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Periosteum tissue engineering in an orthotopic *in vivo* platform

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ABSTRACT

The periosteum plays a critical role in bone homeostasis and regeneration. It contains a vascular component that provides vital blood supply to the cortical bone and an osteogenic niche that acts as a source of bone-forming cells. Periosteal grafts have shown promise in the regeneration of critical size defects, however their limited availability restricts their widespread clinical application. Only a small number of tissue-engineered periosteum constructs (TEPCs) have been reported in the literature. A current challenge in the development of appropriate TEPCs is a lack of pre-clinical models in which they can reliably be evaluated.

In this study, we present a novel periosteum tissue engineering concept utilizing a multiphasic scaffold design in combination with different human cell types for periosteal regeneration in an orthotopic *in vivo* platform. Human endothelial and bone marrow mesenchymal stem cells (BM-MSCs) were used to mirror both the vascular and osteogenic niche respectively. Immunohistochemistry showed that the BM-MSCs maintained their undifferentiated phenotype. The human endothelial cells developed into mature vessels and connected to host vasculature. The addition of an *in vitro* engineered endothelial network increased vascularization in comparison to cell-free constructs.

Altogether, the results showed that the human TEPC (hTEPC) successfully recapitulated the osteogenic and vascular niche of native periosteum, and that the presented orthotopic xenograft model provides a suitable *in vivo* environment for evaluating scaffold-based tissue engineering concepts exploiting human cells.

1. Introduction

Despite being only a few cell layers thick, the periosteum plays an important role in bone development and maintenance. The periosteum is bilayered consisting of an outer fibrous layer and a highly cellularised inner cambium layer. It is highly vascularized and contains an osteoprogenitor niche that acts as a reservoir for bone-forming cells. These characteristics make it an important tissue for bone regeneration. The periosteum's pivotal role in bone metabolism can be displayed by the fact that its removal from bone graft results in a reduced osteogenic capacity of the graft and high risk of graft necrosis due to the missing re-vascularization stimulus [1]. One component of the multimodal concept of large bone defect repair is periosteal substitution in the form of autografts or allografts. Of note, the availability of periosteum itself is limited, as it can only be surgically separated from bones of growing individuals [2,3] (Supplementary Fig. 1). This limitation underlines the clinical need to develop an easily available and simply applicable substitute that can mimic periosteal functions.

The emerging field of tissue engineering and regenerative medicine (TE&RM), which combines cells, scaffolds and/or growth factors, offers new strategies to develop novel periosteal substitutes. Surprisingly, only a small number of studies have focused on the development of periosteal grafts via tissue engineering strategies [4–7]. In addition, the few reported studies primarily

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examined the restoration of the bone compartment and failed to assess whether vital components of the periosteum, such as the osteoprogenitor and vascular niche, had been re-established.

Another current challenge in clinical translation is the lack of appropriate *in vivo* models to evaluate tissue-engineered periosteum constructs (TEPCs) assembled with human cells. In this study, we have developed a novel orthotopic xenograft model to study human TEPCs. NOD scid gamma (NSG) mice have the ability to engraft cells and tissues from other species which allows for the evaluation of more clinically relevant constructs using human primary cells [8]. The use of xenograft model allows researchers to follow up the fate of the transplanted human periosteal osteprogenitor and vascular niche cells using human specific markers. In our newly developed model a small bone window (1 mm in diameter) is created in the lateral side of the femur to expose the bone marrow. The human TEPC (hTEPC) is positioned and secured around the femur and is in direct contact with the exposed bone marrow. A major benefit of this surgical concept is that the construct are exposed to a unique site-specific mixture of growth factors and cytokines that are released from the bone marrow compartment and surrounding soft tissue during bone healing, which may influence the efficacy of the hTEPC [12]. Moreover, both the bone and the surrounding soft tissue have been shown to work in conjunction with each other, and are equally important, in the establishment of the periosteum. Locating the constructs directly onto the bone and suturing the muscle and soft tissue over the hTEPC guides the integration and development of a muscle/ligament-to-bone interface. Furthermore, implantation of hTEPC at orthotopic sites is more physiological when compared to ectopic sides as they are exposed to shear and traction forces created during movement. This is important as the periosteum is a mechanosensitive tissue as it has been shown that external mechanical stimulus can regulate the proliferation and differentiation of periosteal cells [8].

The incorporation of endothelial cells in TEPCs is not only important in order to recapitulate the vascular niche in the periosteum, it also has been shown to improve cell viability and engraftment of donor tissues in a number of tissue-engineered applications including skin, heart, kidney, and bone regeneration [9–13]. Furthermore, star-PEG heparin hydrogel systems have been reported to be suitable platforms for establishing capillary-like structures from endothelial cells *in vitro* allowing for the loading and slow release of pro-angiogenic growth factors with heparin-binding affinity, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) [14]. Our approach consists of mimicking the multiphasic morphology of the periosteum by combining a star-shaped PEG heparin hydrogel system loaded with human umbilical vein endothelial cells (HUVECs) with a medical-grade poly (ε-caprolactone) (mPCL) tubular scaffold seeded with human bone marrow mesenchymal stem cells (BM-MSCs).

2. Materials and methods

2.1. Scaffold preparation

Tubular scaffolds (2 mm inner diameter and 5 mm length) were 3D printed using mPCL with a custom built melt electrospinning writing device (Supplementary video 1) equipped with a rotating collector as previously described [15]. The morphology of the scaffolds was analyzed using a field emission scanning electron microscope (FE-SEM, **Zeiss Sigma**, Carl **Zeiss**, Germany) operating at 10 kV (Fig. 1**A**). The thickness of the tube walls and fiber diameter were assessed using ImageJ. In preparation for seeding, scaffolds were treated with 1 M sodium hydroxide for 30 min to make the scaffolds hydrophilic and then washed with ddH_2O six times. Scaffolds were sterilized by immersion in 70% ethanol followed by overnight evaporation in a biosafety hood and UV irradiation on each side for 20 min. Scaffolds were cut longitudinally and flattened during hydrogel cell seeding, and then allowed to return to shape.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.11.016.

2.2. HUVEC and BM-MSC primary cell culture

HUVECs were purchased from Stemcell Technologies (Canada). HUVECs were transduced with mCherry lentivirus and positive cells were isolated using a FACS Aria III cell sorter (BD Biosciences, USA). The mCherry signal for the HUVECs following sorting was confirmed by fluorescence microscopy. Cultures were maintained in EGM-2 (Promocell, Germany) supplemented 100IU/mL penicillin-streptomycin (Invitrogen, USA). Bone marrow mesenchymal stem cells (BM-MSC) were provided by Dr. Darwin Prockop (Tulane University, USA). BM-MSCs were transduced with pLenti6/V5-dTOPO_Luciferase lentivirus and selected with 5 μg/mL Blasticidin (Invitrogen). A subset of these cells were fixed in 4% Paraformaldehyde (PFA) in order to confirm luciferase expression by immunofluorescence using a rabbit anti-Firefly luciferase antibody (Abcam, UK; ab21176, 1:200) followed by an Alexa Fluor® 488 goat anti-rabbit secondary antibody (Invitrogen, USA; A11008, 1:200). Cultures were maintained in MEM-α (Invitrogen, USA) supplemented with 16.5% FBS (Sigma, USA) and 100IU/mL penicillin-streptomycin (Invitrogen, USA) as per supplier's instructions (Fig. 1**B**).

2.3. Animal studies and experimental groups

All animal studies were conducted according to the Australian Code of Practice for care and use of animals for scientific purposes and approved by the University of Queensland Ethics committee (QUT/IHBI/376/14/ARC). Fifteen 6-week old male NOD-SCID gamma (NSG) mice were purchased from Animal Resources Centre (Australia). The animals were housed at the animal facility within the Translational Research Institute and allowed to acclimatize for 2 weeks. Three study groups were utilized and consisted of either cell-free constructs or constructs seeded with BM-MSC only or with BM-MSC:HUVECs.

2.4. Preparation of a pre-vascularized construct

For the BM-MSC:HUVEC group, tubular scaffolds were seeded with HUVECs at a density of 6×10^6 cells/mL dispersed in 40 μL star-PEG heparin hydrogels (600 Pa) at a cross-linking degree of 0.75 and with 2:1 M ratio of linear RGD. The hydrogels were loaded with 5 μg/mL VEGF (Prospec Bioscience, Israel) and cultured under angiogenic conditions in EGM-2 media for 7 days prior to implantation. All other sample groups were seeded with 40 μL of cell-free star-PEG heparin hydrogel. In order to ensure even distribution of the hydrogel throughout the construct, the longitudinally-cut scaffolds were flattened during the seeding process and allowed to return to their original tubular shape following polymerization of the hydrogel. Additional samples of the hydrogel constructs were fixed at day 7 in order to assess the formation of capillary-like structures (Fig. 1**C**–**D**). Briefly, the constructs were washed with PBS twice and fixed in 4% PFA for 20 min. Samples were permeabilized with 0.2% Triton-X in 2%BSA/PBS for 45 min, washed twice with PBS and then incubated with vWF (Dako, Denmark; A0082, 1:500) and Collagen IV (DSHB, USA; M3F7, 1:100). The constructs were washed in PBS for 30 min three times and incubated with either Alexa Fluor® 488 goat anti-rabbit secondary