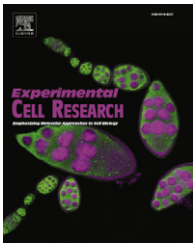
EXPERIMENTAL CELL RESEARCH 318 (2012) 1492–1507

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Direct conversion of tenocytes into chondrocytes by *Sox9*

*Aki Takimoto, Miwa Oro, Yuji Hiraki, Chisa Shukunami**

Department of Cellular Differentiation, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

ARTICLE INFORMATION

Article Chronology:
Received 24 October 2011
Revised version received 5 March 2012
Accepted 2 April 2012
Available online 10 April 2012

Keywords:
Sox9
Scx
Tenomodulin
Tenocytes
Chondrocytes

ABSTRACT

Sox9 is a high-mobility group box-containing transcription factor that functions as a key regulator of chondrogenesis. We here report that Sox9 mediates the direct conversion of tenocytes to chondrocytes through an intermediate state in which both differentiation programs are active. *Sox9* is abundantly expressed in cartilage but is undetectable in limb tendons that express *Scleraxis* (*Scx*) and *Tenomodulin* (*Tnmd*), tendon-specific early and late molecular markers, respectively. Upon forced expression of *Sox9* in the chick forelimb, ectopic cartilage formation is preferentially observed in fibrous tissues including the tendons, ligaments, perichondrium/periosteum, dermis, and muscle connective tissues. *Tnmd* expression in tenocytes isolated from leg tendons was markedly upregulated by forced expression of basic helix–loop–helix (b-HLH) activators including *Scx*, *Paraxis*, *Twist1* and *Twist2*. In contrast, the overexpression of *Sox9* in monolayer tenocytes resulted in the downregulation of *Tnmd* and *Scx* expressions during passaging in culture, and the induction of cartilage molecular markers such as *type II collagen* (*Col2a1*) and *Chondromodulin-I* (*ChM-I*). This *Sox9*-driven switching from a tenocytic to a chondrocytic gene expression profile was associated with a dramatic change from a spindle to a polygonal cellular morphology. The extracellular accumulation of cartilage-characteristic proteoglycans was also observed. These data suggest that tenocytes have a strong potential for conversion into chondrocytes through the activities of *Sox9* both *in vitro* and *in vivo*.

Introduction

In the locomotive organs, ectopic cartilage or bone formation is often observed in tendons and ligaments under pathological conditions such as tendinopathy and ligament disorders [1,2]. Progenitor cells for the tendons, ligaments, cartilage, and bone are all derived from the same origins including the sclerotome, lateral plate mesoderm, and neural crest [3]. In the early stages of organ development, these progenitor populations migrate and populate their prospective regions to give rise ultimately to each primordium [4]. During limb formation, lateral mesoderm-derived tendon and ligament progenitors, as well as chondroprogenitors, express a key transcription factor for chondrogenesis, Sox9 [5]. Upon cell-fate determination, *Sox9* expression is markedly upregulated in chondroprogenitors and promotes the progression of chondrogenic differentiation, whereas in tendon and ligament progenitors this expression diminishes upon cell differentiation [6].

Fibroblasts are cells of mesenchymal origin that produce a wide variety of matrix proteins and contain subpopulations of cells with unique phenotypes and functions. Among these, differentiated tendon fibroblasts, known as tenocytes, are specialized cells that align longitudinally between the thick regular parallel collagen fibers that confer strong tensile resistance. Tenocytes can be readily identified through the expression of *Tnmd*, a gene which is positively regulated by the basic helix–loop–helix transcription factor Scx [7–9]. Although tendons have been shown to contain a minor population of stem cells that may be involved in ectopic cartilage/bone formation [10], it is still

* *Corresponding author at:* 53 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81 75 751 4633.
E-mail address: shukunam@frontier.kyoto-u.ac.jp (C. Shukunami).

0014-4827/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2012.04.002

unclear whether tenocytes are also capable of direct switching to become chondrocytes.

In our current study, we observed ectopic cartilage formation in various fibrous tissues including tendons, ligaments, perichondrium, dermis, and muscle connective tissues, through the forced expression of *Sox9* in chicken embryonic forelimbs. These results demonstrate the chondrogenic potential of tenocytes in various connective tissues. We further demonstrate that *Tnmd*-positive mature tenocytes have the capacity for conversion into chondrocytes by the forced expression of *Sox9* alone, a gene which is virtually undetectable in mature tenocytes. Moreover, while *Tnmd* expression was found to be upregulated by the overexpression of several b-HLH transcription factors including *Scx*, *Paraxis*, *Twist1*, and *Twist2*, *Sox9*-overexpressing tenocytes showed a gradual loss of *Tnmd* transcripts and eventually expressed cartilage markers including *Col2a1*, *Aggrecan* (*Agg*), and *ChM-I*.

Materials and methods

Animals and embryos

C57BL/6 mice were purchased from SHIMIZU Laboratory Supplies (Kyoto, Japan). Fertile white leghorn chicken eggs were obtained from Takeuchi Farm (Nara, Japan). Chick embryos at different stages of development were obtained by incubating fertilized eggs at 38 °C and staging was in accordance with the method of Hamburger and Hamilton [11]. All of these procedures were approved by the Animal Care Committee of the Institute for Frontier Medical Sciences, Kyoto University, and conformed to institutional guidelines for the study of vertebrates.

Cell culture

For the isolation of chick tenocytes and chondrocytes, minced embryonic tissues were treated with EDTA. Following trypsin and collagenase digestion, tenocytes and chondrocytes were isolated from the leg tendons and distal tibiotarsal cartilage, respectively, of chick embryos at stage 41 [8]. Isolated tenocytes and chondrocytes were subsequently grown in α minimum essential medium (αMEM) (Sigama, St. Louis, MO) containing 10% fetal bovine serum (FBS) and in a 1:1 mixture of DME and Ham's F-12 (DF) medium (Mediatech, Manassas, VA) containing 10% FBS, respectively. For isolation of limb bud mesenchymal cells, limb buds of chick embryos at stage 24 were minced and digested with 0.01% trypsin (BD Difco, Franklin Lakes, NJ) and 0.005% collagenase (Roche, Basel, Switzerland), as previously reported [12]. For isolation of dermal fibroblasts, after the removal of the epidermis from the back skin dissected from embryos at stage 37, dermis was digested with 0.01% trypsin and 0.05% collagenase, as previously reported [13]. For isolation of myoblasts, the thigh muscle of embryos at stage 37 was cut into small segments, suspended in Dulbecco's modified Eagle medium (DMEM) **(Sigma)** containing 10% FBS in a centrifuge tube, and agitated on a vortex mixer for 40 s, as previously reported [14]. Osteoblasts were isolated from the calvariae of chick embryos at stage 42, using four sequential 15 minute digestion in 0.05% trypsin and 0.1% collagenase, as previously reported [15]. Calvarial cells from fractions of 2–4 were collected. Limb bud mesenchymal cells were grown in DF medium containing 10% FBS. Dermal fibroblasts were grown in αMEM containing 10% FBS. Myoblasts and calvarial osteoblasts were grown in DMEM containing 10% FBS. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

In vitro differentiation assays

For induction of chondrogenic differentiation, 2×10^5 cell aliquots of tenocytes or limb bud mesenchymal cells were pelleted in 15-ml conical tubes and maintained for 21 days in 0.5 ml of DMEM containing 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml sodium selenite, 1.25 mg/ml bovine serum albumin, 50 μg/ml L-ascorbic acid, 1 μM dexamethasone, and 10 ng/ml recombinant human TGF-β1, as previously reported [16]. For induction of adipogenic differentiation, confluent tenocytes were maintained with DMEM containing 10% FBS, 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, 60 μM indomethacin, and 10 μg/ml insulin, as previously reported [17]. For induction of osteogenic differentiation, confluent tenocytes were treated with DMEM containing 10% FBS, 50 μg/ml L-ascorbic acid, 10 nM dexamethasone, and 2 mM β-glycerophosphate, as previously reported [18].

Retrovirus vector construction

The entire coding sequence of chicken *Sox9* cDNA was amplified using primers listed in Table 1. The amplified products were then subcloned into the *pCRII-TOPO* (Invitrogen, Carlsbad, CA) and sequenced. The ClaI site in the chicken *Sox9* gene was disrupted by mutagenesis using primers listed in Table 1 (*Sox9 mt.* forward and reverse). In the construction of the RCAS (replication competent ALV LTR with a splice acceptor) retroviral vector, mutated *Sox9* cDNA with ClaI sites newly added at both ends was subcloned into the ClaI site of the *RCASBPA* vector. The *RCAS-Myogenin*, *RCAS-EGFP*, and *RCAS-Scx* vectors were constructed as described previously [8]. cDNAs of the entire coding region of the chicken *Twist2*, *Paraxis*, and *Id3* genes were amplified using primers listed in Table 1 and cloned into the *RCASBPA* vector. The full-length human *peroxisome proliferator activated receptor γ* (*PPARγ*) cDNA was purchased from Kazusa DNA Research Institute (Chiba, Japan). The full-length human *Twist1* [19] and chicken *runt-related transcription factor 2* (*Runx2*) [20] cDNAs were generously provided by Dr. Akira Kudo (Tokyo Institute of Technology) and Dr. Masahiro Iwamoto (Children's Hospital of Philadelphia), respectively, and subcloned into *RCASBPA* vector.

Electroporation and transfection

Electroporation of each RCAS retroviral vector into tenocytes was performed using the Amaxa Basic Nucleofector Kit for Primary

Table 1 – Primers used for retrovirus vector construction.

Primer	Sequence
Sox9 forward	5′-TCGCATGAATCTCCTAGACC-3′
Sox9 reverse	5′-GCCTTCACGTGGCTTTAAG-3′
Sox9 mt. forward	5′-GACATCGGCGAGCTCAGCAGC-3′
Sox9 mt. reverse	5′-CACGTCTCGGAAATCAATGTGG-3′
Twist2 forward	5′-TTGCAGAAAGCATGGAAGAAAG-3′
Twist2 reverse	5′-GTGGCTAGTGTGAGGCCGAC-3′
Paraxis forward	5′-GGCCATGGCTTTCACCATGC-3′
Paraxis reverse	5′-TGGGGCTGGCTGTGACAGG-3′
Id3 forward	5′-ATGAAAGCCATCAGCCCGGTGCG-3′
Id3 reverse	5′-GTTAGTGACACAAACTTCTCTCG-3′