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Snail1 Expression Is Required for Sarcomagenesis[1,2]

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Abstract

Snail1 transcriptional repressor is a major inducer of epithelial-to mesenchymal transition but is very limitedly expressed in adult animals. We have previously demonstrated that Snail1 is required for the maintenance of mesenchymal stem cells (MSCs), preventing their premature differentiation. Now, we show that Snail1 controls the tumorigenic properties of mesenchymal cells. Increased Snail1 expression provides tumorigenic capabilities to fibroblastic cells; on the contrary, Snail1 depletion decreases tumor growth. Genetic depletion of Snail1 in MSCs that are deficient in p53 tumor suppressor downregulates MSC markers and prevents the capability of these cells to originate sarcomas in immunodeficient SCID mice. Notably, an analysis of human sarcomas shows that, contrarily to epithelial tumors, these neoplasms display high Snail1 expression. This is particularly clear for undifferentiated tumors, which are associated with poor outcome. Together, our results indicate a role for Snail1 in the generation of sarcomas.

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Abbreviations: EMT, epithelial-to-mesenchymal transition; MSC, mesenchymal stem cell; SMA, smooth muscle actin; PyrK, pyruvate kinase

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[2] This article refers to supplementary materials, which are designated by Table W1 and Figure W1 to W2 and are available online at www.neoplasia.com.

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1. Introduction

Snail1 is a transcriptional factor induced at early phases of epithelial-to-mesenchymal transition (EMT) and required for the initiation of this process [1–3]. Snail1 ectopic expression promotes significant changes in gene expression downregulating epithelial markers, such as E-cadherin, and increasing the synthesis of genes of the mesenchymal lineage. Snail1 genetic depletion prevents EMT induced by several extracellular stimuli both in cell lines and during early embryo development [1]. However, Snail1 expression is not maintained in most adult mesenchymal cells: Snail1 is not expressed by tissue-resident fibroblasts and is only detected in these cells when activated, such as during the process of wound healing or in the stroma of several types of epithelial tumors [4]. Actually, although the study of this factor has been limited by the poor quality of the antibodies recognizing it, most reliable studies show a limited expression of this transcriptional factor in epithelial neoplasias. In these tumors, Snail1 is restricted to few cells in the tumor-stroma interface in areas of invasion or in cells next to areas of inflammation [4,5].

We have recently demonstrated that Snail1 is expressed by mesenchymal stem cells (MSCs) [6]. In these cells, Snail1 is required for the maintenance of the undifferentiated state; Snail1 depletion facilitates their differentiation to adipocytes or osteoblasts and precludes the block on these processes caused by transforming growth factor β (TGF-β). Accordingly, Snail1 obliteration in adult animals promotes a down-regulation in the number of MSCs. Snail1 is also required for the expression of markers specific for MSCs or activated fibroblasts: thus, Snail1 depletion downregulates the protein levels of S100A4, CD29, or TGF-β [6]. Because MSCs have been demonstrated to be involved in the generation of sarcomas [7–9; see 10 as a review], in this article, we have studied the relevance of Snail1 expression in this neoplasia.

2. Materials and Methods

2.1. Mice

The generation of a murine line containing a *Snail1*-conditional allele (*Snail1*^{flox}), a *Snail1*-null allele (*Snail1*$^{-}$), and a *Cre recombinase–Estrogen Receptor* fusion gene under the control of *β-Actin* promoter (*β-Actin CreER*) has been described [6]. These animals were crossed with a *p53*-deficient line [11] to obtain *β-Actin CreER, Snail1*$^{-}$*/Snail1*^{flox}*, p53*$^{-}$ mice. Animals carrying a wild-type (WT) allele of *Snail1*, *Snail1*$^{+}$*/Snail1*^{flox}, were used as controls.

All mice involved in this study were maintained in a rodent barrier facility to guarantee the specific pathogen-free health status of the animals. All animal experiments were previously approved by the Animal Research Ethical Committee from the Parc de Recerca Biomèdica de Barcelona.

2.2. Cell Culture and Generation of Stable Cell Lines

MSCs were obtained as indicated [12] and cultured in Dulbecco's modified Eagle's medium (DMMEM) plus 10% fetal bovine serum (FBS). As previously reported [12; see also 6], these cells were adherent to plastic when cultured in these conditions, expressed CD105 and CD90 (and not CD45 or CD19), and were able to differentiate to adipocytes, osteoblasts, or chondrocytes. Snail1 depletion was obtained transfecting pMX-Cre or the empty plasmid and selecting with 2 μg/ml puromycin for 1 week. Cell lines (3T3-L1, C2C12, NIH-3T3.5, and 1BCR3-G) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Biological Industries, Kibbutz Beit Haemek, Israel), 1 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C in 5% CO_2. Snail1 stable transfectants were obtained by transfection of 6 μg of pcDNA3 Snail1 tagged with hemagglutinin (HA) epitope or control pcDNA3 vector using Lipofectamine reagent (Invitrogen) and selecting with G418 (1 mg/ml) for 3 to 4 weeks. The depletion of Snail1 expression was generated by stable infection with retroviruses using the pRETRO-SUPER vector and oligonucleotide 5′-GATCCCC**GATGCACATCCGAAGCCAC**TTCAA**GAGAGTGGCTTCGGATGTGCATC**TTTTTA-3′ or the corresponding antisense oligo. The interfered murine *Snail1* sequence is shown in bold. A scrambled sequence cloned in the same vector was used as control. Stable transfectants were obtained after selection with 1 μg/ml puromycin.

2.3. Preparation of Cell Extracts and Western Blot Analysis

Cells were washed with phosphate-buffered saline, scraped, and lysed by the addition of 100 μl of lysis buffer [50 mM Tris-HCl (pH 6.8) and 2% sodium dodecyl sulfate]. Thirty micrograms of proteins was fractionated by 10%, 12%, or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blot using antibodies corresponding to S100A4 (Thermo Scientific, Waltham, MA, USA), CD29, CD44 (both from Abcam, Cambridge, UK), Fibronectin (Dako, Glostrup, Denmark), p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-6243), Snail1 [4], Smooth muscle actin (SMA), Pyruvate Kinase (PyrK), and Tubulin (all from Sigma, St Louis, MO, USA).

2.4. RNA Extraction and Analysis

RNA was extracted from 1×10^6 cells with a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) and analyzed by quantitative reverse transcription–polymerase chain reaction (PCR). Retrotranscription was carried out with First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany); cDNA was amplified by quantitative PCR using SYBR Green and a LightCycler 480 (Roche). Reactions were performed according to the manufacturer's directions, using the following primers: murine *TGF-β1*, forward—5′-CTGCAAGACCATCGACATGG-3′ and reverse—5′-GTTCCACATGTTGCTCCACA-3′; and murine *Snail1*, forward—5′-GCGCCCGTCGTCCTTCTCGTC -3′ and reverse—5′-CTTCCGCGACTGGGGGTCCT-3′. *Hypoxanthine-guanine phosphoribosyltransferase* (forward—5′-GGCCAGACTTTGTTG-GATTTG-3′ and reverse—5′-TGCGCTCATCTTAGGCTTTGT-3′) and *Pumilio* (forward—5′-CGGTCGTCCTGAGGATAAAA-3′ and reverse—5′-CGTACGTGAGGCGTGAGTAA-3′) were used as controls. Each reaction was performed using 100 ng of total RNA.

2.5. Tumor Xenografting

Four or five million cells were either subcutaneously or intramuscularly (i.m.) injected into the flank of 8-week-old athymic nude or Severe Combined Immunodeficiency mutant (SCID) mice. Growth of the tumor was followed every other day after the first week. Mice were killed by cervical dislocation when tumors reached a size of 1 cm^3 (4 to 10 weeks after the injection, depending on the cell lines used). At that time, the tumor area was collected, fixed in formalin, and embedded in paraffin. Sections (4 μm) were dewaxed, rehydrated, and stained with hematoxylin and eosin. The study was approved by the Animal Research Ethical Committee from the Parc de Recerca Biomèdica de Barcelona. Analysis of Snail1 expression in tumors was carried out as indicated below for human samples.