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Snail1 controls TGF-β responsiveness and differentiation of Mesenchymal Stem Cells

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Abstract

The Snail1 transcriptional repressor plays a key role in triggering epithelial to mesenchymal transition. Although Snail1 is widely expressed in early development, in adult animals it is limited to a subset of mesenchymal cells where it has a largely unknown function. Using a mouse model with inducible depletion of Snail1, here we demonstrate that Snail1 is required to maintain mesenchymal stem cells (MSCs). This effect is associated to the responsiveness to TGF-β1 which shows a strong Snail1 dependence. Snail1-depletion in conditional knock-out adult animals causes a significant decrease in the number of bone marrow-derived MSCs. In culture, Snail1-deficient MSCs prematurely differentiate to osteoblasts or adipocytes and, in contrast to controls, are resistant to the TGF-β1-induced differentiation block. These results demonstrate a new role for Snail1 in TGF-β response and MSC maintenance.

Keywords

Snail1; mesenchymal stem cells; TGF-β; Akt

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INTRODUCTION

Snail1 is a transcriptional factor that initiates the epithelial to mesenchymal transition (EMT) and enables epithelial cells to acquire migratory properties (1, 2). Accordingly, Snail1 ectopic transfection represses the expression of epithelial genes such as E-cadherin (3, 4), a key element for maintaining the epithelial phenotype. Snail1 also participates in the expression of mesenchymal genes, both indirectly, releasing the inhibition caused on transcriptional activators by E-cadherin, and also directly through the binding to mesenchymal promoters (5, 6). Besides the effects on EMT, Snail1 provides additional functions since it bestows epithelial cells with a higher resistance to apoptosis and cancer stem cell characteristics (1, 2). Probably reflecting these pleiotropic functions, Snail1 is subjected to a tight control, affecting both the transcription of the gene and the stability of the protein (2).

Although Snail1 is expressed at different stages during embryo development and is required for specific processes, such as gastrulation or generation of left-right asymmetry (7, 8), the expression of this protein is very restricted in adult mice (9). For instance, Snail1 is not expressed by tissue-resident fibroblasts and is only detected in these cells when activated; thus, during the process of wound-healing or in the stroma of several types of epithelial tumors (9). Even in these neoplasias, Snail1 is only detected in the tumor-stroma interface in areas of invasion, or in cells next to areas of inflammation (9-11). These results indicate that Snail1, although required for triggering EMT, is not necessary for maintain the mesenchymal phenotype. Accordingly, in cultured primary fibroblasts Snail1 expression is not constitutive and is dependent on serum (8, 12).

Therefore, the specific properties that Snail1 confers to mesenchymal cells are still a matter of research. Genetic Snail1 depletion in murine embryo fibroblasts has revealed a role for this gene in the control of genes essential for invasion, such as membrane type-1 matrix metalloprotease (12). However, other functions of Snail1 in these cells have not been characterized and evidence that Snail1 controls stem cell-like properties has not been reported yet. Here we have analyzed the role of Snail1 in mesenchymal stem cells (MSCs) using a mouse model with inducible depletion of Snail1. Our results indicate that Snail1 expression is required to maintain MSCs in adult animals and for the complete response to TGF-β in these cells.

RESULTS

Snail1 induced the expression of activation markers in fibroblasts

We analyzed the expression of Snail1 protein in a panel of fibroblastic lines in culture. The basal levels of Snail1 in these cells differ, with the highest expression observed in NIH-3T3 (Fig 1A). Moreover, as reported in epithelial cells (13), Snail1 protein increased following treatment with TGF-β1 in all the cell lines examined (Fig 1A). A kinetic analysis performed in L1 cells indicated that Snail1 was up-regulated after 30 minutes of incubation with TGF-β1, much faster than S100A4, a marker of active fibroblasts (Fig 1B).

To check if Snail1 over-expression mimics the effects of TGF-β1, Snail1 was ectopically expressed in 3T3-L1 and C2C12 and the selected populations were analyzed by western blot. Snail1 transfectants showed elevated expression of S100A4 as well as *TGFB1* and *TGFB2* genes and TGF-β1 and -β2 precursor proteins (Figs 1C and D). Therefore Snail1 is not only activated by TGF-β1 but also induces the expression of this cytokine.

Snail1 is relevant for TGF-β1-induced stimulation in the expression of *TGFB1* gene (Fig 1E). Preventing Snail1 up-regulation with a specific shRNA impaired the increased *TGFB1* expression caused by TGF-β1 (Fig 1E), indicating that Snail1 is required for perpetuating this cycle. Moreover, Snail1 shRNA also blocked the TGF-β1-induced up-regulation in S100A4 mRNA (Fig 1E).

Snail1 prevents the differentiation of mesenchymal cells

Under appropriate conditions, 3T3-L1 cells rapidly differentiate to an adipocytic phenotype, a conversion blocked by incubation with TGF-β1 (14). Ectopic expression of Snail1 to levels comparable to those obtained by treatment with the cytokine prevented the morphological differentiation of these cells, as assessed by Oil Red-O staining (Fig 2A). Similar to TGF-β1, ectopic Snail1 blocked the induction of adipocytic markers, such as Glut4 or C/EBP2, although did not modify the levels of other proteins, such as the glucocorticoid receptor or pyruvate kinase (PyrK) (Fig 2B). A transcriptionally inactive Snail1 mutant with a proline to alanine substitution in position 2 (P2A), unable to repress E-cadherin and other target genes (3), did not block differentiation. Although not as much as the L1 cells transfected with the control plasmid, L1 cells expressing Snail1-P2A mutant were stained with Oil Red-O and showed Glut 4 expression (Figs 2A and C).

Similar assays were performed with the C2C12 cell line that differentiates to myotubes after reaching confluence and serum starved. The Snail1 protein levels inversely correlated with differentiation: four days after reaching confluence the levels of the protein were down-regulated with respect to day 0, in contrast to those of the myotube marker myogenin, which was up-regulated (Fig S1B). Ectopic expression of Snail1 also blocked the differentiation of these cells, since it prevented the formation of myotubes (Fig S1A) and the increase in two characteristic markers, myogenin and Myf-6 (Fig S1C).

A similar inhibition was also observed in the differentiation of cultured murine MSC obtained from mouse bone marrow. TGF–β1 induced a rapid increase in Snail1 expression in these cells (Fig S2A) and blocked its conversion to osteoblasts or adipocytes (see below). MSCs differentiation into these two phenotypes was accompanied with a down-regulation in Snail1 protein (Fig 3A). When ectopically expressed, in non-differentiated MSCs Snail1 induced an up-regulation of markers of activated fibroblasts, such S100A4, and of MSCs, such as CD29, CD44 or CD105 (15) (Fig 3B, top panel, and Fig 3H). *TGFB1* and *TGFB2* RNA levels were also slightly increased (Fig 3B, bottom panel) as well as active TGF-β1 in the cell medium (97 ± 9 versus 70 ± 15 pg/ml, as calculated by ELISA). Even although differences in active TGF-β1 were low, MSC-Snail1 cells showed increased endogenous activation of TGF-β receptor with respect to controls, determined analyzing Smad2 phosphorylation (Fig 3C). This result was due to a greater sensitivity to TGF-β1 in MSC-Snail1 cells than in control MSCs (Fig S2B). The relevance of endogenous TGF-β signaling

for Snail1 expression was also examined using two TGF-β receptor inhibitors, SB505124 (SB) (16) and LY-2157299 (LY2) (17), These compounds did not modify the Snail1 protein levels in preconfluent C2C12 and only did it very slightly in L1 or MSCs cells (Fig S3A), Their effect on markers of activated fibroblasts, such as S100A4 or CD29, was also low. As a control, both inhibitors prevent the up-regulation in phosphoSmad2, Snail1 or CD29 caused by TGF-β (Fig S3B). Therefore, these results indicate that autocrine TGF-β did not significantly contribute to Snail1 expression in these cells.

Overexpression of Snail1 completely prevented MSCs from differentiating to osteoblasts, as determined by staining with the specific dye Alizarin Red (Fig 3D), measuring the activity of the osteoblast marker alkaline phosphatase (AP) (Fig 3E) or analyzing the expression of Osterix (Osx) transcriptional factor (Fig 3E, side). Similar results were obtained when these cells were challenged to differentiate to adipocytes: Snail1-expressing MSCs did not show evidence of differentiation, as assessed by Oil Red-O staining and Glut 4 levels (Fig 3F and G).

Up-regulated expression of TGF-β1 was retained in Snail1-transfected MSCs when induced to differentiate to osteoblasts (Fig 3E). Since TGF-β1 prevented MSCs osteogenic differentiation (Fig S4A), we asked whether the ability of Snail1 to block this process was due to the expression of this cytokine. Supplementation of the differentiation medium with the TGF-β receptor inhibitors SB or LY2 did not prevent the block caused by Snail1 over-expression on MSC differentiation to osteoblasts, as shown by Alizarin Red staining (Fig S4A) or measuring AP activity (Fig S4B). As expected, these inhibitors counteract the TGF-β block of osteoblast conversion (Figs S4A and B). SB did not affect the up-regulation in CD44 or S100A4 promoted by Snail1 although it prevented that caused by TGF–β (Fig S4C). As shown above, both inhibitors also avoided TGF-β-dependent up-regulation in Snail1 or CD29 or the increase in Smad2 phosphorylation (Fig S3B).These results indicate that Snail1 block of osteoblastic differentiation is not due to TGF-β1 secretion.

Next we examined the relevance of other signaling elements in Snail1-induced block of differentiation. Activation of PI3Kinase/Akt pathway is required for TGF-β1 expression in MSCs (18). As in epithelial cells (19), Snail1 over-expression down-regulated PTEN levels and stimulated Akt activity, determined measuring the extent of the phosphorylation at the key residue Ser473 (Fig 4A). The widely used PI3 Kinase (PI3K) inhibitor LY-294002 (LY) blocked the Snail1-dependent activation of Akt and also the induction of CD44 and S100A4 but not that of CD29 or CD105 (Fig 4A). PI3K and Akt inhibition significantly reversed the effects of Snail1 on osteogenic differentiation; upon incubation with LY Snail1-transfected MSCs were stained with Alizarin Red and showed up-regulated AP activity with respect to untreated Snail1-MSCs (Figs 4B and C). Two other inhibitors of PI3K/Akt pathway, wortmannin and the Akt-inhibitor MK2066 (MK) also avoided the Snail1 action (Figs 4B and C). Conversely, ectopic expression of an activated form of Akt prevented osteoblast differentiation (Figs 4C and D) although it did not stimulate Snail1 expression (Fig 4E), further indicating that Akt works downstream Snail1 in the prevention of MSCs differentiation.

The ability of MSCs to differentiate to chondrocytes was also analyzed. Ectopic expression of Snail1 in the MSCs did not affect the number of chondrocytes generated from them, as determined by staining with the specific dye Alcian Blue (Fig S5A). Expression of Snail1 was not decreased but rather up-regulated during differentiation into chondrocytes, concomitantly to Col II, a marker of this process (Fig S5B). The detection of Snail1 in embryonic or adult chondrocytes (Fig S5C and D) also supports the conclusion that, in contrast to the observed inhibitory effects on MSC conversion into adipocytes or osteoblasts, Snail1 does not prevent the process of differentiation to chondrocytes. Snail1 was also expressed by primary chondrocytes in culture and declined when the cells became senescent (Fig S5E).

Snail1 depletion affects the number of bone marrow MSCs in vivo

We next analyzed MSCs obtained from murine bone marrow of mice deficient for Snail expression. For this, we engineered a mouse bearing Snail1 null and Snail1 floxed alleles, combined with a tamoxifen inducible Cre recombinase under the control of the ubiquitously active β-Actin promoter (β-act/Cre-ER). To avoid embryonic lethality (7), Snail1 was depleted by injecting tamoxifen in two month-old Snail1$^{Flox/-}$, β-Act-Cre-ER mice (adult animals); Snail1$^{Flox/+}$, β-Act-Cre-ER were used as controls. After one week, MSCs were harvested from femur marrow. FACS analysis revealed that the number of cells negative for the hematopoietic marker CD45 and positive for CD105 or CD90, two markers of MSCs (15, 20), was much lower in Snail1-KO mice than in controls (Fig 5A). Snail1-depleted animals presented lower number of bone marrow cells capable to attach to the plate, a characteristic of MSCs (only 33 % ± 11 of the number obtained in control animals). As expected, Snail1 protein was not detected in cells obtained from Snail1-null animals (Fig 5B); two other MSC markers, CD29 and CD44, were also absent from these cells. A similar down-regulation was also detected in S1004A protein (Fig 5B) and in the expression of *TGFB1* and *TGFB2* genes (Fig 5C).

To check whether the effect of Snail1 was cell autonomous, similar analyses were performed in MSCs isolated from Snail1$^{Flox/-}$ mice prior to injection of tamoxifen and treated with hidroxi-tamoxifen (TAM-OH) in culture. Three days after addition of this drug, Snail1 protein was totally down-regulated as well as CD29, CD44 and S100A4 (Fig 5D). *TGFB1* and *TGFB2* RNAs were also markedly decreased in Snail1-KO cells (Fig 5E). Snail1-depleted cells also showed a slower proliferation with a higher number of cells in G1 than control cells (76 compared with 52%), without signs of apoptosis.

Snail1-KO cells (Snail1$^{Flox/-}$ treated with TAM-OH) differentiated faster than controls (Snail1$^{Flox/+}$). Snail1 KO MSCs were stained with the osteoblast specific dye Alizarin Red (Fig 6A) and showed increased levels of AP activity (Fig 6B) three days after addition of the differentiation medium. After ten days of incubation the differences were lower, since control cells had also undergone differentiation (see Fig 3D and E). Similar effects were obtained when these cells were challenged to differentiate into adipocytes. At day four, when no accumulation of fat was detected in control cells, Snail1-KO cells were strongly stained with Oil Red-O (Fig 6C), and showed up-regulated expression of Glut4 with respect to control cells (Fig 6D).

Snail1-depleted MSCs showed signs of spontaneous differentiation even when cultured in DME medium. Fifteen days after reaching confluency these cultures presented areas with fat droplets (Fig S6A) and had an up-regulated expression of the adipocytic protein Glut4 (Fig S6B). In contrast, these cells did not differentiate to chondrocytes, even when cultured in the optimal conditions, as visualized staining with the specific dye Alcian Blue (Fig S6C).

Snail1 depletion prevents TGF-β1-block of MSCs differentiation

As mentioned above (Fig S2A), Snail1 was quickly up-regulated by TGF-β1 in MSCs, much faster than S100A4. We examined the relevance of Snail1 for the TGF-β1 response in these cells. Addition of TGF-β1 (one day before challenging the MSCs with the differentiation medium) prevented the conversion of MSCs into osteoblasts. On the contrary, MSCs deficient for Snail1 were resistant to TGF-β1 block and showed elevated AP (Fig 6B) and Alizarin Red stainning (Fig 6E). We observed similar results when MSCs were induced to differentiate to adipocytes: in contrast to control cells, MSCs deficient for Snail1 differentiated even in the presence of TGF-β (Fig S7).

TGF-β1 responses were examined in undifferentiated MSCs. Although initial effects of TGF-β1, such as Smad2 phosphorylation, were detected, Snail1-depleted cells showed no or severely impaired up-regulation of different TGF-β1 targets, such as CD44, CD29, CD105, S100A4, PAI-1, *TGFB1* or *TGFB2*, as determined by RT-PCR (Fig 6F) or Western Blot (Fig 6G). However, unresponsiveness to TGF-β1 was not general, since Smad7 expression was stimulated by TGF-β1 in Snail1-KO MSCs. Snail1-depleted MSCs did not show increased Akt activity or PTEN down-regulation upon TGF-β1 stimulation, in contrasts to control MSCs (Fig 6H, upper panel). This PTEN down-regulation by TGF-β1 was associated to lower mRNA levels (Fig 6H, lower) and Snail1 binding to PTEN promoter (Fig 6I); as expected, these two TGF-β1 effects were not observed in Snail1-depleted cells.

Addition of PI3K/Akt inhibitors blocked the TGF-β1-induced up-regulation of a subset (CD44, S100A4, PAI-1) of Snail1-dependent genes (Fig 7A). More importantly, both LY and MK precluded the inhibition of osteoblast differentiation caused by TGF-β1 (Fig 7B and C), suggesting that Akt activation was also required for the TGF-β induced block of this conversion. We noticed that TGF-β only stopped differentiation when added before starting this process since, when supplemented one day after the differentiation medium, cells matured normally (Fig 7B and C). Therefore, in these experiments, and in those previously described for Snail1 transfectants (see Figure 4), PI3K/Akt inhibitors were supplemented to the medium only during the first days of the differentiation (from D-1 to D+2). When added at later times (D+4), they did not prevent the TGF-β block; on the contrary, LY down-regulated the accumulation of differentiation markers in control cells (Fig 7C and D), suggesting an additional role for Akt in the late steps of differentiation. In accordance with its lack on effect on differentiation, at D+1 TGF-β1 did not up-regulate Akt activity (Fig 7E). The initial responses to this cytokine, such as Smad2 phosphorylation were identical at both times.

DISCUSSION

In this article we have analyzed the relevance of Snail1 expression in fibroblastic cells. Our results show that besides increasing markers of activated fibroblasts, Snail1 controls the differentiation of mesenchymal cell lines. Snail1 declines during the process of differentiation; a modest Snail1 ectopic up-regulation, similar to that obtained upon incubation with TGF-β, was sufficient to block differentiation of 3T3-L1 or C2C12 to adipocytes or myotubes, respectively. Similar and more relevant results were obtained in murine MSCs. In these cells, Snail1 ectopic expression prevented their conversion into osteoblasts or adipocytes, whereas Snail1 depletion accelerated them. In contrast, Snail1 ectopic expression did not block the differentiation of MSCs to chondrocytes whereas this process did not happen in Snail1-depleted MSCs. These results are compatible with those published by Nieto and co-workers showing that a forced expression of Snail1 modulates bone growth by controlling chondrocyte proliferation and inhibiting osteoblast differentiation (21, 22). Actually, MSCs conversion into osteoblast or chondrocytes, besides being inversely controlled by Snail1, is also differently sensitive to TGF- β; whereas osteoblastic differentiation was inhibited by TGF-β, chondrocytic conversion required the addition of TGF-β to the differentiation medium. Accordingly, and contrary to what happens in the other cases, Snail1 expression is up-regulated in chondrocytes with respect to MSCs, and is required for chondrocytic differentiation (Fig S6C). Therefore, our results indicate that, as discussed below, Snail1 is necessary for a full TGF-β response in MSCs and for the control of MSC differentiation by this cytokine.

Genetic depletion of Snail1 expression in adult mice confirmed these results. Upon tamoxifen injection and Snail1 obliteration, the number of bone marrow MSCs was markedly decreased. The effect of Snail1 on bone marrow MSCs is cell autonomous, since depletion of Snail1 in vitro also affected the expression of MSCs makers in this population and accelerated their differentiation to osteoblasts or adipocytes when challenged with the appropriate stimulus. These results strongly indicate that Snail1 is required for the maintenance of MSCs. Curiously, another member of the Snail1 family, Slug (Snail2), has been implicated in adipogenesis since Snail2 knock out animals show decreased adipose tissue and Snail2 over-expression enhances adipogenesis (23). These results suggest a different action of both Snail proteins in this process: whereas Snail1 would be required for the maintenance of the undifferentiated phenotype by MSCs, Snail2 would be necessary for the specific induction of the adipogenic program. It is possible that this Snail2 effect would be dependent on the repression of Snail1 expression, since Snail1 promoter contains a functional E-box, putative binding site for Snail2 (24). In any case, these results point to an important role of Snail proteins in the control of adipogenesis.

Snail1 expression is required for a full TGF-β response. In our assays,1) Snail1 was rapidly up-regulated by this cytokine and stimulated TGF- β synthesis and sensitivity; 2) it was required for the activation of many TGF- β targets in MSCs; and 3) it was necessary for the control by this cytokine of MSCs differentiation. However, although Snail1 plays a role in TGF- β-induced gene activation, this does not imply that Snail1-depleted cells are totally resistant to this cytokine. Snail1-KO cells showed initial responses, as indicated by the Smad2 phosphorylation and Smad7 up-regulation in response to the cytokine.

Our results show that Snail1 and TGF-β1 participate in a self-stimulatory loop amplifying the expression of both proteins, since TGF-β1 stimulates its own synthesis and Snail1 enhances the expression of its inducer, TGF-β1. TGF-β1 is a potent inhibitor of adipogenesis and osteoblastogenesis; however, the effect of Snail1 on MSCs differentiation was not dependent on the expression of this cytokine. Although the increase in active TGF-β1 in the supernatant in MSCs upon Snail1 transfection was relevant since it stimulated Smad2 phosphorylation, inhibition of TGF-β receptor did not prevent Snail1 block of osteoblastic differentiation.

The lack of response to TGF-β1 in gene induction and differentiation is associated to the inability to down-regulate PTEN and activate Akt. As previously reported in other cell systems (25-27), TGF-β1 stimulated Akt activity in MSCs, as well as it decreased the levels of PTEN, a direct Snail1 transcriptional target (13). Snail1-dependent PTEN depletion contributes to Akt up-regulation in other cells systems (13, 28), although a transcriptional-independent activation of Akt has also been reported (29) and is probably necessary for the complete stimulation of this protein kinase. In Snail1-depleted MSCs TGF-β was unable to stimulate Akt. Pharmacological inhibition of PI3K/Akt pathway prevented Snail1 or TGF-β activation of several target genes and, more importantly, impeded the Snail1 or TGF-β1 block of osteogenic differentiation. It is noteworthy that TGF-β effects on differentiation are temporally controlled, and when cells had initiated the conversion, they became insensitive to TGF-β block. Concomitantly, TGF-β did not stimulate Akt activity at these later times.

The specific role of Akt in MSCs differentiation is still controversial and has been suggested to regulate it both positively and negatively (30, 31). Our results suggest a dual action of this protein kinase since, besides being relevant for the maintenance of MSCs, it is also required for completion of the differentiation (see Fig 7). It is possible that, as shown for another Snail1-governed cellular conversion, EMT, different Akt isoforms play a contrary role in the control of this process (32). Recent results indicating an opposite action of Akt1 and Akt2 on osteoblastic differentiation (33) support this hypothesis. . Alternatively, the same Akt isoform might play both roles, cooperating with Snail1 in the nucleus (29) in the maintenance of the undifferentiated phenotype, and acting in the cytosol, independently of Snail1 to complete the process. More research should be performed to characterize the specific function of Akt isoforms in the maintenance and differentiation of MSCs, as well as to characterize the TGF-β- and Snail1-sensitive genes controlling MSC conversion in the different phenotypes. In any case, our results indicate a crucial role for Snail1 in MSCs, integrating TGF-β and PI3K/Akt signaling pathways and maintaining these cells in the multipotent state.

MATERIALS AND METHODS

Mice

The targeted null allele of Snail1 gene (7) and the Snail1^{flox} conditional allele (34) have been described. A murine line carrying a Cre recombinase-Estrogen Receptor fusion gene under the control of β-Actin promoter (β-Actin CreER) was obtained from the Jackson Laboratory. Male mice containing the β-Actin-CreER and a Snail1 null allele were crossed with Snail1 $^{flox/flox}$ females. Newborn animals were genotyped by PCR, and animals β-

Actin-CreER, Snail1 null (Snail1^{-}), Snail1 ^{flox} were further analyzed. Animals bearing one copy of the wild-type Snail1 gene were used as controls. Activation of Cre recombinase was induced in 2 months-old animal by four peritoneal injections of tamoxifen (0.1 mg per g of mouse weight, dissolved in corn oil) at alternative days. All mice involved in this study were maintained in a rodent barrier facility in order 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 PRBB.

MSCs Culture and Differentiation

Murine Mesenchymal Stem Cells (MSCs) were obtained as indicated (35) and cultured in DMEM plus 10% fetal bovine serum (FBS). When indicated, cell medium was supplemented with TGF-β1 (5 ng/ml) (Peprotech), SB-505124 (5 μM), LY-294002 (25 μM), Wortmannin (1 μM) (all from Sigma), MK-2206 (10 μM) (Selleck), or LY-2157299 (2 μM) (a kind gift of Dr. E. Batlle, IRB, Barcelona). Osteogenic differentiation of MSCs was performed adding 100 nM dexamethasone, 10 mM β–glycerophosphate, 50 μM L-ascorbic acid-2- phosphate to a confluent culture for three days. Cells were cultured in DMEM plus 10% FBS for ten additional days and either stained with by Alizarin Red or used to prepare cell extracts in 0.1 M Tris buffer, pH 7.2, containing 0.1% Triton X-100 to assay Alkaline Phosphatase activity. Enzymatic activity was determined using *p*-nitrophenylphosphate as substrate in 50 mM 2-amino-2- methylpropanol and 2 mM $MgCl_2$ at pH 10.5.The amount of p-nitrophenyl released was estimated measuring the absorbance at 410 nm.

For adipogenic differentiation, cell were incubated with 1 μM dexamethasone, 0.2 mM indomethacin, 0.1 mg/ml insulin, 1 mM 3-isobutyl-1-methylxanthine in DME medium plus 10% FBS. Stimulation was started when cells reached full confluency; cells were grown for three days in induction medium, for five days in maintenance medium (DMEM plus FBS and insulin) and switched again to the induction medium for three additional days. After 14 days, adipocytic differentiation was assessed staining with by Oil Red-O or determining Glut4 expression by western blot.

MSCs were differentiated to chondrocytes using the micromass culture technique. 2.5×10^5 cells were pelleted under low-speed centrifugation, and the mass of cells was formed at the bottom of the conical centrifuge tube was maintained in the chondrogenic medium for 2 weeks. The chondrogenic medium consists in DMEM (high glucose) supplemented with 10 ng/ml TGF-β3 (Peprotech), 1.25 mg/ml BSA, 0.2 mM ascorbic acid, 0.1 μM dexamethasone and 5 μg/ml Insulin. Regular DMEM was used as control. After 14 days, the specimens were fixed, dehydrated with ethanol, and embedded in paraffin. Alcian blue staining was used to assess the formation of extracellular matrix, which is the mark of chondrogenic differentiation. Cells were counterstained with Nuclear Red (Sigma). Alternatively, cell extracts were prepared and analysed by western blot with the indicated antibodies.

Other methods are detailed in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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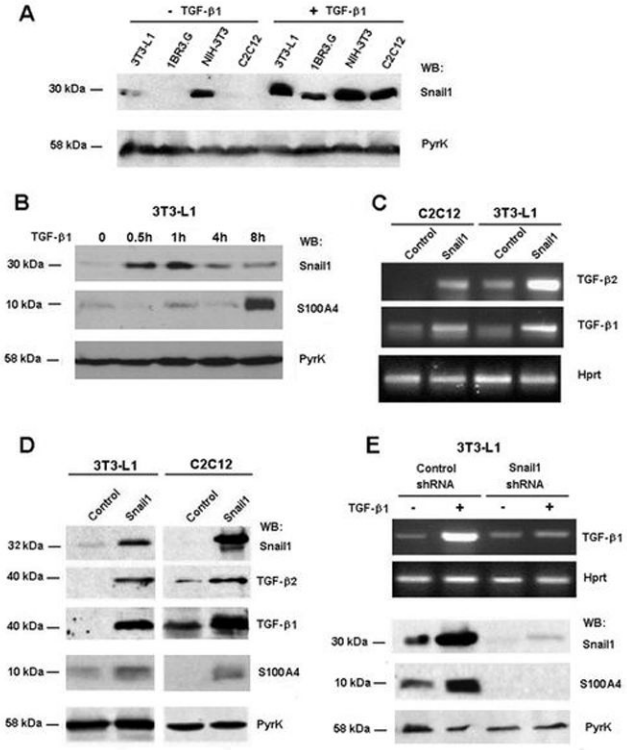

Fig 1. Snail1 induces the expression of markers of activated fibroblasts

Cell populations were transfected with either pcDNA3-Snail1-HA or pcDNA3 control, or with shRNAs specific for Snail1 or a scrambled control. Where mentioned, cell lines were treated with TGF-β1 (5 ng/ml) for 24 hours or for the time indicated. Protein (A, B, D and E, lower panel) or RNA (C, and E upper panel) levels were determined by western blot o semi-quantitative RT-PCR, respectively. Pyruvate Kinase (PyrK) was used as loading control in the western blots; HPRT, in the RT-PCR analysis. The results are representative of three experiments.

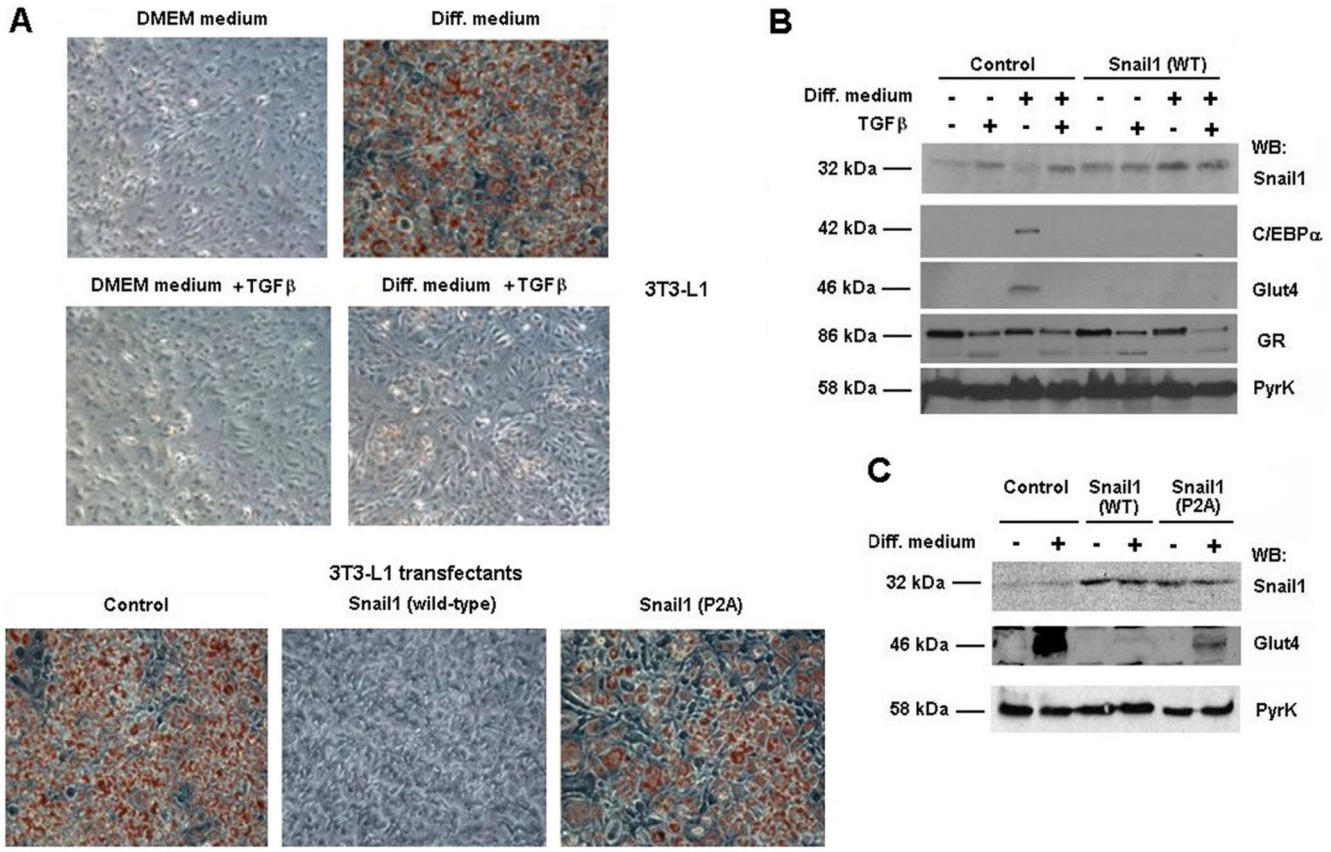

Fig 2. Snail1 prevents the differentiation of 3T3-L1 pre-adipocytes

Control cells or 3T3-L1 transfected with control plasmid, Snail1 wild-type or P2A mutant were cultured in regular DME medium or in differentiation medium for two days after the cells arrived to confluence. Culture medium was replaced by DME medium plus 10% FBS (see Methods) and after two more days cells were either stained with Oil Red (panel A) or homogenized in order to get protein cell extracts (panels B and C). Protein expression was determined using antibodies against Snail1, C/EBPα, Glut4, Glucocorticoid receptor (GR) or PyrK as loading control. When indicated, TGF- β1 (5 ng/ml) was supplemented to the medium at the same moment that the cells arrived to confluence and two days later. The figure shows representative results of at least three experiments performed.

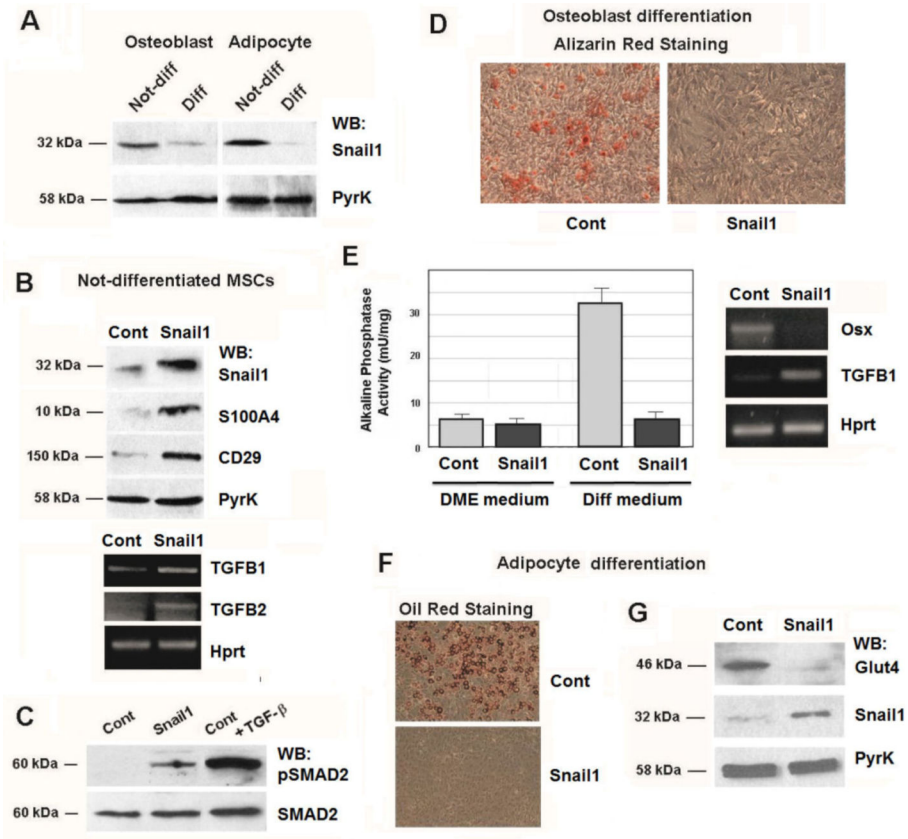

Fig 3. Snail1 blocks MSCs differentiation to osteoblasts or adipocytes

Murine MSCs were transfected with pcDNA3-Snail1-HA or the empty plasmid as control. Protein expression was determined by western blot with the indicated antibodies from extracts prepared from MSCs differentiated to osteoblasts (for 10 days) or adipocytes (14 days) as indicated in Methods (A), or not-differentiated (B top, and C). As control for differentiated cells in panel A (not differentiated), cells were incubated with DMEM plus FBS rather than differentiation medium. RNA was also extracted from MSCs transfected or not with Snail1-HA before being challenged to differentiate and analyzed by RT-PCR for *TGFB1* and *TGFB2* expression (B, bottom). In panel C, TGF-β1 (5 ng/ml) was added to the cell medium 24 hours before preparing the extracts. (D-G); cells were differentiated to osteoblasts (D and E) or adipocytes (F and G); to determine osteoblast differentiation, cells were stained with Alizarin Red (D); alternatively, cell extracts were prepared and AP activity was determined (E, left) or *Osx* RNA analyzed (E, right). Adipocytes were stained with Oil Red-O (F); the presence of the differentiation marker Glut4 was determined by western blot (G).

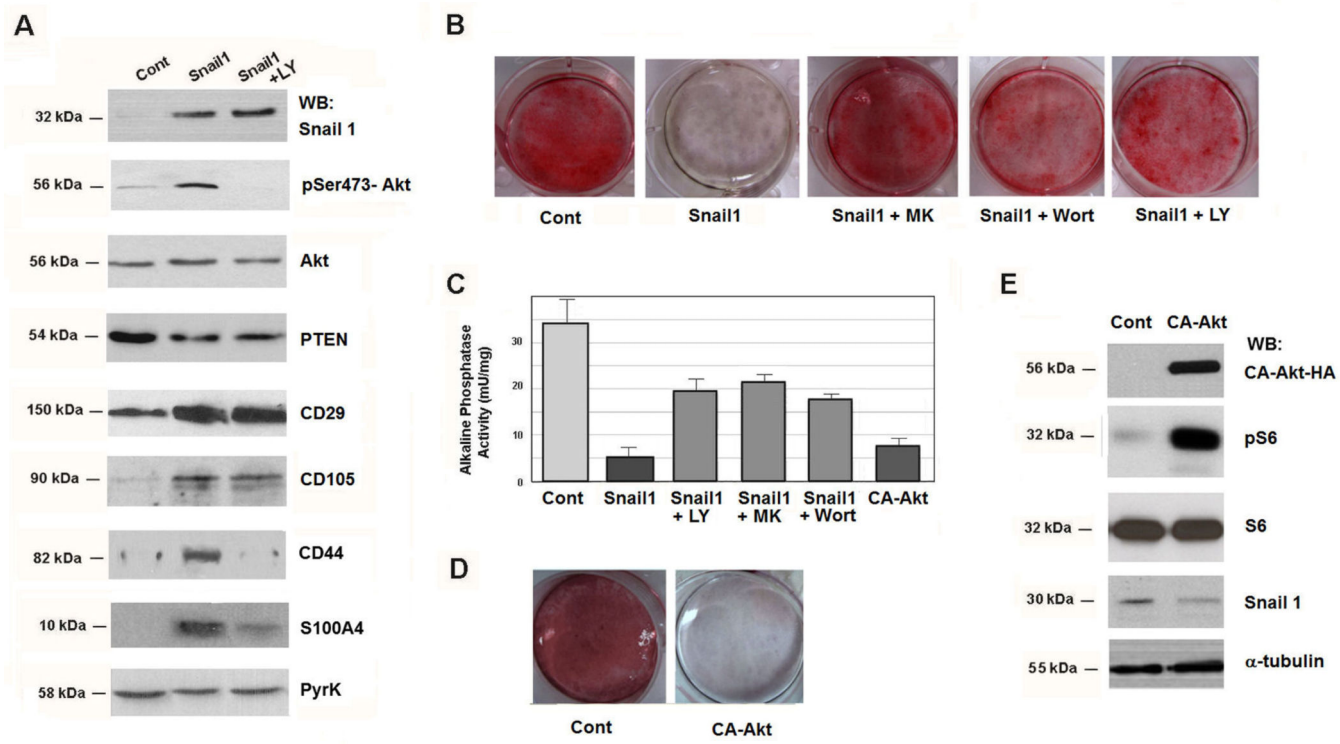

Fig 4. Akt is activated by Snail1 ectopic expression in MSCs

(A); the expression of the indicated proteins was determined in undifferentiated MSCs treated with LY (25 μM) for 24 hours before preparing the extracts. As in Figs 3A and B, cells were pretreated for 15 hours in DMEM medium plus 2% FBS. (B and C); Snail1-transfected MSCs were incubated in osteoblasts differentiation medium for 10 days in the presence of LY, MK, or Wortmannin from one day before adding the medium until two days later (see also Fig 7). Differentiation was determined staining with Alizarin Red (B) or measuring AP activity (C). Results are representative, or show the average ± range of three experiments. (D, E); a constitutively active form of Akt (CA-Akt), tagged with an HA-epitope, was transfected to MSCs. Cells were selected, challenged to differentiate for ten days and stained with Alizarin Red (D). The expression of CA-Akt was checked by western blot (E); since Thr308 and Ser473 are mutated in this form (36), CA-Akt activity was determined analyzing S6 phosphorylation.

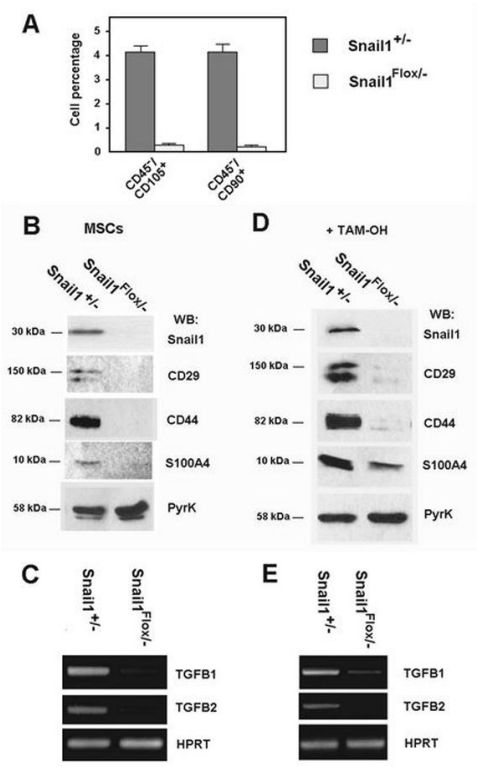

Fig 5. Snail1 controls the number of bone marrow MSCs in vivo

Femur marrow cells were isolated from Snail1$^{Flox/+}$ or Snail1$^{Flox/-}$ animals one week after injection of tamoxifen. (A); cells were analyzed by FACS analysis with antibodies against CD45 and CD105 or CD45 and CD90 antibodies. The percentage of CD45-/CD105+ or CD44-/CD90+ cells is presented for both types of animals. The results show the average ± SD of three experiments performed (B and C) Cells were attached to the cell culture plates and grown for other seven days. Protein extracts (panel B) or total RNA (panel C) was prepared and analyzed. In parallel experiments, bone marrow MSCs were obtained from FloxSnail1/del animals prior to treatment with tamoxifen (TAM); cells were then incubated with hidroxi-tamoxifen (TAM-OH) (1 μM) for two days. Twenty four hours later, the indicated proteins (D) or RNAs (E) were analyzed as above.

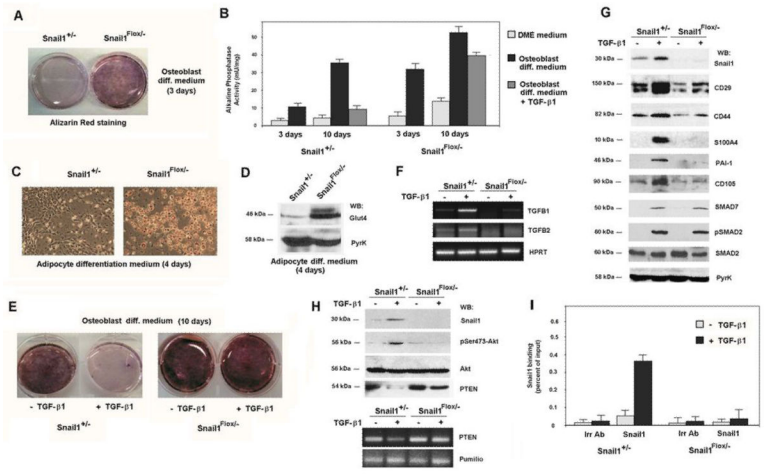

Fig 6. Snail1 depletion promotes the premature differentiation of MSCs and inhibits the response to TGF-β1

MSCs were obtained from Snail1$^{Flox/-}$ animals prior to treatment with tamoxifen, grown to confluency and incubated with TAM-OH for two days. TAM-OH was removed and, after 24 hours, cells were supplemented with the osteoblasts differentiation medium (A, B and E). The extent of differentiation was determined staining with Alizarin Red (A, E) or measuring AP (B). When indicated cells were treated with TGF-β1 (5 ng/ml) one day before adding TAM-OH as indicated in Methods. (C, D); similar experiments were carried out but differentiating cells to adipocytes as described in Fig 3. Differentiation was determined by Oil Red-O staining (C) four days after initiating the process (note that the result presented in Fig 3 was obtained after 14 days) or analyzing Glut4 expression. (F-H); two days after removal of TAM-OH cells were treated with TGF-β1 for 24 hours; the levels of the indicated RNAs (F and H, lower panel) or proteins (G and H, upper panel) were determined as previously described. (I); chromatin immunoprecipitation (ChIP) assays were performed in control or Snail1-depleted MSCs treated or not with TGF-β as indicated in Methods, to determine Snail1 binding to PTEN promoter. The results show the average ± SD of three experiments performed.

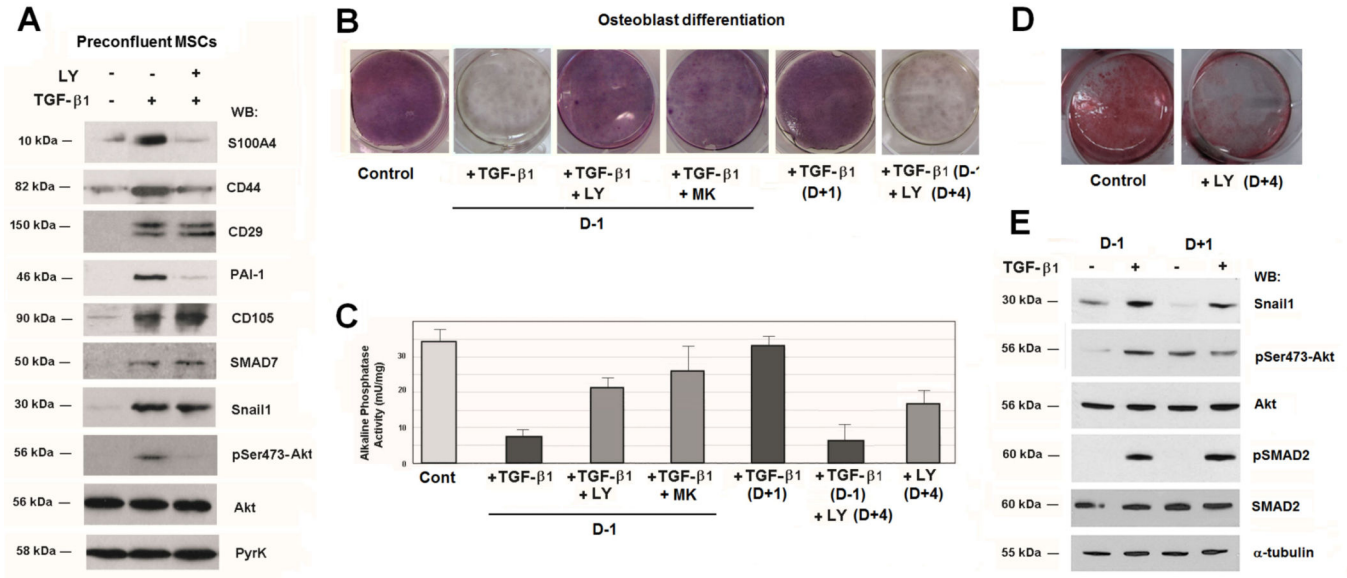

Fig 7. PI3K/Akt inhibitors prevent TGF-β effects on MSC markers and osteogenic differentiation

Panel A; control MSCs were pretreated with DMEM plus 2% FBS for 15 hours and incubated with TGF-β1 (5 ng/ml) or TGF-β plus LY-294002 (25 μM) for 20 hours. Cells extracts were prepared and analyzed by western blot. Panels B, C and D; MSCs were induced to differentiate to osteoblasts; one day before adding the differentiation medium (D-1) TGF-β1 was supplemented with LY or MK when indicated and maintained until D+2. Differentiation was assessed by Alizarin Red Staining (B, D) or measuring AP activity (C) at D+10. Alternatively, TGF-β1 was added at D+1 and maintained until D+8, or LY added at D+4. Panel E; MSCs, one day before reaching confluence and adding the differentiation medium (D-1) or one day after (D+1), were incubated with TGF-β for 20 hours. Cell extracts were prepared an analysed by western blot.