Biomaterials 86 (2016) 106–118

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Early BMP, Wnt and Ca^{2+}/PKC pathway activation predicts the bone forming capacity of periosteal cells in combination with calcium phosphates

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ARTICLE INFO

Article history:
Received 7 May 2015
Received in revised form
26 January 2016
Accepted 27 January 2016
Available online 29 January 2016

Keywords:
Bone tissue engineering
Osteogenesis
Modelling
Progenitor cell
Cell signalling
Calcium phosphate

ABSTRACT

The development of osteoinductive calcium phosphate- (CaP) based biomaterials has, and continues to be, a major focus in the field of bone tissue engineering. However, limited insight into the spatiotemporal activation of signalling pathways has hampered the optimisation of *in vivo* bone formation and subsequent clinical translation. To gain further knowledge regarding the early molecular events governing bone tissue formation, we combined human periosteum derived progenitor cells with three types of clinically used CaP-scaffolds, to obtain constructs with a distinct range of bone forming capacity *in vivo*. Protein phosphorylation together with gene expression for key ligands and target genes were investigated 24 hours after cell seeding *in vitro*, and 3 and 12 days post ectopic implantation in nude mice. A computational modelling approach was used to deduce critical factors for bone formation 8 weeks post implantation. The combined Ca^{2+}-mediated activation of BMP-, Wnt- and PKC signalling pathways 3 days post implantation were able to discriminate the bone forming from the non-bone forming constructs. Subsequently, a mathematical model able to predict *in vivo* bone formation with 96% accuracy was developed. This study illustrates the importance of defining and understanding CaP-activated signalling pathways that are required and sufficient for *in vivo* bone formation. Furthermore, we demonstrate the reliability of mathematical modelling as a tool to analyse and deduce key factors within an empirical data set and highlight its relevance to the translation of regenerative medicine strategies.

1. Introduction

Well-characterized cell-based constructs are required for skeletal tissue engineering (STE) in order to replace, thus avoiding the complications associated with, the standard clinical practice of autograft or allograft transplants [1]. Recent work has focussed on novel means of developing osteoinductive biomaterials and cell populations with unique functionalities. However, this is often carried out without sufficient appreciation of how these modalities interact to form bone *in vivo*. An understanding of these processes is crucial for the development of new and improved osteoinductive constructs, considering a biomimetic approach is now accepted as essential for efficient tissue formation and a successful outcome within the field of STE [2].

In non-compromised fracture healing, key growth factors such as bone morphogenetic proteins (BMP)s, β-catenin/wingless-related factors (Wnts), transforming growth factor beta 1 (TGFβ1) and fibroblast growth factors (FGFs) are released from cells at the fracture site to recruit and trigger skeletal progenitor cells in the periosteum to aid the healing process. This is the primary source of

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http://dx.doi.org/10.1016/j.biomaterials.2016.01.059
0142-9612/

cells that form new tissues during fracture repair. Furthermore, we have previously reported the efficacy of human periosteum derived cells (hPDCs) to form bone *in vivo* when combined with calcium phosphate (CaP) scaffolds, where implanted cells contribute to 65% of the newly formed bone [3]. In this process, Ca^{2+} as well as activated BMP and Wnt-signalling were reported as essential requirements for bone tissue formation, since disruption of any of these factors abrogated bone formation by the CaP/hPDC-construct [4]. Subsequently, we reported that it is not only the presence of Ca^{2+} that is important, rather, to trigger osteogenesis from hPDCs, a precise release profile is required [3]. To investigate the interaction of Ca^{2+} with the cell population and the *in vivo* environment we then used full genome profiling and detailed histomorphometry to define a number of genes, proteins, signalling pathways and *in situ* stem cell properties that are of importance for CaP-mediated osteoinduction [5,6]. Empirically, these activated pathways were linked to BMP, β-Catenin/Wnt, cAMP Response Element-Binding Protein (CREB), TGFβ, Endothelial Growth Factor (EGF) and Extracellular Signal-Regulated Kinase (Erk) signalling.

However, at this stage it is unclear how these signalling pathways interact and are activated (directly or indirectly) during CaP-mediated osteoinduction, and whether these can be used to predict *in vivo* tissue formation. It is therefore clear that further understanding of the mechanism of CaP-mediated osteoinduction by hPDCs from a molecular signalling perspective is essential for the successful development of effective STE constructs. This can be achieved by comprehensively analysing the phenomenological differences pertaining to the *in vivo* activation and inhibition of relevant signalling pathways on various CaP-based scaffolds with known bone forming capacities *in vivo*. One of the hurdles in understanding these signalling pathways and their interactions is the potential size of the experimental dataset. To deal with this complexity, computational modelling, using techniques such as Partial least square regression (PLSR), is an efficient methodology to identify a predictive model based on experimental data [7]. Furthermore, the use of computational evaluation of the *in vivo* outcome is a possible means to gain in-depth knowledge on cell–biomaterial interactions in a cost- and time-effective manner. It is hypothesised that robust and accurate computational models can be developed based on the early activation of cellular signalling pathways that are regulated in response to a bio-instructive matrix. Indeed, the feasibility of this approach has been successfully demonstrated in the medical field where computational modelling has played a significant role in understanding the dynamic pathophysiology of diseases in complex biological systems [8–11]. Of note, the US Food and Drug Administration (FDA) has recently approved *in silico* simulations as valid preclinical evidence, especially in the prediction of safety and efficacy of therapies [12,13]. With respect to skeletal regenerative medicine, computational techniques have been used to gain biological knowledge and understanding of stem cell dynamics and material design [3,7,14–17]. However, as of yet, computational modelling has not been used to predict *in vivo* outcome based on the activation/inactivation of signalling pathways.

Herein, we describe the detection of temporally activated signalling molecules that represent the activation of crucial signalling pathways for bone formation by hPDC-seeded CaP/collagen scaffolds. These pathways were differentially activated in response to the introduction of hPDCs to three types of clinically approved CaP scaffolds with limited (Copios®), mid (VitOss™) and high (NuOss™) bone forming capacity. A computational modelling approach was used to deduce key factors from empirical data thus allowing the correlation of the constructs to their bone forming capacity. Subsequently, a mathematical equation was developed that allows the prediction of *in vivo* bone forming capacity by CaP/collagen-based biomaterials seeded with hPDCs. This predictive model was validated on additional CaP-based biomaterials with a predictability of 96%. The major consequence of this is over a 10-fold reduction in experimental time when investigating new biomaterials *in vivo*, hence expediting the time required for the translation of results from bench to bedside. In addition, the described model also serves to refine the use of animals in research and begins a process of developing computational models for their eventual replacement. Finally, this study highlights the significance and reliability of mathematical modelling as a tool to analyse empirical data and deduce key factors in complex biological systems such as *in vivo* bone formation. Moreover, we define the importance of the ordered and balanced activation of critical pathways during the early stages of bone formation, and how this can be related to Ca^{2+} release from the biomaterial.

2. Materials and methods

2.1. Isolation and expansion of human periosteum-derived cells (hPDCs)

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. Specimens were transported in growth medium (GM) consisting of high-glucose Dulbecco's Modified medium (DMEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% foetal bovine serum (FBS) (Gibco, Merelbeke, Belgium) and antibiotics-antimyocotics solution (100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B; Invitrogen, Merelbeke, Belgium). The biopsies were digested over night at 37 °C in 0.2% type IV collagenase (Invitrogen, Merelbeke, Belgium) in GM. Subsequently, periosteal cells were collected by centrifugation and seeded in T25 flask in GM. Non-adherent cells were removed after 5 days by changing the medium, and the remaining cells were expanded in monolayer in GM. Upon confluence, hPDCs were trypsin-released (0.25% trypsin, 1 mM EDTA; Invitrogen, Merelbeke, Belgium) and re-plated with a seeding density of 5000 cells/cm^2. From passage 2, hPDCs from six different donors were pooled (age 14.9 ± 2.1), to reduce noise from genetic variability. For cryopreservation, hPDCs were suspended in DMEM with 20% FBS and 10% DMSO (Sigma, Bornem, Belgium) and stored in liquid nitrogen. Cells were thawed, sub-cultured upon confluence and further processed for analysis. The ethical committee for Human Medical Research (KU Leuven) approved all procedures, and the patient informed consents were obtained.

2.2. In vivo osteogenesis

Three clinical grade CaP-based scaffolds with known bone forming capacity in combination with hPDCs [i.e. high: NuOss™ (ACEuropa, Lisbon, Portugal); mid: VitOss™ (Orthovita, Leuven, Belgium); and limited: Copios® (Zimmer, Wemmel, Belgium)] were selected based on previous work [3]. A summary of scaffold characteristics can be found in Supplementary Table 1 and an experimental overview is presented in Supplementary Fig. 1A. Briefly, from each material, 21 mm^3 scaffolds were punched out. Upon confluence, *in vitro* 2D expanded cells were trypsin-released, centrifuged and resuspended. To evaluate the process of cell seeding, cells were labelled with CellTracker™ CM-DiI Dye (1 μg/$1^{*}10^6$ cells) (Thermo SCIENTIFIC, Erembodegem, Belgium) according to the manufacturer's instructions. Subsequently, labelled (n = 4) and non-labelled (n = 20) cells (50 000/mm^3) were drop-seeded onto each scaffold. To improve cell attachment, the cell-