miRNAs control tracheal chondrocyte differentiation

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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 de-represses 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.

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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., 2003; 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., 2003; 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.
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′-CCTGACAGTGACTCCAAAG-3′ & 5′-CATGACTCTCACTCAAATC-3′. The Col2a1-Cre was genotyped as in Amarilio et al. (2007). Mutant Col2a1−/−;Dicer1flo−;+/−;R26R(YFP) and Col2a1−/−;Dicer1flo−;+/+;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-bromo-2′-deoxyuridine pulse (BrDU, 30 mg/kg, I.P.). The fraction of BrDU-positive nuclei in tracheal chondrocytes, was studied 48 h post-transfection, with dual luciferase assay (Promega).

**Expression analysis by arrays and real-time PCR**

Tracheal cartilage rings of E18.5 embryos were manually dissected and incubated in Trypsin-EDTA (Biond) 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 DyNamino HS SYBR Green (Finnzyme) for miRNA 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; GenBank accession code NT 039210, the kind gift of Olivier Pourquié) or murine Sox9, Col2a1 and Aggrecan (as in Amarilio et al., 2007)).

**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′; 3’7°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;Dicer1fl/fl mice (named later Col2DCRmut) died as neonates (early after birth), whilst Col2a1−/−;Dicer1−/− littermates (‘controls’) seems to be phenotypically normal. Surviving Col2a1-Cre; Dicer1−/− 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−/−;Dicer1−/− exhibited dental malocclusion, fenestrated skull vault cartilage, and malformed transverse processes of lumbar vertebrae (Figure S2A,B).

Post mortem exploration revealed that col2DCRmut lungs failed to inflate and that the alveoli did not fill with air (Fig. 1A,B). This is consistent with the apparent cyanosis of col2DCRmut 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 alcin-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, col2DCRmut 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 col2DCRmut mice. In the resultant col2DCRmut*R26R mice, cells in which recombination occurred, lost Dicer1 activity and are additionally labeled with GFP. This analysis revealed that chondrocytes are indeed present in the col2DCRmut trachea. Furthermore, the tracheal cartilage is properly patterned into multiple semi-lunar structures in both col2DCRmut and in Col2a1−/−;Cre; Dicer1fl/fl*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.

Reduced Col2a1 and Aggrecan expression in Col2DCRmut tracheal Rings

Col2DCRmut 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 col2DCRmut tracheal rings (Fig. 2B,D). Direct evaluation of mRNA encoding for primary ECM proteins, brings us to

Fig. 1. Tracheomalacia and disinflected alveoli in Col2DCRmut mice. Hematoxylin and Eosin analysis of thoracic transverse section of P1 WT and Col2DCR mutant (A, A’ bar = 1 mm). Disinflected alveoli observed on higher magnification of the same slides (B, B’). Alcian blue/Alizarin red staining reveals normally patterned rib cage in both WT and Col2DCRmut (C, C’). Whole larynx and trachea Alcian blue staining reveals poor deposition of ECM in Col2DCRmut 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 Col2DCRmut tracheal rings and larynx (E, E’).

Fig. 2. Col2a1 and Aggrecan mRNA levels are lower, while Sox family transcription factors are normally expressed in Col2DCRmut. 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 Sox5, Sox6 and Sox6 (E). Pooled tracheal ring RNA of 8 individual embryos; three repeats per genotype. * = P value <0.05.

conclude that low ECM likely results from an intrinsic defect in regulating Col2a1 and Aggrecan expression in Col2DCRmut mice.

To examine the basic differentiation properties of the mutant Col2DCRmut tracheal chondrocytes, we performed RNA in-situ hybridization for Sox9, the chief transcription factor in chondrocyte differentiation. Sox9 was unchanged in col2DCRmut 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 Col2DCRmut 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 col2DCRmut 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 Col2DCRmut tracheal chondrocytes.

Why is Col2a1 and Aggrecan transcription downregulated in chondrocytes that express the three principal Sox transcriptional activators? One potential reason may be because of abnormal upregulation of transcriptional repressors in col2DCRmut 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 Col2DCRmut 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 col2DCRmut 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 Col2DCRmut 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 Col2DCRmut tracheal chondrocyte proliferation relative to controls (Fig. 3D). 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 Col2DCRmut tracheal chondrocytes.

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 adenoaviral vector misexpressing Snail1. Snail1 was overexpressed four fold relative to control, in levels comparable with Snail1 expression in Col2DCRmut tracheal chondrocytes.
The misexpression of Snail1 from the viral vector recapitulated the down-regulation of Col2a1 and Aggrecan expression observed in the col2DCRmut 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 col2DCRmut trachea.

We next studied additional Col2DCRmut 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 4C,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 Col2DCRmut 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. We found potential seed matches on the 3'UTR of Snail1 for miR-30, miR-125b and miR-153 (Fig. 5A).

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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 antisense 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 knockdown 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 Col2DCRmut pups leads to lung disinflation and lethal asphyxia.

Loss of structural cartilage is conceivably the reason for the Col2DCRmut tracheomalacia. Such pathology could result for example from massive chondrocyte death. However, lineage tracing revealed apparently-normal morphogenesis of semi-lunar rings in the col2DCRmut trachea and apoptosis was not detected.

Surprisingly, col2DCRmut 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 col2DCRmut 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
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 col2DCRmut. 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 seem 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. 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/miR-30 – dependent repression of Snail1 is the normal developing tracheal cartilage (D). Loss of miRNA activity in Col2DCRmut trachea or in chondrocytes transfected with miR-125/miR-30/c inhibitors, de-represses Snail1 and consequently represses the transcription of Col2a1 and Aggrecan (E).
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 Colagen 2a1 and Aggrecan expression.

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References


Appendix A. Supplementary data

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