Collagen GFP Reporter Mice Reveal Unique Subsets Of Cells Within The Tendon Midsubstance

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Introduction

Heterogeneity within tendons and ligaments has traditionally been defined at the tissue level. Although, cells residing within the tendon fascicle, known as internal tendon fibroblasts or tenocytes, have classically been regarded as a homogenous population. Recent work has suggested that cells at multiple stages of the lineage exist within the internal population [1]. *In vivo* tools are needed to identify the progenitors and more mature cell types to better understand this lineage. To this end, our lab has identified lineage tracing and GFP reporter mouse lines that map to certain subset of cells within the tenocyte population. We previously reported that Col1a1(3.6kb)-CFP mice containing a 3.6kb fragment of the Col1a1 promoter display similar expression to Scx-GFP within multiple tendons and ligaments [2]. In addition, we demonstrated that Col1a1(2.3kb)-GFP, with a truncated 2.3kb region of the Col1a1 promoter, and Col6a1-GFP transgenic mice display expression in only a subset of cells within the tendon fascicle. The objectives of the current study are 1) to quantify the number of Col1a1(2.3kb)-GFP+ and Col6a1-GFP+ cells within multiple tendons and ligaments at different stages of growth and 2) to measure endogenous gene expression profiles of laser captured GFP+ cells using a microfluidic qPCR array to further define the level of cellular heterogeneity.

Methods

Transgenic Mice

All animals and procedures were approved by UPenn's IACUC. Two transgenic mouse lines were used in this study: 1) **Col2.3GFP** - Col1a1(2.3kb)-GFP mice contain 2.3kb of the Col1a1 promoter driving GFP expression [3] and 2) **Col6GFP** – BAC containing Col6a1 promoter driving GFP expression (acquired from MMRRC).

Experimental Design

Fore- and hindlimbs were isolated from P4, P14, and P28 mice for cryohistological analysis while knees from P14 mice were isolated for LCM and qPCR. The patellar tendon (**PT**), cruciate ligaments (**ACL/PCL**), Achilles tendon (**AT**), and supraspinatus tendon (**ST**) were analyzed for histology (n=4-5/group). For LCM, **Col2.3+** and **Col6+** cells were isolated from the PT and compared to **ACL/PCL**, articular cartilage (**AC**), and *whole* PT midsubstance controls from Col2.3GFP (**Col2.3PT**) and Col6GFP (**Col6PT**) sections (n=4/group).

Cryohistology

Limbs were fixed in formalin, embedded, counterstained with DAPI, and imaged on the Zeiss Axio Scan.Z1.

Laser capture microscopy (LCM)

Knees were fixed in 4% PFA, embedded, and sectioned using CryoJane system. Slides were dehydrated and GFP+ cells or larger regions of tissue were isolated using the ArcturusXT laser capture microscope.

Microfluidic qPCR Array

RNA was extracted from LCM samples, converted to cDNA, and preamplified for 93 targets and 3 housekeeping genes. qPCR reactions for 96 samples and 96 genes were run on Fluidigm's 96.96 Dynamic Array IFC yielding 9,216 individual Ct reactions.

Image Quantification

The GFP intensity was recorded for each cell within the tendon/ligament midsubstance. An equivalent minimum threshold was applied and the percentage of GFP+ cells was computed.

Statistics

One-way ANOVAs with either tissue type or age as fixed factors were used to analyze the number of GFP+ cells in the histological sections. Principal component analysis and hierarchical clustering were used to summarize the qPCR data.

Results

Age and tissue dependence of fluorescent reporters

Both tissue type and age had an effect on Col2.3GFP and Col6GFP expression (Fig. 1; $p<0.05$). Col2.3GFP expression increased in both the AT and PT with age (Fig. 1A). Conversely, Col2.3GFP was higher in the ACL/PCL and ST at P4 and P14 but significantly dropped at P28 ($p<0.05$). Col6GFP expression was highest in

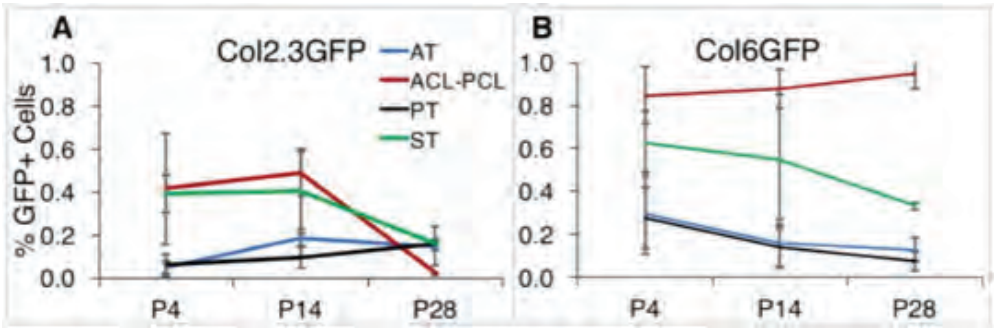

Figure 1. Percentage of Col2.3GFP+ **(A)** and Col6GFP+ **(B)** cells in AT, ACL=PCL, PT, and ST at postnatal days 4, 14 and 28.

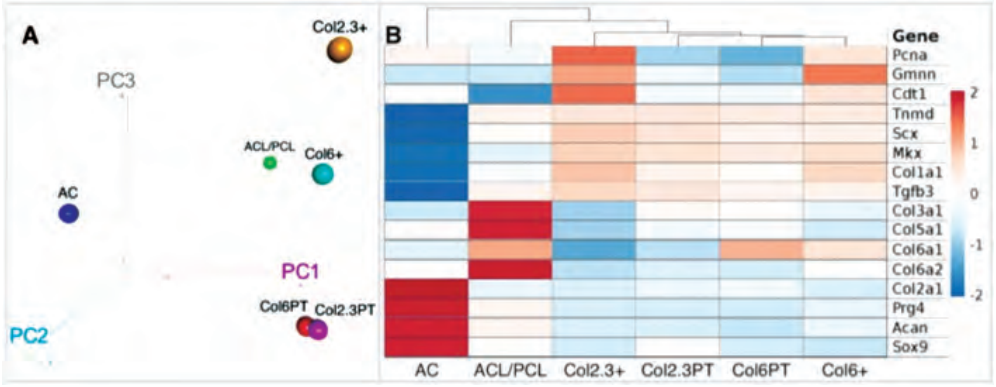

Figure 2. Principal component scores **(A)** of 6 tissue/cell groups and hierarchical clustering of groups **(B)** for subset of genes from dCt values of Fluidigm 96.96 dynamic assay.

the cruciate ligaments (ACL/PCL) at all three ages (Fig. 2B). There was an increasing trend in Col6GFP expression in the ACL/PCL with age while the three tendons all decreased with age. Interestingly, the AT showed a regional variation in both reporter lines (data not shown). The region of the AT midsubstance proximal to the enthesis but adjacent to the calcaneus displayed elevated Col2.3GFP expression at P4 and P14 with a significant drop at P28, similar to the ACL/PCL. Additionally, this region of the AT had the highest Col6GFP expression at all time points.

Endogenous gene expression indicates Col2.3GFP+ cells are more unique than Col6GFP+ cells in the PT

Principal component analysis of the 93 target genes and 6 cell/tissue types (Col2.3+, Col6+, Col2.3PT, Col6PT, ACL/PCL, and AC) revealed that 92% (57+26+9%) of the total variance was accounted for in the first 3 principal components. PC scores indicated that AC was the most different (Fig. 2A). However, Col2.3+ cells were more unique from *whole* PT samples than Col6+ cells (Fig. 2A). In fact, Col2.3+ cells expressed higher levels of cell cycle genes (Pcna, Gmnn, Cdt1) (Fig. 2B). The tendon samples expressed higher levels of tenogenic markers (Tnmd, Scx, Mkx, Col1a1, Tgfb3) while the ACL/PCL expressed higher levels of other collagens (Col3a1, Col5a1, Col6a1, Col6a2). Finally, the AC samples had the highest levels of cartilage-related genes (Col2a1, Prg4, Acan, Sox9).

Discussion

In order to better understand tendon pathologies and to develop improved repair strategies, we must first improve our understanding of the tendon lineage, including markers that define cells at multiple stages of the lineage and signaling pathways that regulate the differentiation of progenitors into mature tenocytes. We utilized two GFP reporter strains in this study to demonstrate a level of cellular heterogeneity within the internal tendon fibroblast (i.e., tenocyte) population that has not been appreciated previously. The Col2.3+ cells may be of particular interest as they display significant changes in expression with age (Fig. 1A) as well as larger differences in endogenous gene expression compared to *whole* PT controls than the Col6+ population (Fig. 2). Col2.3GFP's age-related expression changes correlate with changes in mineral apposition rate in these tissues during growth [4]. In addition, these cells also express higher levels of cell cycle genes. Therefore, we hypothesize that Col2.3GFP is a marker of an actively growing cell phenotype with increased proliferation, metabolic activity, and ECM production. A hypothesis that we will test in future studies.

Significance

An improved understanding of the cellular markers and signaling pathways that define and regulate the tendon lineage will be crucial to developing new therapies to attenuate the progression of pathologies and improve repair outcomes following injury. The anatomical and temporal differences in GFP expression found in this study indicate that cells within the tendon midsubstance are not as homogeneous as previously thought. Using these model systems, we aim to identify the phenotype and function of these cells during normal processes of growth, homeostasis, and repair.

Acknowledgements

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