-peptide small molecule agonist of the PTH1 receptor inhibits adipocyte differentiation in human bone marrow stromal cells. Bone. 2006;39:1361-72.

30. Laflamme C, Rouabhia M. Effect of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers on osteoblast adhesion and growth following culture on a collagen scaffold. Biomed Mater. 2008;3:015008.

31. Song I, Kim BS, Kim CS, Im GI. Effects of BMP-2 and vitamin D_3 on the osteogenic differentiation of adipose stem cells. Biochem Biophys Res Commun. 2011;408:126-31.

32. Bayliss MT, Urban JPG, Johnstone B, Holm S. In vitro method for measuring synthesis rates in the intervertebral disc. J Orthop Res. 1986;4:10-7.

33. Chen J, Dosier CR, Park JH, De S, Guldberg RE, Boyan BD, et al. Mineralization of three-dimensional osteoblast cultures is enhanced by the interaction of 1a,25-dihydroxyvitamin D_3 and BMP2 via two specific vitamin D receptors. J Tissue Eng Regen Med. 2016;10:40-51. doi:10.1002/term.1770.

34. McCarthy HS. The Regulation of Osteoprotegerin and Dickkopf-1 in Osteoblastic Cells [dissertation]. Chester, UK: University of Chester; 2012.

35. Sakai D, Nakamura Y, Nakai T, Mishima T, Kato S, Grad S, et al. Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. Nat Commun. 2012;3:1264.

36. Tekari A, Chan SC, Sakai D, Grad S, Gantenbein B. Angiopoietin-1 receptor Tie2 distinguishes multipotent differentiation capability in bovine coccygeal nucleus pulposus cells. Stem Cell Res Ther. 2016;7:75.

37. Wagner ER, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, *et al*. Defective osteogenic differentiation in the development of osteosarcoma. Sarcoma. 2011;2011:325238.

38. Hunter GK, Hauschka PV, Poole AR, Rosenberg LC, Goldberg HA. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. Biochem J. 1996;317:59-64.

39. Sambrook P, Cooper C. Osteoporosis. Lancet. 2006;367:2010-8.

40. Walters MR, Rosen DM, Norman AW, Luben RA. 1,25-Dihydroxyvitamin D receptors in an established bone cell line. Correlation with biochemical responses. J Biol Chem. 1982;257:7481-4.

41. Boyan BD, Bonewald LF, Sylvia VL, Nemere I, Larsson D, Norman AW, *et al*. Evidence for distinct membrane receptors for 1 α,25-(OH)$_2$D$_3$ and 24R,25-(OH)$_2$D$_3$ in osteoblasts. Steroids. 2002;67:235-46.

42. Chen J, Olivares-Navarrete R, Wang Y, Herman TR, Boyan BD, Schwartz Z. Protein-disulfide isomerase-associated 3 (Pdia3) mediates the membrane response to 1,25-dihydroxyvitamin D$_3$ in osteoblasts. J Biol Chem. 2010;285:37041-50.

43. Jorgensen NR, Henriksen Z, Sorensen OH, Civitelli R. Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an in vitro model for human bone marrow-derived primary osteoblasts. Steroids. 2004;69:219-26.

44. Fujita T, Fukuyama R, Izumo N, Hirai T, Meguro T, Nakamuta H, *et al*. Transactivation of core binding factor alpha1 as a basic mechanism to trigger parathyroid hormone-induced osteogenesis. Jpn J Pharmacol. 2001;86:405-16.

45. Madiraju P, Gawri R, Wang H, Antoniou J, Mwale F. Mechanism of parathyroid hormone-mediated suppression of calcification markers in human intervertebral disc cells. Eur Cell Mater. 2013;25:268-83.

46. Kim SH, Kuh SU, Kim KN, Park JY, Cho KH, Chin DK, *et al*. Biologic response of degenerative living human nucleus pulposus cells to treatment with cytokines. Yonsei Med J. 2015;56:277-86.

47. Willems N, Bach FC, Plomp SG, van Rijen MH, Wolfswinkel J, Grinwis GC, *et al*. Intradiscal application of rhBMP-7 does not induce regeneration in a canine model of spontaneous intervertebral disc degeneration. Arthritis Res Ther. 2015;17:137.

48. Chan SC, Tekari A, Benneker LM, Heini PF, Gantenbein B. Osteogenic differentiation of bone marrow stromal cells is hindered by the presence of intervertebral disc cells. Arthritis Res Ther. 2015;18:29.

49. Tekari A, May RD, Frauchiger DA, Chan SC, Benneker LM, Gantenbein B. The BMP2 variant L51P restores the osteogenic differentiation of human mesenchymal stromal cells in the presence of intervertebral disc cells. Eur Cell Mater. 2017;33:197-210.

50. Melrose J, Burkhardt D, Taylor TK, Dillon CT, Read R, Cake M, *et al*. Calcification in the ovine intervertebral disc: a model of hydroxyapatite deposition disease. Eur Spine J. 2009;18:479-89.

51. Karamouzian S, Eskandary H, Faramarzee M, Saba M, Safizade H, Ghadipasha M, *et al*. Frequency of lumbar intervertebral disc calcification and angiogenesis, and their correlation with clinical, surgical, and magnetic resonance imaging findings. Spine. 2010;35:881-6.