

Decellularized Tendon-Derived Stem Cell Sheet Exhibited Direct Osteogenic and Immunomodulatory Effects for the Promotion of Graft Healing after Anterior Cruciate Ligament Reconstruction

Hin Cheuk KOT¹, Yuk Wa LEE¹, Patrick Shu Hang YUNG^{1,2}, Ssu Chi CHEN¹, Pauline Po Yee LUI^{1,2,*}

¹ Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Hong Kong SAR, China

² Center for Neuromusculoskeletal Restorative Medicine, Hong Kong Science Park, Hong Kong SAR, China (* Correspondence)

Email of Presenting Author: cheukhinkot@cuhk.edu.hk

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INTRODUCTION: The outcome of anterior cruciate ligament reconstruction (ACLR) is not satisfactory with graft failure and graft laxity, requiring a second revision surgery. We showed that decellularized tendon-derived stem cell (dTDCS) sheets promoted graft healing in a rat ACLR model. However, the underlying mechanism remains unclear. We have shown that the transplantation of dTDCS sheet promoted tunnel bone formation, angiogenesis, increased M2 macrophages but decreased M1 macrophages, enhanced TIMP1 expression but reduced MMP1 and MMP13 expression. We therefore hypothesized that dTDCS sheet might promote graft healing by enhancing bone formation and modulating the inflammatory environment. This study aimed to examine the osteogenic and immunomodulatory effects of dTDCS sheets *in vitro*.

METHODS: Animal research ethics committee approved the study. Rat TDSCs were isolated from the Achilles tendons from male Sprague-Dawley rats (6-week-old; weight, 200-220 g) and treated with our proprietary combination of growth factor and biologicals for 2 weeks to induce cell sheet formation. The TDSC sheet was then decellularized according to our published protocol. The content of bioactive factors including SDF-1 and POSTN in the dTDCS sheet and TDSC sheet were compared by ELISA. MC3T3 pre-osteoblasts were seeded on dTDCS sheet or plastic surface. The viability and proliferation of MC3T3 pre-osteoblasts were examined by AlamarBlue reduction assay and *Ki67* mRNA expression while the migration of MC3T3 cells was studied by the transwell assay. The osteogenic effects of dTDCS sheet were examined by calcium nodule formation using Alizarin red S assay and dye quantification at day 28 after induction while the mRNA expression of *Bsf*, *Bglap* and *Osx* was assessed by qRT-PCR at day 7 after induction. The effects of dTDCS sheet on the polarization and expression of inflammatory cytokines in macrophages were assessed by seeding LPS-treated RAW264.7 cells on the dTDCS sheet or plastic surface for 6 hours. The mRNA expression of CD86, CD206, pro-inflammatory (*Il1b*, *Il6*, *Cxcl10*) and anti-inflammatory cytokines (*Il10*) in macrophages was evaluated by qRT-PCR. The effect of dTDCS sheet in modulating the transcriptomes and hence functions of the treated macrophages was examined by RNA sequencing. The top differential biological processes and signalling pathways were further explored.

RESULTS: dTDCS sheet expressed similar levels of SDF-1 and POSTN compared to the TDSC sheet ($p > 0.05$) (both $n = 4-6$ /group). dTDCS sheet increased the viability and proliferation ($p = 0.050$) ($n = 3$ /group), expression of *Ki67* ($p < 0.05$) ($n = 6$ /group), and migration ($n = 4$ /group) of MC3T3 cells. More Alizarin red S-stainable calcium nodules ($p = 0.050$) ($n = 3$ /group) and higher osteogenic marker expression (all $p < 0.05$) ($n = 6$ /group) were observed after seeding MC3T3 cells on the dTDCS sheet compared to seeding on the plastic surface (**Figure 1**). There was a significant lower mRNA expression of CD86 but higher expression of CD206 after culturing LPS-treated RAW264.7 cells on dTDCS sheet compared to cells seeded on plastics (both $p < 0.01$) ($n = 6$ /group) (**Figure 2**). Seeding of RAW264.7 on dTDCS sheet also suppressed LPS-induced increase of *Il1b*, *Il6* and *Cxcl10* as well as increased the expression of *Il10* (all $p < 0.01$) ($n = 6$ /group). RNA sequencing showed that 86 genes were upregulated while 149 genes were downregulated after culturing LPS-treated RAW264.7 on dTDCS sheet (**Figure 3**). DEG analysis showed upregulation of anti-inflammatory *Il33* and *Il10* and downregulation of pro-inflammatory *Il23a* in LPS-treated macrophages seeded on dTDCS sheet compared to cells seeded on plastics. GO analysis showed downregulation of negative regulation of peptidase activities in LPS-treated macrophages seeded on dTDCS sheet compared to cells seeded on plastics. Moreover, processes related to cell division were upregulated as shown by GO, KEGG and Reactome analyses. Reactome analysis also identified MHC class II antigen presentation as one of the key biological processes upregulated in LPS-treated macrophages seeded on dTDCS sheet compared to cells seeded on plastics.

DISCUSSION: The dTDCS sheet expressed osteogenic and chemotactic factors. It enhanced viability, proliferation, migration, and osteogenic differentiation of pre-osteoblasts. The expression of SDF-1 and periostin in dTDCS sheet suggested that it could attract stem/progenitor cells to the injury site and promoted their osteogenic differentiation for tissue repair. The osteogenic effect of dTDCS sheet supported increased bone formation at the tendon-bone junction (TBJ) in ACLR after dTDCS sheet transplantation as reported in our previous study. The dTDCS sheet inhibited LPS-induced M1 polarization and enhanced its M2 polarization, with concomitant reduced expression of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokine in macrophages. Transcriptome analysis showed that dTDCS sheet enhanced the proliferation, MHC class II presentation and peptidase activities of LPS-treated macrophages. The findings were consistent with the lower M1 macrophages and higher M2 macrophages observed at the TBJ and graft mid-substance reported previously in our ACLR animal model. The suppression of pro-inflammatory cytokines in macrophages might contribute to higher graft integrity in the dTDCS sheet group compared to the untreated group observed in our ACLR model. Further study is required to identify the bioactive factors in dTDCS sheet and the signalling pathway that promoted M2 macrophage polarization. In conclusion, the dTDCS sheet expressed key growth-promoting factors similar to TDSC sheet. It showed direct osteogenic and immunomodulatory effects, which might explain increased bone formation and lower inflammation and hence better healing outcomes after its transplantation in the ACLR animal model. We will confirm these *in vitro* findings in the ACLR animal model by immunohistochemistry staining in future.

CLINICAL SIGNIFICANCE: A better understanding of the key biological processes and signalling pathways underlying graft healing effects of dTDCS sheet would provide insight for its optimization and the development of new treatment strategies for the promotion of graft healing after ACLR.

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