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Fabrication, vascularization and osteogenic properties of a novel synthetic biomimetic induced membrane for the treatment of large bone defects

Liling Ren [a,b,1], Yunqing Kang [a,1], Christopher Browne [a], Julius Bishop [a], Yunzhi Yang [a,c,*]

[a] Department of Orthopaedic Surgery, Stanford University, 300 Pasteur Drive, Stanford, CA 94305, USA
[b] School of Stomatology, Lanzhou University, 199 Donggang West Road, Lanzhou, Gansu 730000, China
[c] Department of Materials Science and Engineering, Stanford University, 300 Pasteur Drive, Stanford, CA 94305, USA

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ABSTRACT

The induced membrane has been widely used in the treatment of large bone defects but continues to be limited by a relatively lengthy healing process and a requisite two stage surgical procedure. Here we report the development and characterization of a synthetic biomimetic induced membrane (BIM) consisting of an inner highly pre-vascularized cell sheet and an outer osteogenic layer using cell sheet engineering. The pre-vascularized inner layer was formed by seeding human umbilical vein endothelial cells (HUVECs) on a cell sheet comprised of a layer of undifferentiated human bone marrow-derived mesenchymal stem cells (hMSCs). The outer osteogenic layer was formed by inducing osteogenic differentiation of hMSCs. *In vitro* results indicated that the undifferentiated hMSC cell sheet facilitated the alignment of HUVECs and significantly promoted the formation of vascular-like networks. Furthermore, seeded HUVECs rearranged the extracellular matrix produced by hMSC sheet. After subcutaneous implantation, the composite constructs showed rapid vascularization and anastomosis with the host vascular system, forming functional blood vessels *in vivo*. Osteogenic potential of the BIM was evidenced by immunohistochemistry staining of osteocalcin, tartrate-resistant acid phosphatase (TRAP) staining, and alizarin red staining. In summary, the synthetic BIM showed rapid vascularization, significant anastomoses, and osteogenic potential *in vivo*. This synthetic BIM has the potential for treatment of large bone defects in the absence of infection.

Introduction

The treatment of large bone defects caused by trauma or infection remains a substantial clinical challenge in modern orthopedics. Currently available treatments mainly include vascularized bone transfer [1], cancellous autografting [2], and bone transport [3]. Most recently a two-stage induced membrane technique pioneered by Masquelet and colleagues has received extensive attention [4,5]. This technique involves the temporary implantation of a polymethyl methacrylate (PMMA) cement spacer into the bony defect which induces the formation of a bioactive membrane. 4 to 8 weeks after cement spacer implantation, the spacer is removed and a cancellous autograft is placed into the membrane. This technique has shown great promise in the management of large bone defects with case reports of successful treatment [4,6,7], even greater than 20 cm [8]. Several mechanisms of action are thought to facilitate bone healing in these difficult situations. The presence of the membrane itself compartmentalizes the bone graft, separating it from the surrounding muscle. Previous research has demonstrated that this compartmentalization of bone defects improves bone healing and limits graft resorption [9]. In addition, pluripotent stem cells are present in the membrane along with various growth factors such as the vascular endothelial growth factor (VEGF) and the bone morphogenetic protein-2 (BMP-2) [10], which are known to be important in fracture healing. Studies have also demonstrated that the membrane is well vascularized and a large number of vessels are present in the fibrous inner layer [11,12]. These studies suggest that the induced membrane plays a role in osteogenesis as well as vascularization of the bone graft [10,11].

However, there are some limitations using the induced membrane technique for bone regeneration. Patients have to undergo at least two surgical procedures to implant the PMMA cement spacers and then remove them in addition to any additional procedures required to treat frequently associated soft tissue injuries. The temporary implantation of the PMMA does not guarantee the induction of a robust and appropriately proportioned membrane, and the biologic viability of the membrane appears to degrade over time, peaking 4 weeks after PMMA spacer implantation [10,13]. One alternative approach to addressing

* Corresponding author at: Department of Orthopaedic Surgery, Stanford University, 300 Pasteur Drive, Edwards R155, Stanford, CA 94305, USA. Fax: +1 650 724 5401.
E-mail address: ypyang@stanford.edu (Y. Yang).
[1] These authors contributed equally to this work.

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these limitations is to use tissue engineering techniques to create a synthetic bioactive membrane that mimics the structure of an induced membrane. The synthetic membrane could contain and compartmentalize an autogenous or synthetic bone graft to optimize healing. Theoretically, this synthetic membrane could facilitate single-step reconstruction of large bone defects when combined with appropriate internal fixation and autogenous bone graft or synthetic bone scaffold [14]. Successful development of such a synthetic membrane has the potential to decrease the number of procedures required for the reconstruction of large bone defects while optimizing the biologic microenvironment at the fracture site and accelerating healing. Other synthetic membranes fabricated from bioresorbable polylactide [14], collagen barrier [15] or polylactide [16] have been reported for use in reconstruction of segmental bone defects. However, these synthetic membranes lack both the multiple layered structures and the osteo and angiogenic capacity of a biologically induced membrane.

A novel cell sheet engineering technique developed by Okano and colleagues has proved effective for engineering tissues such as cornea, skin, myocardium, and mucous membrane [17–19], and has also been used to create an osteogenic cell sheet for bone tissue [20–22]. More recently, the pre-vascularization of cell sheet-based constructs has been proposed as a way to circumvent vascularization of 3D thick grafts [23]. Therefore, cell sheet engineering technique has the potential to create a complex 3D synthetic biomimetic induced membrane (BIM).

The purpose of this study is to determine the feasibility of using a cell sheet engineering technique to fabricate a BIM with a similar biological profile to the biologically induced membrane. We first produced a pre-vascularized inner layer by seeding human umbilical vein endothelial cells (HUVECs) on an undifferentiated human bone marrow-derived mesenchymal stem cell (hMSC) sheet, and then an osteogenic outer layer by inducing the osteogenic differentiation of hMSCs. These two cell sheet layers were wrapped together to form a BIM with similar structural configuration to a biologically induced membrane (Fig. 1). We hypothesized that this pre-vascularized BIM would rapidly anastomose with host vasculature and demonstrate potential osteogenic properties. To this end we prepared a pre-vascularized BIM and characterized its *in vitro* pre-vascularization abilities and *in vivo* functional vascularization in the subcutaneous site of mouse. At the same time, we characterized the osteogenic potential of this BIM.

Materials and methods

Cell culture

An MSCGM™ BulletKit™ was utilized to culture hMSCs. The hMSCs have been shown to express CD105, CD166, CD29 and CD44 more than 90% of the time while they express CD14, CD34 and CD45 less than 10% of the time (Lonza Inc.). The hMSCs can be differentiated into adipogenic, chondrogenic, and osteogenic lineages [24]. The hMSCs were cultured in MSCBM (Lonza), which is a non-differentiating growth medium containing 10% fetal bovine serum (FBS) and 1× glutamine–penicillin–streptomycin (GPS; Invitrogen). HUVECs which continuously express GFP were provided by the late Folkmann laboratory at Children's Hospital (Boston) and cultured in EBM™ (endothelial basal medium) and an EGM™ (endothelial growth media) SingleQuots™ Kit (Lonza). The cell medium was changed every 3 days. Cells below passage 9 were used in all the experiments.

Production and characterization of pre-vascularized cell sheets

To make the pre-vascularized composite cell sheet, hMSCs were first cultured in a cell culture dish at a cell density of $9 \times 10^4/cm^2$ in MSCGM medium. After the cells reached confluence, the medium was changed to MSCGM with the addition of 50 μg/ml ascorbic acid and 30 mM glucose to promote the production of extracellular matrix [25]. After 14 days of culture, hMSCs formed a thick cell sheet, referred here as UM.

HUVECs were cultured in EBM-2 and trypsinized after confluence. At day 14 a HUVEC cell suspension was seeded onto the surface of the hMSC sheet at a cell density of $5 \times 10^4/cm^2$. After a designated incubation period, the morphology of the cell sheets was observed under a fluorescent microscope and images were captured. We named this HUVEC/hMSC cell sheet layer HUVEC/UM and intended for it to function as the inner pre-vascularized layer. To investigate the patterns of HUVEC pre-vascularized networks on the UM sheet, immunofluorescent staining was performed. Platelet–endothelial cell adhesion molecule (PECAM-1, or CD31), an endothelial-specific adhesion protein and a specific marker of HUVECs, was used to assess HUVEC pre-vascularized networks. After the sheets were washed three times in PBS, a 5% goat serum-PBS buffer solution was used to block the cell

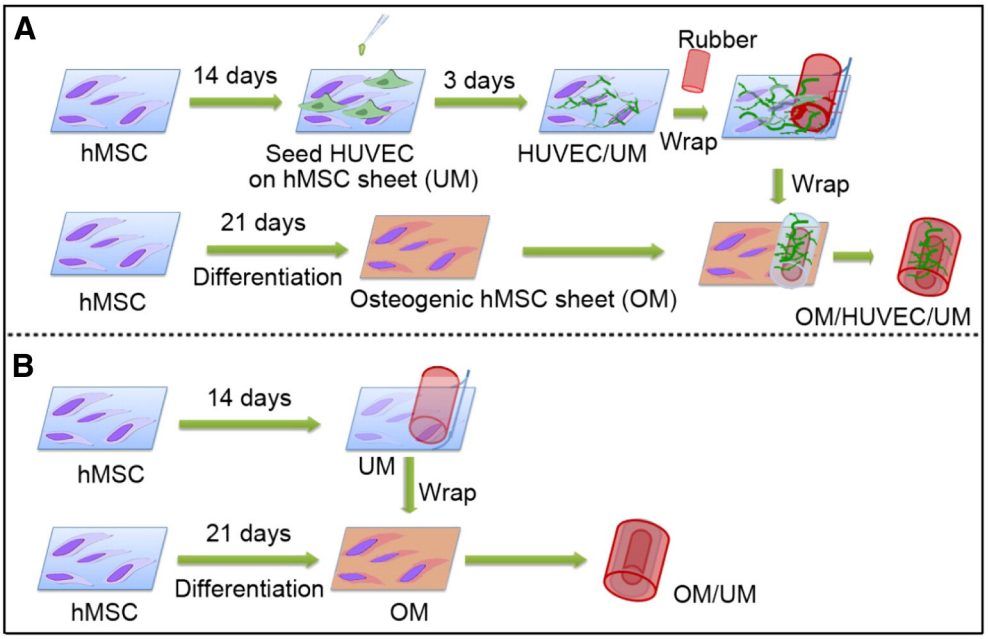

Fig. 1. The schematic of the fabrication of a compound construct OM/HUVEC/UM (A) and OM/UM (B) based on cell sheet engineering.