Ligament-to-bone Interface Tissue Regeneration Using a Functionalized Biphasic Silk Fibroin Scaffold

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Abstract—The emergence of the tissue engineering approach has shown to be a game changer in ligament reconstruction, making it possible to create multi-phasic scaffold structures for multi-specific tissue regeneration. To regenerate the bone-ligament-bone tissue, mimicking hard to soft tissue transition, we propose the use of a functionalized biphasic silk scaffold. Hydroxyapatite nanoparticles (nHA) and bone morphogenetic protein 2 (BMP2) were loaded in the ends of Bombyx mori silk fibroin (SF) scaffold system to enhance enthesis regeneration and bone tunnel healing, while the central one-third supported ligament regeneration. Two groups of biphasic scaffolds, distinguished by the different ends’ additive, were fabricated: nHA only (Ctrl) and n-HA/BMP2 (Exp). A series of bench work, small animal study and large animal preclinical trial was performed using MSC-seeded constructs for the reconstruction of excised ACL. The bioactivity of BMP2 was ascertained and shown to be eluting with an initial burst, followed by a lowered sustained release. Osteogenic genes were upregulated in both groups compared to pure SF. By 24 weeks in vivo, the ACL was regenerated and bone tunnel narrowing was observed with histological evidences indicating new bone and enthesis regeneration in Exp. Better graft to bone integration was observed in Exp compared to Ctrl. From this study, it was demonstrated that the BMP2 eluting biphasic silk scaffold is promising as an advanced tissue engineering treatment modality for complete bone-ligament-bone reconstruction.

Keywords—Tissue engineering, anterior cruciate ligament, enthesis, multi-phasic scaffold, bone.

I. INTRODUCTION

The restoration of ruptured Anterior Cruciate Ligaments (ACL) has constantly been an important field for orthopaedic research, given the chain reaction of more serious degenerative joint diseases that will follow should it be left untreated. The current gold standard for ACL reconstruction has been with the use of tendon autografts. However, problems persist in this solution, primarily due to the inherent limitation of donor site morbidity and the lack of graft integration with the bone tunnel. This integration site is known as the enthesis and its dysfunction has led to 3000 - 10000 revision surgeries annually in the United States alone [1].

With advancement in medical sciences and maturation of supporting technologies, regenerative approach towards treatment of tissue trauma, which is the basis for tissue engineering, is now regarded superior over the current reparative approach. Previous studies in ligament tissue engineering have been focused on the regeneration of the ligament tissue solely [2-5]. Particularly in our previous studies [4, 5], we had implanted the mesenchymal stem cells (MSC) seeded silk scaffolds for ligament-alone regeneration in rabbit and porcine models. 6 months post-implantation, it was observed that the MSC-seeded constructs could regenerate the ACL and epiligament with the existence of vascularity. Nevertheless, one major drawback observed was the limited regeneration of the bone insertion points, which had directly affected the functionality of the regenerated ligament in terms of its load bearing capacity. Clinically, this limitation of non-fusion or non-anchorage of the regenerated soft tissue (ligament) with the hard tissue (native bone) will affect clinical outcome and recovery duration.

In view of the morbidity issue due to graft harvesting and the need for robust graft integration, we propose the use of a functionalized biphasic silk scaffold for complete tissue regeneration of the ligament and bone tunnel tissues. The ends of this hybrid silk scaffold system are incorporated with nanoparticles of hydroxyapatite (nHA) and loaded with bone morphogenetic protein 2 (BMP2) to stimulate bone tunnel and enthesis regeneration, while the central one-third supports ligament regeneration. It is hypothesized that the osteoinductive BMP2 will synergistically complement the osteoconductive nHA to encourage bone regeneration. It is further hypothesized that the in vivo biophysical cues presented in the rabbit and porcine models will stimulate and condition the construct ends to lead to formation of fibrocartilaginous tissues with enhanced load bearing capacity.

II. MATERIALS AND METHODS

A. Scaffold Fabrication

Knitting and Degumming: Knitted scaffolds (240 fibroins, 60 × 20 mm for in vitro characterization and rabbit
implantation; 480 fibroins, 100 × 20 mm for pig implantation) were first fabricated from raw Bombyx mori silk (Thailand) and subsequently degummed as previously described [6].

**Fabrication of the osteogenic and tenogenic zones:** Aqueous SF solution was first obtained through a dissolution process as previously described [7]. Three different types of aqueous SF-based solutions were made by blending: pure aqueous SF (2.6 %w/v), aqueous SF (2.6 %w/v) with nHA (0.78 mg/end), and aqueous SF (2.6 %w/v) with nHA (0.78 mg/end) and BMP2 (29 μg/end). These solutions were then sequentially casted and frozen over the knitted SF sequentially, with the central one-third of the scaffolds casted in pure aqueous SF solution and the ends casted in either SF/nHA solution (control group, Ctrl) or the SF/nHA/BMP2 solution (experimental group, Exp). Scaffold fabrication was completed upon lyophilization and methanol treatment. The blank scaffolds were characterized for scaffold morphology, mechanical properties at the two phases and elution kinetics.

**Sterilizations:** Prior to cell seeding, the scaffolds were sterilized by means of formaldehyde (37%) (JT Baker) gassing for 24 hours. All other equipment was sterilized by autoclaving.

**B. In vitro Characterization**

For in vitro characterizations, the scaffolds were cut at the phase boundaries to form 3 hybrid scaffold groups for characterization: pure SF, SF with nHA (SF/nHA), and SF with nHA/BMP2 (SF/nHA/BMP2). Rabbit MSCs derived from bone marrow (P3, 1 × 10^6/scaffold) were then seeded onto the scaffolds and statically cultured over 28 days. Characterization assays (n = 3) were performed to determine the cellular viability, proliferation, gene expression and collagen deposition levels.

**C. In vivo Characterization**

In vivo characterization of the biphasic silk scaffold was performed in the small and large animal models. The animal experiments were approved by Institutional Animal Care and Use Committee of National University of Singapore.

**Rabbit Model:** For in vivo characterization using the rabbit model, the two groups of complete biphasic scaffolds (Exp and Ctrl) were each seeded with rabbit MSCs (P3, 3 × 10^6/scaffold) and statically cultured to allow cell adhesion for a day prior to implantation. Forty-eight New Zealand White rabbits (12 weeks old, 2.5–3.0 kg) were divided into Exp and Ctrl groups, with standard ACL reconstruction performed [4]. The rabbits were sacrificed at 2, 4, and 6 months postoperatively with knee joints collected immediately and kept at -80 °C. The samples were then scanned using micro-CT prior to histological preparations (n = 3) and mechanical tests (n = 5).

**Porcine Model:** The large animal model of choice is the ACL of Yorkshire pig. Native ACL of the right knees were cleanly removed prior to reconstruction with the biphasic silk scaffold using typical bone tunnel methods [5]. Similarly, two groups of complete biphasic scaffolds (Exp and Ctrl) were each seeded with porcine MSCs (P2, 10 × 10^6/scaffold) and implanted into a total of 14 randomly grouped pigs (~60 kg) and implanted into a total of 14 randomly grouped pigs (~60 kg) and implanted into a total of 14 randomly grouped pigs (~60 kg) and implanted into a total of 14 randomly grouped pigs (~60 kg). The pigs were sacrificed 6 months postoperatively. The knee joints were collected and scanned using micro-CT prior to histological preparations (n = 3) and mechanical tests (n = 4).

**D. Statistical Analysis**

Data were expressed as means ± standard deviation (SD). Single factor analysis of variance (ANOVA) technique was used to assess the statistical significance of results between groups. For pair-wise comparisons, two-tailed, unpaired Student’s t tests were used. A p<0.05 was considered statistically significant.

**III. RESULTS**

**A. Scaffold Morphology, Mechanical Properties and Elution Profile**

The fabricated biphasic silk scaffolds (Fig. 1A) were shown to be porous with interconnected pores (Fig. 1B). nHA and BMP2 were observed to be securely incorporated in the lyophilized SF sponges. The bioactivity of BMP2 was ascertained after the fabrication process and was shown to be eluting with an initial burst release, followed by a lowered sustained release (Fig. 1C).

Fig. 1 Gross observation of the biphasic silk scaffold with osteogenic ends (A), SEM of SF/nHA/BMP2 sponge, mag: 100×, scale bar: 100μm (B), Elution profile of BMP2 from SF/nHA/BMP2 scaffold over 45 days (C).