Tenogenic Differentiation of Mesenchymal Stem Cells and Their Applications in Tendon Tissue Engineering

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Anatomy of Tendon and Muscle Tendon Junction

Muscle-Tendon Junction van Gieson

- collagen fibres
- fibrocyte nuclei

Tendon

skeletal muscle fibres

Muscle
## Tendon has unique composition

<table>
<thead>
<tr>
<th>Composition</th>
<th>Ligament</th>
<th>Tendon</th>
<th>Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (w/w)</td>
<td>60%</td>
<td>55%</td>
<td>9%</td>
</tr>
<tr>
<td>Mineral (w/w)</td>
<td>0</td>
<td>0</td>
<td>69%</td>
</tr>
<tr>
<td>Collagen (% dry weight)</td>
<td>75%</td>
<td>85%</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>(90% type I; 10% type III)</td>
<td>(&gt;90% type I, &lt;10% types III, V, etc.)</td>
<td>(80% type 1 and 20% others)</td>
</tr>
<tr>
<td>Fibroblasts (% volume)</td>
<td>20%</td>
<td>15%</td>
<td>10%</td>
</tr>
<tr>
<td>Extra-cellular matrix (% volume)</td>
<td>80%</td>
<td>85%</td>
<td>91%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(predominant proteoglycan is decorin)</td>
<td>(mainly calcium and phosphate)</td>
</tr>
<tr>
<td>Collagen fibrils diameter (nm)</td>
<td>40-75</td>
<td>60-175</td>
<td>70-100</td>
</tr>
</tbody>
</table>
Normal tendon contains multi-potent stem cells

Tendon-derived Stem Cells (TDSCs) in Normal Rat Tendon Tissue

Isolation and Characterization of Multipotent Rat Tendon-Derived Stem Cells

Yun-Fong Rui, M.Phil., 1,2 Pauline Po Yee Lui, Ph.D., 1,2 Gang Li, Ph.D., 1,2 Sai Chuen Fu, M.Phil., 1,2 Yuk Wa Lee, M.Phil., 1,2 and Kai Ming Chan, M.D. 1,2
Isolation and Characterization of TDSCs

- **GFP-Rat BMSCs**
- **Fluorescent Image**
- **Calcium Nodule Staining** ---- Osteogenisis
- **Oil O Red Staining** ---- Adipogenisis
- **Alcian Blue Staining** ---- Chondrogenisis
TDSCs have the potential of spontaneous tenogenic differentiation
Crucial transcription factors in tendon development and differentiation: their potential for tendon regeneration

Huanhuan Lin · Shouan Zhu · Can Zhang · Ping Lu · Jiajia Hu · Zi Yin · Yue Ma · Xiao Chen · Hongwei Ouyang

Regulation of Tenomodulin Expression Via Wnt/β-catenin Signaling in Equine Bone Marrow-derived Mesenchymal Stem Cells

Shihori MIYABARA¹, Yoshei YUDA¹, Yoshinori KASASHIMA², Atutoshi KUWANO² and Katsuhiko ARAI¹*¹
¹ Department of Tissue Physiology, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

Table 2. Comparison of mRNA level between the tenodon and monolayer BMSC by qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tenodon</th>
<th>Monolayer BMSC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tenomodulin</em></td>
<td>0.00057 ± 0.00012*</td>
<td>0.00006 ± 0.00001</td>
</tr>
<tr>
<td>Col1a2</td>
<td>0.11810 ± 0.03612</td>
<td>0.15139 ± 0.02522</td>
</tr>
<tr>
<td>Col3a1</td>
<td>0.00880 ± 0.00251</td>
<td>0.13373 ± 0.02121</td>
</tr>
<tr>
<td>Col12a1</td>
<td>0.07856 ± 0.01431</td>
<td>0.24827 ± 0.03142</td>
</tr>
<tr>
<td>Col14a1</td>
<td>0.01458 ± 0.00373*</td>
<td>0.00003 ± 0.00001</td>
</tr>
<tr>
<td><em>Decorin</em></td>
<td>29.65080 ± 2.85643*</td>
<td>0.70031 ± 0.14381</td>
</tr>
<tr>
<td><em>Fibromodulin</em></td>
<td>0.11311 ± 0.02413*</td>
<td>0.00599 ± 0.00143</td>
</tr>
<tr>
<td>Lumican</td>
<td>1.16473 ± 0.28143</td>
<td>0.94606 ± 0.14877</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>0.01858 ± 0.00143</td>
<td>0.01010 ± 0.00131</td>
</tr>
</tbody>
</table>
# Tenogenic Markers (Tenocytes Vs. TDSCs)

<table>
<thead>
<tr>
<th></th>
<th>A Tendon</th>
<th>A TDSC</th>
<th>P Tendon</th>
<th>P TDSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scx</td>
<td>1</td>
<td>1.52</td>
<td>1</td>
<td>0.11</td>
</tr>
<tr>
<td>pMkx</td>
<td>1</td>
<td>0.17</td>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>Egr1</td>
<td>1</td>
<td>0.12</td>
<td>1</td>
<td>0.53</td>
</tr>
<tr>
<td>Egr2</td>
<td>1</td>
<td>0.98</td>
<td>1</td>
<td>1.69</td>
</tr>
<tr>
<td>Eya1</td>
<td>1</td>
<td>0.00011</td>
<td>1</td>
<td>***</td>
</tr>
<tr>
<td>TNMD</td>
<td>1</td>
<td>0.002</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>Col1a1</td>
<td>1</td>
<td>0.56</td>
<td>1</td>
<td>0.60</td>
</tr>
<tr>
<td>Col3a1</td>
<td>1</td>
<td>5.08</td>
<td>1</td>
<td>4.81</td>
</tr>
<tr>
<td>Fomd</td>
<td>1</td>
<td>0.07</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Decorin</td>
<td>1</td>
<td>0.00</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>Lox</td>
<td>1</td>
<td>4.62</td>
<td>1</td>
<td>9.54</td>
</tr>
<tr>
<td>EphA4</td>
<td>1</td>
<td>0.95</td>
<td>1</td>
<td>1.78</td>
</tr>
<tr>
<td>TenC</td>
<td>1</td>
<td>2.19</td>
<td>1</td>
<td>1.03</td>
</tr>
<tr>
<td>Six</td>
<td>1</td>
<td>0.92</td>
<td>1</td>
<td>6.73</td>
</tr>
<tr>
<td>CD90</td>
<td>1</td>
<td>34.42</td>
<td>1</td>
<td>276.00</td>
</tr>
<tr>
<td>CD73</td>
<td>1</td>
<td>1.36</td>
<td>1</td>
<td>4.11</td>
</tr>
<tr>
<td>NS</td>
<td>1</td>
<td>1.66</td>
<td>1</td>
<td>1.58</td>
</tr>
</tbody>
</table>
miR-124 is involved in regulation of tenogenic differentiation by targeting tenogenic transcription factors.

<table>
<thead>
<tr>
<th>miR-124</th>
<th>✓Mkx</th>
<th>✓Egr1</th>
<th>✓Eya1</th>
</tr>
</thead>
</table>

### Predicted Consequential Pairing of Target Region (Top) and miRNA (Bottom)

<table>
<thead>
<tr>
<th>Position</th>
<th>miRNA Sequence</th>
<th>Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position 262-268 of MKX 3’ UTR</td>
<td>5’...UUGCCAUUACAGUAAGUGCCUUUG... 3’ CCGUAAGUGGCG-CACGGAAU</td>
<td>5’... 3’</td>
</tr>
<tr>
<td>mo-miR-124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Position 774-780 of EGR1 3’ UTR</td>
<td>5’...AAGUUCACGUCUUGGUGCCUUU... 3’ CCGUAAGUGGCG---CACGGAAU</td>
<td>5’... 3’</td>
</tr>
<tr>
<td>mo-miR-124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Position 843-849 of EYA1 3’ UTR</td>
<td>5’...GCACAAACUCCUGCAGUGCCUUUA... 3’ CCGUAAGUGGCGCACGGAAU</td>
<td>5’... 3’</td>
</tr>
</tbody>
</table>
miR-124 is up-regulated in TDSCs and reduced during spontaneous tenogenic differentiation of TDSCs.

The mRNA expression level of miR-124-3p was much higher in TDSC compared with that in tendon (A), and down-regulated during the process of spontaneous tenogenic differentiation (B).
Tenogenic Differentiation of BM-MSCs

In vitro tensile loading to promote tendogenic differentiation (unpublished data)
The use of growth factors to control tenogenic differentiation of BM-MSCs

Tendon Related Markers:
- Scleraxis (Scx)
- Tenomodulin (TeM)
- Tenascin C (TnC)
- Collagen Type I
- Decorin
- Biglycan
- Smad8
- Epha4

GDF-6, GDF-7 and CTGF can increase TeM expression
CTGF & Ascorbic Acid

CTGF (*Lee CH, 2010*)
- In vivo CTGF promoted postnatal connective tissue to undergo fibrogenesis rather than ectopic mineralization.
- CTGF promoted fibroblastic differentiation of MSCs.

Ascorbic Acid (*Omeroğlu S, 2009*)
- Vitamin C could stimulate the Achilles tendon healing because of early angiogenesis and increased collagen synthesis in a healthy rat model.
CTGF and Vit C Promoted Tendon-specific Markers Expression in TDSCs in vitro

1. Higher Tenogenic Differentiation Potential of TDSCs compared to BMSCs
2. CTGF (25ng/ml) did not promote Osteogenesis and Chondrogenesis of TDSCs and BMSCs
CTGF and Vit C Promoted ECM Production in TDSCs

Sirius red staining, 2 weeks,
*p<0.05, N=6, Non-Parameter Tests, Mann-Whitney Test
Limitations & Challenges of Tendon Regeneration

**Limitations:**

Tendon healing is poor:
1. The tendon healed with poor tissue quality.
2. The regenerated fibrotic scar tissue could not regain its original mechanical strength.

*(Miyashita et al., 1997)*

**Challenges:**

*How to improve tendon healing outcome?*

*Tissue Engineering – can we use BM-MSCs or TDSCs to promote tendon healing or regeneration?*
Tendon-derived stem cells (TDSCs), may be used to produce tendon tissues through tenogenic differentiation in vitro; and form neo-tendon and promote tendon healing in vivo.

Tendon-Derived Stem Cells (TDSCs) Promote Tendon Repair in a Rat Patellar Tendon Window Defect Model

Ming Ni,1,2 Pauline Po Yee Lui,1,2,3 Yun Feng Rui,1,2 Yuk Wa Lee,1,2 Yuk Wai Lee,1,2 Qi Tan,1,2 Yin Mei Wong,1,2 Siu Kai Kong,4 Pui Man Lau,4 Gang Li,1,2,3 Kai Ming Chan1,2

1Department of Orthopaedics and Traumatology, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China, 2The Hong Kong Jockey Club Sports Medicine and Health Sciences Centre, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong SAR, China, 3Program of Stem Cell and Regeneration, School of Biomedical Science, The Chinese University of Hong Kong, Hong Kong SAR, China, 4Programme of Biochemistry, School of Life Sciences, The Chinese University of Hong Kong, Hong Kong SAR, China

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1. GFP-TDSCs were isolated from the intact patellar tendons of GFP rats and characterized following our previous study protocol.

2. Fibrin glue constructs with or without GFP-TDSCs was transplanted into the SD rat patellar tendon window defect.

3. The patellar tendons were harvested for ultrasound imaging, histology, ex vivo fluorescent imaging and biomechanical test at various time points.
TDSCs Promotes Tendon Healing - H&E staining

1. The cellularity was higher in the TDSCs group than control groups at week 1.
2. The healing cells became elongated at 2 and 4 weeks in the TDSCs group.
3. More extracellular matrices were produced in the TDSCs group than control groups at all time points.
1. The collagen birefringence increased with healing in both groups.
2. Higher collagen birefringence was observed in the TDSCs group than control groups at all time points, suggesting better collagen fiber alignment.
Biomechanical Test

Control VS TDSCs:
◆ At 4 weeks post-op, the Ultimate Stress and Young’s Modulus in the TDSCs group was significantly higher than that of control group.

![Ultimate Stress and Young's Modulus Graphs](image-url)
The histology results showed the TDSCs group and BMSCs group had similar healing quality in tendon healing.
At all time points, the Ultimate Stress and Young’s Modulus in the TDSCs and BM-MSCs groups had no significant difference.
Engineered TDSCs Cell Sheets *in vitro*

TDSCs were treated by CTGF and Vit C for 2 weeks.
Engineering Tendon Using TDSCs Cell Sheets

Intact Patellar Tendon

Engineered Tendon
ESFTT formed neo-tendon tissues in nude mice

Engineered tendon sheets by TDSCs formed neo-tendon tissues in nude mice.
ESFTT Forms Neo-tendon in Nude mouse.
TDSCs Cell Sheet for Tendon Repair

(A) GFP-TDSCs

(B) Cell Sheet Formation
- CTGF
- Ascorbic Acid

(C) Tendon Tissue Engineering

(D) Engineered Scaffold-free Tendon Tissue

(E) Characterization:
- mRNA Expression of:
  - Tenogenic markers
  - Osteogenic markers
  - Chondrogenic markers
  - Tendon ECM markers

Animal Model Study

Assessments:
1. Gross Observation
2. In vivo Fluorescence Imaging
3. Histology
4. Immunohistochemistry

Neo-tendon formation in nude mouse.

Promote tendon healing in SD rat patellar tendon window injury model.

Assessments:
1. Gross Observation
2. Histology
3. Immunohistochemistry
4. Biomechanical Test
**TDSCs Cell Sheet for Tendon Repair**

<table>
<thead>
<tr>
<th>Control Group</th>
<th>ESFTT Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>B1</td>
</tr>
<tr>
<td>C1</td>
<td>D1</td>
</tr>
<tr>
<td><strong>4 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>B2</td>
</tr>
<tr>
<td>C2</td>
<td>D2</td>
</tr>
<tr>
<td><strong>8 weeks</strong></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>B3</td>
</tr>
<tr>
<td>C3</td>
<td>D3</td>
</tr>
</tbody>
</table>
TDSCs
Cell Sheet in Tendon Repair
TDSCs Cell Sheets for Tendon Repair

Non-treatment Group

Negative Control

Osteocalcin

Type II collagen

ESFTT Group

Negative Control

Osteocalcin

Type II collagen

2 weeks

4 weeks

8 weeks

GFP staining

GFP staining

GFP staining

Non-treatment Group

Negative Control

GFP staining

GFP staining

GFP staining

ESFTT Group

Negative Control

GFP staining

GFP staining

GFP staining
TDSCs Cell Sheet for Tendon Repair

A  Ultimate Stress  

B  Young’s Modulus  

Time Point

Groups
Control Group  
TDSCs sheet Group  

*\(p=0.001\)  
*\(p=0.01\)  
*\(p=0.012\)

*\(p=0.004\)  
*\(p=0.004\)  
*\(p=0.001\)
Engineered scaffold-free tendon tissue produced by tendon-derived stem cells

Ming Ni, Yun Feng Rui, Qi Tan, Yang Liu, Liang Liang Xu, Kai Ming Chan, Yan Wang, Gang Li

Conclusions

1. TDSCs is a new cell source for tendon regeneration.
2. TDSCs showed its superiority in promoting tendon healing than that of BMSCs after stimulating with CTGF and ascorbic acid for 2 weeks.
3. The engineered TDSCs cell sheets formed neo-tendon tissues in vivo.
4. The engineered TDSCs cell sheets promoted tendon healing in a rat acute patellar tendon injury model, it may be a new strategy for tendon repair.
Summary

1. More mechanistic studies of tenogenic differentiation are needed: epigenetic and genetic regulations; mechanical and environmental cues, etc.

2. The use of TDSCs or growth factors to guide or promote tendon regeneration in tendon disorders and injury.

3. Clinical studies: clinical trials and samples for further confirmation studies.
Thank You!

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