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Small molecule stimulation enhances bone regeneration but not titanium implant osseointegration

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ABSTRACT

The osteogenic and osseointegrative potential of a small molecule was examined to assess its usefulness in regenerative procedures. Purmorphamine was used to stimulate bone growth and repair in an *in vitro* cell-based assay and an *in vivo* chick embryo CAM-assay with and without the presence of an implant. Purmorphamine adhered to precipitated hydroxyapatite coating, could activate the sonic hedgehog pathway and thereby stimulated osteodifferentiation. Porous calcium phosphate beads were used to deliver this small molecule *in vivo* and showed that purmorphamine increased the trabecular bone to bone area significantly. The assay showed purmorphamine failed to induce any significant difference in osseointegration on titanium coated PTFE implants. This suggests that, while a small molecule can enhance osteogenesis and might be useful in regenerative procedures, it failed to enhance the osseointegration of a Ti coated implant, suggesting that this sort of stimulation might be useful for enhancing bone regeneration where bone loss due to disease exists, but not for enhancing early stability of an implant.

Introduction

Replacement of bone is an on-going challenge for surgeons in skeletal and craniofacial restoration and applied scientists in bone engineering. The need for dental or craniofacial restoration exists as bone loss in the jaws often occurs due to disease or to the removal of large sections of bone due to cancer or injury. There are currently two main approaches: the use of autologous bone from elsewhere in the body (*e.g.* fibula or iliac crest) [1], or the use of implants *e.g.* titanium prostheses. In fact, the approach required depends upon whether mechanical strength and structure are needed, or whether the bone needs to be regenerated in a non-load-bearing situation (*e.g.* alveolar bone loss). Tissue engineering and regenerative medicine have sought to provide alternatives, with only limited success, since the demands are very stringent—especially where the synthetic tissue must possess mechanical strength.

Prostheses and implants restore excellent function when fully integrated and are unlikely to be replaced by tissue engineered constructs; however, there remains considerable room for improvement at the level of integration [2]. Methods for the filling of defects have been less successful as it has not been possible to trigger the required biological response using hydroxyapatite, de-cellularized bone, or other packing materials. Although these two approaches are very different, they share the common element of needing to generate a new bone to fix or integrate an implant or to replace a resorbable matrix during void filling. Failure of this bone generation leads to the formation of fibrous tissue due to movement at the bone–material interface of an implant or due to failure of osteogenic differentiation in the scaffold [3].

The aim of this research is to examine whether small molecules which stimulate the hedgehog pathway can accelerate the formation of bone and improve the integration of titanium implants [4].

Long bone fracture repair is mediated by a cartilaginous soft callus that affects bony union through the stimulation of bone formation around the callus and replacement of the cartilage itself by marrow and bone tissue [5]. The same process of endochondral ossification occurs in the embryo [6]. There are two recognized processes occurring: the induction of the peripheral bone (cortical) which will ultimately be the load-bearing cylinder of a long bone, and the replacement of the cartilage scaffold by internal bone and marrow. These two processes match those required to enhance the integration of an implant (a peripheral bone layer fused to the existing bone) and also the replacement of a 3-D scaffold by bone. In order to replace a resorbable construct, it must first be invaded by angiogenic sprouts followed by the formation of the bone collar around the periphery of the cartilage. Paracrine hedgehog signaling is necessary for both of these events [7–10]. Manipulation of this pathway is therefore a good target for the stimulation of

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bone growth in humans [11,12]. It is of interest that in the absence of the one molecule necessary for both these processes during embryogenesis, Indian hedgehog, neither part of the endochondral ossification occurs [7]. This process represents bone formation in trans – one cell type induces the formation of another (cartilage inducing bone) – the cells that give rise to the inducing signals (and extra-cellular matrix) do not themselves produce the bone.

Previously, purmorphamine (Pur) that selectively induces osteogenesis in multipotent mesenchymal progenitor cells was identified [13]. Purmorphamine has been shown to increase alkaline phosphatase (ALP) activity in both cell lines C3H10T1/2 and MC3T3-E1 and enhances osteoblastic differentiation of human bone marrow mesenchymal cells in culture and also when grown on titanium [14,15]. Further, it also seems to inhibit adipocyte maturation [16,17]. Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. The transmembranic protein smoothened (Smo) is normally suppressed by another transmembranic protein patched (Ptch); this suppression is inhibited by sonic hedgehog protein in the developmental stage. It has been shown that Smo can be artificially targeted by Pur and the suppression by Ptch on Smo is stopped, leading to an activation of Smo and thereby the hedgehog signaling pathway leading to stimulation of bone formation. In this way Pur can replace the function of sonic hedgehog (Fig. 1a) [18].

When the Smo inhibition is blocked by a hedgehog protein, Smo can activate members of the Gli-family. Genetic studies have shown that mutations in Gli2 and/or Gli3 result in severe defects in skeletal development in mice and humans [19–22]. Ablating the hedgehog genes in postnatal chondrocytes leads to dwarfism, showing that the hedgehog is essential for maintaining the growth plate and articular surface and is required for sustaining trabecular bone and skeletal growth [23]. It has been shown that Gli2 is a powerful transactivator of the BMP-2 gene *in vitro* and *in vivo* and that overexpression of Gli2 in osteoblast precursor cells induces osteoblast differentiation [24]. This and the combined effect of BMP-2 [25], explain the osteogenic induction by the hedgehog pathway activation [26–28].

The mode of delivery of Pur is as important as the biology of its effect as diffusion makes a simple injection ineffective. Delivering sonic hedgehog or purmorphamine by binding it to a calcium phosphate layer should stimulate differentiation and proliferation locally and spread in a controlled manner by the release of calcium phosphate. This delivery system avoids the immediate burst-release of the active molecule and allowing the osteogenesis of the surrounding precursor cells.

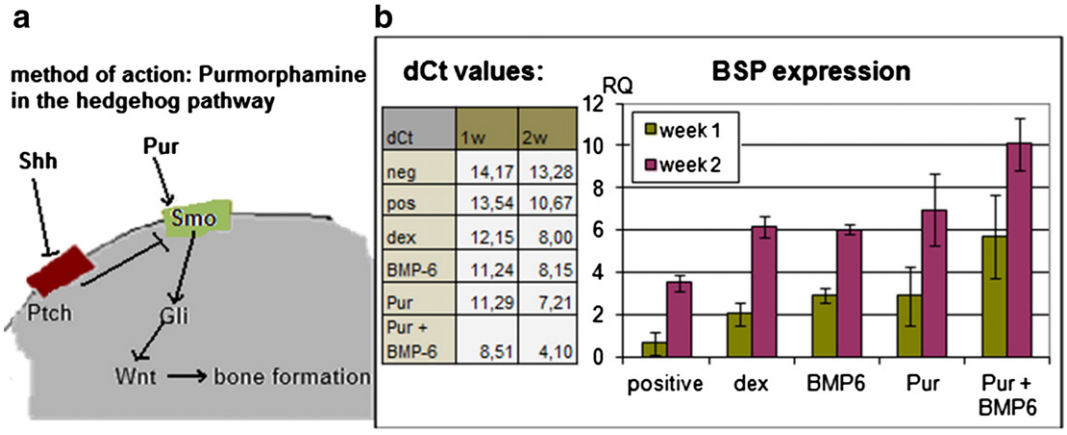
Calcium phosphate (CaP) is a natural component of the bone, making a CaP or CaP coated delivery system ideal for the delivery of signal molecules to enhance bone formation [29]. A CaP coating can be made by sintering or in a biomimetic way, with the latter having the advantage of being able to incorporate bioactive molecules into the coating without destroying their biological activity. Since purmorphamine has never been tested when adhered on an HA-coating, preliminary *in vitro* experiments were performed in order to study if its ability to increase the Gli expression is maintained.

Some bone agonists have been implanted in ectopic sites to demonstrate their osteogenic properties [30–32], but purmorphamine's potential has not been tested, let alone delivered in an *in vivo* bone defect. The assay system that was developed for this study, uses the chorioallantoic membrane (CAM) of the chick to support the growth and repair of explanted calvarial bone tissue [33]. This method shows chondrocyte-derived agonists can stimulate the pathways involved in endochondral bone formation and these agonists can be replaced by a small molecule. The same assay is used to evaluate the integration of an implant; the effect of a titanium coating and the addition of purmorphamine are examined histologically and mechanically.

Material and methods

Purmorphamine activity in medium and adhered onto CaP

Cells were isolated from the calvaria of neonatal mice (ICR-CD1, Harlan, Oxon, UK) at P5, as previously described [34] based on the original method [35]. In brief, sequential digests with crude Type IA collagenase (Sigma, UK) were used on pooled calvaria (from 10 to 20 pups), those cells being released first were discarded and subsequent fractions (up to 4) were collected and pooled. Cells were maintained and expanded for a maximum of 2 passages and cultured in LG DMEM (Invitrogen, Paisley, UK), 10% FBS (PAA, Farnborough, UK), p/s (PAA) and ascorbate-2-phosphate (50 μg/ml; Fluka) (=negative medium). Real-time Q-PCR analyses were used to check the upregulation of the osteoblast differentiation marker *Bsp* after 1 and 2 weeks of culture in neg. medium, pos. medium (=neg. medium + 10 mM β-glycerophosphate (Invitrogen)), Dex (=pos. medium + 10^{-8} M dexamethasone) [36–38], BMP-6 (=pos. medium + 100 ng/ml BMP-6 (R&D Systems, UK)) [39,40], Pur (=pos. medium + 2 μM purmorphamine (Calbiochem, Beeston, UK)) and Pur + BMP-6 (=pos. medium + 2 μM purmorphamine + 100 ng/ml BMP-6). RNA was extracted using Trizol (Invitrogen) according to the manufacturer's guidelines; cDNA was prepared using a cDNA archive kit (Applied Biosystems) and Q-OPCR was carried out according to the protocols for the ABI 7300 Real-time PCR machine in 96 well formats. Taqman gene expression primer details were as follows: *GapdH*: Mm_99999915-g1; *Bsp*:



dCt	1w	2w
neg	14,17	13,28
pos	13,54	10,67
dex	12,15	8,00
BMP-6	11,24	8,15
Pur	11,29	7,21
Pur + BMP-6	8,51	4,10

Fig. 1. [a] Purmorphamine activates the hedgehog pathway by stimulating Smoothened instead of inhibiting Patched which inhibits Smoothened like Sonic hedgehog protein does; this leads to more Gli expression, upregulating several other pathways including Wnt pathway, leading to more bone formation; [b] RNA analysis showed the upregulation of Bsp when purmorphamine was administered to a similar degree as Dex and BMP-6, but following another route as a combination of Pur and BMP-6 showed a synergistic effect; dCt = Ct values relative to Ct(GapdH), RQ; relative to dCt of Bsp of cells in negative medium (DMEM + 10%FCS + p/s + Asc).

Mm_00492555-m1 (Applied Biosystems); Q-PCR was analyzed using the relative expression software tool (REST) [41].

Calcium phosphate precipitation and purmorphamine adhesion

In the following *in vitro* tests, plastic Thermanox® coverslips (Nalge Nunc Int., Naperville, IL, USA) coated with calcium phosphate were compared to plain plastic of the 6 and 24-wells, following the method suggested as a carrier for osteoinductive agents described by Liu et al. [42]. The coverslips were cleaned in acetone, then in 70% ethanol, and in demineralized water subsequently. Before being immersed in simulated body fluid (SBF: 142 mM Na^+, 5 mM K, 1.5 mM Mg^{2+}, 2.5 mM Ca^{2+}, 147.8 mM Cl^-, 4.2 mM HCO_3^-, 1.0 mM HPO_4^{2-}, 0.5 mM SO_4^{2-} using NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 (Sigma, UK)) (5×) for 24 h at 37 °C, the pH had first been adjusted to 6.5 by the passage of gaseous carbon dioxide through the SBF (5×) [42]. The slow rise of pH by the release of CO_2 and the addition of Mg-molecules stimulated the high nucleation precipitation of calcium phosphate on the coverslips. After rinsing with PBS, a second coating was performed in lower nucleation conditions using Hank's Balanced Salt Solution HBSS with 3.5 mM $CaCl_2$ added for 48 h at 37 °C. In this second slower coating signal molecules can be added to incorporate them into the Crystal lattice. Purmorphamine molecules were thereby adhered with a simple heat immobilization procedure; after the CaP coating is added, 1 ml of distilled water with 200 μM purmorphamine per disc was allowed to evaporate on the surface by heating to 60 °C for several hours.

Raman analysis of the CaP discs

A Raman spectrum of the CaP coated sample was obtained using a LabRam spectrometer (Horiba Jobin Yvon, Stanmore, UK). This was equipped with a 633 nm laser, grating of 1800 and ×50 objective. Wavenumber range of 800 to 1650 cm^{-1}, scan time of 5 s and sample number of 20 were used. After smoothing and background subtraction the sample spectra were compared with those obtained for hydroxyapatite and thermanox.

Light II reporter cells

Light II reporter cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM with 4 mM L-Glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate supplemented with 0.4 mg/ml G-418 (Autogen Bioclear, Calne, UK) and 0.15 mg/ml Zeocin (Autogen Bioclear) and 10% fetal calf serum (PAA). G-418 was used to select for the firefly and Zeocin for the Renilla luciferase reporter gene. After being cultured to a maximal density, 10,000 cells/ml Light II cells were seeded on plastic or on CaP discs using an assay DMEM-medium supplemented with 0.5% fetal calf serum, 5 mM HEPES buffer (pH 7.4) and the signal molecules if not already adhered on the CaP. To measure activity of the adhered purmorphamine after release in the medium, CaP coated discs with the agonist were put in DMEM for 24 h or 2× 24 h before the Light 2 cells were seeded onto them.

The cells growing on the CaP coated discs were visualized using ^^a toluidine blue stain after fixation in 4% PFA for 24 h and photographed with a digital camera (Nikon Coolpix 4500) attached to a stereomicroscope (Zeiss Gmbh, Jena, Germany). To visualize the ability of cells to attach onto the CaP surface and how this might influence the shape of the cell, the discs were prepared for imaging by SEM. CaP coated discs with adherent cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (Agar Scientific Ltd., Stansted, Essex, UK) overnight and then dehydrated through a series of ethanol solutions, 20%, 50%, 70%, 90% and 3 changes in absolute ethanol. The discs were then placed for 2 min in Hexamethyldisilazane (Agar Scientific Ld, UK), removed and allowed to dry. They were then attached to aluminium stubs with adhesive carbon tabs (both Agar Scientific Ltd, UK), sputter coated with gold/palladium (Polaron E5OO, Bio-Rad, Richmond, Surrey UK) and viewed in a JEOL JSM-5410LV SEM microscope (JEOL UK Ltd, Welwyn, Herts, UK) operating at 10 kV and 10 mm working distance. SEM images would also reveal the roughness of the coating; which might influence the cell's shape and ability to differentiate.

Dual firefly and Renilla luciferase Gli-reporter

After 48 h of growth on the test samples the cells were lysed with Passive lysis buffer (Promega), the lysate was brought in a black 96-well and the Dual Luciferase Reporter™ assay was performed using a Labsystems Luminoskan Ascent Plate Luminometer [43]. The Gli-responsive firefly luciferase was measured manually and immediately after adding the Luciferase Assay Reagent II. Subsequently, the Stop&Glo component was added to measure the constitutive Renilla expression. A relative Gli expression was obtained by dividing the firefly by the Renilla luminescence.

In vivo agonist delivery by CaP in fetal chick femurs

Preparation of beads and adhesion of agonists

As described in Paul and Sharma, 1999; the HA-beads were prepared by mixing 5 g hydroxyapatite (Sigma-Aldrich, Dorset, UK) with 10 ml of a 2% chitosan (Sigma) solution in 2% (v/v) acetic acid. The solution was poured in sunflower-oil and stirred to dispense the chitosan-HA-solution into small bubbles. The bifunctional cross-linking reagent gluturaldehyde (Sigma) was added to cross-link the chitosan and the formed beads were filtered, washed with acetone and sintered at 1300 °C for 2 h. As the chitosan was burned away, pure porous HA-beads were left over [44]. The beads were soaked in 200 μM purmorphamine in PBS for 24 h and control beads were soaked in PBS only; while this Pur concentration is 100× higher than the *in vitro* concentration tested it was expected that the amount would be sufficient to achieve a measurable effect.

In vivo implantation of the beads in a fetal chick femur defect

Fertilized eggs (J.K. Needle and Co., Herts, UK) were incubated at 39 °C within the first week upon arrival. A host egg was windowed at day 3 [45] to be able to use the chicken chorioallantoic membrane (CAM) as a culture substrate at day 7. The femurs were isolated from donor eggs at day 14. All soft tissues were removed from the femur and a small defect was made with a tip of a needle (BD Microlance 3). 10 beads were taken with a micropipette and injected onto the defect and pushed further into the defect with a needle-tip. This was performed using beads soaked in purmorphamine and control beads without purmorphamine ($n = 3$). The femur with the implant was brought on the CAM of the host egg, the window was sealed with plastic tape and the host egg was incubated for another 7 days. In the following experiments the femurs of the fetal chicken from the host egg could be used as donors. All small animal experiments were carried out as described in project license PPL 70/6269 by researchers with a personal license (K. Gellynck: PIL 70/20356), both according to the Animals (scientific procedures) Act 1986, Home Office, UK.

Histology and analysis of the bone growth at the implant-site

After 7 days of further growth on the CAM the femurs were cut away from the CAM and fixed in 4% Paraformaldehyde (PFA) for 24 h. No decalcification was done to leave the CaP beads intact; as the bone was immature, the decalcification was not necessary. Subsequently to an alcohol and xylene series the femurs were embedded in wax and cut with a microtome (HM 330) at 8 μm. The sections were stained with a 1% toluidine blue staining for 1 min. To be able to quantify the difference in bone growth at the implant-site between the different agonists and controls the Pro-Image-software (Pro-Image, Boulder, CO, US) was used to calculate the percentage of bone marrow and bone-less area towards the total bone area.

Bone marrow osteogenesis; alkaline phosphatase and mineralization

To clarify if the extra bone and bone cells could be bone marrow derived, the bone marrow of 18 day old chicken embryo femurs was

flushed out, cultured in a 6-well for one day, before the medium was changed into a negative medium (DMEM + 10%FCS + p/s + Asc), a positive medium (negative + ß-glycerphosphate) and medium where 10^{-8} M dexamethasone, 100 ng/ml BMP-6, 0.1 M pamidronate (Sigma Chemical Co, St Louis, USA) or 2 μM purmorphamine was added. After 14 days of cell culture with these media, one well was measured for alkaline phosphatase activity using the standard PNPP assay from Sigma. This develops a soluble yellow reaction product relative to the amount of alkaline phosphatase measured at an absorbance of 410 nm; cells were lysed with 150 μl 1% Triton-X, 50 μl of the lysate was added to 50 μl of the paranitrophenolphosphate (PNPP, Sigma) assay buffer. The reaction was terminated after 30 min by the addition of 150 μl 1 M NaOH. ALP activity was measured at 410 nm using the Titertek Multiskan [46,47].

Stimulation of osseointegration

Ten 100 μm thick, 3 mm wide strips were cut coated from a PTFE block. A titanium coating was added to 7 of them by Institut Straumann AG (Basel, Switzerland) and 4 of these got an additional 200 μM purmorphamine dried onto them. Similarly to the CaP bead implants, these strips were pushed in a defect up to the bone marrow cavity of a 14 day old embryonic chick femur and placed on the CAM of a 7 day old host egg for 7 days (Fig. 4a). The femurs were fixed in 4% PFA and immersed in LR white resin according to the manufacturer's protocol and sectioned (10 μm) with a Reichert-Jung/Leica Polycut S microtome (Heerbrugg, Switzerland). The trabecular bone was visible without staining. To quantify the mechanical strength of the integration of the PTFE strips, a metal hook was attached to the bottom clamp of the dynamic mechanical analyzer (DMA, Perkin-Elmer) to hold the bone, using the top clamp to pull the PTFE strip out of the bone (Fig. 4d). A static force scan was performed using a constantly increasing force (200 mN/min) until the strip (PTFE only n = 2, titanium coated PTFE n = 3, titanium coated PTFE + purmorphamine n = 3) was pulled out of the bone (breaking point) on which point the required force was a quantification for the integration.

Results

In vitro purmorphamine activity

The hedgehog pathway works over 2 transmembranic proteins; patched (Ptch) and smoothened (Smo), where Smo is activating the Gli protein function and transcription which will further regulate the transcription of proteins important in bone formation like Wnt. In the inactive state, Smo is inhibited by Ptch. The sonic hedgehog protein, during bone formation in the developmental stage produced by chondrocytes, will stop this inhibition and start bone formation (Fig. 1a). Purmorphamine works by directly activating the Smo transmembrane protein regardless whether Ptch is inhibiting Smo or not. This activation was analyzed through the expression of the bone marker *Bsp*.

Q-PCR dCt values using GapdH as an internal control: in negative medium (control): 1w: 14.17, 2W: 13.28; in positive medium: 1w: 13.53, 2W: 10.67; adding dexamethasone to positive medium: 1w: 12.14, 2W: 8.00; using BMP-6: 1w: 11.24, 2W: 8.14; using purmorphamine: 1w: 11.29, 2W: 7.21; using both purmorphamine and BMP-6: 1w: 8.51, 2W: 4.10. Thereby Q-PCR-data showed that the administration of 2 μM purmorphamine had similar effect on the expression of *Bsp* as both dexamethasone and BMP-6. The upregulation was greater than when positive

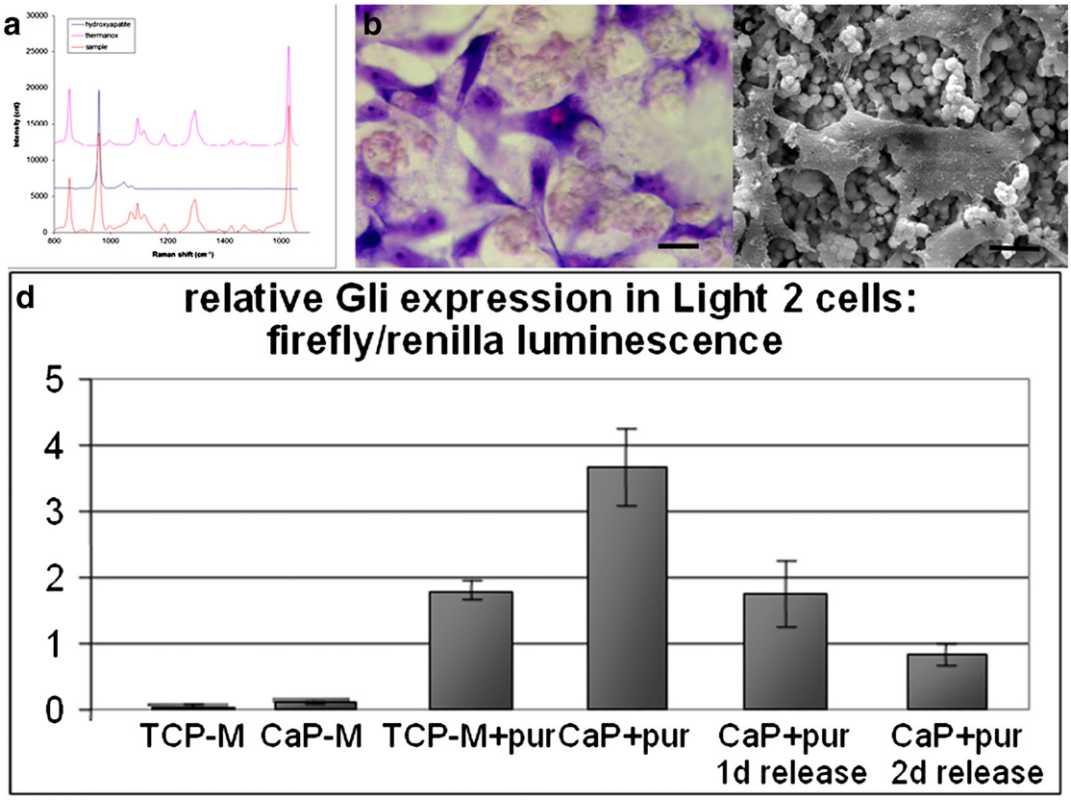


Fig. 2. [a] Raman spectroscopy of the calcium phosphate precipitated sample; thermanox coverslip; and hydroxyapatite; showed that the precipitated calcium phosphate was primarily hydroxyapatite, peak at 960 cm^{-1}. Light 2 cells growing on precipitated CaP; toluidine blue stained [b] and SEM picture [c]; scale bar: 10 μm. [d] The Gli expression in the Light 2 cells on the CaP, relative to the cell number was measured using the Promega kit, and calculated by dividing the firefly luminescence by the Renilla luminescence. Cells grown on tissue culture plastic (TCP) in medium (M) without purmorphamine (Pur) showed almost no expression; When cells were grown in medium with 2 μM purmorphamine (TCP-M + pur) or on CaP discs with 20 μM purmorphamine adhered onto it (CaP + pur), the cells showed a significantly higher expression. When these CaP + pur discs where presoaked in medium for 1 or 2 days before the cells were seeded onto them, the Gli expression became gradually smaller, but still higher than when no Pur was added onto the CaP.

medium (DMEM + 10%FCS + p/s + Asc + ß-glycerphosphate) was used without extra agonists. This activation by purmorphamine had an additive effect compared to BMP-6 stimulation as the addition of both simultaneously showed a higher upregulation than each on their own (Fig. 1b). This shows that purmorphamine is a small molecule (=non-protein molecule) that can activate the hedgehog pathway and thereby stimulate bone formation.

Activity of purmorphamine adhered onto calcium phosphate coated discs

The strong Raman peak at 960 cm^{-1}, (PO stretch) in the spectrum of pure hydroxyapatite (dark blue spectrum, Fig. 2a) was clearly observed in the Raman spectrum of the CaP coated plastic disc (light blue spectrum, Fig. 2a), but not in the spectrum of the plastic disc without CaP (green spectrum, Fig. 2a). Almost all other peaks from the CaP coated plastic disc were coincident with and therefore attributed to Thermanox® plastic peaks. Only a shoulder-peak around 1065 cm^{-1} was not identifiable. This provides strong evidence that the biomimetically precipitated CaP is primarily hydroxyapatite. Further analysis would be required to confirm purity but for our purpose as an agonist delivery mechanism the verification of the CaP coating is sufficient (Fig. 2a). A Raman spectrum of a coated disc with purmorphamine added did not show any detectable differences compared to the spectrum of the coated disc without purmorphamine. This is to be expected given the low level of drug and confirms this technique is not suitable for quantifying Pur levels on the discs.

Whether the adhered small molecule kept its activity was analyzed with a dual luciferase cell-based bioassay. As the Light 2 cells are made to express firefly luciferase when the Gli-inducible promoter is upregulated, the stimulation of the hedgehog pathway can be calculated by measuring this firefly luciferase luminescence; the constitutive Renilla luciferase is a measure for the number of cells (Figs. 2b and c show the cell attachment and spreading onto the CaP coating). The ratio of the two gives the Gli expression per cell, a quantification of the bio-activity of the adhered Pur. In Fig. 2d, it is shown that the cells growing on the CaP coated discs with Pur expressed more luciferase or Gli compared to those where no CaP was adhered. Soaking the Pur CaP discs in medium once or twice for 24 h showed that not all of the agonist molecules were released immediately but that there was a gradual release up to 2 days and after soaking for 2 days the Gli expression was still being upregulated.

In vivo agonist delivery by CaP beads in fetal chick femurs

The size of the HA-porous beads (+/− 30–150 μm) could be adjusted by increasing the speed of the stirring, the faster the stirring; the smaller the beads. A uniformity of the bead-size was not a necessity as beads with a similar size (+/− 50 μm) could be selected afterwards. The CaP beads with an appropriate size could easily be pushed in the defect with the tip of a 27 gage needle.

The chick femurs with the implants inserted were overgrown by vessels from the chicken CAM and could thereby remain vital. The femurs had even grown in thickness during the 7 days they were on the CAM incubated at 37 °C. During the sectioning of the middle part of the bone care was taken to ensure that the bones were not over-decalcified and

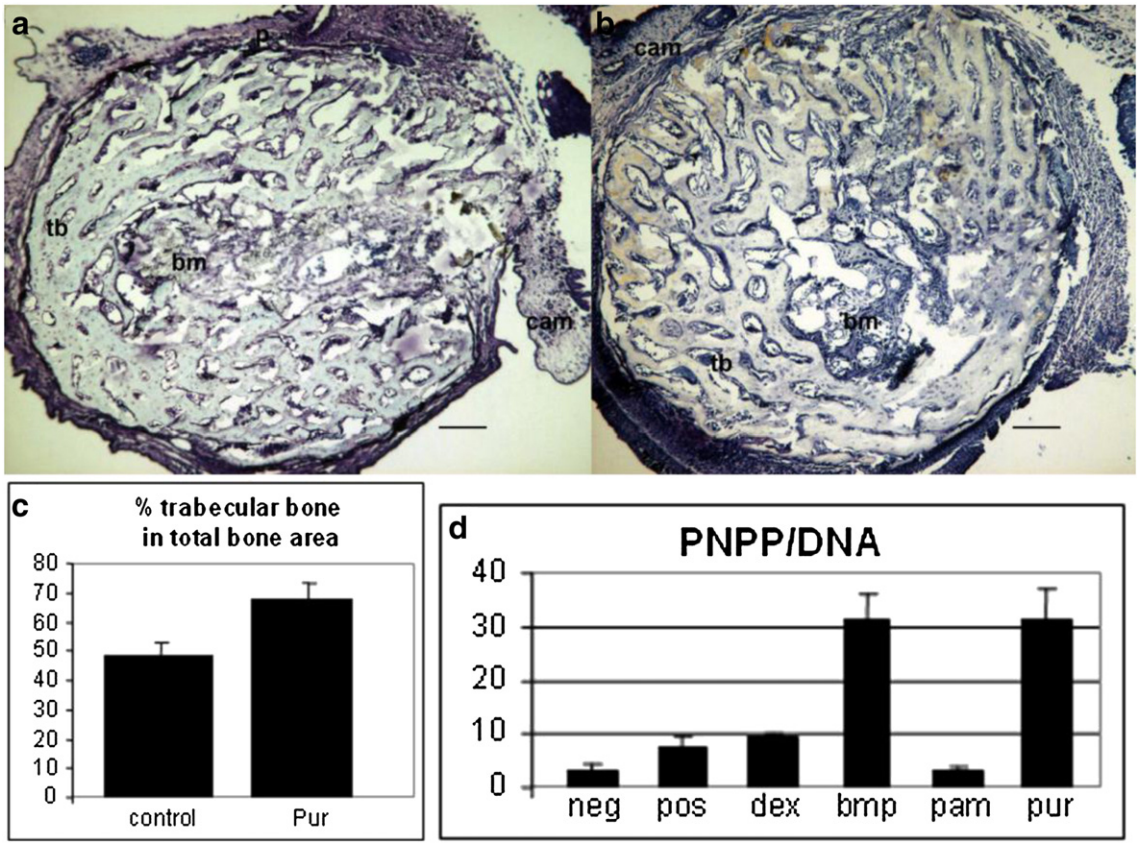


Fig. 3. CaP beads soaked in 200 μM purmorphamine for 24 h, were implanted in 14 day old embryonic chick femurs, empty control beads were implanted in others. These femurs were kept alive for 7 days by grafting them onto the chorioallantoic membrane (cam) of a 7 day old host egg. [a] Shows the control bone and [b] where purmorphamine was added, scale bar: 100 μm. These toluidine blue stained sections were used to calculate trabecular bone (tb) mass by quantifying the percentage of trabecular bone using image-Pro Plus software. The comparison [c] showed a significant increase (48.25 +/− 6.52% to 68.19 +/− 7.13%) when purmorphamine (Pur) was added to the beads. Showing this extra bone is bone marrow (bm) derived, the bone marrow of 18 day old chicken embryo femurs was flushed out, and cultured in negative medium (DMEM + 10%FCS + p/s + Asc), a positive medium (negative + ß-glycerphosphate) and positive medium plus dexamethasone, BMP-6, pamidronate or purmorphamine. After 14 days, alkaline phosphatase activity was measured using the PNPP methodology showing that addition of ß-glycerphosphate, BMP-6 and purmorphamine both increased the Alp activity significantly, dexamethasone insignificantly increased and pamidronate even decreased the Alp activity.

the site of implant and the CaP beads could be retrieved. The toluidine blue stained sections showed the difference in bone growth between the controls and the femurs where beads with agonists had been implanted (Figs. 3a and b). To quantify the bone growth the size of the overall bone area, and the trabecular bone area were measured and the proportion of trabecular bone area to the bone marrow was compared between the different samples, the average 68.19 +/− 7.13% trabecular bone to overall bone area of the test (Pur) samples was significantly higher than the 48.25 +/− 6.52% of the control samples, showing the *in vivo* effect of the adhered small molecule in and on the implanted CaP beads.

Bone marrow osteogenesis; alkaline phosphatase

The only selection from the bone marrow cell population was made by removing the non-sticky cells when the medium was refreshed, making it a rather stem-cell rich cell-mixture, similar to the bone marrow. But a significant increase in alkaline phosphatase activity (this is a marker for osteodifferentiation of the cells) was seen when BMP-6 (31.44 +/− 4.63) or Pur (31.27 +/− 5.86) was added to the positive medium (7.37 +/− 2.07) as shown by the PNPP-spectroscopy results in Fig. 3d; the addition of dexamethasone showed a small non-significant increase (9.10 +/− 0.81) and pamidronate even showed a decrease (2.72 +/− 1.10) and was similar to the cells grown in negative medium (2.97 +/− 1.41).

Stimulation of osseointegration

Even with the PTFE strips implanted in the embryonic femurs the CAM was able to embed the bones and keep them alive for 7 more days (Fig. 4a). Histology shows the presence of soft tissue in between the space where the PTFE implant was and the trabecular bone (Fig. 4b), whereas with Ti coated and Ti coated Pur adhered strips the trabecular bone was touching the implant on both sides (Fig. 4c). The DMA tensile tests (Fig. 4d) showed that the constantly increasing force only slightly moved the implant up to a breaking point when the implant was pulled out of the femur (Fig. 4e). As a constantly increasing force of 200 mN/min was applied, the time of breaking was a measure of the force required to pull the implant out of the femur and as a consequence the osseointegration of the implant. One PTFE implant got detached during processing, illustrating its low strength. The average force required was lower for PTFE on its own compared to the Ti coated strips, but no difference could be seen between the Ti coated implants with or without purmorphamine (Fig. 4f).

Discussion

Calcium phosphate is already used frequently as a coating material for bone implants showing good biocompatibility and bio-activity. It also has been shown to increase the osteoconductivity and speed of healing on implant placement [48]. The Raman spectra showed that by precipitating CaP onto plastic discs, a hydroxyapatite-like calcium phosphate coating could be formed. Using another method CaP beads were produced. These beads can then be saturated with the small molecules of interest and then be used as a vehicle for delivering them to a site of bone damage.

The results, using light II cells, show that purmorphamine attached to the CaP coating kept its activity and could activate the hedgehog pathway for several days and that adhering or incorporating it to the CaP surface attached cells can also be guided into the osteogenic differentiation. These coated beads were used to deliver purmorphamine *in vivo* in chicken embryo femur defects. Comparing the bone growth showed that there is a significant difference between the bone growth

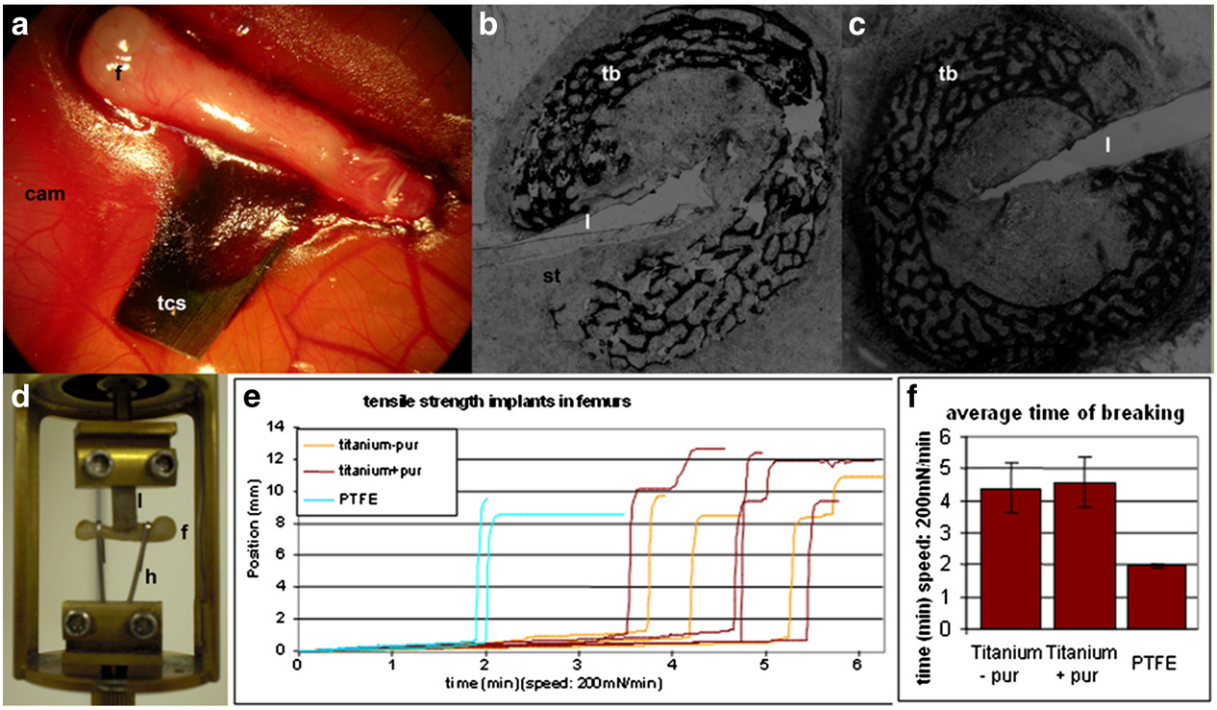


Fig. 4. To investigate the effect of a titanium coating +/− purmorphamine on osseointegration, 14 day old embryonic chick bones were isolated and PTFE strips, with or without titanium coating (tcs), well or not soaked in 200 μM purmorphamine were implanted through the bone into the bone marrow. The whole was embedded in a chorioallantoic membrane (cam, [a]) of a 7 day old host egg for 7 days. LR white resin was used to make 10 μm sections: [b]; section where a blank PTFE strip was implanted showing soft tissue (st) between trabecular bone (tb) and implant (I). [c]; Section where a titanium coated PTFE strip with purmorphamine was implanted; the trabecular bone is touching the implant on both sides. To quantify the strength of the integrations, tensile tests [d] were performed on a dynamic mechanical analyzer, a constantly increasing force was applied and set out against the position, showing the time (or force) needed to pull the implant (I) out (=breaking) of the femur (f) held by a metal hook (h) in the bottom clamp. A sharp incline in the position *versus* time graph [e] showed the moment of breaking when the implant was detached out of the femur. As a constantly increased force (200 mN/min) was used, this time of breaking was equivalent to a certain force. A significant difference was found between the PTFE controls (1.95 +/− 0.07 min) and the titanium coated strips (4.5 +/− 0.75 min), but not between the ones with (4.58 +/− 0.79 min) and without purmorphamine (4.39 +/− 0.78 min) [f].

at the implant-site of the beads soaked in agonists and the control beads. This is the first time the activity of purmorphamine is shown *in vivo*; although there is already significant research out there showing the ability of this small molecule to induce osteogenic differentiation in mesenchymal stem cells [49,50].

This research proved that purmorphamine can be delivered with hydroxyapatite based biomaterials or that hydroxyapatite can be made bioactive by soaking it in a purmorphamine solution. As the bone marrow was narrowed down by the delivered agonist it was questionable whether this was due to an overstimulation of the periosteum or whether this extra bone formation could come from within the bone marrow or an endosteal layer. As it was shown that bone marrow cells flushed out of the chicken embryo bones can be mineralized *in vitro* just as hMSC can, it is possible that the extra bone formation is formed in this way.

The use of the chick femur model as a novel method to evaluate implant integration is presented and showed the difference between PTFE and titanium coated implants; but no difference in the strength of the bone to implant bond was detected when the hedgehog agonist was added. This could be explained by the possibility that purmorphamine was not well enough taken up by the titanium or that the effect on the integration was too small to be detected by the method used here. In this method the bone-implant construct had to be transferred to the mechanical analyzer and clamped and hooked using a self-made device. This clamping can affect the construct, but once clamped the metal device will not bend compared to the bone-implant construct; hence all movement and breaking is in the bone-implant construct. The results from this study suggest that as purmorphamine is a cheap and stable substitute for recombinant sonic hedgehog protein, it could be used in bone regenerative medicine, but it has not been shown to be an effective adjunct to implant placement to enhance osseointegration.

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References

[1] Gamradt SC, Lieberman JR. Bone graft for revision hip arthroplasty. Clin Orthop Relat Res 2003;417:183–94.
[2] Albrektsson T, Johansson C. Osteoinduction, osteoconduction and osseointegration. Eur Spine J 2001;10:S96–S101.
[3] Aspenberg P, Goodman S, Toksviglarsen S, Ryd L, Albrektsson T. Intermittent micromotion inhibits bone ingrowth—titanium implants in rabbits. Acta Orthop Scand 1992;63(2):141–5.
[4] Costantino PD, Hiltzik D, Govindaraj S, Moche J. Bone healing and bone substitutes. Facial Plast Surg 2002;18(1):13–26.
[5] Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: the cellular picture. Semin Cell Dev Biol 2008;19(5):459–66.
[6] Tuan RS. Biology of developmental and regenerative skeletogenesis. Clin Orthop Relat Res 2004;427:S105–17.
[7] St Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 1999;13(16):2072–86.
[8] Colnot C, Romero DM, Huang S, Helms JA. Mechanisms of action of demineralized bone matrix in the repair of cortical bone defects. Clin Orthop Relat Res 2005;435:69–78.
[9] Day TF, Yang YZ. Wnt and hedgehog signaling pathways in bone development. J Bone Joint Surg Am 2008;90A:19–24.
[10] Mak KK, Chen MH, Day TF, Chuang PT, Yang YZ. Wnt/beta-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation. Development 2006;133(18):3695–707.
[11] Edwards PC, Ruggiero S, Fantasia J, Burakoff R, Moorji SM, Paric E, et al. Sonic hedgehog gene-enhanced tissue engineering for bone regeneration. Gene Ther 2005;12(1):75–86.
[12] Kinto N, Iwamoto M, Enomotolwamoto M, Noji S, Ohuchi H, Yoshioka H, et al. Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. FEBS Lett 1997;404(2–3):319–23.
[13] Wu X, Ding S, Ding Q, Gray NS, Schultz PG. A small molecule with osteogenesis-inducing activity in multipotent mesenchymal progenitor cells. J Am Chem Soc 2002;124(49):14520–1.
[14] Beloti MM, Bellesini LS, Rosa AL. Purmorphamine enhances osteogenic activity of human osteoblasts derived from bone marrow mesenchymal cells. Cell Biol Int 2005;29(7):537–41.
[15] Beloti MM, Bellesini LS, Rosa AL. The effect of purmorphamine on osteoblast phenotype expression of human bone marrow mesenchymal cells cultured on titanium. Biomaterials 2005;26(20):4245–8.
[16] Cousin W, Fontaine C, Dani C, Peraldi P. Hedgehog and adipogenesis: fat and fiction. Biochimie 2007;89(12):1447–53.
[17] Fontaine C, Cousin W, Plaisant M, Dani C, Peraldi P. Hedgehog signaling alters adipocyte maturation of human mesenchymal stem cells. Stem Cells 2008;26(4):1037–46.
[18] Sinha S, Chen JK. Purmorphamine activates the hedgehog pathway by targeting Smoothened. Nat Chem Biol 2006;2(1):29–30.
[19] Buscher D, Grotewold L, Ruther U. The Xt(J) allele generates a Gli3 fusion transcript. Mamm Genome 1998;9(8):676–8.
[20] Hardcastle Z, Mo R, Hui CC, Sharpe PT. The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. Development 1998;125(15):2803–11.
[21] Miao DS, Liu HL, Plut P, Niu MJ, Huo RJ, Goltzman D, et al. Impaired endochondral bone development and osteopenia in Gli2-deficient mice. Exp Cell Res 2004;294(1):210–22.
[22] Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HHQ, et al. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development 1997;124(1):113–23.
[23] Maeda Y, Nakamura E, Nguyen MT, Suva LJ, Swain FL, Razzaque MS, et al. Indian hedgehog produced by postnatal chondrocytes is essential for maintaining a growth plate and trablecular bone. Proc Natl Acad Sci U S A 2007;104(15):6382–7.
[24] Zhao M, Qiao M, Harris SE, Chen D, Oyajobi BO, Mundy GR. The zinc finger transcription factor Gli2 mediates bone morphogenetic protein 2 expression in osteoblasts in response to hedgehog signaling. Mol Cell Biol 2006;26(16):6197–208.
[25] Yuasa T, Kataoka H, Kinto N, Iwamoto M, Enomoto-Iwamoto M, Iemura SI, et al. Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2. J Cell Physiol 2002;193(2):225–32.
[26] Spinella-Jaegle S, Rawadi G, Kawai S, Gallea S, Faucheu C, Mollat P, et al. Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. J Cell Sci 2001;114(11):2085–94.
[27] Nakamura T, Aikawa T, IwamotoEnomoto M, Iwamoto M, Higuchi Y, Maurizio P, et al. Induction of osteogenic differentiation by hedgehog proteins. Biochem Biophys Res Commun 1997;237(2):465–9.
[28] van der Horst G, Farih-Sips H, Lowik CWGM, Karperien M. Hedgehog stimulates only osteoblastic differentiation of undifferentiated KS483 cells. Bone 2003;33(6):899–910.
[29] Peter B, Pioletti DP, Laïb S, Bujoli B, Pilet P, Janvier P, et al. Calcium phosphate drug delivery system: influence of local zoledronate release on bone implant osteointegration. Bone 2005;36(1):52–60.
[30] Mori S, Yoshikawa H, Hashimoto J, Ueda T, Funai H, Kato M, et al. Antiangiogenic agent (TNP-470) inhibition of ectopic bone formation induced by bone morphogenetic protein-2. Bone 1998;22(2):99–105.
[31] Wozney JM. The potential role of bone morphogenetic proteins in periodontal reconstruction. J Periodontol 1995;66(6):506–10.
[32] Wozney JM. Engineering new bone with bone morphogenic proteins. Ann Endocrinol (Paris) 2006;67(2):180–1.
[33] Moore R, Ferretti P, Copp A, Thorogood P. Blocking endogenous FGF-2 activity prevents cranial osteogenesis. Dev Biol 2002;243(1):99–114.
[34] Buxton PG, Bitar M, Gellynck K, Parkar M, Brown RA, Young AM, et al. Dense collagen matrix accelerates osteogenic differentiation and rescues the apoptotic response to MMP inhibition. Bone 2008;43(2):377–85.
[35] Peck WA, Birge SJ, Fedak SA. Bone cells—biochemical + biological studies after enzymatic isolation. Science 1964;146(365):1476–7.
[36] Tenenbaum HC, Heersche JNM. Dexamethasone stimulates osteogenesis in chick periosteum in vitro. Endocrinology 1985;117(5):2211–7.
[37] Igarashi M, Kamiya N, Hasegawa M, Kasuya T, Takahashi T, Takagi M. Inductive effects of dexamethasone on the gene expression of Cbfa1, osterix and bone matrix proteins during differentiation of cultured primary rat osteoblasts. J Mol Histol 2004;35(1):3–10.
[38] Takada J, Chevalley T, Baylink DJ, Lau KHW. Dexamethasone enhances the osteogenic effects of fluoride in human TE85 osteosarcoma cells *in vitro*. Calcif Tissue Int 1996;58(5):355–61.
[39] Wozney JM. The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 1992;32(2):160–7.
[40] Yamaguchi A, Ishizuya T, Kintou N, Wada Y, Katagiri T, Wozney JM, et al. Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem Biophys Res Commun 1996;220(2):366–71.
[41] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30(9).
[42] Liu YL, Layrolle P, de Bruijn J, van Blitterswijk C, de Groot K. Biomimetic coprecipitation of calcium phosphate and bovine serum albumin on titanium alloy. J Biomed Mater Res 2001;57(3):327–35.
[43] Sherf BA, Navarro SL, Hannah RR, Wood KV. Novel reporter gene technology integrating firefly and Renilla luciferase assays for internal control. Mol Biol Cell 1996;7:925.
[44] Paul W, Sharma CP. Development of porous spherical hydroxyapatite granules: application towards protein delivery. J Mater Sci Mater Med 1999;10(7):383–8.
[45] Andacht T, Hu W, Ivarie R. Rapid and improved method for windowing eggs accessing the stage X chicken embryo. Mol Reprod Dev 2004;69(1):31–4.

[46] Piche JE, Carnes DL, Graves DT. Initial characterization of cells derived from human periodontia. J Dent Res 1989;68(5):761–7.
[47] Hasselgren G. Alkaline-phosphatase in developing teeth and bone of man and macaque monkey. Acta Odontol Scand 1978;36(3):143–8.
[48] Kuroda Kensuke, Okido Masazumi. Hydroxyapatite coating of titanium implants using hydroprocessing and evaluation of their osteoconductivity. Bioinorg Chem Appl 2012;2012 [Article ID 730693, 7 pages].
[49] Granéli C, Karlsson C, Lindahl A, Thomsen P. Virtual ligand-based screening reveals purmorphamine analogs with the capacity to induce the osteogenic differentiation of human mesenchymal stem cells. Cells Tissues Organs 2013;197(2):89–102.
[50] Faghihi F, Baghaban Eslaminejad M, Nekookar A, Najar M, Salekdeh GH. The effect of purmorphamine and sirolimus on osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. Biomed Pharmacother 2013;67(1):31–8.