Biochemical and Biophysical Research Communications 435 (2013) 356–360

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The SNAI1 and SNAI2 proteins occupy their own and each other's promoter during chondrogenesis

Ying Chen [a,b], Thomas Gridley [a,*]

[a] Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, ME 04074, USA
[b] Graduate School of Biomedical Sciences, University of Maine, Orono, ME 04469, USA

ARTICLE INFO

Article history:
Received 22 April 2013
Available online 7 May 2013

Keywords:
Snail
Slug
Transcriptional repressor
Chondrogenesis
Chromatin immunoprecipitation

ABSTRACT

Two Snail family genes, *Snai1* and *Snai2*, encode E2 box-binding transcriptional repressors that are important for cartilage development during long bone formation in mice. We demonstrated previously that the *Snai1* and *Snai2* genes function redundantly, and compensate for each other's loss during mouse chondrogenesis in vivo. A prediction from this genetic data is that the SNAI1 and SNAI2 proteins can bind to each other's promoter to regulate gene expression. Here we demonstrate that expression of *Snai1* and *Snai2* RNA and protein is induced during chondrogenic differentiation of cultured mouse ATDC5 cells. Using chromatin immunoprecipitation assays, we then show that endogenous SNAI1 and SNAI2 proteins bind to a subset of E2 boxes in both their own and each other's promoter in differentiating ATDC5 cells. Together with our previous genetic data, these results support the model that expression of the *Snai1* and *Snai2* genes is negatively regulated by their protein products occupying each other's promoter during chondrogenesis, and help provide an explanation for the genetic redundancy observed in the mouse loss of function models.

1. Introduction

Snail family genes encode zinc finger-containing proteins that function primarily as transcriptional repressors [1,2]. To date, three members of the Snail gene family have been described in vertebrates: *Snai1* (also known as Snail), *Snai2* (Slug) and *Snai3* (Smuc). Snail family proteins possess a highly conserved carboxy-terminal region, containing four or five Cys2-His2 (C2H2)-type zinc finger regions and a more divergent amino-terminus that contains the evolutionarily conserved SNAG domain. The zinc finger regions are sequence-specific DNA-binding domains that bind E2-box sequences (CAGGTG and CACCTG). Both the SNAI1 and SNAI2 proteins recruit other proteins, such as histone deacetylase-1 (HDAC-1), to the E2 boxes of target genes to form a transcriptional repression complex that suppresses the transcription of Snail target genes [3,4].

The *Snai1* and *Snai2* genes have been widely studied for their ability to trigger the epithelial to mesenchymal transition during multiple processes of developmental and cancer biology. In addition to their role in epithelial cells, *Snai1* and *Snai2* gene function is important in some non-epithelial cells, such as chondrocytes and osteoblasts. A previous gain-of-function study using a tamoxifen-inducible *Snai1* transgenic line demonstrated that upregulation of *Snai1* activity in mouse long bones caused a reduction in bone length [5], and several genes involved in cartilage and bone development have previously been demonstrated or implicated as Snail target genes [6–9]. We demonstrated recently that the *Snai1* and *Snai2* genes functioned redundantly during embryonic long bone development [10]. Our results demonstrated that the *Snai1* and *Snai2* genes transcriptionally compensate temporally, spatially, and quantitatively for each other's loss, and demonstrated an essential role for Snail family genes during chondrogenesis in mice. A prediction from this genetic data is that the SNAI1 and SNAI2 proteins can bind to each other's promoter to regulate gene expression. Here we examine, using chromatin immunoprecipitation, the binding patterns of the SNAI1 and SNAI2 proteins to their own and each other's promoter during chondrogenic differentiation of the mouse ATDC5 cell line.

2. Materials and methods

2.1. Cell culture and Alcian blue staining

The ATDC5 cell line was obtained from Abgent (Cat# CL1016). The cells were grown and maintained in Iscove's DMEM medium (Cellgro) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2 and 95% air. When cells reached confluency (designated Day −7), they

* Corresponding author. Address: Center for Molecular Medicine, Maine Medical Center Research Institute, 81 Research Drive, Scarborough, ME 04074, USA.
E-mail address: gridlt@mmc.org (T. Gridley).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.086

were maintained in growth media for an additional seven days. To initiate chondrogenic differentiation (Day 0), growth medium was supplemented with 10 ml/L of insulin, transferrin and selenium supplement (ITS-G; Gibco) and 37.5 μg/ml of ascorbate 2-phosphate (Sigma). Cells were incubated in differentiation media at 37 °C for up to 21 days, and the medium was changed every other day to allow the cells to differentiate into mature and hypertrophic chondrocytes. Alcian blue staining was performed at various time points (Day 0, Day 7, Day 14, Day 21), as described previously [10].

2.2. Reverse transcriptase-PCR (RT-PCR) and quantitative RT-PCR analyses

Total RNA from cells was isolated using the Qiagen Mini mRNA Extraction kit. RNA (2 μg of each sample) was reverse-transcribed with random hexamer primers (Ambion). Ten nanograms of cDNA were used for real-time quantitative PCR amplification for each well. qRT-PCR was performed using Super SYBR Green PCR Master Mix on a MyiQTM single color Real-Time PCR detection system (Bio-RAD) using Bio-RAD iQ5 software. qRT-PCR was performed using the primer pairs described in our previous study [10]. For each gene tested, three experimental replicates and four biological replicates were performed. Gene expression levels were normalized to the β-actin mRNA level.

2.3. Identification of E2 boxes in the Snai1 and Snai2 promoters

Promoter sequences between −2500 bp and +500 bp of the mouse *Snai1* and *Snai2* genes were exported from the Cold Spring Harbor Laboratory promoter database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process = searchPromForm) and from Genomatix (http://www.genomatix.de/applications/ChIP-Seq.html). The Sequencher program was utilized to search for E2 boxes (CACCTG and CAGGTG) in the *Snai1* and *Snai2* promoter regions. To determine whether these E2 boxes are conserved among different species, we employed Sequencher to search for E2 boxes in 3 kb genomic DNA (−2500 bp to +500 bp) of the *Snai1* and *Snai2* genes of different mammalian species.

2.4. Western immunoblot analysis

Protein was extracted using the M-PER mammalian protein extraction reagent (Pierce). Immunoblots were incubated with the appropriate antibodies, anti-SNAI1 antibody (1:50, Cell Signaling) or anti-SNAI2 antibody (1:100, Santa Cruz), and visualized using an Immobilon Western blot kit (Millipore) and peroxidase-conjugated affinity-purified secondary antibody. To confirm equal loading for each sample, blots were stripped (10% SDS, 0.5 M Tris pH 6.8, 0.8% β-mercaptoethanol) and the membranes were re-probed with anti-actin antibody (1:1000, Santa Cruz).

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with a ChIP assay kit (MilliPore), according to the manufacturer's instructions. ATDC5 cells at Day 7 of differentiation were cross linked with formaldehyde at 37 °C for 10 min. To shear genomic DNA, cell lysates were sonicated on ice eight times for 8 s each. For immunoprecipitation, anti-SNAI1 antibody (1:50, Cell Signaling) or anti-SNAI2 antibody (1:50, Santa Cruz) was used, with normal rabbit IgG (1:50, Santa Cruz) and normal goat IgG (1:50, sc-2028 Santa Cruz) used as antibody negative controls. Immunoprecipitated and input DNA were subjected to PCR using primers specific to each E2 box within the *Snai1* or *Snai2* promoter region. Two sets of primers amplifying regions within the *Snai1* or *Snai2* promoter region that do not include any E2 boxes were also designed as negative controls.

For the *Snai1* gene, the primers used were: negative control 1 (NC1) (Forward: GTCCTGCTTGTTGTGTGGAA; Reverse: TGGAGGCAGAGCTAGAAAGC); negative control 2 (NC2) (Forward: GTGCTCTCCCTCTGGACTCA; Reverse: AGTGGGAGGATGGTCAGACA); region 1 (Forward: TGTTTTGTTTGGGATTTCTGC; Reverse: GCTGCCCTTGAACTCAGAGA); region 2 (Forward: GGAGCCAGGTGAATCTCTGA; Reverse: GGTTGTTGGCATCGAATTTT); region 3 (Forward: GAGGAAAAGCTTGGCTGAAA; Reverse: GAGGCCTGTTCACAACCTCA); region 4 (Forward: GTGGGCTTCATTTGCCTTT; Reverse: CTTGCTTGGTACCTGCCTTC); region 5 (Forward: TGTGAACGTTCCAACACGAT; Reverse: GTCACGGAAGGACTTTCAGC); region 6 (Forward: CTGGAACCTGCTCTCAAAGG; Reverse: GAAAGGAGGTGGGGAGAGAC); region 7 (Forward: GAGCCCAAGCGGAATCTC; Reverse: CTACGATCCCCTAGCAGCAG); region 8 (Forward: AGCCCAACTATAGCGAGCTG; Reverse: GATCCCAACTGCCAAGACC); and region 9 (Forward: CGGGAGTTGAAGACTCGAAG; Reverse: CTCTGCGGGAAGAGAAGAGA).

For the *Snai2* gene, the primers used were: negative control 1 (NC1) (Forward: TGCAAATGCAAGTGAAGTGAC; Reverse: CACATCCCTGGTTTGCATTA); negative control 2 (NC2) (Forward: TCCAGACGCAACTTCCAAAT; Reverse: AAACGTGTTTCCGCTAGGTG); region 1 (Forward: CTAAAAGCAGCACCCCAATC; Reverse: GGCACAGCAGAGGAAAATTG); region 2 (Forward: TTCCCTGTTGTCATGTG TGG; Reverse: GAGCTGGAGTCTGAGTTGACC); region 3 (Forward: CAGACTCCAGCTCTGATCCA; Reverse: CGCAGACTTCCTGGGTTAAT); region 4 (Forward: CGTTTTCTCATCCCCAGATT; Reverse: ACCCTGACTCTTCCCAACAA); and region 5 (Forward: GAGCCGGGTGACTTCAGAG; Reverse: TCGCTGTAGTTGGGCTTCTT.

3. Results

3.1. Expression of the Snai1 and Snai2 genes during chondrogenic differentiation of ATCD5 cells

ATDC5 cells are a mouse cell line, originally derived from a teratocarcinoma cell line, which is capable of chondrogenic differentiation in culture [11,12]. To assess ATDC5 cell differentiation, cells that had been confluent for seven days were placed in differentiation media (designated as Day 0), and Alcian blue staining of the cell cultures was performed at various time points, including Days (D) 0, D7, D14, and D21. Alcian blue stains proteoglycans that are a component of the cartilage-specific extracellular matrix. Over this time course, the cell cultures exhibited increased numbers of Alcian blue-stained cartilaginous nodules with an increased intensity of Alcian blue staining (Fig. 1A). We also assessed ATDC5 differentiation by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to determine the mRNA expression levels of several markers characteristic of chondrogenic differentiation, such as Aggrecan, collagen II alpha 1 (*Col2a1*), Indian hedgehog (*Ihh*), and collagen X alpha 1 (*Col10a1*). Expression of these markers increased during the time course of the cultures (Fig. 1B).

We then investigated the patterns of *Snai1* and *Snai2* mRNA and protein expression during differentiation of the ATDC5 cell line. The relative mRNA expression levels of both the *Snai1* and *Snai2* genes increased six to twelve fold over the first 7–14 days of differentiation (Fig. 2A and B). Western immunoblotting analyses demonstrated that SNAI1 and SNAI2 protein levels also increased over the differentiation time course (Fig. 2C and D).

3.2. Specific binding of the SNAI1 and SNAI2 proteins to E2 boxes of their own and each other's promoters during differentiation of ATDC5 cells

We searched the regions from −2500 bp to +500 bp of the *Snai1* and *Snai2* genes for E2 box sequences (CACCTG and CAGGTG), and