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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.

2.6. Immunohistochemical Analysis of Human Tumors

One hundred and nine formalin-fixed, paraffin-embedded tissue specimens, consisting of different histologic type and grade sarcomas, were selected from the Departments of Pathology Tumor Banks of the Hospital del Mar (Barcelona, Spain), Hospital Virgen de la Salud (Toledo, Spain), and Fundación Jiménez Díaz (Madrid, Spain). The analysis of the samples was approved by the Ethical Committees of Clinical Experimentation of the three institutions. Clinical data and follow-up were obtained from the review of 101 patients' medical records. Follow-up of the patients was carried out for at least 8 years after surgery. Microscopic confirmation of diagnosis, tumor type, and histologic grade was carried out by pathologists of the four institutions. Specific survival was calculated from time of surgery of the primary tumor to patient death secondary to its cancer.

Immunohistochemical analysis of Snail1 protein was performed as previously described by using monoclonal antibody (mAb) EC3 [4,13,14], using 4-μm sections. For antigen unmasking, sections were immersed in Tris EDTA buffer (pH 9) and boiled for 20 minutes. Immunohistochemical staining was carried out with anti-Snail1 mAb EC3 supernatant at 1:300 dilution using the CSA II Amplification System (Dako), in a Dako Link platform. TGF-β expression was determined with a rabbit polyclonal antibody against TGF-β (sc146; Santa Cruz Biotechnology) for 1 hour at room temperature diluted at 1:100. As negative controls, the same sections incubated with nonimmunized serum. Sections were counterstained with hematoxylin.

Immunohistochemical evaluation was conducted by two investigators blinded to clinical data. Snail1 staining was graded as positive only when nuclear staining was detectable. A tissue sample was considered positive when at least 1% of the cells in the analyzed area showed nuclear staining. Snail1 staining was scored semiquantitatively according to a scale of 0 to 300. This was the result of multiplying the percentage of positive cells (from 1% to 100%) and the intensity of immunoreactivity [1–3]. Samples were categorized into the following three groups: negative expression, low expression (lower than 10), and high expression (higher than 10). In the case of TGF-β analysis, cells were scored as positive when cytoplasm was observed. TGF-β expression in malignant cells was compared with stromal cells located away from the tumor: tumors stained stronger than stroma were considered as high expression; tumors stained similar to stroma were considered intermediate expression, whereas those that showed weaker staining than normal cells or did not stain at all were considered weak/negative. Correlations between markers were calculated by χ^2 test. Survival data were analyzed according to the Kaplan-Meier method, for the samples with high Snail1 expression *versus* those with null or low expression, and tested for significance between the groups with the log-rank test. The relation of Snail1 expression on specific survival was also analyzed by multivariate analysis (Cox proportional risk regression model). In all statistical tests, *P* values lower than .05 were considered significant. All statistical analyses were carried out using StatView for Windows version 5.0 (SAS Institute Inc, Cary, NC, USA).

3. Results

3.1. Snail1 Increases the Tumorigenic Properties of Fibroblastic Cell Lines

Snail1 is required for maintenance of MSCs and other mesenchymal cells in an undifferentiated state [6]. We examined the relevance of this factor in the tumorigenic capability of mesenchymal cells. We ectopically expressed Snail1 in 3T3-L1, C2C12, and 1BR3.G cell lines (Figure 1*A*). As previously reported [6], Snail1 upregulated the levels of markers of activated fibroblasts, such as S100A4 (Figure 1*A*). Ectopic Snail1 expression in 3T3-L1 cells increased their tumorigenicity when injected subcutaneously into nude mice: tumors were observed in seven of eight injections with the Snail1-expressing cells but only after one of eight for the control cells (Figure 1*B*). Histologic analyses showed that the tumors were undifferentiated sarcomas, showing different cellular types with nuclei of variable sizes and diameters. Mitoses were atypical. Tumors presented high cellular density and proliferation and displayed an invasive front infiltrating adjacent soft tissues (Figure 1*C*). The tumors did not show any evidence of adipocytic differentiation. Snail1 expression was maintained in the tumors, as assessed by immunostaining (Figure 1*D*). In a similar manner, ectopic expression of Snail1 in C2C12 or 1BR3.G cells also increased tumor formation (Figure 1*B*).

The inverse experiment was carried out with NIH-3T3 cells. We used a clone of these cells, NIH-3T3.5, that expresses high levels of endogenous Snail1 and, when injected in athymic mice, induces tumors with features of pleomorphic sarcomas, thus, with a high apparent mitotic index, nuclei with variable sizes and diameters, atypical mitoses, and presence of cytoplasmic vesicles. Expression of Snail1 was decreased in this cell line expressing a specific Snail1 short hairpin RNA (shRNA) (Figure 1*E*). Snail1 down-regulation caused a decreased expression of markers of MSCs or activated fibroblasts, such as S100A4 or CD29 (Figure 1*E*). Although Snail1 down-regulation did not alter the rate of proliferation *in vitro*, it did when the cells were grafted to nude mice (Figure 1*F*). Analyses of tumors postmortem indicated that the average size in control animals (transfected with scrambled shRNA) was 1.07 cm^3 (±0.18, SD) but only 0.15 cm^3 (±0.10, SD) in animals grafted with NIH-3T3.5 cells transfected with Snail1 shRNA.

3.2. Snail1 Depletion Prevents the Generation of Sarcomas by p53-Deficient MSCs

MSCs are not capable to originate tumors when injected into immune-deficient mice. However, loss of p53 is sufficient to provide tumorigenic potential to these cells [15,16], originating leiomyosarcomalike malignancies. This model of MSC xenografting has been used as a model of *in vivo* sarcomagenesis. We analyzed the relevance of Snail1 in this system. p53-deficient MSCs were generated bearing a floxed copy of *Snail1*. Surprisingly, p53 depletion increased Snail1 RNA (Figure 2*A*) and protein (Figure 2*B*). Expression of *TGF-β1* gene, activated by Snail1 [6], was also increased (Figure 2*A*). CD29, another stem cell marker, and other proteins upregulated in MSCs (CD44) or in activated fibroblasts (Fibronectin and SMA) were also increased in p53-deficient cells (Figure 2*B*). Snail1 expression was eliminated by transfection of Cre; Snail1 depletion decreased the levels of TGF-β1, CD29, CD44, S100A4, SMA, and Fibronectin in p53 WT and null (KO) cells although to different extents (Figure 2, *A* and *B*).

p53-deficient MSCs also showed a higher clonogenic capability than control MSCs when grown *in vitro* in standard culture conditions (Figure 2*C*). Snail1 depletion markedly decreased the number of colonies either in WT or in p53 KO MSCs (Figure 2*C*). Other properties of these cells were also differently altered by *Snail1* or *p53* genetic depletion. Cell migration was upregulated by p53 depletion and decreased by Snail1 deficiency in p53 KO MSCs (Figure W1). Cell attachment to Collagen 1 was also upregulated in these cells but was not sensitive to Snail1 depletion (Figure W1).

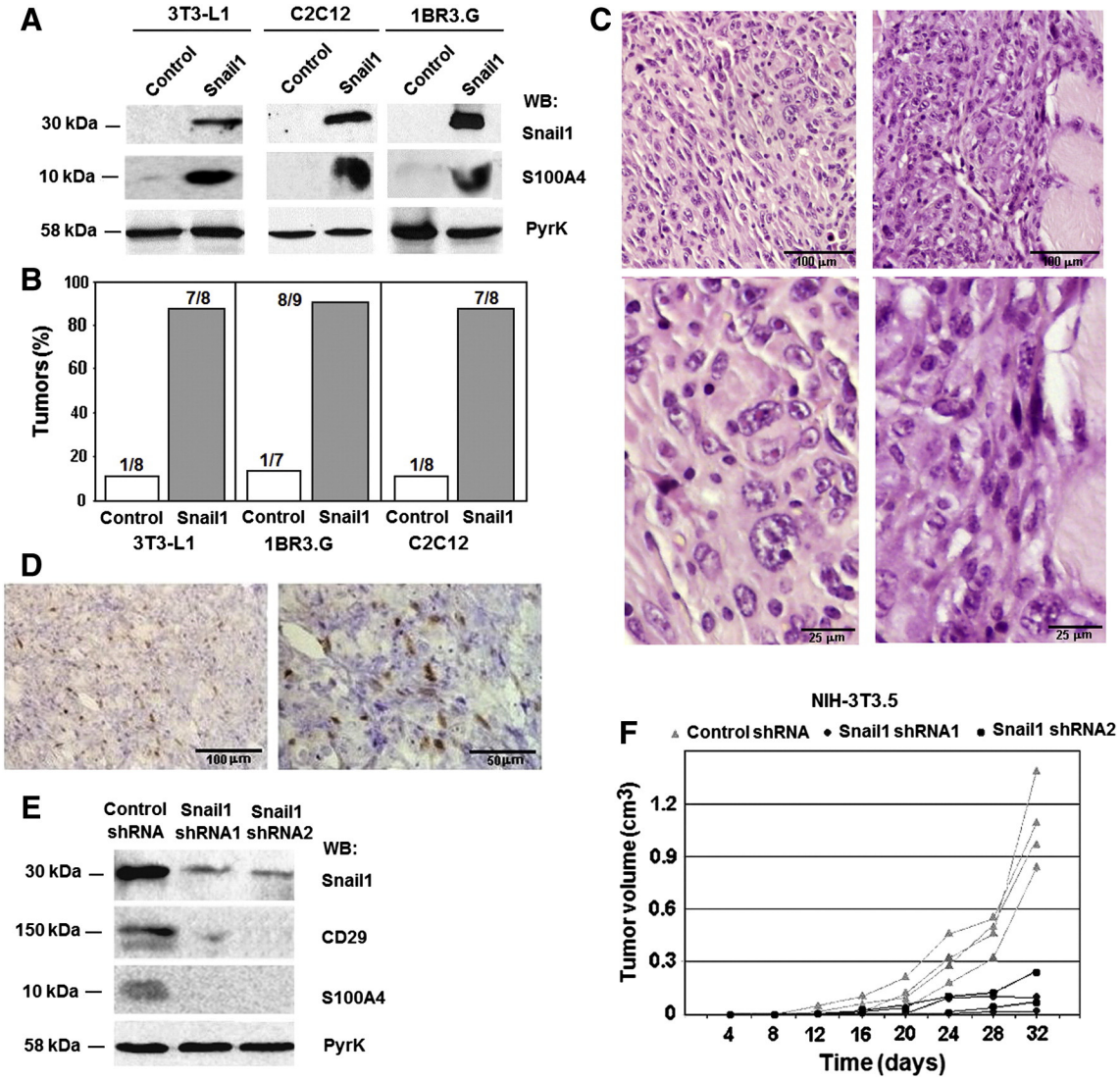

Figure 1. Snail1 increases the tumorigenic capability of fibroblasts in nude mice. Cells (3T3-L1, C2C12, or 1BR3.G) were stably transfected with pcDNA3 Snail1-HA or control plasmid. (A) Expression of Snail1 and S100A4 was determined by Western blot analysis. Four millions of cells were subcutaneously injected in the flanks of nude mice. (B) The presence of the tumors was determined after 30 (3T3-L1 and 1BR3.G) or 60 days (C2C12). The number of tumors relative to the number of injections is indicated for the different cell populations. A histologic analysis of a tumor obtained from ·3T3-L1 Snail1 cells is presented (C); higher magnifications of the micrographs are also shown. (D) Immunostaining with the mAb anti-Snail1 of one of these tumors is also shown. (E and F) Snail1 down-regulation inhibits the tumorigenesis of mesenchymal cell lines. (E) Expression of Snail1 or other markers of fibroblast activation in NIH-3T3.5 fibroblasts transfected with control or Snail1 shRNAs is shown. (F) NIH-3T3.5 transduced with a control or Snail1-specific shRNA were injected in the flanks of nude mice. Tumor size was measured every 4 days.

Snail1 depletion totally prevented the capability of MSCs to form tumors. Two months after implantation, p53 KO MSCs originate tumors in xenografted mice when implanted subcutaneously or i.m. An analysis of these tumors demonstrated that they presented the features of high-grade leiomyosarcomas, with areas of extensive muscular infiltration (Figure 3*A*). These tumors were mostly composed by elongated cells with cigar-shaped nuclei (Figure 3*A*) and expressed SMA (Figure W2); they also show an occasional weak expression of muscle-specific Actin but not of MyoD1 or Desmin (Figure W2). These tumors were detected in all the cases where p53 KO MSCs were injected i.m. and in 60% of xenografted mice when implanted subcutaneously (Figure 3*B*); no tumors were observed with WT MSCs. Depletion of Snail1 totally abrogated the capability of p53 KO MSCs to originate tumors (Figure 3*B*). In accordance with the role of Snail1 for their growth, these tumors showed abundant Snail1 expression (Figure 3*C*).

3.3. Snail1 Is Expressed by Human Sarcomas

We also investigated whether Snail1 expression was detected in human sarcomas. The analysis was carried out with mAb specific for Snail1 and not reacting with other members of this family [4]. This molecular tool has been previously used in several analyses [12,13,17–19]. The analysis of a panel of sarcomas revealed that Snail1 was expressed by 84 of 109 tumors. The reactivity was localized in the nucleus in tumoral cells with a faint expression in the cytosol that was not considered because Snail1 is not active in this compartment. Of the 84 Snail1-expressing tumors analyzed, 33 expressed high levels of Snail1, and 51 expressed low levels. Representative results are

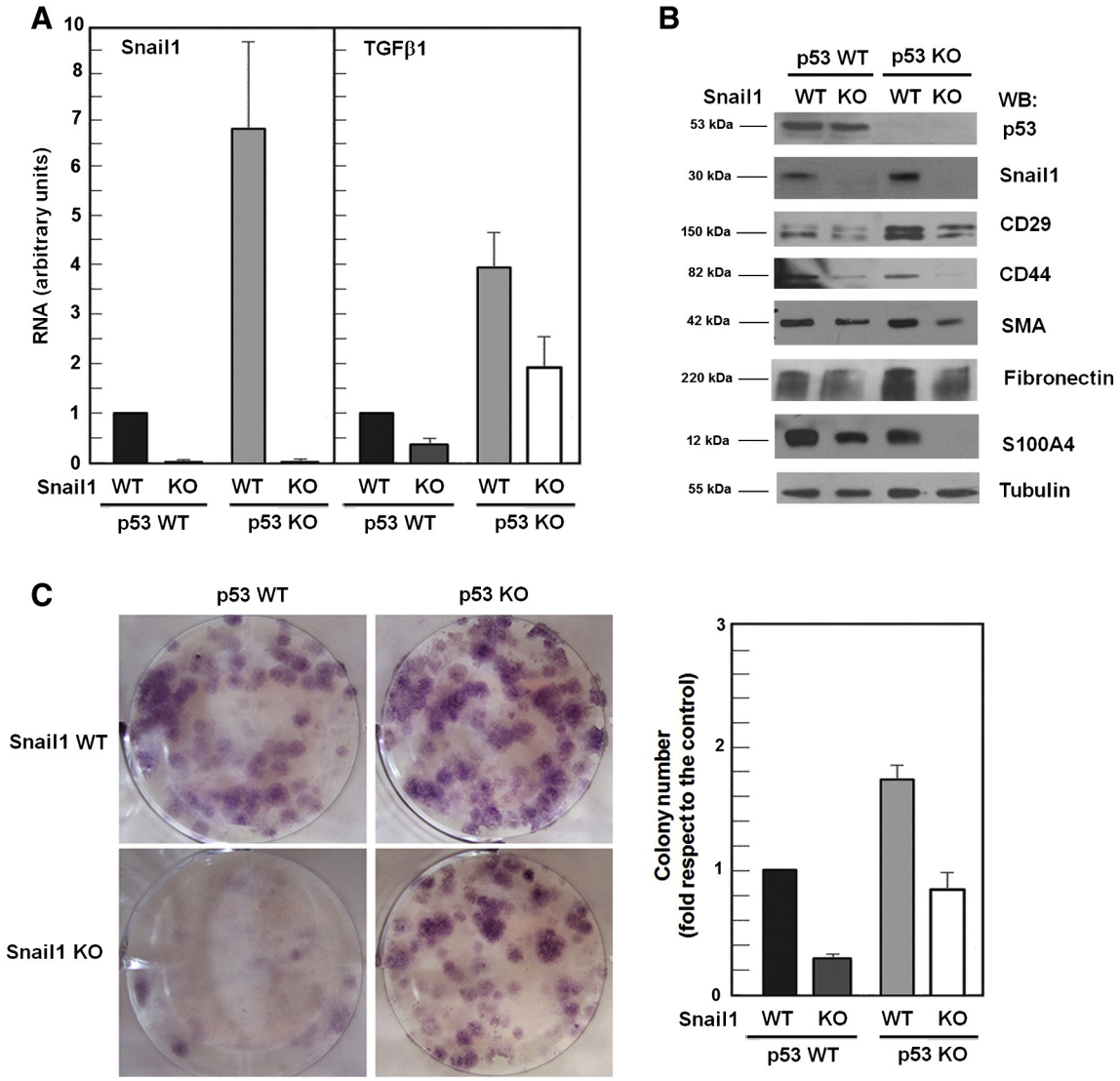

Figure 2. Effect of Snail1 depletion on gene expression in WT or p53-deficient MSCs. (A and B) The expression of Snail1 and the indicated markers was determined by quantitative reverse transcription–PCR (A) or Western blot analysis (B) in control or p53-deficient MSCs either WT or KO for Snail1. (C) Cells (10^3) were seeded on 10-cm diameter plates in DME plus 10% FBS medium; 10 days later, they were washed with phosphate-buffered saline, fixed with 4% p-formaldehyde, and stained with crystal violet. The figure shows a representative experiment of two performed and the average ± range of the colony number, referred to the value obtained with WT MSCs.

presented in Figure 4 showing tumors classified as negative (*A*), Snail1 low (*B* and *D*), or Snail1 high (C, *E*, and *F*). High Snail1 expression was particularly observed in areas of invasion (Figure 4, *E* and *F*).

Snail1 expression inversely correlated with the grade of differentiation ($P < .001$). In 19 (44%) from the 43 grade 3 sarcomas, Snail1 was strongly expressed. In contrast, none of the 31 low-grade sarcoma showed high Snail1 levels. The presence of Snail1 also varied among the different types of sarcomas (Table W1). For instance, 10 of 15 malignant fibrohistiocytomas showed a high expression of Snail1, whereas only 2 of 14 malignant adipocytic sarcomas were classified in this category. Importantly, only 1 of 17 locally aggressive tumors (i.e., fibromatosis and solitary fibrous tumors) highly expressed Snail1.

We also determined whether Snail1 expression in sarcomas modified the clinical outcome. A Kaplan-Meier analysis indicated that high expression of Snail1 was associated with lower disease-free survival (specific survival) of the patients with sarcoma with a $P = .035$ (Figure 4*G*). Univariate and multivariate analyses were also performed to verify the influence of Snail1 expression in specific survival. As shown in Table W1, in the multivariate Cox regression model, Snail1 expression showed an independent prognostic factor with respect to specific survival ($P = .011$) as well as histologic subtype, tumor grade, or tumor location.

A Kaplan-Meier analysis was also carried out with the 43 patients in whom the sarcomas were located in visceral, thoracic, retroperitoneum, or intrabdominal (trunk) locations, with a worse prognosis than the overall sarcomas. Although the number of cases was low, high expression of Snail1 in these tumors was also associated to a lower accumulative survival ($P = .016$) (Figure 4*H*).

Because expression of Snail1 and TGF-β are interrelated [13], we also determined expression of TGF-β in these samples. Tumors were also classified as TGF-β negative/low, intermediate, or high; representative staining are shown in Figure 5, *A* to *C*. TGF-β expression was closely correlated with that of Snail1 ($P < .001$); more than 80% of the tumors classified as Snail1 high also showed an

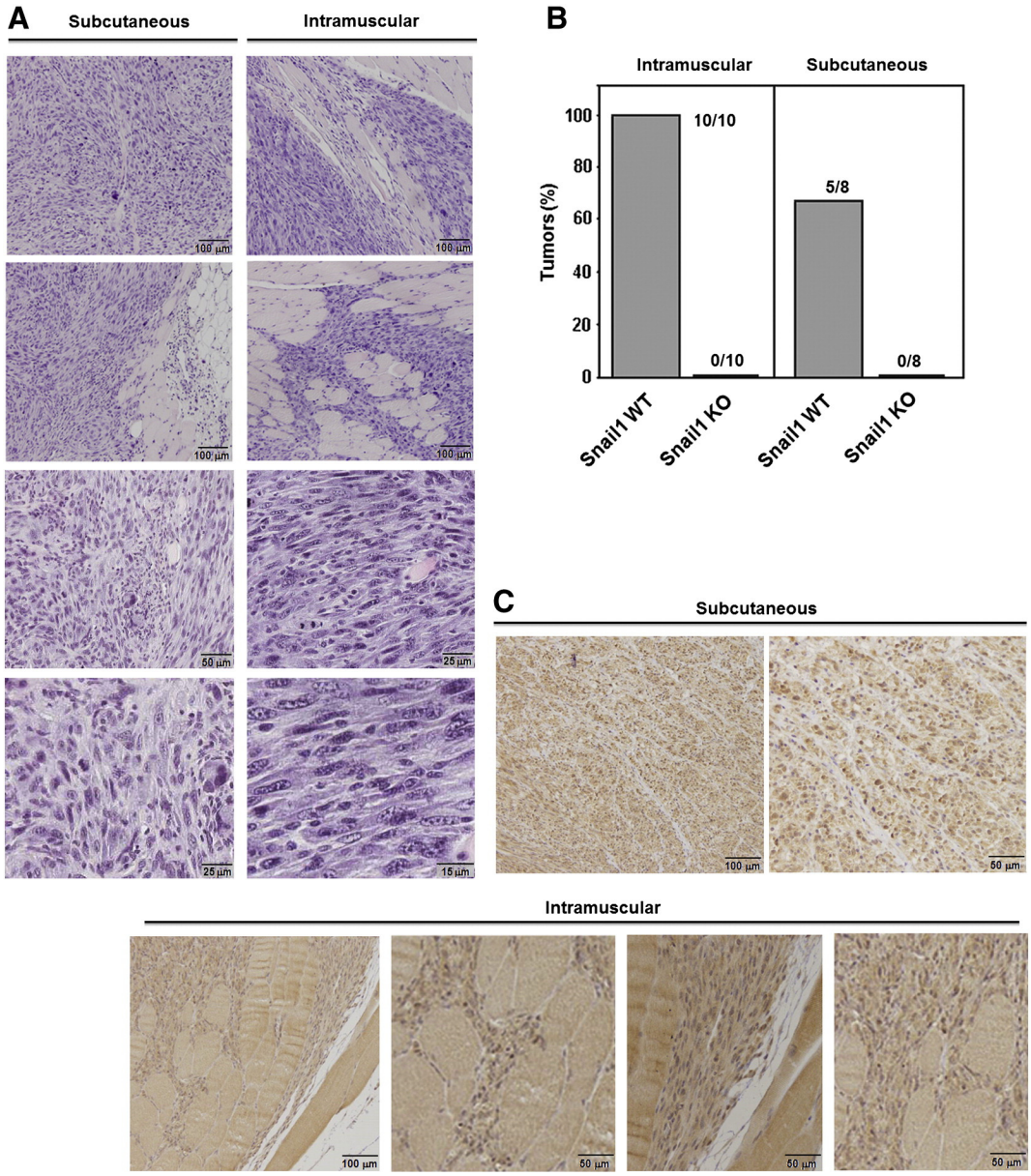

Figure 3. Snail1 prevents the generation of sarcomas by p53-deficient MSCs. Five million of p53-deficient MSCs, either WT or KO for Snail1, were injected into SCID mice either in subcutaneous or i.m. locations; presence of the tumors was determined 60 days later. A histologic analysis of a tumor obtained is presented in A; details of the micrographs are also shown in rows 2 and 4. The number of tumors relative to the number of injections is indicated for the two implantation locations (B). (C) Immunohistochemical analysis of Snail1 expression in the tumors obtained with p53-deficient MSCs injected either subcutaneously or i.m. Bars indicate magnification.

elevated expression for TGF-β (Figure 5*D*; see also Table W1). The expression of another MSC marker, CD29, was also examined. A significant proportion of the tumors analyzed also showed expression of this marker (Figure 5*E* and Table W1) that correlated with that of Snail1 (Figure 5*F*).

4. Discussion

Although Snail1 plays a well-studied function triggering EMT and inducing the complex genetic reprogramming associated to this transition, its role is not limited to epithelial cells. Recent evidences indicate that Snail1 is also required for activation of fibroblasts and for driving invasion [6,20], indicating a relevant function also in mesenchymal cells. Although some actions of this transcriptional factor in both cell lineages might be common, some effects considerably differ.

In epithelial cells, Snail1 expression promotes the acquisition of migratory and invasive features [21], a trait also provided to mesenchymal cells. However, its action on proliferation is different. Although Snail1 down-regulation decreases the tumorigenic capability of some cell lines [22,23], Snail1 transfection retards the proliferation of most epithelial cell lines [24] and inhibits cancer-initiating traits in prostate tumor cells [25]. Similar results are also observed in glioblastoma multiforme: Snail1 expression in glioblastoma multiforme cell lines increases migration, whereas it down-regulates tumorigenesis [26]. Probably related to this effect retarding cell growth, Snail1 expression in epithelial neoplasias is very limited,

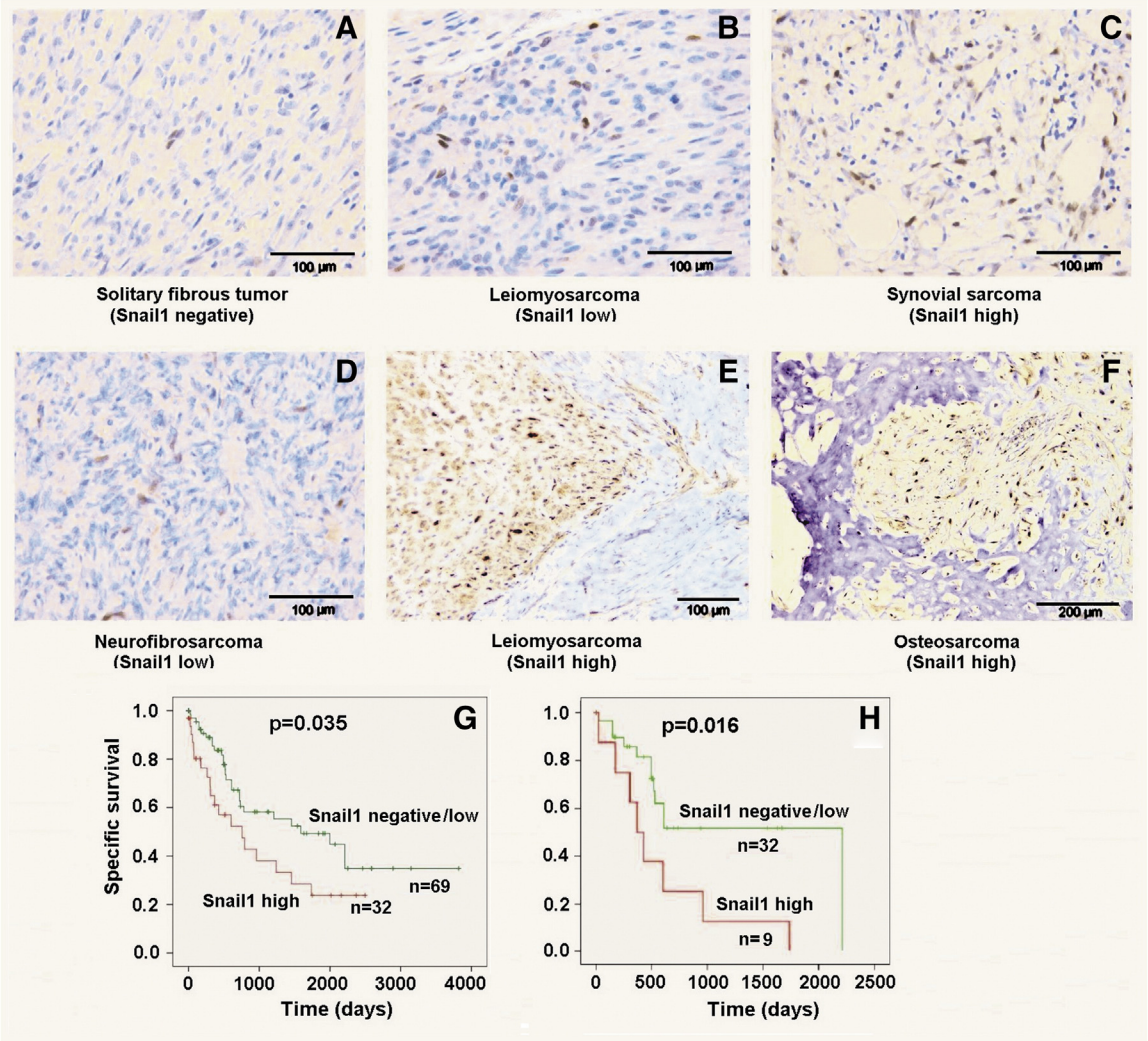

Figure 4. Snail1 expression is associated to a lower specific survival in human sarcomas. Expression of Snail1 was determined as indicated in Materials and Methods section in samples from human sarcomas. Micrographs of several representative stained sections corresponding to Snail1 (A–F) are shown. Bars indicate magnification. Kaplan-Meier analyses of specific survival with respect to Snail1 expression (high *vs* low or null) in 109 sarcomas or in 33 visceral, thoracic, retroperitoneum, and intrabdominal (trunk) sarcomas are presented in G and H. The *P* values are indicated.

with the exception of few cases [18], and it is normally detected in stromal cells close to areas of invasion or inflammation [4,27].

Snail1 action in mesenchymal cells is different because Snail1 overexpression in cell lines does not inhibit cell proliferation [6] but provides to these cells with tumorigenic potential when grafted to nude mice, where they induce sarcomas. We have recently described that Snail1 prevents the differentiation of fibroblastic cell lines and MSCs and maintains these cells in an undifferentiated state [6]. Increasing evidences indicate that MSCs carrying specific mutations are the cause of sarcomas [7–10]; accordingly, MSCs can be transformed depleting p53, a common alteration in human sarcomas [28]. MSCs deficient for p53 originate high-grade sarcomas when transplanted to nude animals [15]. It is noteworthy that p53 depletion in MSCs increases Snail1 protein and RNA. A Snail1 inhibition by p53 has been described in other systems, where p53 represses Snail1 expression through the stimulation of miR-34 [29]. According to our preliminary results, a sustained Snail1 up-regulation by ectopic expression is not sufficient to confer tumorigenic properties to MSCs, suggesting that additional effects dependent on p53 depletion are also necessary for MSC transformation. In any case, it is evident that Snail1 is required for tumorigenesis, because Snail1 depletion in p53 KO MSCs totally abolishes their capability to originate sarcomas.

Snail1 expression was observed in human sarcomas. Compared with our studies in other neoplasms, Snail1 presents a much more abundant expression. This might be the consequence of p53 depletion, as shown above, although it is likely that the cooperation with other signaling pathways can enhance this up-regulation. For instance, activation of PDGF receptor or c-Met signaling pathways has been detected in a variety of sarcomas [30]. These pathways activate Snail1 expression in different cellular systems [2]. We detected differences between the various sarcomas because, in some subtypes, such as malignant fibrohistiocytomas, the percentage of tumors with high Snail1 expression was greater than for other pathologies, such as liposarcomas or solitary fibrous tumors. The elevated expression of Snail1 in the higher graded and more aggressive sarcomas was probably responsible for the lower survival of these patients. These results suggest a putative role for this transcriptional

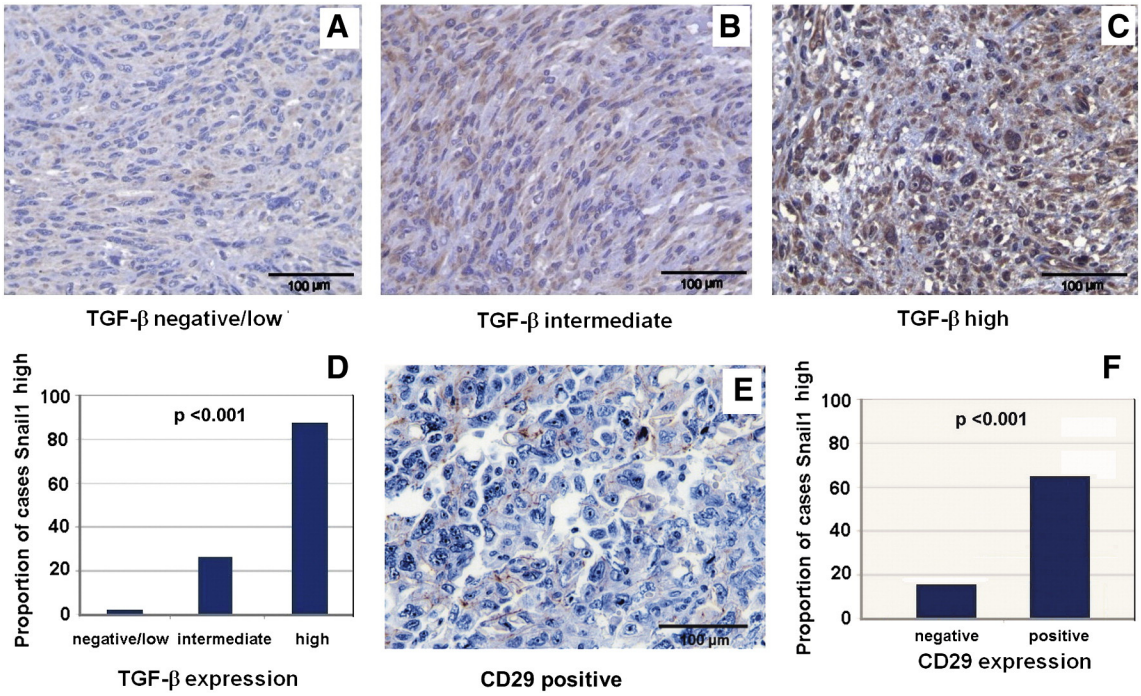

Figure 5. Snail1 expression correlates with that of TGF-β and CD29 and is associated to a lower specific survival in human sarcomas. Expression of TGF-β and CD29 was determined as indicated in Materials and Methods section in samples from human sarcomas. Micrographs of several representative stained sections corresponding to TGF-β are shown in A to C, and a representative staining of a CD29-positive tumor is shown in E. Bars indicate magnification. The correlation between the expression in tumors of TGF-β and Snail1 is presented in D; that of CD29 and Snail1 is presented in F. The *P* values are indicated.

factor in the genesis of at least some of these tumors, such as those more undifferentiated and associated with a more aggressive clinical behavior. Our data also indicate that this Snail1 expression in tumors is also associated with increased expression of TGF-β. Therefore, an elevated Snail expression in sarcomas would be indicative of a higher number of tumor cells with stem cell characteristics and would induce tumors with worse prognosis. It is likely that Snail1 expression confers higher metastatic potential to mesenchymal cells, probably through the up-regulation of integrins required for homing or of cytokines, such as TGF-β, that activate the metastatic niche favoring colonization, as recently shown for colon cancer [31].

Although the involvement of Snail1 in tumorigenesis seems to be valid for most sarcomas, it might be not for a specific type, chondrosarcoma. It should be remarked that, unlike other conversions, differentiation of MSCs to chondrocytes is not prevented by Snail1 overexpression and is dependent on the incubation with TGF-β3; actually, primary chondrocytes express Snail1 [6]. We have analyzed a small number of chondrosarcomas (five), included in the Table W1 in the category of other tumors. In these, Snail1 expression associated with that of TGF-β and did not correlate with a lower survival. Although the low number of cases precludes getting conclusions, the presence of Snail1 in these sarcomas is likely to be related with a differentiated phenotype, unlike most sarcomas. At this respect, Snail1 has been shown to be downregulated in chondrosarcoma cell lines with respect to normal chondrocytes [32].

Our results on the analysis of human tumors also indicate that determination of Snail1 expression might be very informative and useful in clinical routine, because it is more relevant than localization and even grade. Moreover, the identification of inhibitors of Snail1 action is being actively pursued in many laboratories with the goal of preventing invasion of epithelial tumors; our results suggest that these compounds might be even more effective on sarcomas, providing new therapies against these neoplasms.

Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.05.002.

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