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Snail1 Is a Transcriptional Effector of FGFR3 Signaling during Chondrogenesis and Achondroplasias

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SUMMARY

Achondroplasias are the most common genetic forms of dwarfism in humans. They are associated with activating mutations in FGFR3, which signal through the Stat and MAPK pathways in a ligand-independent manner to impair chondrocyte proliferation and differentiation. Snail1 has been implicated in chondrocyte differentiation as it represses *Collagen II* and *aggrecan* transcription in vitro. Here we demonstrate that Snail1 overexpression in the developing bone leads to achondroplasia in mice. Snail1 acts downstream of FGFR3 signaling in chondrocytes, regulating both Stat and MAPK pathways. Moreover, FGFR3 requires Snail1 during bone development and disease as the inhibition of Snail1 abolishes its signaling even through achondroplastic- and thanatophoric-activating FGFR3 forms. Significantly, Snail1 is aberrantly upregulated in thanatophoric versus normal cartilages from stillborns. Thus, Snail activity may likely be considered a target for achondroplasia therapies.

INTRODUCTION

Endochondral ossification is a multistep process in which cartilage is replaced by bone. During this process, bones grow in length at the growth plate, where chondrocytes sequentially undergo proliferation, prehypertrophy, and hypertrophy. When the proliferating chondrocytes stop dividing, they differentiate into hypertrophic chondrocytes before undergoing apoptosis. This population promotes the mineralization of the surrounding matrix, directing perichondrial cells to become osteoblasts and attracting blood vessels that allow the osteoblasts to invade the cartilage mold and lay down a true bone matrix (Karsenty and Wagner, 2002; Kronenberg, 2003). Thus, the control of longitudinal growth in long bones is mediated by a complex interplay of regulatory networks that involve the chondrocytes in the growth plate and the perichondrial cells in the perichondrium.

FGF signaling is one of the main pathways that control bone development (Ornitz and Marie, 2002). In particular, signaling through the FGFR3 is instrumental in controlling bone size by inhibiting chondrocyte proliferation and differentiation (L'Hôte and Knowles, 2005). Indeed, disruption of FGFR3 in mice causes severe bone dysplasia with enhanced growth, due to the expansion of proliferating chondrocytes within the growth plate (Colvin et al., 1996; Deng et al., 1996). In contrast, activating mutations lead to the principal forms of dwarfism in humans such as achondroplasia (ACH), thanatophoric dysplasia types I and II (TDI and TDII), and hypochondroplasia (HCH) (Rousseau et al., 1994, 1995, and 1996; Shiang et al., 1994; Bellus et al., 1995a and 1995b, Prinos et al., 1995; Tavormina et al., 1995a and 1995b). Studies in mouse models have revealed that signaling through FGFR3 inhibits chondrocyte proliferation and differentiation (Chen et al., 1999; Naski et al., 1996; Wang et al., 1999). This growth inhibition associated with FGF signaling is specific to chondrocytes (Wang et al., 2001) and is mediated by STAT1, the phosphorylation and subsequent nuclear translocation of which activates cell cycle inhibitor $p21^{Waf1/Cip1}$ (Su et al., 1997; Li et al., 1999; Sahni et al., 1999; Legeai-Mallet et al., 2004). The milder cartilage and bone phenotype of Stat1 null mice when compared to that of FGFR3 null mice suggested that FGFR3 signaling also interacts with other pathways during skeletal development. Indeed, FGFs also activate the MAPK pathway in chondrocytes, which affects longitudinal growth by regulating hypertrophic chondrocyte differentiation and matrix deposition (Murakami et al., 2000 and 2004).

The expression of *Snail1* in mesenchymal condensations and hypertrophic chondrocytes, coupled with the fact that Snail can be induced by FGF in other developmental systems (Nieto et al., 1992; Seki et al., 2003; Nieto, 2002), prompted us to study the role of Snail1 in chondrogenesis. We show that not only is *Snail1* expressed in

hypertrophic chondrocytes during normal development, but it is also found in the prehypertrophic population. To further understand Snail1 function, we generated a transgenic mouse model in which we can activate Snail1 protein function. We show that deregulation of Snail1 activity in the developing bone leads to achondroplasia in mice. Indeed, we demonstrate that Snail1 lies downstream of FGF signaling in chondrocytes and that in the absence of Snail1, signaling through FGFR3 is abolished even when the receptor is constitutively active as in the ACH and TDII mutant forms. Thus, our data indicate that Snail1 is essential to transduce FGFR3 signaling during chondrogenesis.

RESULTS

Aberrant Snail1 Activation at Fetal Stages Induces a Dwarfism-like Phenotype

To gain further insight into the role of Snail in skeletal development, we generated a transgenic mouse model expressing a chimeric construct in which Snail1 could be activated by tamoxifen (Snail1-ER; see Experimental Procedures). This inducible system is appropriate to analyze transcription factors' activity, since despite its constitutive expression the exogenous protein is only active after nuclear translocation upon tamoxifen administration (Feil et al., 1996; Boutet et al., 2006).

Taking advantage of the strong repression of *E-cadherin* transcription by Snail1 (Cano et al., 2000), we assessed the activity of the chimeric protein in preimplantation transgenic embryos where E-cadherin is crucial for compaction of the morula (Riethmacher et al., 1995). Snail1 activation completely repressed *E-cadherin* transcription, mimicking the *E-cadherin* mutant phenotype and confirming that the chimeric protein was fully active in embryos (see Figure S1 in the Supplemental Data available with this article online). We selected a transgenic line with significant exogenous protein expression in the developing bones to analyze the effect of Snail1 aberrant activation during chondrogenesis (Figure 1). Snail1 was activated by administering tamoxifen to pregnant mothers from 12.5 dpc, and embryos were then analyzed at 18.5 dpc. All embryos developed short limbs but a normal-sized head (Figure 1A). Closer examination of the dissected limbs confirmed the reduced length of the long bones, and Alcian blue/Alizarin red staining of the femurs showed a reduced growth plate (brackets in Figures 1B and 1C). Exogenous Snail1 protein was readily translocated to the nucleus within 2 days of tamoxifen administration (Figures 1D–1G). Hematoxylin staining of paraffin sections revealed a highly disorganized growth plate, probably reflecting altered proliferation and maturation (Figures 2A–2D). Moreover, the columnar structure of proliferative chondrocytes was lost (see insets in Figures 2C and 2D). We observed graded phenotypes that correlated with the expression level of the exogenous Snail1 protein (Figure S2). The bones in Figure 2 correspond to embryos of intermediate phenotype, where the size of both the proliferating and hypertrophic zones was greatly reduced (Figure 2E). This phenotype is reminiscent of the achondroplasia-type dwarfism (Horton, 2006).

The reduced length might reflect diminished proliferation or increased cell death. To assess whether proliferation was affected in these mice, we analyzed the phosphorylation of histone H3 to visualize the mitotic cells (Prigent and Dimitrov, 2003). There was significant phospho-H3 staining in the growth plate of the control embryos in a zone adjacent to the hypertrophic chondrocytes (Figures 2F–2H). However, in transgenic embryos that were administered tamoxifen, the phospho-H3-stained cells were scattered (Figure 2H) as they were reduced in number to around 30% in the same zone (Figure 2I). Cell death analysis by TUNEL staining and quantification of labeled cells did not reveal differences in wild-type and transgenic embryos regardless of tamoxifen administration (Figure S3). These results indicate that Snail1 activation impairs cell proliferation in the growth plate without affecting cell survival.

The Endogenous Expression of *Snail1* Is Compatible with Its Inhibiting Chondrocyte Proliferation

Since *Snail1* is normally expressed during chondrogenesis (Nieto et al., 1992; Seki et al., 2003), we assessed whether its endogenous expression pattern is coincident with the stages of diminished cell proliferation. When *Snail1* expression was compared with that of other markers for different cell populations, we observed that in addition to the condensing mesenchyme and hypertrophic chondrocytes (Figures 3A–3C), *Snail1* is also transcribed in the prehypertrophic chondrocyte population (Figure 3D). *Snail1* transcripts coincided with those of the parathyroid hormone-related peptide receptor gene (*PPR*; Figure 3F), a specific marker of the prehypertrophic chondrocytes (Lee et al., 1995) and importantly, also with high levels of *Fgfr3* gene expression (Figure 3E), crucial in controlling bone size by inhibiting chondrocyte proliferation (L'Hôte and Knowles, 2005). Similarly, there was colocalization of *Snail1* expression with the last stage of *type II collagen* transcription (Figure 3H) that is then lost before hypertrophic differentiation, and which is concurrent with a switch to Type X collagen secretion (Karsenty and Wagner, 2002). These data indicated that the endogenous expression of *Snail1* is compatible with its exerting an influence on chondrocyte proliferation.

Snail1 Inhibits Chondrocyte Proliferation by Activating the Stat1 Pathway

Achondroplasia in humans and mice is associated with activating mutations in FGFR3, which signals through Stat1. This transcription factor is the mediator of the cell-type-specific growth-inhibitory activity of FGFR3 on chondrocytes (Sahni et al., 1999; Wang et al., 2001). Since tamoxifen-treated transgenic embryos displayed a dwarf phenotype, we analyzed whether Stat1 activation was responsible for the low proliferation observed in the developing bones. Indeed, activation of Stat1 can be detected by its nuclear translocation (Figure 4D, see inset), and it is accompanied by a strong increase in *p21* transcription

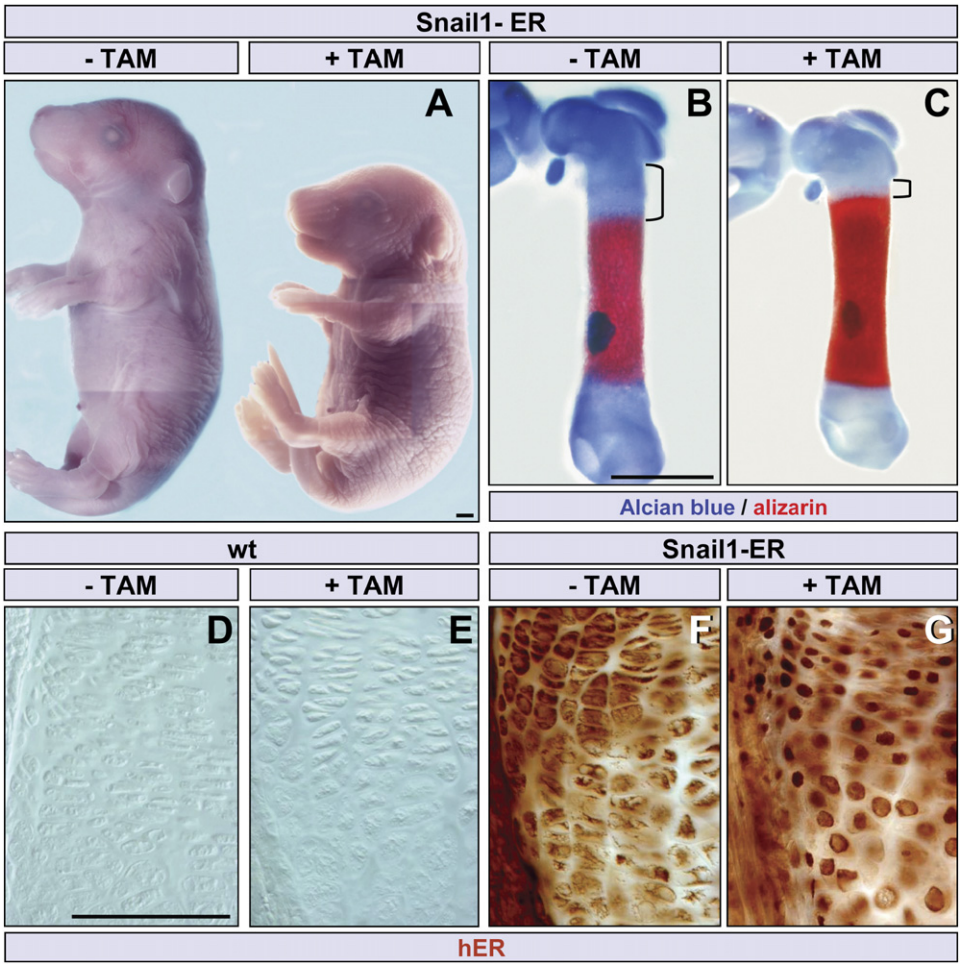

Figure 1. Snail1 Activation at Fetal Stages Induces a Dwarfism-like Phenotype

(A) Transgenic embryos expressing a tamoxifen-inducible Snail construct (Snail1-ER) display shorter limbs and body but a normal-sized head when analyzed at 18.5 dpc after tamoxifen administration (see Experimental Procedures).

(B and C) Alcian Blue-Alizarin Red S staining shows a reduced growth plate in the femur of tamoxifen-treated Snail1-ER embryos (brackets).

(D–G) Exogenous Snail1 protein is expressed in the cytoplasm of the transgenic bones (F) and is translocated to the nucleus upon tamoxifen (TAM) administration (G), as seen with an anti-human estrogen receptor antibody (hER). Scale bar indicates 1 mm in (A), 500 μm in (B), and 100 μm in (D)–(G).

(Figure 4E), the cell cycle inhibitor target of Stat1 in chondrocytes (Su et al., 1997; Sahni et al., 1999). However, the transcription of the *Stat1* gene is not altered in the tamoxifen-treated transgenic bones, suggesting that the regulation of Stat activity is at the level of nuclear translocation (Figure S4). Interestingly, endogenous *Snail1* expression was associated with high levels of nuclear Stat1 both in the perichondrium (compare Figure 4G with Figure 4J) and the hypertrophic chondrocytes (compare Figure 4H with Figure 4K). These data indicate that Stat1 activation appears to be coupled to Snail1 activity during normal chondrogenesis.

Snail1 Is Downstream of FGFR3 Signaling in Chondrocytes

Sustained Snail1 activation induces a strikingly similar phenotype to that observed in humans and mice carrying activating mutations in FGFR3. Therefore, we wondered whether Snail1 might be a target of FGF signaling during chondrogenesis. This possibility was analyzed in cultures of dissociated 14.5 dpc hindlimb cells from wild-type embryos, which differentiated to chondrocytes upon exposure to BMP-2 (Figure 5A; Valcourt et al., 1999).

Differentiated chondrocytes form prototypical nodules after 5 days in culture, and they express Type II but not Type I collagen (Figure 5A). When differentiated chondrocytes were treated with FGF, the transcription of *p21* increased, indicating that the cells expressed FGFR3 in culture (Figure 5B). This increase was significantly higher when FGF was added after transfecting the cells with a wild-type version of FGFR3 (Figure 5B). When chondrocytes were transfected with the ligand-independent mutant versions of FGFR3 present in ACH (G374R; Wang et al., 1999) or TDII (K644E; Li et al., 1999), constitutively high expression of *p21* was registered. The levels of *Snail1* expression followed a similar trend of those of *p21* after

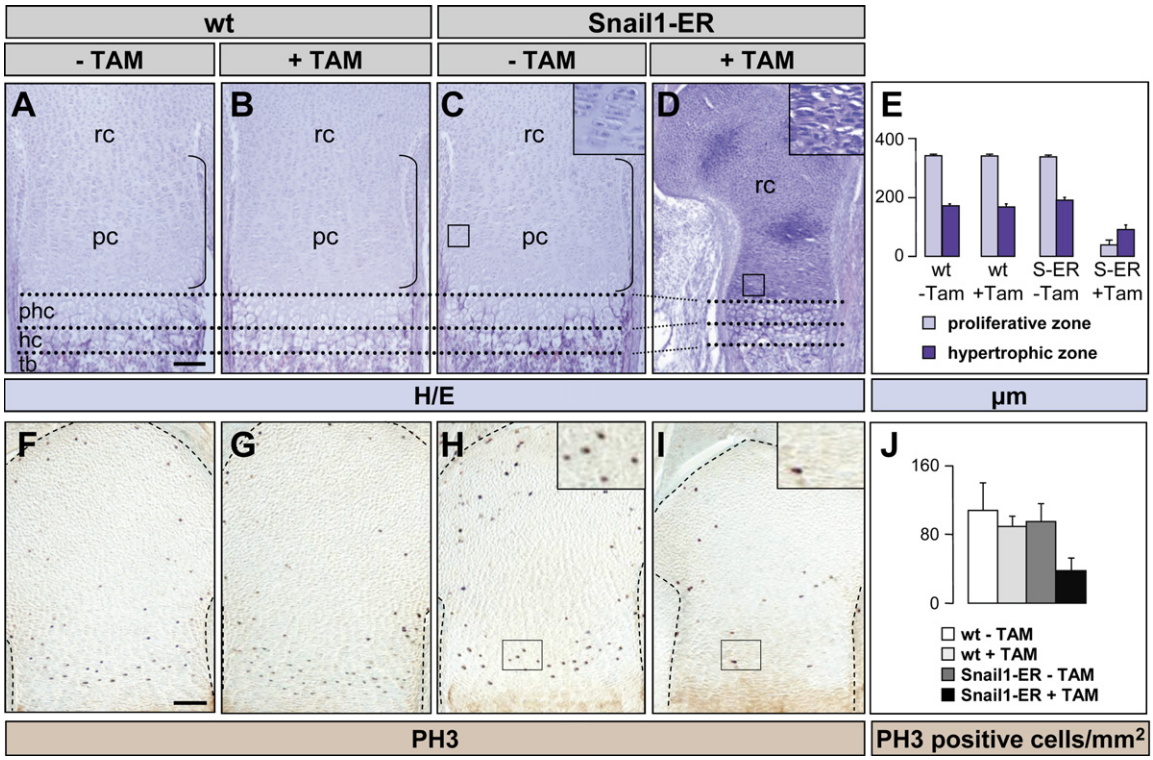

Figure 2. The Dwarfism-like Phenotype following Snail1 Aberrant Activation Is Associated with a Reduced Growth Plate and a Decrease in Chondrocyte Proliferation

(A–D) Histological sections of transgenic bones after tamoxifen administration reveal the disorganization of the growth plate in which the typical chondrocyte columns are almost completely absent (compare insets in [C] and [D]).

(E) The proliferating and hypertrophic chondrocyte populations are very much reduced.

(F–I) The phospho-histone-3-labeled cells are greatly reduced in the growth plates of tamoxifen-treated transgenic embryos, indicating a much lower proliferation rate (insets in [H] and [I]).

(J) Phospho-histone-3-positive cells were quantified and represented as the percentage of total cell number. Results are the mean values ± SD from 12 tamoxifen-treated transgenic embryos plus five per each control condition (three sections per embryo; [E]) and from five embryos per experimental condition (five sections each; [J]). Scale bars indicate 100 µm.

transfection with the wild-type or the mutant FGFR3 forms (Figure 5C). In addition, *Snail1* expression was highest when chondrocytes expressed the TDII FGFR3 mutant, as were the levels of *p21* transcription (Figure 5B). Considering that p21 was induced in the transgenic mice upon Snail activation and that this activation does not modify *Fgfr3* expression (Figure S5), our results indicate that Snail1 is a downstream target of FGFR3 signaling that regulates the Stat1 pathway. The regulation is at the level of nuclear translocation as described during FGFR3 signaling (Su et al., 1997). Indeed, the levels of *Stat1* transcripts remain unaffected (Figure 5E) as they do after Snail1 activation in transgenic mice (Figure S4).

Differentiated collagen II-positive chondrocytes express *Snail1* transcripts as they do in the developing bone (see Figures 3D and 3H), and the protein is localized in the nucleus (Figure 5F). The upregulation of *Snail1* expression observed upon TDII-FGFR3 transfection results in an increase in nuclear Snail1 protein (250% increase; Figure 5F). *Snail2*, the other very similar Snail family member, was not activated by FGF or TDII-FGFR3 (Figure 5D). This activating form of the receptor was unable to induce *Snail1* or *p21* expression in the epithelial kidney MDCK cell line (data not shown), suggesting that the pathway may be specific for chondrocytes.

Snail1 Activation Impairs Hypertrophic Chondrocyte Differentiation by Activating the MAPK Pathway

In addition to reduced proliferation, we also observed a narrower hypertrophic zone in the growth plates of tamoxifen-treated Snail1-ER embryos (Figure 2E), as well as a delay in the formation of the secondary ossification centers when the transgenic animals were treated postnatally (Figure S6). Both these defects, impaired hypertrophic chondrocyte differentiation and diminished proliferation, have also been associated with activating mutations of FGFR3 in mice and humans (Iwata et al., 2000; Legeai-Mallet et al., 2004). Indeed, FGF signaling coordinates the onset of chondrocyte differentiation with the arrest of chondrocyte proliferation in the developing growth plate (Dailey et al., 2003). Thus, we assessed the degree of chondrocyte differentiation by analyzing the expression of *Type X collagen*, a marker of the hypertrophic

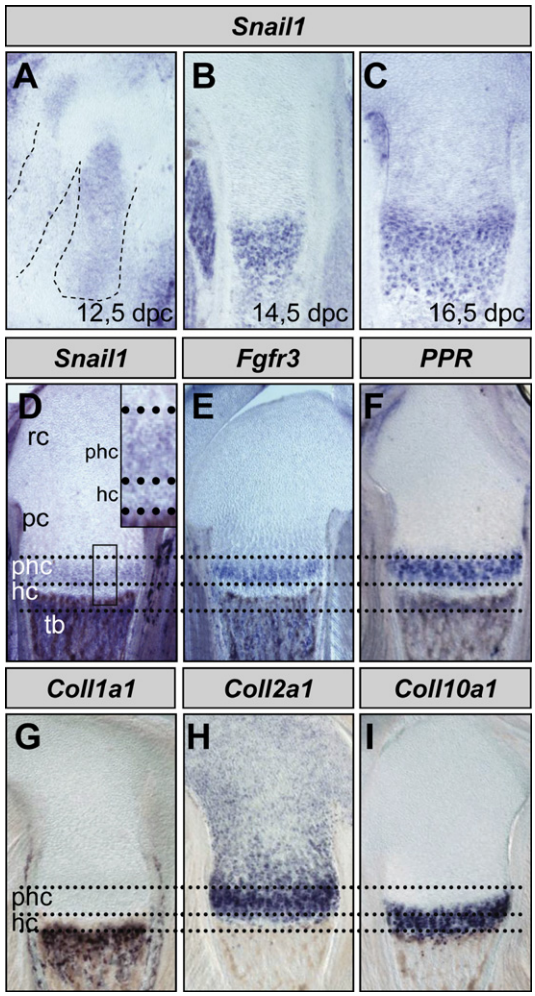

Figure 3. *Snail1* Expression in Developing Hindlimbs

(A–C) *Snail1* expression in embryonic hindlimbs from 12.5 dpc to 16.5 dpc. *Snail1* is first expressed in the mesenchymal condensations that will form the cartilage and bone. From 14.5 dpc, *Snail1* transcripts are detected in prehypertrophic and hypertrophic chondrocytes in a gradient fashion and in the perichondrium.

(D–I) *Snail1* expression in 18.5 dpc embryonic hindlimbs compared with that of specific markers of the main cell populations in the growth plate. A side-by-side comparison indicates that *Snail1* is expressed in both prehypertrophic and hypertrophic chondrocytes (see inset in [D]). Markers: *Fgfr3*; *Coll1a1* (osteoblats), *Coll2a1* (proliferating and prehypertrophic chondrocytes), and *Coll10a1* (hypertrophic chondrocytes) corresponding to Type I, Type II, and Type X collagens, respectively; *PPR*, the parathyroid hormone-related peptide receptor. rc, resting chondrocytes; pc, proliferative chondrocytes; phc, prehypertrophic chondrocytes; hc, hypertrophic chondrocytes; tb, trabecular bone; p, perichondrium. Scale bar indicates 100 μm.

population, and that of *Sox9*, which at this stage is restricted to prehypertrophic chondrocytes. The expression of the *Type X collagen* was significantly weaker in the developing bones of transgenic embryos in which Snail1 was activated (Figures 6A–6D). This decrease may be due to a direct effect of Snail1 on *Type X collagen* expression or to its role on chondrocyte differentiation. In addition, *Sox9* expression failed to be downregulated (Figures 6E–6J) in the resting and proliferating chondrocytes (compare Figure 6F with Figure 6I) or in the reduced hypertrophic population (Figures 6G and 6J, red stars). Other differentiation markers such as Indian hedgehog (*Ihh*) and parathyroid hormone-related peptide receptor (*PPR*) were also analyzed upon Snail1 activation, and *Ihh* transcripts were downregulated (Figure S7) as in other mouse models of achondroplasia (Naski et al., 1998; Zhang et al., 2006). Hence, as well as diminishing chondrocyte proliferation, Snail1 activation also impairs hypertrophic chondrocyte differentiation.

FGF induces *Sox9* expression in chondrocytes through the MAPK (MEK1/Erks) pathway (Murakami et al., 2000). Considering Snail1 as a downstream target of FGF signaling in chondrocytes and that Sox9 is not downregulated in hypertrophic chondrocytes upon sustained Snail1 activation, we analyzed whether the defects on differentiation observed in the transgenic mice might be due to an increase in the activity of the MAPK pathway. We differentiated chondrocytes by adding BMP2 to cultures of hindlimbs from 14.5 dpc Snail1-ER transgenic embryos (Figures 6K and 6L). After 24 hr in culture in the absence of tamoxifen, the basal levels of phospho-Erks were maintained although they increased after FGF administration. This indicates that in-vitro-differentiated chondrocytes respond to FGF signaling. In the presence of tamoxifen and following the nuclear translocation of the activated Snail1 transgenic protein (Figure 6K), the levels of phospho-Erks in chondrocytes were constitutively high, regardless of the presence of FGF (Figure 6L). As expected, the increased level of activated Erks was correlated with elevated levels of Sox9 protein (Figures 6M and 6N), confirming that Snail1 also regulates the MAPK pathway during chondrocyte differentiation.

Snail1 Is Required for FGFR3 Signaling in Chondrocytes

Having determined that Snail1 is downstream of FGF signaling in chondrocytes and that it modulates both the Stat1 and MAPK pathways in the control of chondrocyte proliferation and differentiation, we asked whether Snail1 was required for FGF signaling through FGFR3. Accordingly, we cotransfected chondrocyte primary cultures similar to those shown in Figure 5 with the mutant ACH or TDII FGFR3 forms alone or in combination with different siRNAs against *Snail1* (Figure 7 and Figure S8). Both *Snail1* transcripts and Snail1 protein were highly upregulated upon transfection of activating forms of FGFR3 in the absence of FGF (Figure 5F and Figures 7A and 7C). This expression returned to near-basal levels after transfection of *Snail1* siRNAs, either individually or in combination (Figures 7A and 7C and Figure S8). Interestingly, the elevated expression of *p21* observed in cells transfected with ACH or TDII FGFR3 also decreased when Snail1 expression was inhibited by the different siRNAs (Figure 7B and Figure S8). Similarly, Erks were activated in ACH- or TDII FGFR3-expressing chondrocytes, and their activation levels decreased to the basal state when Snail1 expression was blocked (Figure 7C). Overexpression of a construct

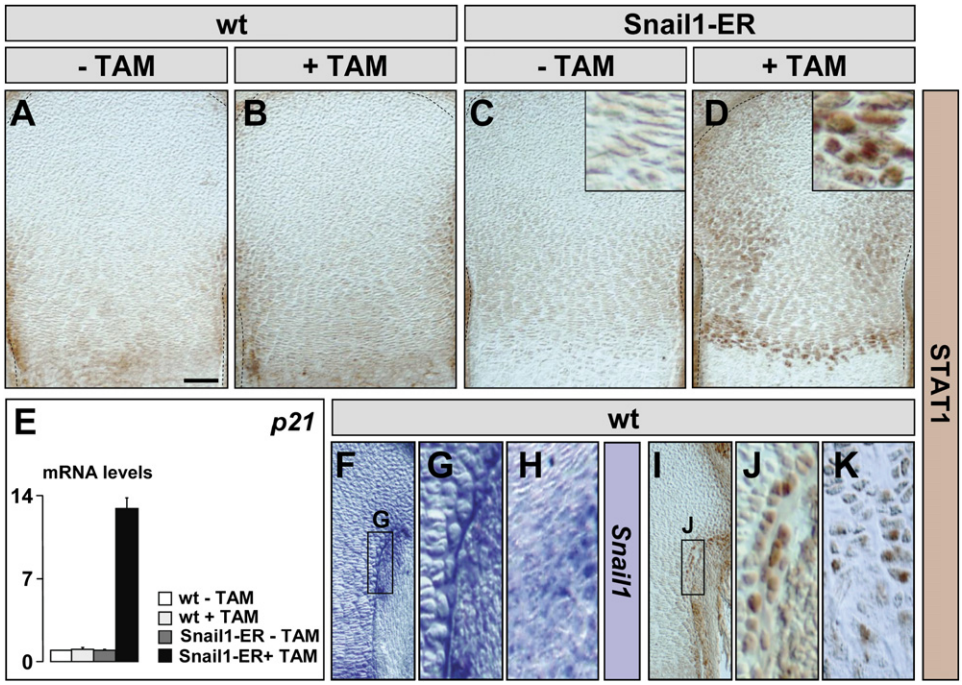

Figure 4. Decreased Proliferation in the Growth Plate of Snail1-ER Embryos Correlates with STAT1 Nuclear Localization and p21 Activation

(A–D) STAT1 is localized in the nucleus of the growth plate cells in tamoxifen-treated transgenic embryos (D).

(E) Quantitative PCR from developing bones shows the increase in *p21* transcription upon Snail1 activation (black bar). Results are the mean ± SD of triplicates from a representative experiment (n = 5).

(F–K) *Snail1* expression coincides with STAT1 nuclear localization in both the perichondrium (G and J) and the hypertrophic chondrocytes ([H] and [K] taken from the same hyperprophic region not visible in [F] and [G]) of wild-type developing bones. Scale bar indicates 100 μm.

containing the Snail1 coding region resulted in increased p21 and pERK expression. When this construct was expressed together with a Snail1 siRNA designed to target 3′ untranslated sequences (siRNA-3), it rescued the action of the siRNA (Figures 7A–7C). Our data indicate that Snail1 mediates the FGFR3 activity that modulates the two signaling cascades which coordinate chondrocyte proliferation and differentiation (Figure 7D) even in the situation that reflects the most severe achondroplastic condition (TDII) in humans. Indeed, the analysis of TDII (bearing the K650E mutation in the *Fgfr3* gene) versus normal cartilages from stillborn individuals indicated that Snail1 was aberrantly activated (around 12-fold) in the TDII fetus (Figure 7E). This result demonstrates that Snail1 induction occurs when FGFR3 signaling is activated in vivo and that high Snail1 levels occur in achondroplasia.

DISCUSSION

The results of this study reveal unexpected and important roles for Snail1 in the control of longitudinal bone growth under normal and pathological conditions. We demonstrate here that aberrant Snail1 activation in transgenic mice is sufficient to induce achondroplasia, the most common form of human dwarfism. The class of chondrodysplasias known as achondroplasia is produced by activating point-mutations in FGFR3. These are usually de novo mutations that generate phenotypes of different severity, from the mildest hypochondroplastic condition (HC) to the lethal form of dwarfism, thanatophoric dysplasia (TD) (see Ornitz and Marie, 2002; Brodie and Deng, 2003 for reviews). We show here that Snail1 lies downstream of FGFR3 signaling, which requires Snail1 for regulating chondrocyte proliferation and differentiation through the activation of the Stat1/p21 and MAPK/Erks pathways, respectively. Moreover, Snail1 is an important transcriptional effector of FGFR3 since even the signaling through the constitutively active ACH or TDII-FGFR3 is abolished when Snail1 expression is silenced, as assessed by the read-outs of both pathways: the cell cycle inhibitor p21 and activated Erks. In addition, as aberrant Snail1 activation is sufficient to induce achondroplasia, genetic or epigenetic alterations influencing Snail expression or activity may cause this disease independently of mutations in the *FGFR3* gene.

Snail1 and the Control of Chondrocyte Proliferation

Our data demonstrate that Snail1 activation in developing mouse bones induces Stat1 nuclear translocation, which increases the expression of the cell cycle inhibitor *p21*. This pathway is known to be mediated by FGFR3 in chondrocytes, and it leads to a blockage of cell division and the initiation of chondrocyte differentiation. *Snail1* is not only

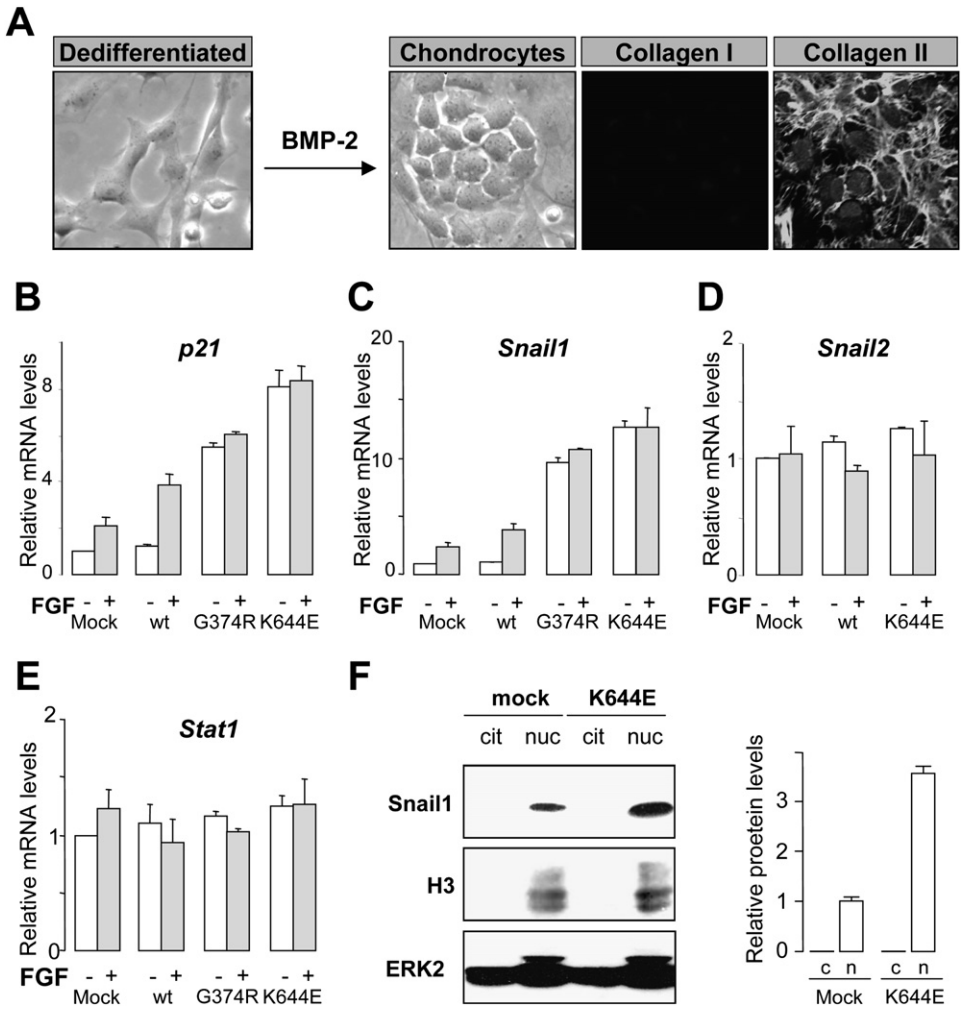

Figure 5. Activation of the FGF Pathway Induces *Snail1* Expression

(A) Primary cell cultures from 14.5 dpc embryonic limbs were differentiated to chondrocytes by exposure to BMP2. Phase contrast micrographs show the cells just after setting up the culture and following differentiation. Cells express the chondrocyte marker Collagen II but not the osteoblast marker Collagen I.

Relative mRNA levels for *p21* (B) and *Snail1* (C) after activating the FGFR3 pathway by administering FGF either before or after transfection with a wild-type (wt) or activating ACH-FGFR3 (G374R) or TDII-FGFR3 (K644E) constructs. FGF induces *p21* transcription, which is constitutively activated after transfection with the ligand-independent mutant receptors. The regulation of *Snail1* transcription correlates with that of *p21*, and the TDII mutant induces the highest levels of *p21* and *Snail1* transcripts.

(D) FGFR3 signaling does not induce *Snail2* expression.

(E) FGFR3 signaling does not upregulate *Stat1* transcription.

(F) The increase in *Snail1* expression upon FGFR3 K644E transfection results in increased levels of nuclear Snail1 protein. Results are the mean values ± SD of triplicates from a representative experiment (n = 4).

expressed in the condensing mesenchyme and the hypertrophic chondocytes (Nieto et al., 1992; Seki et al., 2003) but also in the prehypertrophic cell population, compatible with a role of Snail1 in the attenuation of chondrocyte proliferation. Inhibiting cell proliferation seems to be a common role for Snail1 in different cell contexts, since it also attenuates the growth of epithelial cells in culture by increasing the level of p21 expression and directly repressing *Cyclin D2* transcription (Vega et al., 2004). The effects of Snail1 on proliferation have been considered in the framework of the epithelial-to-mesenchymal transition (EMT) both in development and cancer progression (Barrallo-Gimeno and Nieto, 2005). As such, the cytoskeletal modifications and changes in cell shape associated with the Snail-induced EMT may be incompatible with proliferation. A decrease in proliferation would favor cell migration versus division during embryonic development and tumor invasion versus tumor growth during cancer progression (Vega et al., 2004). In the developing bone, Snail1 does not induce an EMT, although it still downregulates cell division. These data indicate that Snail1 can influence the induction of cell movement and the attenuation of cell proliferation independently depending on the cell context.

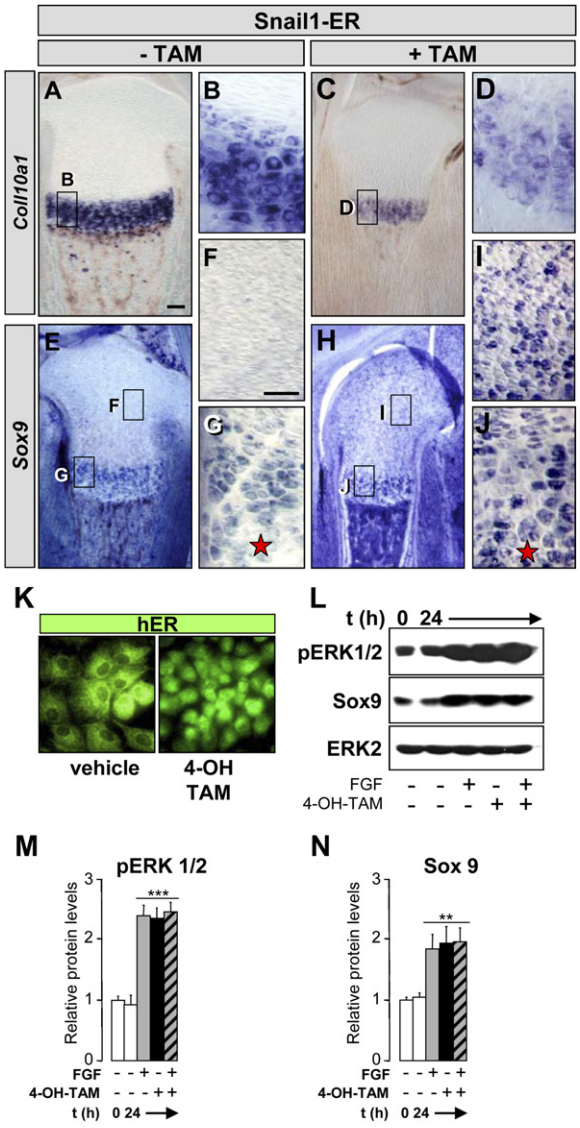

Figure 6. Sustained Snail1 Expression Impairs Hypertrophic Chondrocyte Differentiation by Activating the MAPK Pathway

(A–D) The hypertrophic chondrocyte differentiation marker *Type X Collagen* is greatly reduced in the growth plate of tamoxifen-treated Snail1-ER embryos (compare [B] with [D]).

(E–J) *Sox9* expression fails to be downregulated. Note its high expression in the resting chondrocytes (compare [F] with [I]) and in the limits between the prehypertrohic and hypertrophic populations in tamoxifen-treated Snail1-ER embryos compared to the same region in untreated transgenic embryos ([G] and [J], red stars).

(K) Immunofluorescence for hER allows Snail1 transgenic protein visualization. Note its nuclear translocation upon 4OH-TAM administration.

(L) Western blot analysis showing the levels of activated Erks and Sox9 protein in chondrocytes from Snail1-ER embryos differentiated in vitro.

(M and N) Quantification of western blots confirms the increase in the levels of activated Erks and Sox9 protein. Results are the mean ± SD from three independent experiments. ANOVA analysis: ** $p < 0.01$; *** $p < 0.001$.

This study also shows that Snail1 is downstream of the FGFR3 pathway. The pathway seems to be specific for Snail1, since Snail2, the other very similar vertebrate Snail family member, was not induced either by FGF or by activating FGFR3 mutations. Furthermore, the induction of *Snail1* expression by FGFR3 seems to be specific for chondrocytes since TDII FGFR3 is unable to induce *Snail1* or *p21* expression in the epithelial kidney MDCK cell line. Interestingly, MDCK cells upregulate p21 in response to *Snail1* (Vega et al., 2004), indicating that the failure of FGFR3 in upregulating p21 and inhibiting cell proliferation in MDCK cells is due to its failure in inducing Snail1 expression. In these cells, Snail1 is in fact induced by a different signaling pathway, that of TGF-β (Peinado et al., 2003). These results may explain why the growth-inhibitory response to FGF signaling is unique to chondrocytes, as FGF is known as a proliferative factor in other cell types (Wang et al., 2001).

Snail1 is indeed required for the FGFR3-induced proliferation arrest in chondrocytes. Silencing Snail1 blocks the induction of p21 mediated by the constitutively active TDII-FGFR3 in cultured chondrocytes, and the phenotype we observe after Snail1 activation reproduces that of mice with activating mutations in FGFR3 (Horton, 1997; Ornitz and Marie, 2002). In relation to this, it is worth mentioning that in a mouse TDII model bearing the same mutant form of the receptor, FGFR3 activation unexpectedly increased chondrocyte proliferation in embryos at 15.5 dpc (Iwata et al., 2000). However, this effect of the activating mutation was transient as it was not observed at 18.5 dpc or after birth (Iwata et al., 2000), the stages at which we have analyzed our transgenic mice. Since we do not observe any alteration when the mice were analyzed before 16.5 dpc (data not shown), the possibility exists that chondrocytes are resistant to Snail1 activity until FGFR3 takes on its role in inhibiting proliferation. The differential ability of FGFR3 to induce Snail1 and, therefore, the differential proliferative response to FGFR3 may reflect the different targets with which it interacts at different developmental stages.

Snail1 and the Control of Chondrocyte Differentiation

It has long been established that FGFR3 signals through the Stat1 pathway in chondrocytes and that it is activated in achondroplasia (Su et al., 1997). However, although the loss of Stat1 can rescue proliferation in achondroplastic mice, the reduced hypertrophic zone or the delay in forming secondary ossification centers is not recovered, only partly restoring bone length. This suggests the existence of another pathway regulating longitudinal bone growth. Indeed, elegant studies carried out by Murakami and colleagues demonstrated that the achondroplastic phenotype is not only the result of Stat activation but also of MAPK/Mek activation. FGF induces chondrocyte differentiation in a Mek1-dependent manner (Murakami et al., 2000), and constitutive activation of the MEK1/Erks provokes Stat1-independent achondroplasia by impairing hypertrophic chondrocyte differentiation (Murakami et al.,

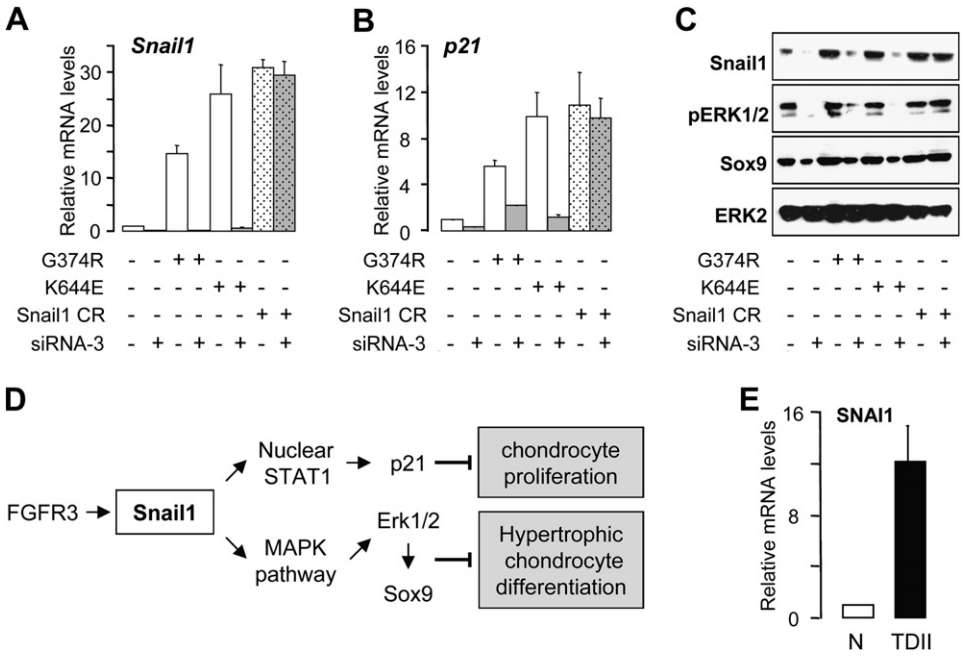

Figure 7. Snail1 Inhibition Abolishes FGFR3 Signaling in Chondrocytes and Is Aberrantly Activated in a Human Thanatophoric Stillborn

Relative mRNA levels for *Snail1* (A) and *p21* (B) in chondrocyte primary cultures cotransfected with the activating mutations of FGFR3 (ACH G374R; TDII K644E) alone or in combination with a siRNA against Snail1 (siRNA-3) and/or a plasmid containing the coding region of Snail1 (Snail1 CR). When Snail1 expression is silenced, the FGF signaling target p21 is not activated (gray bars in [B]). Snail CR overcomes the effect of a *Snail1*-specific siRNA-3 designed to target 3′ untranslated sequences. Snail1 CR increases *p21* transcription up to the level obtained upon transfection with the activating FGFR3 mutants even in the presence of Snail1 siRNA-3 (B). Represented data are the mean ± SD of triplicates from a representative experiment (n = 4).

(C) Western blot showing the levels of Snail1 protein, Sox9, and activated Erks. Total Erk2 was used as a loading control. Note the increased level of Snail1, activated Erks, and Sox9 expression in chondrocytes transfected with the activating versions of FGFR3, and the decrease to basal levels of all three proteins when the cells were cotransfected with siRNA-3. Snail1 CR increases activated Erks and Sox9 expression even in the presence of siRNA-3.

(D) Diagram showing the two signaling cascades triggered by FGFR3 in chondrocytes. Snail lies upstream of the known readouts of the Stat1 and MAPK pathways: nuclear Stat1 and p21 activation on one hand, and activated Erks leading to increased *Sox9* expression on the other. In the wild-type condition, the two pathways coordinate chondrocyte proliferation and differentiation. When overstimulated either by activating FGFR3 mutations or through sustained Snail activation, the pathological inhibition of chondrocyte proliferation and differentiation leads to the dwarfism phenotype.

(E) *Snail1* is aberrantly activated in cartilages from a stillborn bearing the TDII-*Fgfr3* mutant gene (K650E). TDII, thanatophoric displasia type II stillborn; N, non-dwarf stillborn bearing the wild-type *Fgfr3* gene.

2004). Interestingly, the contribution of chondrocyte hypertrophy and matrix synthesis to longitudinal growth is very significant (Wilsman et al., 1996), explaining the achondroplasia-like phenotype observed in mice with constitutively activated Mek-1 in which chondrocyte proliferation is not affected. Our data demonstrate that Snail1 is also required for FGFR3 to activate Erks, indicating that Snail mediates the signaling by FGFR3 through both the described pathways. Very recently, constitutive activation of MKK6, a MAPK that specifically activates p38, has been shown to delay chondrocyte differentiation and inhibit proliferation in mice (Zhang et al., 2006). Although in vitro studies have linked the MAPK/p38 pathway to FGF signaling in chondrocytes (Raucci et al., 2004; Stanton et al., 2004), this pathway has not been directly linked to FGFR3 signaling (L'Hôte and Knowles, 2005). In relation to this, the level of activated p38 remained unaffected in wild-type chondrocytes upon expression of TDII FGFR3, and Snail1 activation did not increase activated p38 in transgenic chondrocytes (Figure S9).

Snail as a Therapeutic Target for Achondroplasia

Snail1 transcripts are very much increased in a stillborn bearing the most severe and lethal achondroplastic condition (TDII; K650E). Considering that viable human achondroplasia is due to a G380R mutation (98% of the viable cases [Shiang et al., 1994]), it will be of much interest to analyze Snail1 expression in a cohort of achondroplastic patients. It seems likely that *Snail1* would also be increased in those patients because, as shown here, the corresponding mutation in mice (G374R) also induces elevated levels of Snail1 expression leading to decreased proliferation and differentiation. If this is the case, the observed correlation between the achondroplastic phenotype and the amount of Snail1 protein in our transgenic

mice suggests that *Snail1* activity can be a target for achondroplasia therapies.

The attenuation of FGFR3 signaling has been long thought to be an effective therapy for achondroplasia, since it could reconstitute normal bone growth (Aviezer et al., 2003; Horton, 2006). As such, the C-type natriuretic peptide (CNP) downregulates the FGF-induced activation of the MAPK pathway and restores the inhibition of ECM production in mice (Yasoda et al., 2004). However, CNP does not affect the Stat1 pathway and thus it will not restore chondrocyte proliferation to normal levels (Yasoda et al., 2004). Thus, identifying Snail as an effector mediating both pathways may help in the design of more effective therapies.

EXPERIMENTAL PROCEDURES

Transgenic Mice and Snail Activation upon Tamoxifen Administration

The pcDNA3-Snail1-ER^{T2} construct contains the Snail1 coding sequence fused to a mutated version of the ligand-binding domain of the human estrogen receptor that recognizes the synthetic ligand 4′-OH-Tamoxifen, obtained from the plasmid pCre-ER^{T2}, kindly provided by P. Chambon (Feil et al., 1996). This construct was used to generate a tamoxifen-inducible Snail1 transgenic mouse according to standard procedures (Hogan et al., 1994). A line was selected that expressed significant levels of the transgenic protein in the developing bone. The subcellular localization of the protein was assessed by immunohistochemistry using a human estrogen-receptor antibody (Santa Cruz). Genotyping was carried out by PCR, using the primers 5′-ACGATAAGCTCGAGCCATCTGC-3′ and 5′-ACCGAGATGATGTA GCCAGCAG-3′. Tamoxifen (Sigma) was dissolved in corn oil at a final concentration of 30 mg/ml. Corn oil or 75 μg of Tamoxifen/g of body weight (Hayashi and McMahon, 2002) was injected intraperitoneally into pregnant females at 12.5 and 14.5 dpc.

Embryo Dissection and In Situ Hybridization

Embryos were removed at 18.5 dpc and fixed overnight in 4% paraformaldehyde. Hindlimbs were decalcified in 22.5% formic acid and 10% sodium citrate at 4°C for 24 hr. Subsequently, they were gelatin embedded, and 30 μm vibratome sections were obtained. In situ hybridization was performed as described in Cano et al. (2000), and the plasmids used to obtain digoxigenin riboprobes corresponded to the following coding sequences: *Snail1* (1–1600 bp), *Collagen1a1* (4271–4691 bp), *Collagen2a1* (3814–4453 bp), *Collagen10a1* (1790–3744 bp), *Pthr1* (1484–4214 bp), and *Fgfr3* (3984–4214 bp).

Cartilage and Bone Staining, Histological and Immunohistochemical Analyses

Embryonic hindlimbs were dissected and fixed overnight in 10% formalin. Specimens were stained with Alcian Blue to visualize the cartilage. Limbs were then trypsinized and stained with Alizarin Red S, cleared in KOH, and stored in glycerol. Histological sections were obtained from hindlimbs fixed in 10% formalin, decalcified as above, and either embedded in paraffin (hematoxylin and eosin) or gelatin (immunohistochemistry). Antibodies: α-human estrogen receptor (α-hER, 1:200; Santa Cruz), α-phospho-histone 3 (α-PH3, 1:1000; Upstate), or α-STAT1 (1:100; Transduction Labs). Immunodetection was carried out with the biotin-streptavidin system (ABC kit, Pierce) according to the manufacturer's instructions.

Chondrocyte Primary Cultures and Transfections

Hindlimbs from wild-type 14.5 dpc mouse embryos were dissected in α-MEM, 1% BSA, 0.1% L-Glutamine, and 0.1% penicillin/streptomycin and cultured overnight. The following day, the bones were trypsinized for 20 min at 37°C and digested for 2 hr in 3 mg/ml Collagenase P in DMEM and 10% FCS at 37°C. The digestion medium was removed and the cells were plated in primary culture media (Woods and Beier, 2006). After 5 days in culture, the cells were seeded at a density of 50,000 per well and differentiated to chondrocytes over 5 days in the presence of 50 ng/ml of BMP2 (Valcourt et al., 1999). Subsequently, cells were transfected using Lipofectamine (Invitrogen) with expression plasmids containing a wild-type or a mutated version of the *FGFR3* cDNA (1.5 μg/well) either alone or in combination with different Snail1 small interfering RNAs (siRNAs) or the random negative control. Cells were cultured with or without FGF to activate the receptor, and 72 hr later the cells were collected. The total RNA was purified from the cells, reverse transcribed, and amplified (Q-RT-PCR), or, alternatively, protein extracts were obtained for western blot analysis. The siRNA duplex oligonucleotides were designed as recommended by Invitrogen, according to the following sequences: siRNA1 (mSna1_454): 5′-AAUAUUUGCAGUUGAAGAUCUUCCG-3′; siRNA2 (mSna1_685): 5′-CAAACCCACUCGGAUGUGAAGAGAU-3′; siRNA3 (mSna1_1023): 5′-CAGCUGCUUCGAGCCAUAGAACUAA-3′; and the Cat. 14750–100 sequence was used as a random siRNA control. For the MAPKs assays, 14.5 dpc embryo hindlimbs from transgenic mice were processed as described above, and the differentiated chondrocytes were cultured in the absence or presence of 200 nM 4-OHT (Sigma) and/or FGF. After 24 hr, the cells were collected, and their RNA or proteins were purified and assayed by Q-RT-PCR or western blot, respectively. Immunofluorescence was performed on differentiated cells seeded on cover glasses and fixed with ethanol at −20°C. The cells were incubated with α-Collagen I (1:20; Calbiochem), α-Collagen II (1:50; Calbiochem), or α-hER (1:200; Santa Cruz) antibodies.

Real-Time PCR

RNA was isolated using the SIGMA Genelute Total Mammalian RNA Kit according to the manufacturer's instructions, and cDNA was synthesized using Super Script III Reverse Transcriptase and Oligo(dT) primers. Q-PCR was performed using an ABI PRISM 7000 sequence detection system with the Sybr Green method (primers available on request). Transcript levels were calculated using the comparative C_t method normalized to GAPDH. The final results were expressed relative to a calibrator (wild-type embryos in the absence of Tamoxifen or mock-transfected cells) using the $2^{-(\Delta\Delta Ct)} \pm$ SD formula.

Western Blotting

For immunoblotting of total protein, cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% SDS, 0.5% Deoxicolate, and 1% Triton X-100 supplemented with protease inhibitor standard mix (1 mM NaF, 1 mM β-glycero-phosphate, 5 mM NaPPi, 5 μg/ml Leupeptine, 1 mM Vanadate, and 100 μg/ml PMSF). For the separation of nuclear and cytosolic proteins, cells were lysed in 10 mM HEPES-K (pH 7.9), 10 mM KCl, 10 mM $MgCl_2$, and 0.5 mM DTT with protease inhibitor mix to obtain the cytosolic extracts. Pellets were then incubated in 20 mM HEPES-K (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and protease inhibitor mix. 50 μg of protein per lane was loaded on a denaturing 12% SDS-PAGE gel. Blots were incubated with α-phosphorylated ERK1/2 (1:2000; Cell Signaling), α-Sox9 (1:200; Santa Cruz), α-Snail1 (1:200; Abcam), α-Phospho-H3 (1:500, Cell Signaling), α-phospho-p38 (1:1000; Cell Signaling [Thr180/Tyr182]), or α-total ERK2 (1:500; Santa Cruz) antibodies. The immunoreactive protein bands were detected using peroxidase-conjugated secondary antibodies. Quantifications were processed with the L Process v2.0 program from Bioimager Fujifilm.

Human Tissue Samples

Human cartilage samples were obtained from TDII and normal cartilages from stillborn patients. The Research Ethics Committees of our Institutions approved the protocols to analyze the tissues, which were processed for real-time RT-PCR as described above.

Supplemental Data

Supplemental data show the characterization of transgene activation in preimplantation embryos, the demonstration that Snail1 expression levels correlate with the achondroplasia phenotype, the absence of Snail1 effects on chondrocyte cell death, the demonstration that p38 levels are not affected in chondrocytes by aberrant levels of Snail1 or ligand-independent FGF signaling, the expression of Ihh and PTHrP-R in transgenic Snail1-ER embryos, the maintenance of FGFR3 expression upon Snail1 activation, the delay in the formation of secondary ossification centers after Snail1 activation in postnatal mice, and additional controls for siRNA Snail1 silencing and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/13/6/872/DC1/.

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