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miRNAs control tracheal chondrocyte differentiation

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ABSTRACT

The specific program that enables the stereotypic differentiation of specialized cartilages, including the trachea, is intrinsically distinct from the program that gives rise to growth plate hypertrophic chondrocytes. For example, Snail1 is an effector of FGF signaling in growth plate pre-hypertrophic chondrocytes, but it derails the normal program of permanent chondrocytes, repressing the transcription of Aggrecan and Collagen type 2a1 (Col2a1). Here we show that miRNA activity is essential for normal trachea development and that miR-125b and miR-30a/c keep Snail1 at low levels, thus enabling full functional differentiation of Col2a1 tracheal chondrocytes. Specific inhibition of miR-125b and miR-30a/c in chondrocytes or Dicer1 knockout in the trachea, de-repress Snail1. As a consequence, the transcription of Aggrecan and Col2a1 is hampered and extracellular matrix deposition is decreased. Our data reveals a new miRNA pathway that is safekeeping the specific genetic program of differentiated and matrix-producing tracheal chondrocytes from acquisition of unwanted signals. This pathway may improve understanding of human primary tracheomalacia and improve protocols for cartilage tissue engineering.

Introduction

microRNA (miRNA) are genome-encoded small RNAs, which regulate gene expression posttranscriptionally (Bartel, 2009). miRNAs hairpin precursors are processed by the RNase III enzymes Drosha and Dicer1, generating a single-stranded ~22nt miRNA, which programs the RNA-induced silencing complex to repress specific targets. Recent evidence indicates that miRNAs have a role in regulating the development and maintenance of skeletal tissues (Hassan et al., 2010; Kobayashi et al., 2008; Lin et al., 2009; Liu, 2009). Furthermore, several miRNA genes, such as miR-140, miR-675, miR-145 and miR-199, appear to be highly relevant to cartilage biology in culture and *in vivo* (Dudek et al., 2010; Eberhart et al., 2008; Lin et al., 2009; Miyaki et al., 2010; Nicolas et al., 2008; Tuddenham et al., 2006; Watanabe et al., 2008; Yang et al., 2011).

The transcription factor Sox9 is the principle activator of the chondrocyte-specific differentiation program (Bell et al., 1997; Bi et al., 1999), whereby it cooperatively functions with L-Sox5 and Sox6 (Lefebvre et al., 1998). Together, these Sox-family factors induce the expression of many effector genes and directly regulate the transcription of extracellular matrix (ECM) proteins such as Col2a1 and Aggrecan.

The trachea is composed of C-shaped hyaline 'rings' that develop in the mesenchyme surrounding the upper respiratory tract. The structural rigidity of the tracheal semi-lunar 'rings' prevents the airway from collapsing during respiration. Several genetic pathways have been shown to affect tracheal cartilage development, including hedgehog (Miller et al., 2004), retinoic acid signaling (Mendelsohn et al., 1994; Vermot et al., 2003) and fibroblast growth factor (FGF) (Eswarakumar et al., 2004; Sala et al., 2011). If signaling in any of these pathways goes awry, a structurally weak trachea develops, giving rise to potentially fatal airway collapse during respiration. This pathology is known as primary tracheomalacia (for reviews see (Boogaard et al., 2005; Carden et al., 2005; McNamara and Crabbe, 2004)).

FGF signaling plays multiple essential roles in growth plate chondrogenesis (reviewed in (Ornitz and Marie, 2002)) and in the trachea, yet its ectopic activation in the trachea results in poor deposition of extracellular matrix (Eswarakumar et al., 2004; Sala et al., 2011). Snail1 (also known as Snai1) is an important transcription factor downstream of the FGF receptor in pre-hypertrophic chondrocytes, where it inhibits proliferation and represses Col2a1 and Aggrecan expression, through interactions with HDAC1 and HDAC2 (de Frutos et al., 2007; Hong et al., 2009; Seki et al., 2003). However, Snail1 is expressed at low levels in the tracheal cartilage, suggesting that there is an idiosyncratic program for the differentiation of this tracheal cartilage that deviates from related chondrogenic program.

Our current analysis suggests that miRNA function in the trachea is essential for proper synthesis and deposition of extracellular matrix.

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In the normal developing trachea, miRNAs specifically repress Snail1, whose unwanted activity is harmful for the development of normal tracheal cartilage. miR-30a/c and miR-125b function as direct repressors of Snail1 expression and their specific downregulation in culture or in a Dicer1 model *in vivo*, results in Snail1 upregulation, inhibited proliferation and perturbed expression of ECM proteins Col2a1 and Aggrecan.

Materials and methods

Mouse genetics

Dicer1 conditional allele, the gift of Mike McManus, Brian Harfe and Cliff Tabin (Harfe et al., 2005), was PCR genotyped with primers: 5′-CCTGACAGTGACGGTCCAAAG-3′ & 5′-CATGACTCTTCAACTCAAACT-3′. The Col2a1-Cre was genotyped as in Amarilio et al. (2007). Mutant Col2a1-Cre;Dicer1flx/flx;R26R(YFP) and Col2a1-Cre;Dicer1flx/+;R26R (YFP) littermate heterozygous controls were maintained in accordance with the Weizmann Institute of Science IACUC guidelines. The day of the vaginal plug appearance was considered embryonic day 0.5 (E0.5).

Histological studies and RNA in situ hybridization

Alcian blue/Alizarin red staining of E17.5-P1 trachea or whole mount was performed as in Amarilio et al. (2007). For histology, the whole respiratory tree, including the trachea and lungs was fixed in 4% paraformaldehyde-PBS (weight/vol.) over-night at 4 °C, dehydrated into 100% ethanol, embedded in paraffin blocks and sectioned. E18.5 cell proliferation was assayed by a two-hour, 5-bromodeoxyuridine pulse (BrdU, 30 mg/kg, I.P.). The fraction of BrdU-positive nuclei in tracheal chondrocytes, was studied with cell proliferation kit (GE Healthcare). Quantification of cells undergoing apoptosis in the trachea was performed with a TUNEL detection kit (Roche). Paraffin section *in-situ* hybridization was performed as in Brent et al. (2003) with antisense probes corresponding to an intronic fragment of the mouse Snail1 (1004nt–1553nt; GenBank accession code NT 039210, the kind gift of Olivier Pourquié) or murine Sox9, Col2a1 and Aggrecan (as in (Amarilio et al., 2007)).

Expression analysis by arrays and real-time PCR

Tracheal cartilage rings of E18.5 embryos were manually dissected and incubated in Trypsin-EDTA (Bioind);37 °C;30′, twice (see Fig S3). Total RNA was extracted with TRI Reagent (MRC). qPCR, performed in technical triplicates on a LightCycler480 (Roche) using DyNAmo HS SYBR Green (Finnzyme) for mRNA or miScript SYBR Green PCR (Qiagen) for miRNA. mRNA expression was normalized to the ribosomal protein S18 and miRNA to the small RNA, U6. Agilent microRNA microarray [G4471A-021828] hybridized with labeled RNA, extracted from tracheal cartilage rings. The full profile is provided in supplementary Table 1.

Assessment of direct miRNA-Snail1 3′UTR interactions

miRNA vectors for miR-30, miR-125b or miR-7 (Control), were generously given by Reuven Agami. The whole mouse Snail1 3′ UTR (728nt; NM_011427) was subcloned into a psiCHECK-2 Vector (Promega) and transfected into HEK293T cells using JetPeiTM reagent (Polyplus), together with miRNA vectors. Firefly and Renilla luciferase activity was studied 48 h post-transfection, with dual luciferase assay (Promega).

miRNA knockdown

ATDC5 cells were cultured in differentiation medium (DMEM/F12 1:1(vol/vol), with 5% (vol/vol) fetal calf serum, 1% (vol/vol) PSG and 10 μg/ml ITS (Sigma)). 2′-O-methyl miRNA inhibitors against miR-30a/c, mir-125b or miR-7 (irrelevant control) had been purchased from Qiagen and transfected with HiPerFectTM (Qiagen) to ATDC5 cells, and RNA was extracted with TRI reagent (MRC) at multiple time points.

Snail1 over-expression in primary tracheal chondrocytes

The mouse Snail1 cDNA was cloned into an adenoviral vector that harbors a CMV promoter and an IRES GFP detection cassette. Tracheae were dissected from three week old mice, incubated in 0.25% trypsin 2×15′. Individual rings were manually teased, transferred into Hyaluronidase (0.5 mg/ml; Sigma) PSG (2% vol/vol in DMEM) for 30′; 37°c and then into Collagenase type V (1 mg/ml; Sigma) PSG (2% vol/vol in DMEM) for 15′. Next, tracheal rings were transferred into a fresh Collagenase V/PSG solution and aggressively agitated until completely dissociating into single cells. Primary chondrocytes were cultured in DMEM containing fetal calf serum (10% vol/vol) and PSG (2% vol/vol in DMEM). Primary tracheal chondrocytes were infected with the pCMV-Snail1-IRES-GFP virus 12 h after culturing.

Results

Dicer1 deficiency in the tracheal chondrocytes leads to respiratory failure at birth

To investigate the physiological role of miRNA activity in tracheal rings, we crossed a Dicer1 conditional allele (Harfe et al., 2005) to a transgene expressing Cre recombinase under the control of the Col2a1 promoter. This resulted in Dicer1 knockout and in repression of miRNA maturation, specifically in chondrocytes (Figure S1A). Approximately 85% of the Col2a1-Cre:Dicer$^{fl/fl}$ mice (named later 'Col2DCR^{mut}') died as neonates (early after birth), whilst Col2a1-Cre;Dicer1$^{fl/+}$ littermates ('controls') seems to be phenotypically normal. Surviving Col2a1-Cre:Dicer$^{fl/fl}$ mice died before weaning and had significant growth retardation relative to littermate controls as previously described in (Kobayashi et al., 2008). In addition, E18.5 Col2a1-Cre:Dicer$^{fl/fl}$ exhibited dental malocclusion, fenestrated skull vault cartilage, and malformed transverse processes of lumbar vertebrae (Figure S2A,B).

Post mortem exploration revealed that col2DCR^{mut} lungs failed to inflate and that the alveoli did not fill with air (Fig. 1A,B). This is consistent with the apparent cyanosis of col2DCR^{mut} pups. Since Dicer1 was conditionally deleted only in cartilaginous tissues, we further evaluated whether defects in the rib cage and trachea may play a role in the ventilation defect. Whole-mount alcian-blue/alizarin-red examination revealed only minute changes in the rib cage (Fig. 1C), but the tracheal ring and larynx cartilage staining was drastically reduced (Fig. 1D). Thus, col2DCR^{mut} newborns manifest tracheomalacia — a condition characterized by flaccidity of the tracheal support cartilage, which leads to tracheal collapse. Conceivably, the defect in the quantity or quality of the extracellular matrix is the reason for the observed neonatal asphyxia.

Partial chondrocyte differentiation and normal tracheal ring morphogenesis in the Dicer1 mutants

To reveal the tissue composition of the Dicer1 mutant tracheae, we crossed a fluorescent genetic fate tracer (Giel-Moloney et al., 2007) onto the genetic background of the col2DCR^{mut} mice. In the resultant col2DCR^{mut};R26R mice, cells in which recombination occurred, lose Dicer1 activity and are additionally labeled with YFP. This analysis revealed that chondrocytes are indeed present in the col2DCR^{mut} trachea. Furthermore, the tracheal cartilage is properly patterned into multiple semi-lunar structures in both col2DCR^{mut} and in Col2a1-Cre;Dicer1$^{flx/+}$;R26R controls (Fig. 1E). While Dicer1 knockout has been shown to initiate apoptosis in many cell types, loss of Dicer1 function in chondrocyte was not reported to cause apoptosis (Kobayashi et al., 2008). Consistently, we did not identify signs of programmed cell death by searching for nuclear pyknosis or by performing a TUNEL assay.

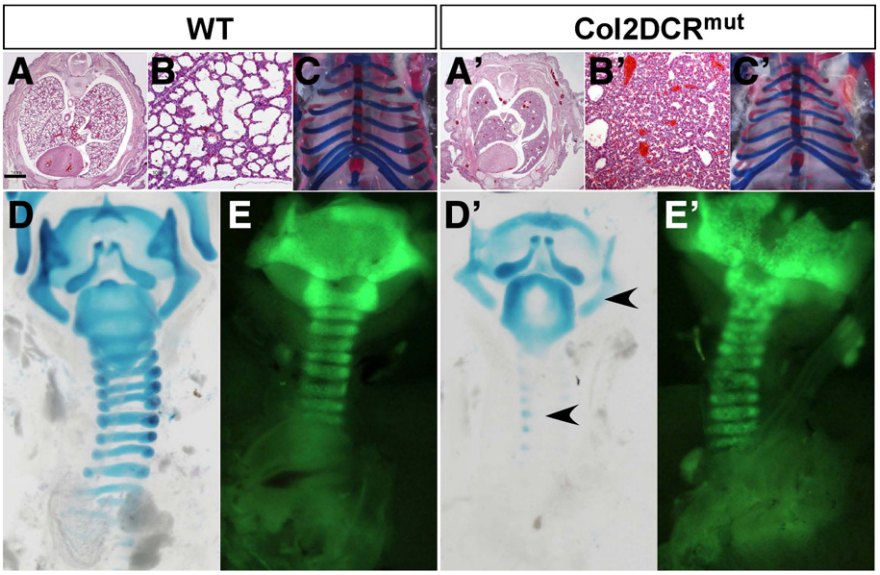

Fig. 1. Tracheomalacia and disinflated alveoli in Col2DCR^{mut} mice. Hematoxylin and Eosin analysis of thoracic transverse section of P1 WT and Col2DCR^{mut} (A,A' bar = 1 mm). Disinflated alveoli observed on higher magnification of the same slides (B,B',). Alcain blue/Alizarin red staining, reveals normally patterned rib cage in both WT and Col2DCR^{mut} (C,C'). Whole larynx and trachea Alcian blue staining reveals poor deposition of ECM in Col2DCR^{mut} tracheal rings and larynx relative to control (D,D' arrow heads pointing to reduced matrix areas). Genetic fate tracing of the Col2a1 lineage by a conditional Rosa26 YFP reporter exemplifies the normal morphogenesis of Col2DCR^{mut} tracheal rings and larynx (E,E').

Reduced Col 2a1 and Aggrecan expression in Col2DCR^{mut} tracheal Rings

Col2DCR^{mut} chondrocytes do not properly deposit ECM as required and therefore poorly stain with alcian-blue. To understand this phenotype better, we evaluated the expression levels of Col2a1 and Aggrecan, two important matrix proteins in the tracheal cartilage. The *in situ* detection of Col2a1 mRNA revealed reduced expression relative to controls and was similarly quantified by qPCR (Fig. 2A,D). Aggrecan mRNA levels, were also downregulated in col2DCR^{mut} tracheal rings (Fig. 2B,D). Direct evaluation of mRNA encoding for primary ECM proteins, brings us to

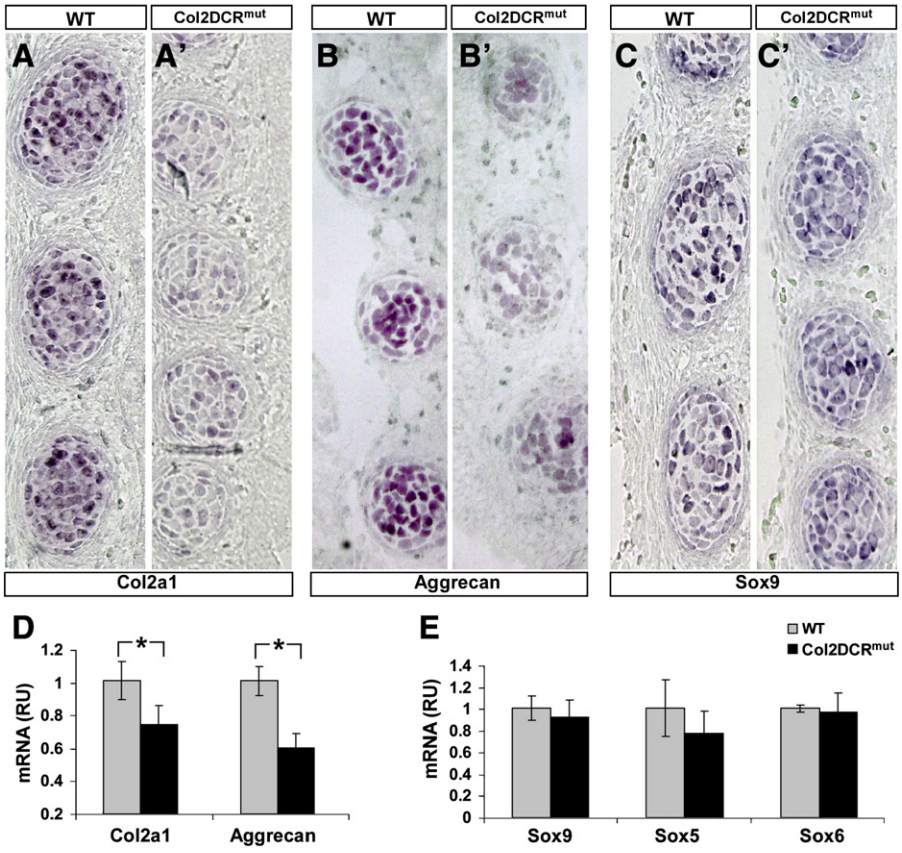

Fig. 2. Col2a1 and Aggrecan mRNA levels are lower, while Sox family transcription factors are normally expressed in Col2DCR^{mut}. Tracheal section *in situ* hybridization obtaining antisense RNA probes for Col2a1 (A,A'), Aggrecan (B,B') and Sox9 (C,C'). qPCR analysis of Col2a1 and Aggrecan (D) and the mRNA encoding for the transcription factors Sox9, Sox5 and Sox6 (E). Pooled tracheal ring RNA of 8 individual embryos; three repeats per genotype. * = P value < 0.05.

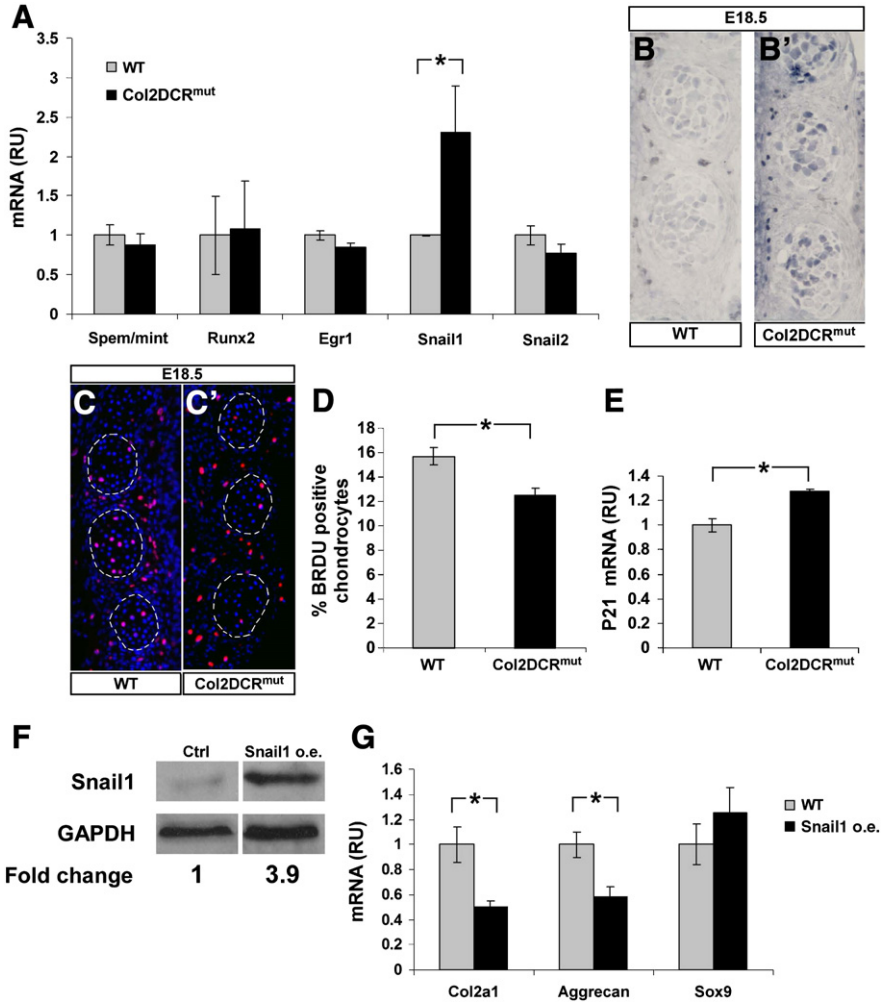

Fig. 3. Snail1 is de-repressed in Col2DCR^{mut} tracheae and is able to represses Col2a1 and Aggrecan expression in primary tracheal chondrocytes. qPCR analysis of potential repressors of Col2a1 and Aggrecan transcription, preformed on RNA isolated from tracheal rings. Snail1 is specifically and significantly upregulated. Pooled RNA sample from 8 Col2DCR^{mut} tracheae relative to controls. (A) Section *in situ* hybridization of tracheae with antisense RNA probes for Snail1 (B,B'). A representative immunofluorescent micrograph from a BrdU incorporation study of E18.5 Col2DCR^{mut} and WT trachea (C,C'). BrdU-red, nuclei-blue. Cartilaginous rings demarcated by dashed lines. Bar graph quantification of the number of BrdU-positive tracheal chondrocytes (D; 500 cells/individual animal; n = 3,3). qPCR analysis of P21 mRNA levels in RNA isolated from tracheal rings (E; n = 3,3). Overexpression of Snail1 in primary tracheal chondrocytes quantified by western-blot analysis relative to a control virus expressing GFP (F). qPCR analysis of Col2a1, Aggrecan and Sox9 mRNA in primary tracheal chondrocytes infected with an adenoviral vector expressing GFP (gray) or Snail1 (black). (G; n = 3,3). * = P value < 0.05.

conclude that low ECM likely results from an intrinsic defect in regulating Col2a1 and Aggrecan expression in Col2DCR^{mut} mice.

To examine the basic differentiation properties of the mutant Col2DCR^{mut} tracheal chondrocytes, we performed RNA *in-situ* hybridization for Sox9, the chief transcription factor in chondrocyte differentiation. Sox9 was unchanged in col2DCR^{mut} tracheal chondrocytes relative to controls (Fig. 2C,E). Furthermore, the qPCR analysis of Sox9 and its two transcriptional co-activators Sox5 and Sox6, was within the normal expression spectrum (Fig. 2E). We conclude from this study that the core transcriptional machinery engaged in chondrogenesis is properly maintained in Col2DCR^{mut} tracheal chondrocytes.

Why is Col2a1 and Aggrecan transcription downregulated in chondrocytes that express the three principle Sox transcriptional activators? One potential reason may be because of abnormal upregulation of transcriptional repressors in col2DCR^{mut} tracheal chondrocytes. We therefore quantified the expression levels of a reported set of repressors of Col2a1 or Aggrecan by qPCR, including Spen/Mint, Egr1, Snail1 (Snai1) and Slug (Snai2) (Seki et al., 2003; Tan et al., 2003; Yang et al., 2005). Runx2, a transcription factor that promotes chondrocyte hypertrophy (Takeda et al., 2001) was also considered. The mRNA levels of Snail1 were upregulated in Col2DCR^{mut} tracheal chondrocytes, while the mRNA levels of other candidate inhibitors were not significantly changed (Fig. 3A). Once Snail1 upregulation was substantiated by mRNA *in-situ* hybridization (Fig. 3B), we considered it a prime candidate repressor of Col2a1 and Aggrecan in col2DCR^{mut} tracheal chondrocytes.

Intriguingly, misexpression of a Snail1 transgene in growth plate chondrocytes induces Sox9 expression, represses chondrocyte proliferation and upregulates the transcription of P21 (de Frutos et al., 2007). Therefore, if Snail1 upregulation is functionally important for the observed phenotype in Col2DCR^{mut} tracheal rings, then P21 levels should be upregulated and proliferation should be decreased relative to wild type chondrocytes. When we performed BrdU incorporation analysis of E17.5 tracheal chondrocytes, we identified a 20% reduction in Col2DCR^{mut} tracheal chondrocyte proliferation relative to controls (Fig. 3C,D). At the same time, the expression levels of P21 were significantly upregulated in the mutants (Fig. 3E). These observations support functional upregulation of Snail1 in Col2DCR^{mut} tracheae.

In order to verify that Snail1 can indeed negatively regulate Col2a1 and Aggrecan synthesis in the trachea, we isolated primary tracheal chondrocytes and infected them with an adenoviral vector misexpressing Snail1. Snail1 was overexpressed four fold relative to control, in levels comparable with Snail1 expression in Col2DCR^{mut} tracheal chondrocytes.

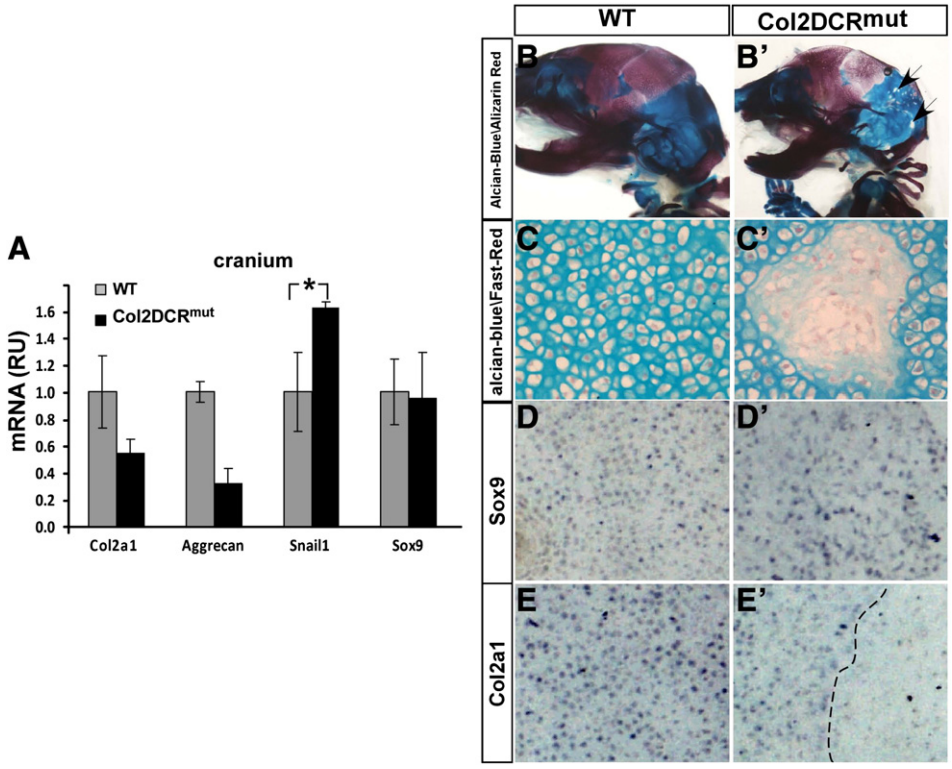

Fig. 4. Col2a1 and Aggrecan mRNA levels are downregulated and Snail1 is up-regulated in the neurocranium cartilage of Col2DCR^{mut}. qPCR analysis of Col2a1, Aggrecan, Sox9 and Snail1 preformed on RNA isolated from neurocranium cartilage, suggest that Col2a1 and Aggrecan levels are reduced, Snail1 levels are up-regulated without change in Sox9 levels (A) n = 3 with 3 technical repeats, * = P value<0.05. Whole mount alcian-blue/Alizarin-red staining of WT (B) and Col2DCR^{mut} (B') cranium. Section alcian-blue/nuclear fast-red staining of WT (C) and Col2DCR^{mut} (C') interparietal cartilage. Sox9 RNA *in-situ* hybridization on WT (D) and Col2DCR^{mut} (D') interparietal cartilage. Col2a1 RNA *in-situ* hybridization on WT (E) and Col2DCR^{mut} (E') interparietal cartilage. Note diminished Col2a1 signal right to the dashed line, where corresponding Sox9 expression in adjacent section (D') is continuous.

The misexpression of Snail1 from the viral vector recapitulated the down-regulation of Col2a1 and Aggrecan expression observed in the col2DCR^{mut} trachea (Fig. 3F,G). Taken together, these data indicate that the upregulation of Snail1 effectively reduces Col2a1 and Aggrecan transcription and is likely responsible also for the reduced proliferation observed in the col2DCR^{mut} trachea.

We next studied additional Col2DCR^{mut} cartilaginous tissues including the neurocranium that was also found to be poorly stained by alcian blue (Fig. 4B,B'). qPCR analysis revealed that Col2a1 and Aggrecan levels are downregulated while Snail1 levels are increased (Fig. 4A). Section analysis of the interparietal neurocranium, revealed reduced matrix staining and fenestrated cartilages (Fig. 4B,B' and 4 C,C'). Furthermore, Sox9 and Col2a1 RNA *in situ* hybridization on adjacent interparietal neurocranium sections, revealed specific tissue compartments, wherein Sox9 is normally detected, yet Col2a1 mRNA is not expressed (Fig. 4D, D', and 4E,E').

The specialized articular cartilage of the joint also produces Col2a1. In additional, it expresses a unique articular isoform, namely Col2b (Kahn et al., 2009; Nalin et al., 1995). Our analysis did not reveal any change in the expression of Col2a1, Col2b, Snail1 or Sox9 in Col2DCR^{mut} articular cartilage, relative to control (Figure S4). We conclude that miRNA are essential for the proper expression of matrix proteins in cartilages but this phenotype is readily evident in the tracheal and cranial cartilage tissues and was not equally-detected in the articular or rib cartilages.

miR-30a/c and miR-125b are regulators of Snail1

As Dicer1 deletion removes the entire set of miRNAs, we next sought the specific miRNA genes that are responsible for the de-regulation of Col2a1 and Aggrecan expression upstream of Snail1. In order to narrow down the possible miRNA candidates we took a bioinformatics approach obtaining *TargetScan* (Lewis et al., 2005) to identify miRNA binding sites (seed-matches) on Snail1 3′UTR. We found potential seed matches on the 3′UTR of Snail1 for miR-30, miR-125b and miR-153 (Fig. 5A).

To further filter potential miRNA candidates upstream of Snail1, we have screened a miRNA expression array (Agilent) with RNA derived from isolated E18.5 tracheal chondrocytes (Fig. 5B). Known cartilage miRNAs such as mir-140, mir-199 and mir-214 (Lee et al., 2009; Lin et al., 2009; Miyaki et al., 2010; Nicolas et al., 2008; Tuddenham et al., 2006; Watanabe et al., 2008) are highly expressed in the tracheal ring cartilage. The detailed list of expressed miRNA may be found at Table S1.

Based on this global analysis, we identified three miRNA genes, which are both highly expressed in tracheal chondrocytes and also potentially bind to complementary sequences on the Snail1 3′UTR, namely, miR-30a, miR-30c and miR-125b. A fourth miRNA, miR-153 that is predicted to target the Snail1 mRNA, is not expressed in tracheal chondrocytes and therefore was omitted from further evaluation.

In order to functionally assess potential direct interactions of the candidate miRNAs with the predicted regulatory sequences on the Snail1 mRNA, we cloned the Snail1 3′UTR into a psiCHECK-2^{TM} vector, that is additionally expressing a Renilla luciferase for internal normalization of transfection efficiency. HEK 293T Cells were then co-transfected with the luciferase-Snail1 3′UTR reporter alone, or in addition to mir-30a or mir-125b expression vectors. The relative luciferase activity in the presence of potential targeting miRNAs was quantified. This analysis revealed that miR-30 represses the expression of the luciferase reporter by 60% and miR-125 dampens the reporter expression by 30% (Fig. 5C,D). The inhibition could not be gained by the expression of miR-7, an irrelevant miRNA. Furthermore, inhibition was completely abrogated when

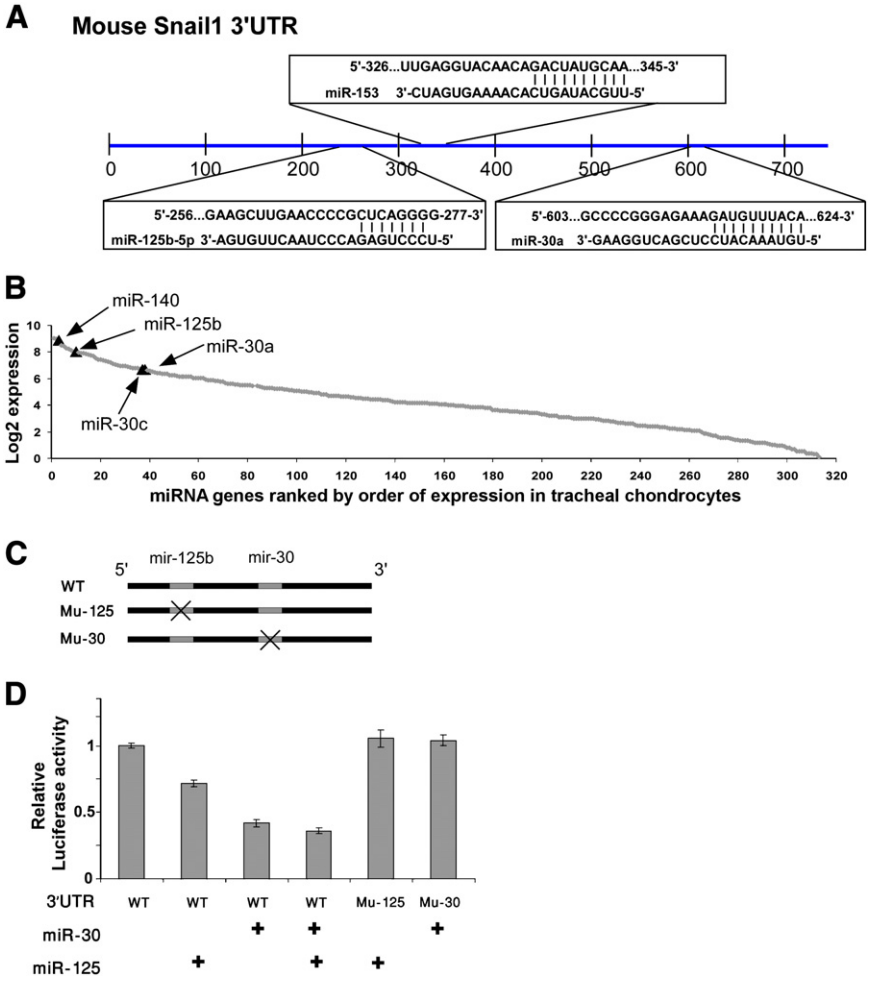

Fig. 5. Snail1 is a target of miR-30a/c and miR-125b in tracheal chondrocytes. The Snail1 3′UTR harbors conserved binding sites for mir-125b, mir-153, and mir-30 (A). RNA extracted from tracheal cartilage rings of wild type mice, hybridized onto an Agilent miRNA expression array. The specific miRNA profile of E18 tracheal chondrocytes is presented along the X-axis, from highly expressed miRNA genes (left) to low expressed genes (right) on a Log2 scale in arbitrary units. miR-125b miR-30a and miR-30c species are annotated by an arrow and a triangle (B). A scheme of the luciferase-Snail1-3′UTR vectors, harboring the Snail1 3′UTR; mu-125 and mu-30 are reporter with mutant binding sites for miR-125 or miR-30, respectively (C). A bar graph of normalized luciferase activity in HEK293T cells, transfected with a luciferase-Snail1-3′UTR and an expression vector for an irrelevant miRNA (miR-7), miR-30, miR-125, or both miR-30/miR-125 together (n = 6, in two technical duplicates).

the binding sites for miR-125 or miR-30 were respectively mutated (Fig. 5C,D). We conclude that Snail1 is regulated by miR-125 and miR-30 through two conserved *bona-fide* sites on its 3′UTR.

Specific miR-30/125b knockdown de-represses Snail1 and downregulates Aggrecan and Col2a1 expression

Snail1 was shown to impair Collagen 2a1 and Aggrecan expression in ATDC5 cells (Seki et al., 2003). To functionally link miR-125b and miR-30a/c to Snail1, we knocked-down these miRNAs, obtaining anti-sense oligos. We first transfected specific miR-125b/30a/c inhibitors, along with the Snail1 3′UTR reporter. The luciferase activity from the Snail1 reporter was upregulated by transfecting miRNA inhibitors, relative to mock transfected ATDC5 cells (Fig. 6A). Next, we monitored the expression of endogenous Snail1, which was upregulated when miR-125b/30a/c inhibitors were introduced to ATDC5 cells. Sox9 levels were not attenuated under these conditions. A few days later, the expression of Col2a1 and Aggrecan was downregulated, relative to control (Fig. 6B). These data suggest Snail1 as a key target of miR-125b/30a/c. To see if Snail1 is indeed a primary target of miR-125b/30a/c in chondrocytes, we designed a combined loss-of-function experiment, wherein we knocked-down simultaneously miR-125b/30a/c and Snail1. Snail1 knock-down in the background of miR-125b/30a/c inhibition restored Collagen 2a1 and Aggrecan expression levels (Fig. 6C). Together, these data provide experimental evidence for miR-125b/30a/c-based repression of Snail1 that is needed for normal expression of Col2a1 and Aggrecan in tracheal chondrocytes.

Discussion

Loss of miRNA activity in the tracheal cartilage manifests with tracheomalacia — a condition characterized by flaccidity of the tracheal support cartilage, which leads to tracheal collapse. Airway collapse in Col2DCR^{mut} pups leads to lung disinflation and lethal asphyxia.

Loss of structural cartilage is conceivably the reason for the Col2DCR^{mut} tracheomalacia. Such pathology could result for example from massive chondrocyte death. However, lineage tracing revealed apparently-normal morphogenesis of semi-lunar rings in the col2DCR^{mut} trachea and apoptosis was not detected.

Surprisingly, col2DCR^{mut} chondrocytes express the transcription factors Sox9, Sox5 and Sox6 that set chondrocyte differentiation in motion and are also required for transcription of ECM proteins such as Col2a1 and Aggrecan. This suggests that col2DCR^{mut} chondrocytes properly initiate their differentiation program (Fig. 2). However, the steps that are required for acquisition of a fully functional chondrocyte state are blocked by the loss of miRNA activity. We provide evidence that chondrocyte differentiation and function is hampered, at least in certain cartilage tissues, because of improper expression of the

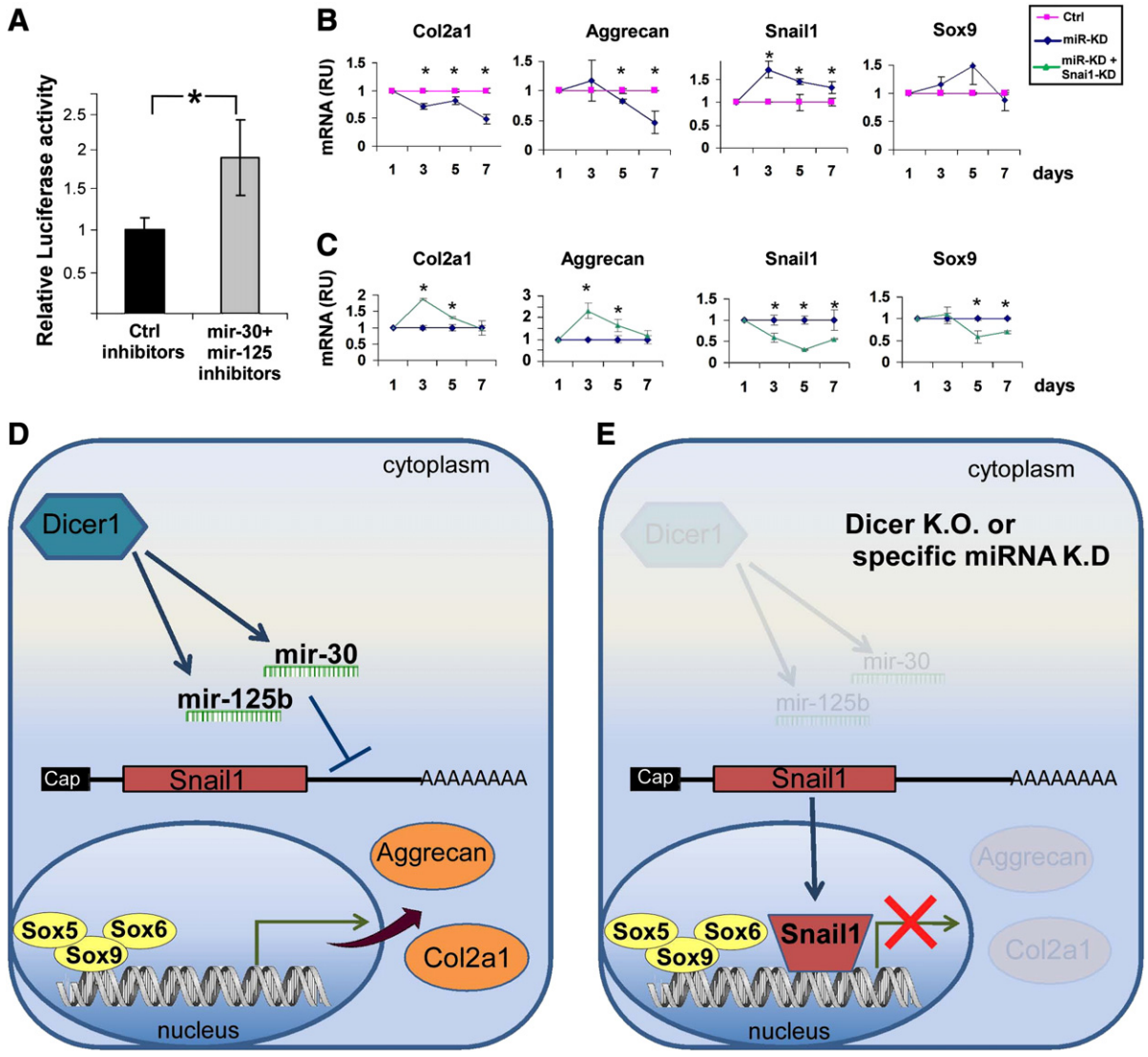

Fig. 6. miR-125b and miR-30 control Col2a1 and Aggrecan synthesis through Snail1 expression and reduces. ATDC5 cells were transfected with 2′-O-methyl antisense inhibitor against miR-125b and miR-30. The expression of a luciferase-Snail1-3′UTR reporter is specifically upregulated by the miR-125b/30 inhibitors (A). The expression of Col2a1, Aggrecan, Snail1 and Sox9 mRNA over several days after transfection with miR-125b/30a/c inhibitors, relative to control (B). The expression of Col2a1, Aggrecan, Snail1 and Sox9 mRNA over several days after transfection with siRNA against Snail1 and miR-125b/30 inhibitors, relative to miR-125b/30a/c inhibitors alone (C). Data in (B,C) is normalized to the expression of GAPDH and of S18 RNA. (n = 3; three technical repeats) * = P value < 0.05. A model for miR-125/miR-30 –dependant repression of Snail1 is the normal developing tracheal cartilage (D). Loss of miRNA activity in Col2DCR^{mut} trachea or in chondrocytes transfected with miR-125b/30a/c inhibitors, de-represses Snail1 and consequently represses the transcription of Col2a1 and Aggrecan (E).

transcriptional repressor Snail1. Snail1 is a mediator of FGF signaling that is necessary in the growth plate (de Frutos et al., 2007; Hong et al., 2009; Seki et al., 2003). The particular developmental program of tracheal chondrocytes requires permanent expression of Col2a1 and ectopic FGF signaling was previously shown to cause mal-genesis of the trachea (Eswarakumar et al., 2004). Overexpression of Snail1, the effector of FGF signaling, downregulates Col2a1 synthesis, represses cell proliferation and upregulates the cyclin-dependent kinase inhibitor P21 (de Frutos et al., 2007).

We have observed Snail1 upregulation and its typical downstream sequella in tracheal and neurocranial chondrocytes of the col2DCR^{mut}. This is particularly intriguing since neurocranial chondrocytes are neural-crest descendants, rooting from the ectoderm. Thus, like with Sox9 and other transcription factors, the importance of miRNAs is commonly shared by chondrocytes of different lineages. However, in some other case, *e.g.*, the articular cartilage, loss of miRNA activity did not seems to result in similar molecular and functional changes, suggesting tissue-specific functions of miRNAs. Taken together these data are suggesting that at least in some cartilages miRNAs function represses FGF/Snail1 signaling, thus supporting proper secretion of matrix proteins.

miR-125b and miR-30 family members are highly expressed in tracheal chondrocytes and regulate Snail1 mRNA through evolutionarily conserved binding sites. In fact, an exceptionally-long paired-recognition sequence makes miR-30 particularly potent in repressing Snail1 expression. Indeed, in cultured chondrocytes, miR-125 and miR-30 knock-down by specific inhibitors resulted in upregulated expression of Snail1. Consistently, Snail1 downstream targets, Col2a1 and Aggrecan were repressed. Furthermore, since the compound knock-down of Snail1 and mir-125/30a/c restored Col2a1 and Aggrecan expression we argue that Snail1 is a primary target downstream of miR-125b/30a/c. Taken together, miR-125b/30a/c specifically function as positive regulators of chondrocyte differentiation by repressing harmful Snail1 expression (Fig. 6 D,E model). Nonetheless, it is likely that additional miRNAs play regulatory roles controlling chondrocyte differentiation.

In summary, Snail1 is an effector of FGF signaling that is important in growth plate cartilage development but is repressed in the trachea to enable the differentiation of chondrocytes of this life-supporting organ. In the intact developing trachea, one important role of miR-30 and miR-125b is to keep Snail1 at low levels and to allow for full functional differentiation of Col2a1 expressing chondrocytes. Loss of this miR-125/30-based safeguard mechanism against unwanted activation of Snail1, uncovers the unique nature of the cartilages such as the trachea that depend on miRNA to permanently maintain Collagen 2a1 and Aggrecan expression.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.09.002.

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