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SHORT REPORT

Enhancement of osteogenic gene expression for the differentiation of human periosteal derived cells

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Abstract The osteogenic differentiation of progenitor populations allows analysis of cell functionality as well as creating a platform for investigating stem cells for bone tissue engineering. Protocols used for osteogenic differentiation of progenitor cells are often identical to those detailed for bone marrow mesenchymal stem cells, however this may be flawed due to cell populations residing in different niches and being in distinct stages of differentiation. We herein describe the individual and combined effects of known osteo-inductive agents; dexamethasone (Dex), 1,25-dihydroxyvitamin D3 (VitD3), all trans-retinoic acid (atRA), cyclic AMP (cAMP) and bone morphogenic protein 2 (BMP2) in combination with fetal bovine serum (FBS) on osteogenesis of human periosteal derived cells (hPDCs). The addition of Dex&FBS was essential for the transition of hPDCs to an ALP positive cell population. Subsequently, atRA, Dex&FBS and BMP2 were required for the expression of transcription factors governing osteogenesis and hence differentiation towards a mature osteoblast. It is also hypothesized that Dex has no direct effect on the differentiation of hPDCs, instead its effect is to augment differentiation in combination with other factors. These data provide a comprehensive assessment of known osteogenic factors, in a novel multiplex system, to evaluate their effect on progenitor cell differentiation.

Introduction

The *in vitro* differentiation of progenitor cells allows researchers to manipulate cellular environment, signaling and interactions without the interference of other cell/tissue types. These differentiation models are not only useful for increasing the understanding of stem cell kinetics but are also applicable to tissue engineering as a system to investigate tissue formation *in vitro*. It has also been previously documented that the treatment of progenitor cells with factors which enhance differentiation *in vitro* can have a positive effect on tissue formation *in vivo* (Siddappa et al., 2008; Song et al., 2009). Thus, to allow the use of stem cells as a cell source for clinical applications, establishment of defined differentiation conditions for specific stem cell populations is of utmost importance.

When considering osteogenic differentiation most work has been carried out on bone marrow mesenchymal stem cells (BM-MSCs). The current gold standard for osteogenic differentiation of this cell type is regarded as basal medium

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containing 10% fetal bovine serum (FBS), dexamethasone (Dex), β-glycerophosphate (β-GP) and L-ascorbic acid-2-phosphate (AsAP) (Jaiswal et al., 1997). This formulation has been shown to exhibit a robust differentiation for BM-MSCs, however some caveats exist for alternative cell populations. For example, Dex causes a dual differentiation of adipose derived cells to both adipocytes and osteoblasts under osteogenic conditions (Arutyunyan et al., 2009). This has been attributed to Dex potentially augmenting differentiation of MSCs and not specifically directing differentiation (Oshina et al., 2007). This effect is unsurprising considering most progenitor cells are developmentally in various stages of differentiation and residing within different niches. Alternative osteogenic stimulators have been described including 1,25-dihydroxyvitamin D3 (VitD3) (Liu et al., 1999), all trans-retinoic acid (atRA) (Wan et al., 2006), cyclic AMP (cAMP) (Siddappa et al., 2008) and bone morphogenic protein 2 (BMP2) (Rickard et al., 1994), however confounding results on osteogenic differentiation have also been documented with many of these. For example Dex has been shown to block osteogenic differentiation of murine osteoprogenitor cells with VitD3 shown to down regulate osteocalcin (OCN) within the same cell type (Lian et al., 1997). Additionally, FBS within cell culture media can also have a negative effect on differentiation due to endogenous growth factors interfering with cellular processes such as BMP signaling (Zilberberg et al., 2007).

A major limitation in studies aiming to elucidate differentiation conditions is the absence of investigating interactions between inductive agents. This limitation is perhaps due to the absence of quantitative experimental designs for the assessment of multiple factors in a multiplex assay, which can be used to define factors and interactions to promote cellular differentiation. We herein provide a novel methodology to elucidate effects of known osteogenic factors on the differentiation of human periosteal derived cells (hPDCs). These are progenitor cells which are known to be involved in bone development and postnatal repair processes and are therefore a promising starting population for clinically relevant skeletal tissue engineering (Roberts et al., 2011). The analysis of differentiation achieved through a factorial design strategy which allows the direct contribution of each factor to measurable outcomes to be evaluated along with potential interactions thereof. This work represents a first step to elucidate specific factors which can enhance the osteogenic differentiation of hPDCs. Additionally, it provides a novel strategy for the investigation of stem cell differentiation for the development of cell-customized *in vitro* conditions as part of a tissue engineering approach.

Results

Morphological changes in hPDC cultures

To investigate the effect of Dex, VitD3, atRA, BMP2, cAMP and FBS on osteogenic differentiation of hPDCs, a factorial design was employed utilizing high and low concentrations of each factor. Sixteen different compositions of media were formulated (Table 1) and used to grow cells for a period of 14 days. Note that differentiating condition 1 (Con-1) contains basal low levels of all factors and condition 16 (Con-16) contains high levels of all factors. Upon analysis of cultures differences were observed in cell morphology, as shown in Fig. 1. Media containing 1% FBS resulted in visibly less cells (Fig. 1, panels 1–8), however cell morphology varied from fibroblastic-like (Fig. 1, panels 1, 2, 6, 7, 8) to cuboidal-like cells (Fig. 1, panels 3, 4, 5; white arrows). High FBS containing media resulted in visibly more cells (Fig. 1, panels 9–16), also with variable morphology including nodule like structures (Fig. 1, panels 10, 14, 15; white stars) and also cuboidal cells (Fig. 1, panel 11; white arrows), indicating a

Table 1 Experimental setup (resolution IV and two-level fractional factorial design (2^{6-2} design)) showing patterns of osteogenic stimulants in each differentiation condition. Dex, atRA, VitD3, cAMP, BMP2 and FBS were added to basal media containing 50 μg/ml ascorbic acid 2-phosphate (AsAP) and 10 mM β-glycerophosphate (β-GP), in the concentration and the pattern indicated, to produce 16 different differentiation conditions (1 to 16).

Condition	Pattern	Dex (nM)	atRA (μM)	VitD3 (nM)	cAMP (μM)	BMP2 (ng/ml)	FBS (%)
1	−−−−−−	0	0	0	0	0	1
2	−++−−−	0	5	100	0	0	1
3	++−+−−	100	5	0	500	0	1
4	+−++−−	100	0	100	500	0	1
5	+−−−+−	100	0	0	0	50	1
6	+++−+−	100	5	100	0	50	1
7	−+−++−	0	5	0	500	50	1
8	−−+++−	0	0	100	500	50	1
9	++−−−+	100	5	0	0	0	10
10	+−+−−+	100	0	100	0	0	10
11	−−−+−+	0	0	0	500	0	10
12	−+++−+	0	5	100	500	0	10
13	−+−−++	0	5	0	0	50	10
14	−−+−++	0	0	100	0	50	10
15	+−−+++	100	0	0	500	50	10
16	++++++	100	5	100	500	50	10

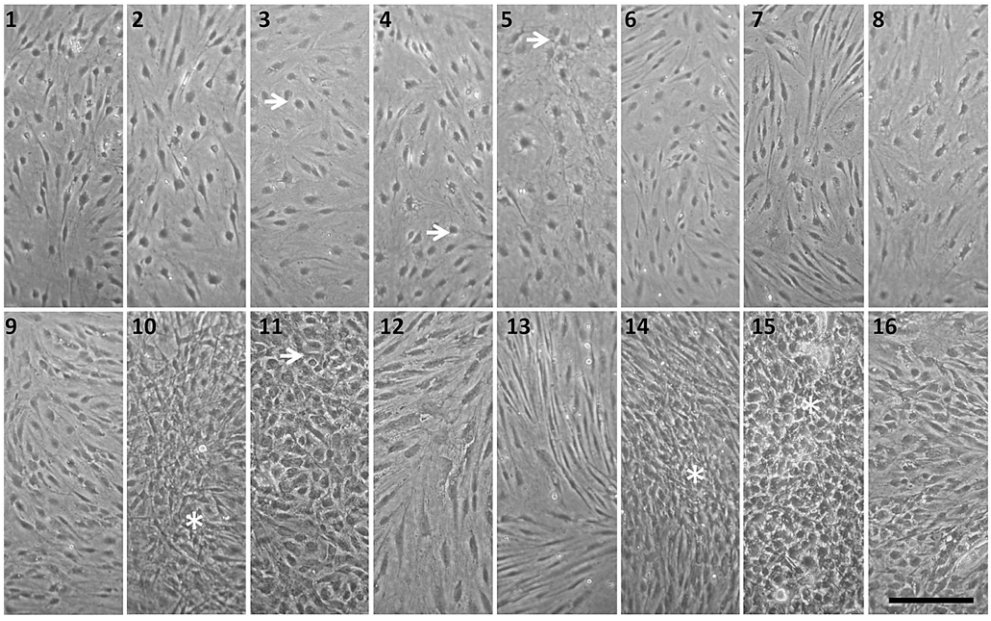

Figure 1 Cell morphology of hPDCs undergoing osteogenic differentiation. hPDCs were cultured and differentiated using each of the differentiation conditions as outlined in Table 1 using the techniques described in Materials and methods. Following 14 days microscopic images depicting cellular morphology were taken. Note the presence of cuboidal cells (white arrows) and nodular-like structures (white stars) in certain conditions. (Scale bar: 200 μm).

range and extent of differentiation when comparing each of the 16 conditions.

Analysis of proliferation/differentiation

Analysis of cell number from each culture confirmed the observations described above. Cultures 1 to 8 (1% FBS) contained between $1.1\times10^4\pm1213$ cells/cm^2 and $1.8\times10^4\pm1234$ cells/cm^2 which, when compared to the condition containing the highest cell number (condition 10), was approximately 10 fold lower (Fig. 2I). The high FBS concentration (10%) allowed greater cell proliferation which allowed for cell numbers between $2.9\times10^4\pm1061$ cells/cm^2 and $12.5\times10^4\pm9145$ cells/cm^2. Alkaline phosphatase (ALP) activity was highest in culture 10 (121.2 ± 12.3 R.U./μg DNA) and lowest in condition 13 (5.6 ± 0.7 R.U./μg DNA) (Fig. 2II). Gene expression for transcriptional co-activator with PDZ-binding motif (TAZ) revealed higher expression in cultures 1 to 8 when compared to 9 to 16 (Fig. 2III). Runx2 expression was variable throughout the 16 culture conditions (Fig. 2IV). Osterix expression peaked in conditions 7, 8 and 13 (Fig. 2V) and OCN expression was high in conditions 2, 8 and 14 (Fig. 2VI).

Contribution of specific factors to osteogenic differentiation

As each of the 16 culture conditions was designed using factorial analysis, the specific contribution of each factor, and potential interactions thereof, could be assessed for significance to each measured output. When considering the 'main effects' from each of the factors it was clear that FBS had the largest positive effect on cell proliferation, conversely atRA had the largest negative effect (Fig. 3I). When considering ALP activity atRA had the largest negative effect; with the largest positive effect for ALP coming from cAMP. However potential interactions were considered, the largest overall positive effect for ALP activity came from the interaction of Dex with FBS (Fig. 3II). TAZ expression was mainly stimulated with atRA, with a potential positive interaction with cAMP, and repressed by FBS (Fig. 3III). Dex and BMP2 had the largest negative effect on Runx2 expression. Only FBS had a significant positive effect on the expression of this gene. Interestingly, when factor interactions were considered Dex had a possible positive effect on Runx2 expression when combined with atRA and/or FBS (Fig. 3IV). BMP2 had the largest positive influence on the expression of Osterix with Dex&FBS having the largest negative effect (Fig. 3V). VitD3 exhibited the greatest positive influence on OCN expression with Dex contributing predominantly to the repression of this gene (Fig. 3VI).

Discussion

Osteogenic differentiation, as observed during intramembranous ossification, involves the following processes: (1) cell proliferation (2) cell migration–aggregation and (3) cell differentiation with the dynamic expression of osteogenic transcription and growth factors (Karner et al., 2009). To replicate this differentiation profile, culture conditions which will allow promotion of these key events would be required. Early osteogenic differentiation is characterized firstly by a proliferative burst, including the formation of nodule-like structures, accompanied by the expression of ALP. ALP induction is a key event in the differentiation of osteoblasts as it mediates matrix mineralization and hence bone tissue formation. It is also one of the earliest markers of osteoblastic differentiation.

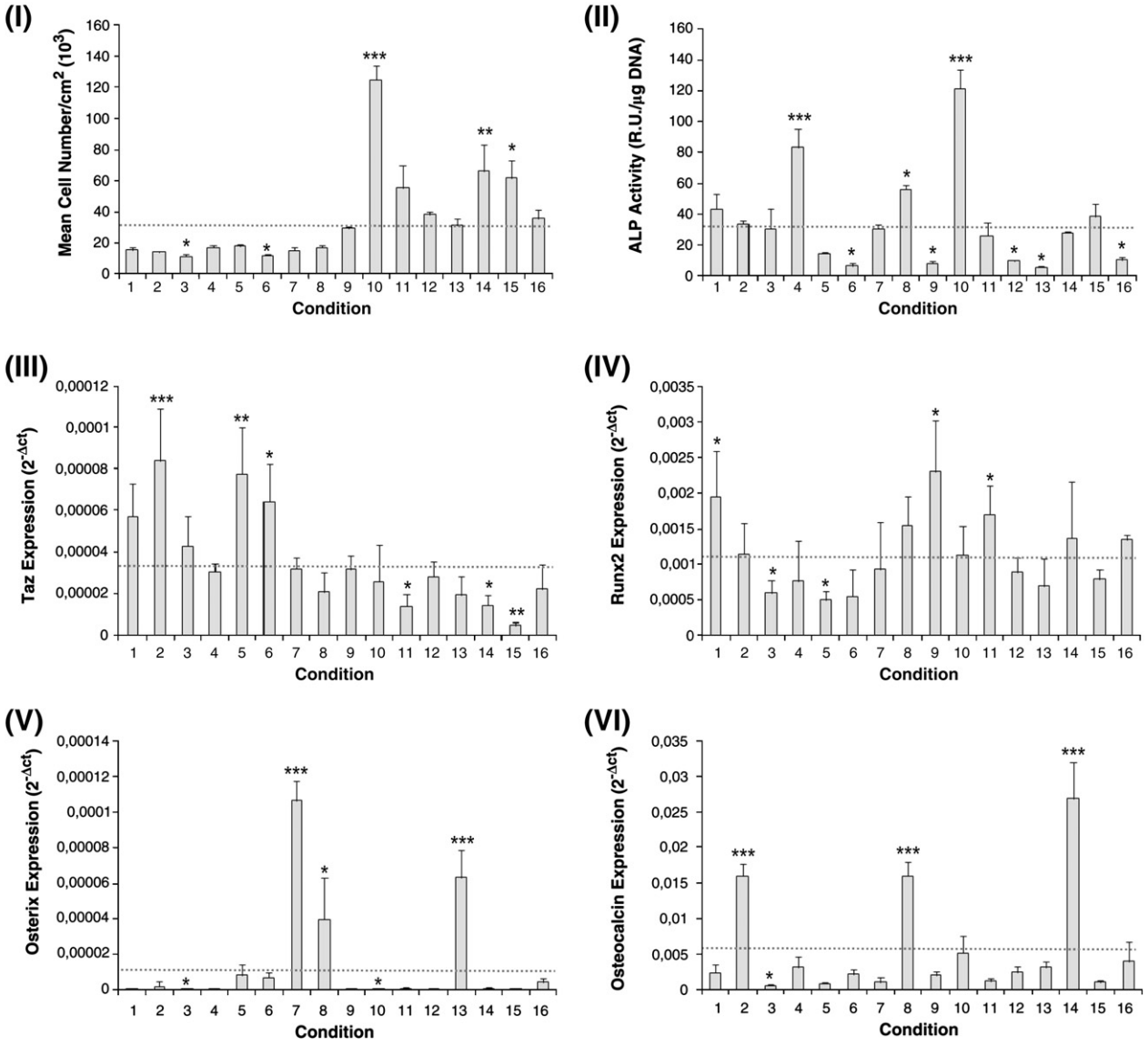

Figure 2 Analysis of hPDC proliferation and osteogenic differentiation. hPDCs were cultured and differentiated using the 16 differentiation conditions outlined in Table 1. Following 14 days DNA was extracted, ALP activity (R.U. = absorbance at 620 nm) monitored and osteogenic gene expression quantified described in Materials and methods. (I) Cell number, (II) ALP activity, (III) TAZ expression, (IV) Runx2 expression, (V) Osterix expression and (VI) Osteocalcin (OCN) expression. Statistical significance is compared to the mean of the data (horizontal dashed line). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Proliferation of hPDCs was positively influenced by FBS and to a lesser extent VitD3. Interestingly, nodule-like structures (Fig. 1) were only observed in FBS-containing media indicating the requirement of proliferation for this effect. atRA has the greatest negative effect on both cellular proliferation and ALP induction, which has been reported previously with alternative cell types including vascular smooth muscle cells (Kosaka et al., 2001) and osteoblast-like cells respectively (Kaji et al., 1995; Ogston et al., 2002). Conversely, atRA has also been reported to have a stimulatory effect (James et al., 2010) on ALP activity suggesting cellular specificity when considering this factor. The addition of cAMP to cultures has the greatest, single factor, stimulatory effect on ALP induction, which has previously been documented with human MSCs. Addition of cAMP to MSC cultures has also previously been shown to improve *in vivo* bone formation (Siddappa et al., 2008). Additionally, the interaction of Dex and FBS (Dex&FBS) has the largest overall positive effect on ALP induction. Indeed it has been previously documented that an ALP-negative cell population could form bone nodules *in vitro* only after Dex treatment, whereas ALP-positive populations could form nodules *in vitro* without Dex (Turksen and Aubin, 1991). VitD3 also had a stimulatory effect on ALP activity within hPDCs, which has been previously reported (Uchida et al., 1988), however this was less than that observed with Dex&FBS and cAMP. Consequently, it is clear that Dex&FBS and cAMP are required to facilitate the differentiation of hPDCs to early osteoprogenitors which display positivity for ALP.

To further elucidate factors which allow differentiation to specific stages of maturation, key transcription factors which

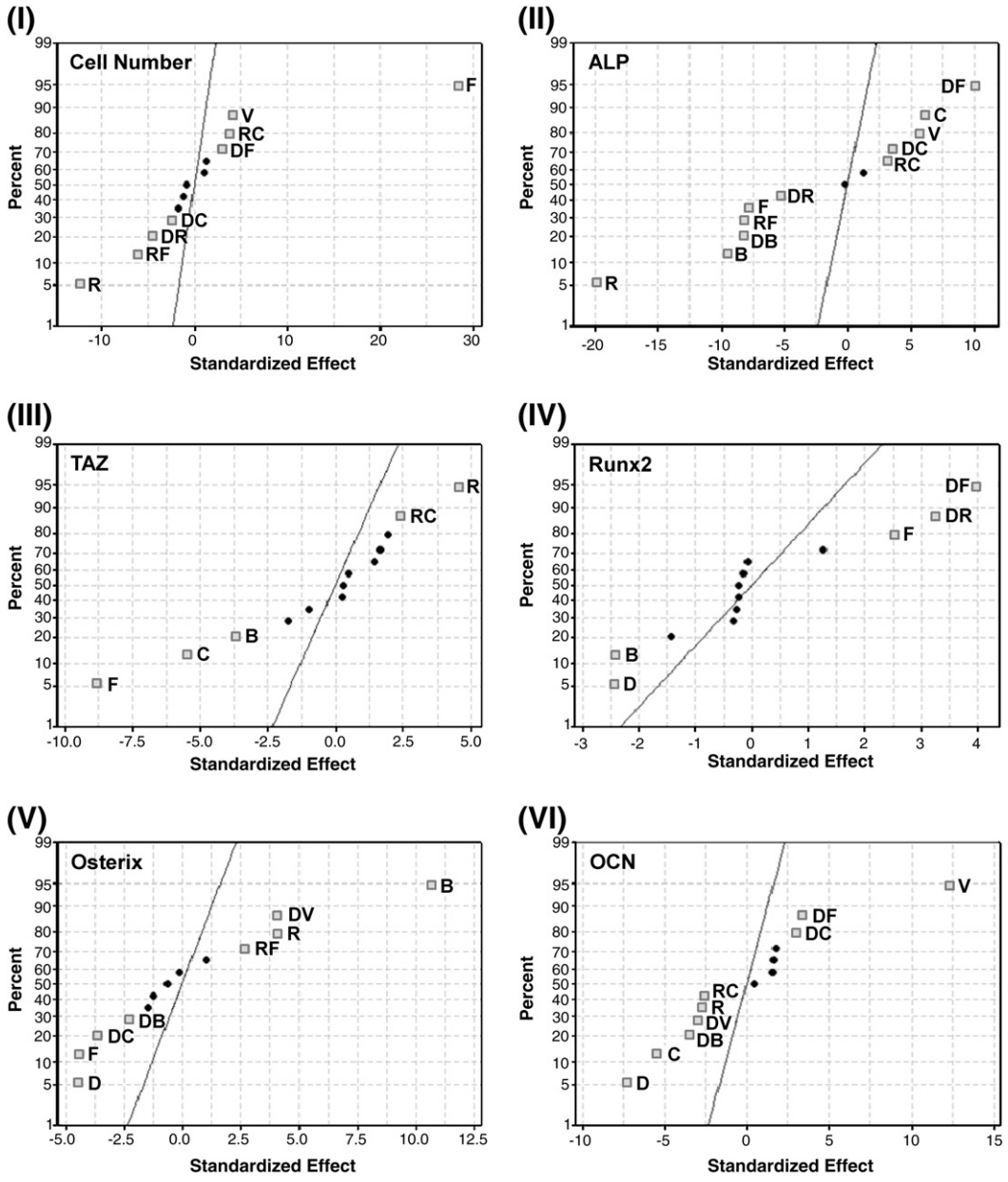

Figure 3 Plots of the standardized effects for each factor. Normal probability plots of main (one) factor effects and potential two-factor interactions for (I) DNA content, (II) ALP activity, (III) TAZ expression, (IV) Runx2 expression, (V) Osterix expression and (VI) OCN expression. Each factor is defined by the following single letter code (D) Dex, (R) atRA, (V) VitD3, (C) cAMP, (B) BMP2, (F) FBS. Statistically significant factors ($p<0.05$) are indicated in gray while black points indicate non-significant factors. The diagonal line transecting each plot indicates perfect non-significance, thus increasing departure from this line implies increasing significance.

govern differentiation of MSCs to osteoblasts were investigated. TAZ expression has been previously hypothesized to indicate the degree of differentiation of MSCs towards the osteoblastic lineage. TAZ acts downstream of Runx2 and assists in secretion of bone matrix-related proteins (Hong et al., 2005). Runx2 is involved in osteoblast differentiation and bone formation. In particular, Runx2 is required for early commitment of MSCs to osteoprogenitors, and also functions later in osteoblast differentiation to regulate the formation of the extracellular matrix (Ducy et al., 1999). Osterix is a zinc finger-containing transcription factor that is specifically expressed in osteoblasts of all skeletal elements and acts downstream of BMP2/p38 signaling (Ulsamer et al., 2008).

atRA has the greatest positive effect on TAZ transcription with FBS having the greatest negative effect, indicating that cellular proliferation is inhibitory to the expression of this transcription factor. It has previously been suggested that atRA may play an important role in the expression of this gene (Wan et al., 2006), however, this has previously not been experimentally defined. Furthermore, FBS stimulated the production of Runx2 while Dex inhibited it. However, the indicated potential interactions with Dex which have a positive effect on Runx2 expression further highlights the complexities of *in vitro* osteogenic differentiation. When considering the expression of Osterix again Dex has an inhibitory effect with the greatest positive effect being

attributed to BMP2. This interaction has previously been described and indeed has been shown to exist in a Runx2 independent fashion (Lee et al., 2003). Interestingly, FBS has a negative effect on Osterix expression which may be due to reduced BMP signaling as Osterix lies downstream from BMP/p38 and FBS has previously been shown to have a detrimental effect on this pathway (Zilberberg et al., 2007). These data suggest that expression of the key regulators of osteogenic differentiation within hPDCs is reliant on the activation of Dex, atRA and BMP mediated signaling pathways. Additionally, BMP2 has a dual function within the final stages of differentiation by inducing Osterix while simultaneously repressing Runx2, as observed developmentally (Komori, 2008). This gives rise to the hypothesis that the sequential addition of these factors may potentially nurture hPDCs through osteoblastic differentiation as observed during intramembranous ossification (a schematic representation of this is shown in Supplementary Fig. 1). Interestingly, synergy between these key regulators of osteoblastogenesis has been previously documented, albeit in established cell lines (Benayahu et al., 1994).

OCN is a marker of late stage osteogenic differentiation and regulates matrix mineralization during bone formation. VitD3 had the greatest positive effect on this transcript while Dex had the greatest negative effect. This effect has previously been documented for VitD3 supplemented osteoblast cultures (Viereck et al., 2002), however not for hPDCs. Interestingly, Dex again has a negative effect on the expression of this gene indicating that perhaps the role of Dex is in early augmentation of differentiation and the effect on late differentiation is negative. This hypothesis is substantiated through reports that Dex can attenuate VitD3 induced OCN expression (Schepmoes et al., 1991). Indeed it has been reported that the main effect of Dex on osteoblasts is through the modulation of cell cycle regulatory elements such as increased p27kip1 and decreased p21waf1/cip1 levels. The attenuation of osteoblast growth to high density by Dex is associated with severe impairment of mineralized extracellular matrix formation (Smith et al., 2000), however this may also be related to the timing cellular stimulation with Dex (McCulloch and Tenenbaum, 1986). Additionally, although VitD3 has the largest positive effect on OCN expression a smaller, but significant, positive effect is observed with the interaction of Dex&FBS. This may be due to the positive effect of the Dex&FBS interaction on Runx2 expression as OCN is a direct target of this transcription factor (Frendo et al., 1998).

In conclusion, we have herein demonstrated a novel methodology for the investigation and customization of stem cell differentiation using multiple factor analysis. From this it is clear that addition of FBS and Dex are essential for the transition of hPDCs to an ALP positive cell population. Subsequently, atRA, Dex&FBS and BMP2 are required to allow expression of the main transcription factors governing osteogenesis and hence differentiation towards a mature osteoblast. Although we propose these factors as key regulators in osteogenic differentiation of hPDCs, further work is required to elucidate the precise mechanism by which their influence is exerted. This is highlighted when considering the negative or no effect of certain factors individually, which is changed to a positive effect when combined with other factors in a proposed interaction. This is hypothesized to be due to multiple signaling pathways being affected simultaneously and emphasizes the complexity of stem cell differentiation. We also propose that the use of specific factors may alter the rate of osteogenic differentiation, as despite using a single time point, a range of early and late markers were apparent within each of the conditions. This study provides the first insight on how to optimize differentiation conditions with respect to specific progenitor cell types. This will allow further study on osteogenic differentiation of hPDCs for bone tissue engineering.

Materials and methods

Harvest of periosteal tissue and isolation of the cells

Periosteal biopsies (0.5 cm^2) were harvested from the medial side of the proximal tibia of male and female adolescent and adult patients during total knee replacement surgery or distraction osteogenesis. The periosteum was stripped from the tibia with a periosteal lifter. The periosteal specimens were transported in growth medium consisting of high-glucose Dulbecco's Modified medium (DMEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% FBS (BioWhittaker, Verviers, Belgium) and antibiotic–antimycotic solution (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen, Merelbeke, Belgium). The biopsies were finely minced and digested overnight at 37 °C in 0.2% type IV collagenase (Invitrogen, Merelbeke, Belgium) in growth medium (as described above). Subsequently periosteal cells were collected by centrifugation and seeded in a T25 flask in growth medium. Non-adherent cells were removed after 5 days by changing the medium. A pool of hPDCs was subsequently created [n = 6, age 14.9 (SD ± 2.1)] to reduce noise from genetic variability. The ethical committee for Human Medical Research (Katholieke Universiteit Leuven) approved all procedures, and patient informed consent forms were obtained.

Cell culture

Cells were expanded in monolayer in growth medium. Upon confluence, hPDCs were trypsin released (0.25% trypsin, 1 mM EDTA; Invitrogen, Merilee, Belgium) and re-plated with a seeding density of 7500 cells/cm^2. For cryopreservation, hPDCs were suspended in DMEM with 20% FBS and 10% DMSO (Sigma, Borne, Belgium) and stored in liquid nitrogen. For the *in vitro* osteogenic differentiation cells were thawed, re-plated and expanded in 12 well plates at a density of 10,000 cells/cm^2 in growth media containing 50 μg/ml AsAP and 10 mM β-GP.

Factorial design and osteogenic stimulation

A resolution IV and two-level fractional factorial design (2^{6-2} design) in triplicate was implemented as experimental design in this study. There were six factors used for this design: Dex, VitD3, atRA, BMP2, cAMP and FBS, resulting in sixteen runs for every medium formulation. Each factor was investigated at a high (+1) and a low (−1) level (design and concentration is indicated in Table 1). Resolution IV means that no main effect is aliased with any other main effect or with any two-factor

interaction, but two-factor interactions are aliased with each other (Box et al., 1978). The experimental data collected from the implemented fractional factorial design was processed by the Minitab 14 statistics package to obtain relative contribution of each factor to the final effect.

Osteogenic stimulants and respective concentrations were selected following a thorough review of literature on osteogenic differentiation of progenitor cells. Sixteen different combinations of osteogenic stimulants were created with alternative high and low levels of each factor as shown in Table 1. All factors were purchased from Sigma. Cells were seeded at 7500 cells/cm^2 and following 48 h culture the different media formulations were added randomly, in triplicate, to individual wells of 12 well plates. The media was changed every 3 days and cells harvested on day 14 for ALP, DNA and RNA analysis.

Biochemical and molecular analysis

Cell cultures were harvested and lysed in 0.05% Triton-X100 (in PBS). The DNA content was quantified using Quant-iT™ dsDNA HS Assay Kit (Invitrogen). The concentration of DNA was converted to a predicted cell number using a pre-determined value of 6.93 pg DNA/hPDC. The ALP levels were measured using BluePhos® Microwell Phosphatase Substrate System at 620 nm (Kirkegaard & Perry Laboratories). The ALP activity was calculated by normalizing the absorbance of each sample to the DNA content of each well (R.U. = absorbance at 620 nm).

Total RNA of each sample was isolated by using Nucleospin® RNA extraction kit (Macherey-Nagel) and quantified by using Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was synthesized by reverse transcription of 250 ng of total RNA using RevertAid™ H Minus First Strand cDNA synthesis kit with Oligo(dT)$_{18}$ as primer (Fermentas). SYBR green qPCR primers were designed to span an intron so only RNA specific amplification was possible. The PCR reaction was cycled in a Rotor-Gene sequence detector as follows: 95 °C for 3 min, 40 cycles of 95 °C for 3 s and 60 °C for 60 s. Each sample was tested in duplicate and compared with β-actin expression as described previously (Chai et al., 2011). Relative differences in expression were calculated using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008).

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was determined using ANOVA with Minitab 14. Statistical significance is indicated as follows: *: $p<0.05$, **: $p<0.01$, and ***: $p<0.001$.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.04.003.

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