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The role of engineered tendon matrix in the stemness of tendon stem cells *in vitro* and the promotion of tendon-like tissue formation *in vivo*

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ABSTRACT

When injured, tendons tend to heal but with poor structure and compromised function. Tissue engineering is a promising approach to enhancing the quality of healing tendons. Our group and others have identified tendon stem cells (TSCs), a type of tendon-specific stem cells which may be optimal for cellular interventions seeking to restore normal structure and function to injured tendons. However, *in vitro* expanding of TSCs on regular plastic cell culture dishes only yields a limited number of TSCs before they lose the stemness, i.e., the self-renewal capability and multipotency. In this study, we developed a substrate material for TSCs, engineered tendon matrix (ETM) from decellularized tendon tissues. We showed that ETM *in vitro* was able to stimulate TSC proliferation and better preserve the stemness of TSCs than plastic culture surfaces. *In vivo*, implantation of ETM-TSC composite promoted tendon-like tissue formation whereas implantation of TSCs alone led to little such tissue formation. Together, the findings of this study indicate that ETM may be used to effectively expand TSCs *in vitro* and with TSCs, to enhance repair of injured tendons *in vivo*.

1. Introduction

Tendon injuries are a common problem in both occupational and athletic settings. However, injured tendons typically heal slowly, especially when the tendon injury is substantial or when rupture with tendon retraction occurs. Natural tendon healing also results in the formation of scar tissue, which has inferior mechanical properties making the healed tendons susceptible to re-injury [1]. In recent years, tissue engineering has emerged as a promising approach towards tendon repair or regeneration [2–7]. As a major component in tendons, autologous tenocytes have been used in repair of injured tendons [8]. However, removal of tendon sections for deriving tenocytes leads to formation of a secondary lesion at the donor site. In addition, tenocytes have a limited proliferative potential and quickly lose their phenotype in culture [9]. As a result, tenocytes may not be an ideal cell source for repair of injured tendons. Also, while implantation of bone marrow mesenchymal stem cells (BMSCs) induced a short term benefit in terms of improved mechanical properties at 4 weeks, it produced no visible improvement in the structure of healing tendons afterwards [10]. Moreover, the use of BMSCs even resulted in ectopic bone formation in tendons in a rabbit tendon injury model [11]. Embryonic stem cells (ESCs) can be a potential source for repair of injured tendons [12], but controlling ESC differentiation fates *in vivo* is more difficult than mesenchymal stem cells (MSCs) due to the pluripotency of ESCs; as a result, implantation of ESCs may risk formation of teratoma [13,14]. Together, these studies indicate that non-tendon derived stem cells may not be optimal for cell therapies seeking to restore the normal structure and function of injured tendons.

Traditionally, tendons are thought to contain mainly tenocytes, the resident cells responsible for tendon maintenance and repair. In recent years, tendon stem/progenitor cells (TSCs) has been identified in humans, mice, rabbits, and rats [15–18]. TSCs differ from tenocytes in that they possess clonogenicity, self-renewal, and multi-differentiation potential, the three universal criteria of stem cells. Also, as tendon-specific stem cells, TSCs by default differentiate into tenocytes [16]. Moreover, when implanted *in vivo*, TSCs were able to form tendon-like tissues [15,16]. Therefore, TSCs may be an ideal cell source for effective repair of injured tendons. However, TSCs *in vivo* are rare and amounts to less than 5% of total tendon cells [15]. Therefore, it is necessary to expand TSC

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populations in order to obtain sufficient number of these cells for cell therapy. However, when TSCs are cultured on plastic dishes, a common practice nowadays, they typically lose stemness after only several passages. A major reason for this problem is that plastic surfaces are foreign to TSCs and do not provide an appropriate environment for maintaining their stemness. Indeed, years of intensive stem cell research has uncovered that stem cells need appropriate "niches" in order to keep their stemness [19]. One of the most important niche factors for stem cells is the extracellular matrix (ECM), which is known to be crucial to the normal growth and function of stem cells [20–22]. Undoubtedly, an ideal ECM for TSCs would be a matrix from tendon tissues, which may retain the crucial niche factors including matrix components (e.g. collagen) and growth factors that are able to regulate TSC function. In a recent study, for example, when BMSCs were seeded on decellularized multilayer sliced tendon tissues, they expressed tenomodulin, a specific marker for tenocytes, suggesting that tendon matrix can prime non-tissue-specific BMSCs to differentiate towards tenocytes [23]. As a further advancement, we have developed an engineered tendon matrix (ETM) using decellularized tendon tissues, which can exist in the form of either film or gel for facilitating *in vivo* implantation. The purpose of this study was to characterize the behavior of TSCs on ETM in terms of self-renewal and multi-potency and to determine their ability to form tendon tissue *in vivo*. We found that TSCs on ETM exhibited enhanced self-renewal capability and multi-differentiation potential *in vitro*, and they were able to form tendon-like tissues *in vivo*.

2. Materials and methods

2.1. Preparation of ETM

Twelve female New Zealand white rabbits (8–10 months old, 3.0–4.0 kg weight) were used for both ETM preparation and TSC derivation. The protocol for the use of the rabbits was approved by the University of Pittsburgh IACUC. All rabbits were fully sedated using intra-muscular Ketamine (10 mg/kg) and Xylazine (3 mg/kg) injections and were then sacrificed. After sacrifice, rabbit patellar tendons were obtained by dissection.

For preparation of ETM, patellar tendons were immersed in liquid nitrogen for 5 min and ground into powder. The powder was then treated with 0.5% trypsin/PBS solution under vigorous agitation at 37 °C. The treatment lasted for 24 h, and the trypsin was changed every 4 h. Then the powder was washed three times with PBS, 30 min each time, and treated with a nuclease solution (50 U/ml DNase and 1 U/ml RNase in 10 mM Tris-HCl, pH 7.5) at 37 °C for 12 h. After nuclease digestion, the powder was treated with 1% Triton X-100 for 24 h, washed with PBS 6 times with 8 h each time, and stored at −80 °C for subsequent cell culture or implantation experiments.

2.2. Fabrication of ETM film and gel

ETM powder was dissolved with 3% acetic acid (HAc, ~0.5 M) to make a 5% solution. An ETM film was formed by adding 1 ml ETM-HAc solution in a 35 mm Petri dish and letting it dry overnight under UV light. ETM was also made in a gel form by the addition of sodium hydroxide to ETM-HAc solution.

2.3. Scanning electron microscopy (SEM) of ETM

ETM was sputter coated with gold/palladium and examined under a JEOL (Tokyo, Japan) SEM with an accelerating voltage of 3.0 kV.

2.4. Isolation of rabbit and human TSCs

TSCs were isolated from rabbit patellar tendons. The procedures for isolation of TSCs were similar to our previously published protocol [24]. Using the same protocol, human patellar TSCs were derived from human tendon samples. The protocol for obtaining human tendons and the subsequent culture study was approved by the Institutional Review Board (IRB) of University of Pittsburgh.

2.5. Cell culture

The ETM film was washed 3 times with PBS, 3 times with 70% ethanol, and finally 5 times with PBS. Rabbit patellar TSCs (rPTSCs) at passage 1 were seeded either on ETM film or in ETM gel in 6-well plates at a density of 4.5×10^4 cells/well and cultured for up to 7 days. The morphology of TSCs grown on ETM film or in ETM gel was either examined directly by using an inverted microscope or histochemically stained with H&E. Population doubling time (PDT) was determined to assess the proliferative capacity of these cells on ETM films according to the method previously published [16].

For H & E staining, TSCs grown on/in ETM were fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the ETM with TSCs was placed in pre-labeled base molds filled with frozen section medium (Neg 50; Richard-Allan Scientific; Kalamazoo, MI). The base mold with ETM and TSCs was quickly immersed in liquid nitrogen cooled 2-methylbutane and allowed to solidify completely. The ETM-TSCs block was cut into 10 μm thick sections, and the sections were placed on glass slides and allowed to dry overnight at room temperature. The sections were rinsed three times with PBS and stained with H&E.

2.6. Characterization of TSCs

TSCs were characterized by immunostaining the following stem cell markers: octamer-binding transcription factor 4 (Oct-4), stage-specific embryonic antigen-1 and -4 (SSEA-1 and SSEA-4), and nucleostemin. The TSCs grown on ETM film or the plastic surfaces of Petri dishes were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and treated with 0.1% Triton X-100 for 30 min for Oct-4 and nucleostemin staining. After washing the cells with PBS, either mouse anti-Oct-4 (1:350) or goat anti-nucleostemin (1:400) was applied for 2 h at room temperature. The cells were washed with PBS for three times, and either Cy-3-conjugated goat anti-mouse IgG antibodies (1:500 for Oct-4) or Cy3-conjugated donkey anti-goat IgG antibodies (1:500 for nucleostemin) was applied for 1 h at room temperature. In order to stain for SSEA-1 and SSEA-4, fixed cells were blocked with 2% mouse serum for 1 h and incubated with mouse anti-human SSEA-1 or SSEA-4 antibodies (1:500) for another hour at room temperature. After washing the cells with PBS, TSCs were treated with either fluorescein isothiocyana (FITC)-conjugated goat anti-mouse secondary antibodies (1:500 for SSEA-1) or Cy3-conjugated goat anti-mouse IgG antibodies (1:500 for SSEA-4) for 1 h at room temperature. The stained cells were examined using fluorescence microscopy. All antibodies used were from Chemicon International (Temecula, CA), BD Biosciences (Franklin Lakes, NJ), or Neuromics (Edina, MN).

To quantify the expression of stem cell markers, the stained samples were examined using an inverted fluorescence microscope and images were taken with a 20× objective using a CCD camera. A total of 60 views from 3 wells of a 6-well plate were randomly chosen for each stem cell marker and the number of positively stained cells was manually counted. The percentage of each stem cell marker expression was determined by dividing the number of positively stained cells by the total number of cells stained by the nuclear stainging reagent Hoechst fluorochrome 33342 (1 mg/ml; Sigma, St. Louis, MO).

2.7. Characterization of multi-differentiation potential of TSCs

Multi-differentiation potential of TSCs on ETM or plastic surfaces was tested *in vitro* for adipogenesis, chondrogenesis, and osteogenesis. TSCs at passage 1 were seeded either on ETM or plastic surfaces in 6-well plates at a density of 2.4×10^5 cells/well in basic growth medium (DMEM plus 10% FBS). To test adipogenic potential, TSCs were cultured in adipogenic induction medium (Millipore, Billerica, MA) consisting of basic growth medium added with dexamethasone (1 μM), insulin (10 μg/ml), indomethacin (100 μM), and isobutylmethylxanthine (0.5 mM). As a test of chondrogenic potential, TSCs were cultured in basic growth medium supplemented with prolin (40 μg/ml), dexamethasone (39 ng/ml), TGF-β3 (10 ng/ml), ascorbate 2-phosphate (50 μg/ml), sodium pyruvate (100 μg/ml), and insulin-transferrin-selenious acid mix (50 mg/ml) (BD Bioscience, Bedford, MA). Finally, the osteogenic potential of TSCs was tested by culturing TSCs in osteogenic induction medium (Millipore, Billerica, MA) consisting of basic growth medium supplemented with dexamethasone (0.1 μM), ascorbic 2-phosphate (0.2 mM), and glycerol 2-phosphate (10 mM).

After 21 days culture, TSCs grown on ETM or plastic surfaces with various differentiation media were stained using Oil Red O for adipogenesis, Safranin O for chondrogenesis, or Alizarin Red S for osteogenesis, respectively (detailed protocols are shown below). The stained samples were examined using an inverted microscope and images were taken with a 20× objective using a CCD camera. A total number of 12 views from each well were randomly chosen. The areas of positive staining were identified manually and computed by a SPOT imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI). The ratio of positive staining was calculated by dividing the stained area by the view area. The values of all views from three duplicate wells (36 views in total) were averaged to obtain the percentage of positive staining, which represents the extent of cell differentiation in the respective induction medium.

2.8. Oil Red O assay for adipogenesis

After removing culture medium, cells were washed with PBS 3 times, for 5 min each time. The cells were then fixed by 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were washed with PBS 3 times each for 5 min and then with distilled water 2 times each for 5 min. Finally, the cells were incubated with 0.36% Oil Red O solution (Millipore, Billerica, MA) for 50 min and then washed 3