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Defects in chondrocyte maturation and secondary ossification in mouse knee joint epiphyses due to Snorc deficiency

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SUMMARY

Objective: The role of Snorc, a novel cartilage specific transmembrane proteoglycan, was studied during skeletal development using two *Snorc* knockout mouse models. Hypothesizing that Snorc, like the other transmembrane proteoglycans, may be a coreceptor, we also studied its interaction with growth factors.
Methods: Skeletal development was studied in wild type (WT) and *Snorc* knockout mice during postnatal development by whole mount staining, X-ray imaging, histomorphometry, immunohistochemistry and qRT-PCR. *Snorc* promoter activity was studied by applying the LacZ reporter expressed by the targeting construct. Slot blot binding and cell proliferation assays were used to study the interaction of Snorc with several growth factors.
Results: *Snorc* expression was localized in the knee epiphyses especially to the prehypertrophic chondrocytes delineating the cartilage canals and secondary ossification center (SOC). Snorc was demonstrated to have a glycosaminoglycan independent affinity to FGF2 and it inhibited FGF2 dependent cell growth of C3H101/2 cells. In *Snorc* deficient mice, SOCs in knee epiphyses were smaller, and growth plate (GP) maturation was disturbed, but total bone length was not affected. Central proliferative and hypertrophic zones were enlarged with higher extracellular matrix (ECM) volume and rounded chondrocyte morphology at postnatal days P10 and P22. Increased levels of *Ihh* and *Col10a1*, and reduced *Mmp13* mRNA expression were observed at P10.
Conclusions: These findings suggest a role of Snorc in regulation of chondrocyte maturation and postnatal endochondral ossification. The interaction identified between recombinant Snorc core protein and FGF2 suggest functions related to FGF signaling.

Introduction

Proteoglycans are integral part of cartilage extracellular matrix (ECM) and many of them are crucial for chondrogenesis, endochondral ossification and cartilage homeostasis[1–3]. Dermatan sulphate, chondroitin sulphate and especially heparin sulphate side chains but also the core proteins can bind, sequester and transport growth factors, and thus, are involved in the generation of defined concentration gradients during development[4,5]. Membrane proteoglycans usually mediate the ECM-cell interactions and all of them function as coreceptors of high affinity growth factor receptors[6–9]. They have been shown to contribute to the interaction of growth factors and their receptors such as FGFs and FGFRs[10–12].

Snorc (Small Novel Rich in Cartilage) is a novel transmembrane proteoglycan. It was identified in a microarray study of novel chondrogenesis-related genes in knee epiphyseal cartilage of developing mouse and its nonannotated transcript is called as 3110079O15Rik[13]. The gene is composed of three exons and it codes for a 121 amino acids long highly conserved type I single-pass transmembrane chondroitin/dermatan sulphate proteoglycan. Interestingly, Snorc core protein has no identified paralogues[13].

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Snorc is expressed in several cartilage tissues during development, including at least cartilage anlage, hyaline cartilage and fibrocartilage tissues. In the knee joint, *Snorc* is expressed from E12.5 onwards with the highest expression levels in resting, proliferative and prehypertrophic zones of the growth plate (GP). In adult mouse and human articular cartilage (AC), the protein is detected only in the uncalcified regions. BMP-2 induces *Snorc* expression during chondrogenesis both *in vivo*, during fracture healing, and *in vitro*, in a limb micromass culture[13].

Based on previous studies, we raised a hypothesis that Snorc has a role in chondrocyte maturation and endochondral ossification during skeletal development. For this purpose, we generated two *Snorc* knockout mouse models, and in the present study investigated the effect of *Snorc* deficiency on cartilage and bone during embryonic and postnatal development. We also searched for its growth factor interaction partners in order to find clues for its functions in skeletal tissue metabolism.

Materials and methods

Generation of Snorc deficient mice and genotyping

An embryonic stem cell clone containing targeted allele 3110079O15Rik$^{tm1a(EUCOMM)Hmgu}$ (*Snorc*^{a}) by a promoter driven "knockout first" EUCOMM/KOMP-CSD was obtained from International Knockout Mouse Consortium[14]. Correct targeting in mice was analyzed by PCR over the 5′- and 3′-homology arms using SequalPrep™ Long PCR Kit (Life Technologies) using several primer pairs [Fig. 1; Supplementary Fig. 1(A); Supplementary Table I] and by sequencing the ends of these PCR products.

Three chimeric males were bred with C57BL/6NCrl females to acquire germline transmitted *Snorc*$^{a/+}$ mice and further bred to obtain homozygous mice (*Snorc*$^{a/a}$). Germline transmission of *Snorc*^{a} allele was analyzed by PCR for the *Snorc*^{a} and *Snorc*$^{+}$ (wild type, WT) alleles (Fig. 1; Supplementary Table I).

Disruption of mRNA expression in *Snorc*^{a} allele is based on the utilization of a novel splice acceptor (En2SA) introduced into the locus, resulting in expression of *LacZ*-reporter gene, and a lack of expression of the exons 2 and 3 [Fig. 1; Supplementary Fig. 1(C)]. Intronic loxP-sites of the targeted allele further allowed the generation of a second *Snorc* deficient allele 3110079O15Rik$^{tm1-b(EUCOMM)Hmgu}$ (*Snorc*^{b}) with the deletion of exons 2 and 3 to ensure gene silencing in the case the inserted novel splice acceptor was not efficiently recognized, or if there was an additional promoter in the intronic region upstream of exon 2[15]. For this purpose, male *Snorc*$^{a/a}$ mice were bred with transgenic female mice expressing Cre-recombinase in mature oocytes, allowing recombination to occur in zygote prior to two-cell stage[16]. Occurrence of preferred site-specific recombination in the offspring was studied by PCR for *Snorc*$^{+}$ and *Snorc*^{b} alleles [Supplementary Fig. 1(B); Supplementary Table I]. In addition, correct deletion was verified by PCR using several other primer pairs (not shown).

Experimental animals

Study plan was approved by the National Animal Experiment Board ELLA (project license ESAVI-2010-04857/Ym-23). *Snorc* deficient mice were crossbred with C57Bl/6NCrl background. Animals were maintained in the Central Animal Laboratory of University of Turku following 3R's principles. Adequate number of animals of WT, *Snorc*$^{a/a}$ and *Snorc*$^{b/b}$ genotypes were collected from heterozygous matings (Supplementary Table II).

Genotyping and gender determination

Genomic DNA was isolated from pieces of ear or tail after digestion at 55°C for 6 h—overnight in 0.2 mg/ml Proteinase K (Thermo Fisher Scientific), 100 mM Tris—HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl. DNA was precipitated in 50% isopropanol and dissolved in sterile water. For genotyping, fragments of knockout (primers F3 and R3) and WT (primers F4 and R4) alleles and for gender, a fragment of male specific *Sry* were amplified using DyNAzyme II (Thermo) DNA polymerase (Fig. 1, Supplementary Table I)[17].

Gene expression analyses

Total RNA was isolated from knee joints including distal femoral and proximal tibial epiphyseal cartilage of P5 (P = postnatal day) and P10 mice and from proximal tibial epiphyseal cartilage of P22

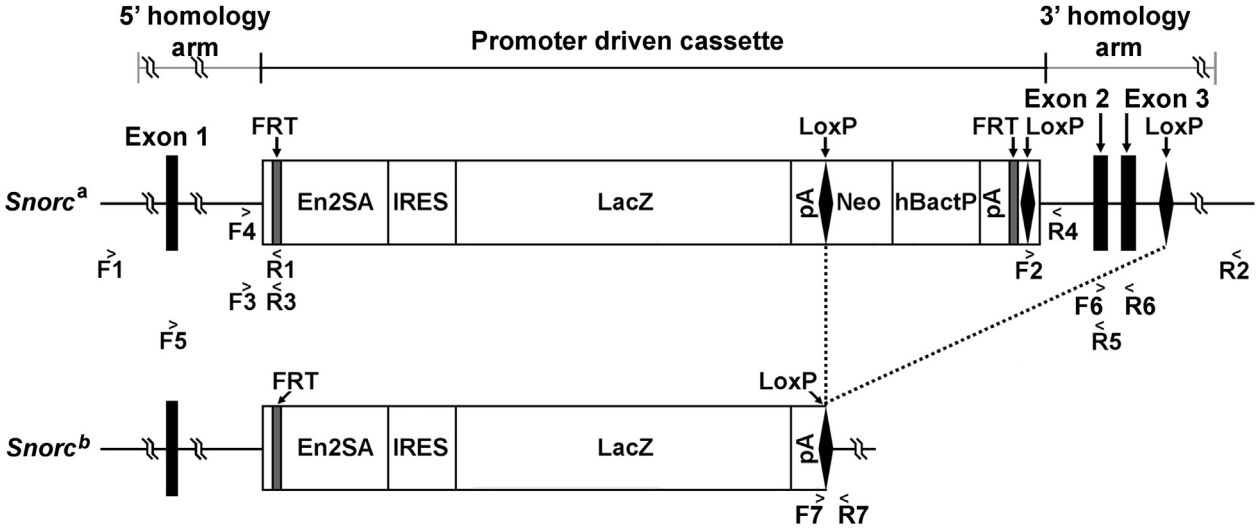

Fig. 1. Structure of targeted *Snorc*^{a} and *Snorc*^{b} alleles. *Snorc*^{b} allele was generated from the *Snorc*^{a} allele by Cre recombination mediated deletion of exons 2 and 3. Locations of the PCR primers are indicated and their sequences are listed in Supplementary Table I.