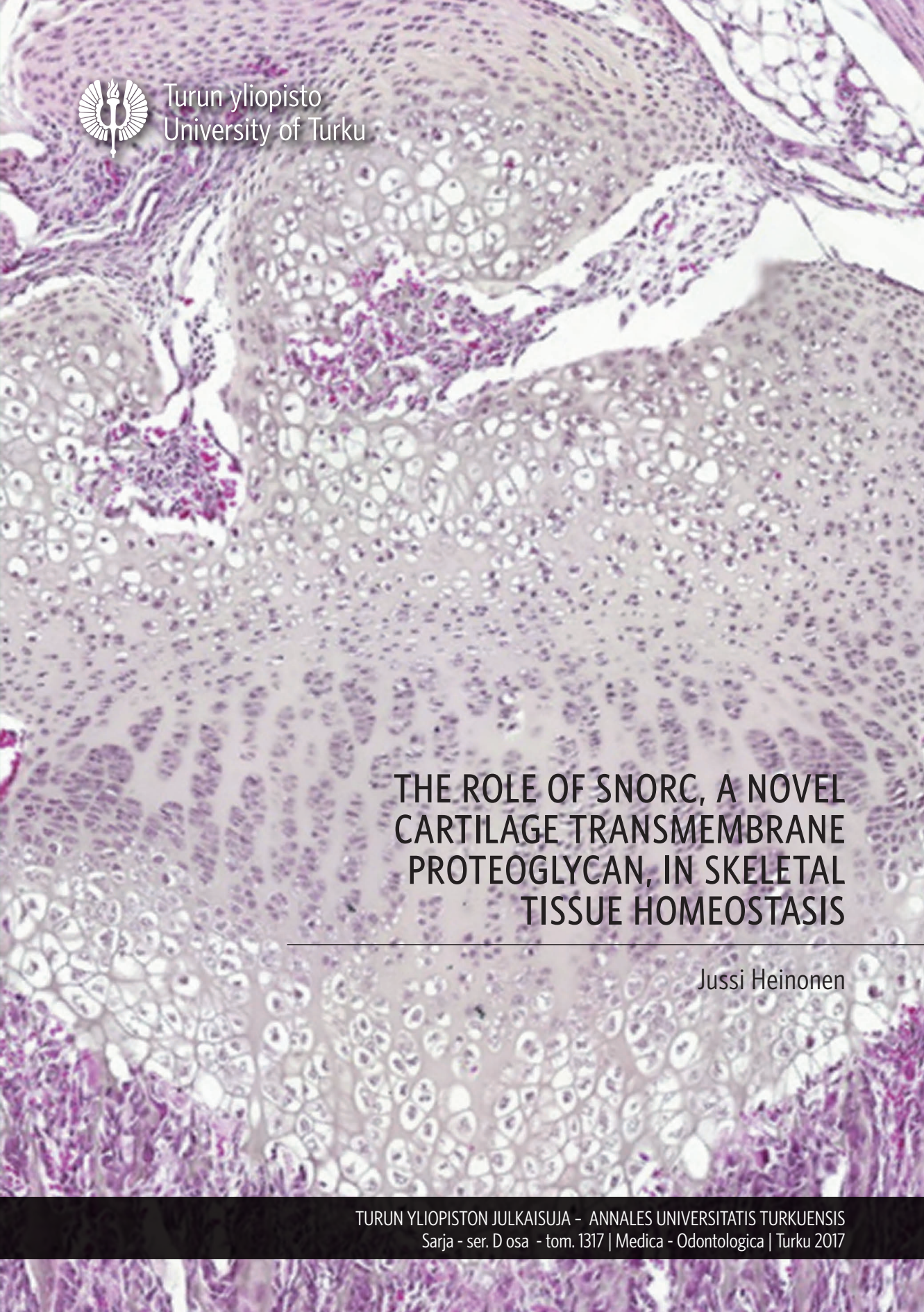

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THE ROLE OF SNORC, A NOVEL CARTILAGE TRANSMEMBRANE PROTEOGLYCAN, IN SKELETAL TISSUE HOMEOSTASIS

Jussi Heinonen

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Science is the knowledge of consequences, and dependence of one fact upon another.
Thomas Hobbes 1588-1679

ABSTRACT

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Role of Snorc, a Novel Cartilage Transmembrane Proteoglycan, in Skeletal Tissue Homeostasis.
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Discovery of molecular mechanisms underlying chondrogenesis is necessary to develop therapies for skeletal disorders and articular cartilage defects or disorders like osteoarthritis (OA). Specific properties of each tissue are derived from tissue-specific gene expression. Therefore, it is likely that genes with tissue-specific expression have an important role in development of structural and functional properties of tissue. Remarkable number of genes associated with OA and genetic skeletal disorders have a cartilage-selective expression pattern. Some of these are proteoglycans (PG), which are important structural components of cartilage extracellular matrix (ECM) and modulators of activity of extracellular signal molecules.

The aim of the study was to discover novel genes essential in chondrogenesis and cartilage homeostasis by transcriptional profiling. Of the large-scale analysis, a promising transcript named *Snorc* (small novel rich in cartilage), was selected for more specific analysis. The aim of the analysis was to define expression and structure of *Snorc* gene and protein, to define interaction partners of Snorc protein and to analyze the role of the *Snorc* gene in mouse skeletal tissue development.

Snorc is a type I single-pass transmembrane chondroitin or dermatan sulfate PG, highly conserved in vertebrates. Expression of *Snorc* was observed throughout life span in mouse epiphysis especially during development. Expression is most intense in proliferative and prehypertrophic cartilage of growth plate (GP) during embryonic development, and in prehypertrophic chondrocytes surrounding secondary ossification center (SOC) during postnatal development. Messenger RNA is cartilage-specific, but immunoreactivity was detected also in ECM of trabecular bone. Size of mineralized SOC is decreased and structure of GP disturbed in *Snorc*-deficient mice knee epiphysis, compared to wild type (WT). Proliferative and hypertrophic zones were enlarged, especially in medial part, and chondrocyte morphology was changed in *Snorc*-deficient mice GP at postnatal day (P) 10 and 22. Indian hedgehog (*Ihh*) and collagen type X alpha 1 chain (*Col10a1*) expression were increased and matrix metalloproteinase 13 (*Mmp13*) decreased in P10 *Snorc*-deficient mouse epiphysis. Peripheral quantitative tomography revealed increase in endosteal and in periosteal perimeter, and in the area of trabecular bone in the cross-section of distal femoral metaphysis in adult *Snorc*-deficient mice compared to WT. However, alterations in long bone length were not observed. Bone morphogenetic protein 2 induced expression of *Snorc* in chondrocytes. Snorc extracellular domain (ECD) binds fibroblastic growth factor 2 (Fgf2) independently of glycosaminoglycan chain of Snorc.

Snorc is a novel cartilage-specific transmembrane PG with a role in maturation of postnatal GP chondrocytes, epiphyseal ossification and metaphyseal bone formation in mouse. *Snorc*-deficiency caused alterations in expression of *Ihh* and *Mmp13* genes in postnatal epiphysis. *Ihh* is important regulator of proliferative zone and *Mmp13* necessary for vascularization and ossification of cartilage. Binding of Fgf2 to Snorc ECD clues that *Snorc* may have a role in Fgf signaling potentially as a coreceptor.

Key words: Membrane proteoglycan, cartilage, growth plate, trabecular bone, Fgf

TIIVISTELMÄ

Jussi Heinonen

Snorcin, uuden rustospesifisen kalvoproteoglykaanin, rooli tukirangan kudosten homeostaasissa.

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Ruston kehityksessä tärkeiden molekyylibiologisten mekanismien tunteminen on välttämätöntä, jotta on mahdollista kehittää hoitoja esim. tukirangan kehityshäiriöihin ja nivelruston vaurioihin ja sairauksiin kuten nivelrikkoon. Kullekin kudokselle ominaiset geenien ilmenemisprofiilit määrittävät kudoksen ominaisuudet, joten on todennäköistä, että kudosspesifisesti ilmenevällä geenillä on erityinen rooli kudoksen rakenteellisten ja toiminnallisten ominaisuuksien kehittymisessä. Merkittävällä määrällä nivelrikkoon ja tukirangan kehityshäiriöihin liitetyistä geeneistä ilmeneminen on rustolle ominainen. Näistä osa koodaa proteoglykaaneja, jotka ovat tärkeitä ruston soluväliaineen rakenteellisia komponentteja ja solunulkoisten signaalimolekyylien toiminnan säätelijöitä.

Tämän työn tavoitteena oli löytää uusia, ruston kehityksessä ja homeostaasissa oleellisia geenejä seulomalla uusia, aiemmin tunnistamattomia RNA-transkripteja, jotka ilmenevät spesifisesti rustossa kehityksen aikana. Lupaava RNA-transkripti, *Snorc* (small novel rich in cartilage), valittiin tarkempaa tutkimusta varten, jonka tavoitteena oli selvittää geenin ja proteiinin ilmenemistä ja rakennetta, proteiinin vuorovaikutuskumppaneita ja geenin roolia hiiren tukirangan kehityksessä.

Snorc on tyypin I integraalinen kondroitiini- tai dermataanisulfaattikalvo-proteoglykaani, joka on säilynyt hyvin selkärankaisten evoluutiossa. Snorc ilmenee hiiren epifyysirustossa läpi elämän, mutta erityisesti kehityksen aikana. Sikiökehityksen aikana ilmeneminen on voimakkainta kasvulevyn proliferatiivisessa ja prehypertrofisessa rustossa ja syntymän jälkeisen kehityksen aikana sekundaarisen luutumiskeskuksen (SOC) ympärillä. Lähetti-RNA ilmenee spesifisesti rustossa, mutta proteiinia nähdään myös hohkaluun soluväliaineessa. *Snorc*-poistogeenisen (KO) hiiren polven epifyysissä SOC oli pienentynyt ja kasvulevyn rakenne häiriintynyt verrattuna villityypin hiireen. Kasvulevyn proliferatiivinen ja hypertrofinen vyöhyke olivat laajentuneet erityisesti keskiosastaan ja rustosolujen muoto oli muuttunut *Snorc* KO hiiressä 10. ja 22. päivä syntymän jälkeen. Indian hedgehog (*Ihh*), ja tyypin 10 kollageeni alfa 1 ketju (*Col10a1*) -geenien ilmeneminen oli lisääntynyt ja matriksin metalloproteinaasi 13 (*Mmp13*) vähentynyt *Snorc* KO-hiiren epifyysissä 10. päivä syntymän jälkeen. Perifeerinen kvantitatiivinen tomografia osoitti endosteaalisen ja periosteaalisen kehän ja hohkaluun pinta-alan kasvamisen reisiluun ulomman metafyysin poikkileikkauksessa aikuisella *Snorc* KO-hiirellä. Luun morfogeneettinen proteiini 2 sai aikaan *Snorcin* ilmenemistä. Fibroblastinen kasvutekijä 2 (Fgf2) sitoutui Snorcin solunulkoiseen rakennealueeseen glykosaminoglykaanista riippumatta.

Tutkimukset osoittivat, että Snorc on uusi rustospesifinen solukalvon läpäisevä proteoglykaani, jolla on rooli syntymänjälkeisen kasvulevyn kehityksessä, epifyysin luutumisessa ja metafyseaalisen luun muodostumisessa hiiressä *in vivo*. *Snorc*in toiminnan esto aiheutti muutoksia *Ihh* ja *Mmp13* geenien ilmenemisessä syntymänjälkeisessä epifyysissä. *Ihh* on tärkeä proliferatiivisen vyöhykkeen säätelijä ja *Mmp13* välttämätön ruston verisuonten muodostumisessa ja luutumisessa. Fgf2 sitoutuu Snorcin solunulkoiseen osaan, mikä viittaa siihen, että Snorcilla olisi rooli Fgf signaloinnissa mahdollisesti koreseptorina.

Avainsanat: Kalvoproteoglykaani, rusto, kasvulevy, hohkaluu, Fgf

TABLE OF CONTENTS

ABBREVIATIONS

AC	articular cartilage
Acan	aggrecan gene
Actb	beta-actin
ActrI	activin receptor type I
AER	apical ectodermal ridge
Alk-1	activin receptor-like kinase 1
Bgn	biglycan
BMD	bone mineral density
Bmp	bone morphogenetic protein
Bmpr	bone morphogenetic protein receptor
BSA	bovine serum albumin
C4st1	chondroitin 4 sulfotransferase 1
cAMP	cyclic adenosine monophosphate
Cd44	cd44 antigen
Col1a1	collagen, type I, alpha I chain
Col2a1	collagen, type II, alpha 1 chain
Col9a2	collagen, type IX, alpha 2 chain
Col10a1	collagen, type X, alpha 1 chain
Col11a2	collagen, type XI, alpha 2 chain
Comp	cartilage oligomeric matrix protein
CS	chondroitin sulfate
Cspg4	chondroitin sulfate proteoglycan 4
Ct.CSA	cortical bone cross sectional area
Ctgf	connective tissue growth factor
CZ	calcified zone
Dcn	decorin
Dhh	desert hedgehog
DMSO	dimethyl sulfoxide
DS	dermatan sulfate
DZ	deep zone
ECD	extracellular domain/ectodomain
ECM	extracellular matrix
EN2SA	engrailed-2 splice acceptor
ER	endoplasmic reticulum
EST	expressed sequence tag
Ext1	exostosin
Fgf	fibroblast growth factor
Fgfr	fibroblast growth factor receptor
Fmod	fibromodulin
FZD	frizzled
GAG	glycosaminoglycan

GP	growth plate
Gpc	glypican
GPI	glycerophosphatidylinositide
GSD	genetic skeletal disorder
HA	hyaluronan
Has	hyaluronan synthase
HE	hematoxylin eosin
HH	hedgehog
Hprt1	hypoxanthine phosphoribosyltransferase 1
HRP	horseradish peroxidase
HS	heparan sulfate
Ihh	indian hedgehog
KO	knockout
KS	keratan sulfate
L/EC	lumenal/extracellular
Lef/Tcf	lymphoid enhancer factor/T-cell factor
Lum	lumican
m	months of age
Mapk	mitogen activated protein kinase
Mmp	matrix metalloproteinase
Mtn	matrilin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZ	middle zone
N-cam	neural cell adhesion molecule
Nrp1	neuropilin
OA	osteoarthritis
O-GalNAc	O-linked N-acetylgalactosamine
O-GlcNAc	O-linked N-acetyl-glucosamine
Osx	osterix
P	postnatal day
Papss2	phosphoadenosine phosphosulfate synthetase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
Pdgf	platelet-derived growth factor
PG	proteoglycan
PKA	protein kinase a
POC	primary ossification center
Ppia	peptidylprolyl isomerase A
pQCT	peripheral quantitative computed tomography
Pthr1	parathyroid hormone-related peptide receptor 1
Pthrp	parathyroid hormone related peptide
Ptprz1	phosphacan
qPCR	quatitative polymerase chain reaction

qRT-PCR	quantitative reverse transcription polymerase chain reaction
RZPD	German Resource Center for Genome Research
SD	standard deviation
Sdc	syndecan
SGBS	Simpson-Golabi-Behmel syndrome
Shh	sonic hedgehog
SLRP	small leucine rich proteoglycan
Smo	smoothened
Snorc	small novel rich in cartilage
SOC	secondary ossification center
Sox	[SRY (Sex-Determining Region Y)-Box]
SZ	superficial zone
Tak1	tgfβ-activated kinase 1
Tb.BMC	trabecular bone mineral content
Tb.BMD	trabecular bone mineral density
Tb.CSA	trabecular bone cross sectional area
TBS	tris-buffered saline
Tgfβ	transforming growth factor beta
Tgfbr3	betaglycan
TH	thyroid hormone
Top2a	topoisomerase II alpha
Tt.CSA	total bone cross sectional area
Vegf	vascular endothelial growth factor
Wif-1	Wnt inhibitory factor 1
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred in the text by their Roman numerals I and II:

I Heinonen J, Taipaleenmäki H, Roering P, Takatalo M, Harkness L, Sandholm J, Uusitalo-Järvinen H, Kassem M, Kiviranta I, Laitala-Leinonen T, Säämänen A-M: Snorc is a novel cartilage specific small membrane proteoglycan expressed in differentiating and articular chondrocytes. Osteoarthritis and Cartilage 2011; 19:1026-1035.

II Heinonen J, Zhang FP, Surmann-Schmitt C, Honkala S, Stock M, Poutanen M, Säämänen A-M: Defects in chondrocyte maturation and secondary ossification in mouse knee joint epiphyses due to Snorc deficiency. Osteoarthritis and Cartilage 2017; 25:1132-1142

Review contains also unpublished data.

1. INTRODUCTION

Understanding of the molecular mechanisms behind the differentiation and maturation of cartilage tissue is crucial for the development of therapeutic approaches for skeletal disorders and degenerative disorders of articular cartilage (AC). Genetic skeletal disorders (GSD) have an overall prevalence of at least 1 per 4,000 children. Although this is individually rare, it extrapolates to a minimum of 225,000 people in the European Union (Briggs et al. 2015). AC injury is a common disorder of the knee joint concerning all genders and ages. For instance, incidence of cartilage lesions has been reported to be 65% in routine arthroscopies (Memon, Quinlan 2012). Knee osteoarthritis (OA) is the most common joint disorder in United States, occurring in 10% of men and 13% of women aged 60 years or over (Zhang, Jordan 2010).

Specific structural and functional features of tissues are caused by their tissue-specific gene expression. Thus, genes with tissue-specific expression profiles are candidates to play an essential role in the development of specific properties of the tissue. Mutations in genes that are actively and specifically expressed in cartilage, are involved in several GSDs, and are risk factors of OA. Some of these genes are proteoglycans (PG) (Spector, MacGregor 2004, Warman et al. 2011). Proteoglycans are abundant and essential molecules in cartilage extracellular matrix (ECM). They consist of core protein to which one or more glycosaminoglycans (GAGs) are covalently attached. They are usually extracellular molecules, but are also bound to plasma membrane (Iozzo, Schaefer 2015). For example, *Col9a2* (collagen, type IX, alpha 2 chain), *Acan* (aggrecan) and *Gpc6* (glypican 6) are genes encoding for PGs with cartilage-selective expression. Their mutations are associated to GSDs, such as multiple epiphyseal dysplasia 2, idiopathic short stature SED Kimberley and omodysplasia 1, respectively (Funari et al. 2007, Warman et al. 2011). *Col9a2* and *Acan* genes encode for ECM PGs and *Gpc6* gene encodes for membrane PG. Mutations causing dysfunction or deficiency of enzymes affecting general sulphation of GAGs, such as phosphoadenosine phosphosulfate synthetase 2 and carbohydrate sulfotransferase 3 are associated to GSDs and cause defects in chondrogenesis and endochondral ossification indicating importance of PGs in these processes (Cortes, Baria & Schwartz 2009, Warman et al. 2011, Hermanns et al. 2008).

In cartilage ECM, PGs are important structural components and they are involved in formation of diffusion gradients of extracellular signal molecules (Heinegard 2009, Cortes, Baria & Schwartz 2009). Coreceptor function is characteristic for membrane PGs and they are, e.g., regulators of function of high affinity growth factor receptors. PGs with transmembrane domain can function also matrix receptors and transduce signals between ECM and cytoplasm (Couchman 2010).

In this study, transcripts that were expressed intensely and in cartilage-specific manner, and which were structurally highly conserved in vertebrates, were

screened to find novel genes potentially important in chondrogenesis and endochondral ossification. *Snorc* (small novel rich in cartilage) was selected for more detailed expressional, structural and functional analyses. This study revealed that Snorc is a novel cartilage-specific small transmembrane PG with affinity to fibroblast growth factor 2 (Fgf2). Snorc has a role in secondary ossification, differentiation of postnatal growth plate (GP) chondrocytes and metaphyseal bone formation in mouse.

2. REVIEW OF THE LITERATURE

2.1 Evolution of vertebrate skeletal tissues

Cephalochordates (Amphioxus) are marine invertebrates and are now considered the most basally branching group within the chordates phylum that includes subphylum vertebrates. Rudimentary collagen matrix producing somites of Cephalochordates are considered to be the evolutional origin of vertebrate skeletal tissues (Yong, Yu 2016). For instance, genetic duplication of fibrillar collagen and evolution of Acan through domain shuffling were involved in evolution of skeletal tissues, such as cartilage and bone of vertebrate endoskeleton (Wada 2010, Shimeld, Holland 2000). In lower vertebrate classes, like Agnatha (e.g. lampreys) and Chondrichtyes (e.g. sharks), the skeleton is predominantly cartilaginous (Eames et al. 2007). The cartilaginous endoskeleton is considered to predate biomineralized endoskeleton (Kawasaki, Weiss 2006). In Osteichtyes (bone fishes) and Tetrapods (Amphibians, Reptiles, Birds and Mammals), the endoskeleton is mineralized and formed primarily by endochondral ossification when cartilage anlage is replaced by bone (Kawasaki, Weiss 2006). Compared to embryonic development, proportion of cartilage is low in skeleton of adult mammals, where it is located, e.g., in articular surfaces, intervertebral discs, trachea, rib cage, nose and ear. Mammalian cartilage can be divided into hyaline (e.g., cartilage anlage, articular cartilage and nasal and tracheal cartilage), fibrocartilage (e.g., annulus fibrosus in intervertebral disc) and elastic cartilage (e.g., ear cartilage).

2.2 Limb bud initiation

During vertebrate embryogenesis, connective tissues including cartilage and other skeletal tissues derive predominantly from mesoderm. However, most of the craniofacial cartilage and bone and all pharyngeal cartilage are of ectodermal origin, derived from neural crest (Couly, Coltey & Le Douarin 1993, Olsen, Reginato & Wang 2000). Mesenchymal cells derived from lateral plate mesoderm migrate under ectoderm, and limb bud formation is initiated at E9.5 of mouse embryo. Mesenchymal interactions between apical ectodermal ridge (AER) and mesenchyme coordinate proliferation and patterning of the mesenchymal cells. AER is a stratum of ectodermal cells located in distal end of limb bud (Mariani, Martin 2003, Zuniga 2015).

2.3 Precartilaginous condensation

2.3.1 Histology and markers

In the middle of the limb bud, the mesenchymal cells form cell condensation that prefigures skeletal elements (Fig. 1A). At the cellular level, this condensation is recognized as closer packing density of cells compared to uncondensed cells. At the molecular level, this condensation is observed at E10.5 in mouse embryonic

limb bud using, e.g., SRY (Sex-Determining Region Y)-Box (Sox9) as a marker gene. Other abundant active genes in condensation are ECM- or cell surface-related molecules, such as hyaladherins, versican, tenascin, syndecan, neural cell adhesion molecule (N-cam) and heparan sulfate (HS) and chondroitin sulfate (CS) PGs. Although aggeregation is initially contiguous, it forms the segments in proximodistal order which can be regarded as precursors of the stylopood, zuegopood and autopood of the future skeleton. These segments are observed at E11.5 in mouse embryos. During condensation, the cells located centrally in condensation commit to a chondrogenic fate, while the cells located peripherally remain undifferentiated and form perichondrium (Shimizu, Yokoyama & Asahara 2007, Hall, Miyake 2000).

2.3.2 Essential adhesion molecules, growth factors and cytokines

Cell adhesion molecules N-cadherin and N-cam facilitate cell-cell contacts during condensation and are important in the initiation of condensation process and in maintenance of condensation, respectively (DeLise, Tuan 2002, Hall, Miyake 2000). Transforming growth factor β (Tgf-β) is involved in initiation of mesenchymal condensation by upregulating several proteins, such as tenascin, fibronectin, N-Cadherin and N-cam that are central in condensation process (White et al. 2003, Hall, Miyake 2000). Syndecan expression sets boundaries for condensation by inhibiting N-cam expression (Hall, Miyake 2000, Shimizu, Yokoyama & Asahara 2007). Role of Sox9 is crucial for mesenchymal condensations. Discernible mesenchymal condensation was not observed in limb buds of mice, from which Sox9 was removed before formation of mesenchymal condensations (Akiyama et al. 2002). Also, bone morphogenetic protein (Bmp) signaling regulates growth of condensation and later noggin inhibits this growth by enabling cell differentiation to chondrocytes (Shimizu, Yokoyama & Asahara 2007, Hall, Miyake 2000).

2.4 Chondrocyte differentiation

Cells located centrally in condensation commit to chondrogenic fate. These cells differentiate to chondrocytes, which is defined by production of cartilage ECM proteins like collagens type II, IX and XI, and Acan. Especially Acan PG and collagen type II are the major structural components of ECM of differentiated cartilage. Cells located peripherally remain undifferentiated and form the perichondrium (Hall, Miyake 2000).

2.4.1 Sox9, Sox6 and LSox5

Sox9 transcription factor is a key regulator of chondrogenesis. In humans, heterozygous mutations in the *Sox9* gene cause campomelic dysplasia (Liu et al. 2017). In mice, haploinsufficiency of *Sox9* induce chondrodysplasia, while homozygous *Sox9* loss-of-function mutations eliminate chondrogenesis altogether in prechondrogenic limb mesenchyme (Bi et al. 2001, Akiyama et al. 2002). *Sox9* transcription factor is expressed early in mesenchymal condensation and its

expression continues during chondrocyte differentiation. Sox9 is necessary for formation of mesenchymal condensations, chondrocyte differentiation and maturation (Akiyama et al. 2002). *Sox9* binds to promoter regions of, e.g., *Acan,* collagen, type II, alpha 1 chain (*Col2a1*), collagen type XI, alpha2 chain (*Col11a2*) and several other cartilage ECM genes and activates their transcription (Bell et al. 1997, Bridgewater, Lefebvre & de Crombrugghe 1998). L-Sox5 and Sox6, distant relatives to Sox9, are also required for chondrocyte differentiation. However, they are not needed for the formation of mesenchymal condensations (Smits et al. 2001). L-Sox5, Sox 6 and Sox9 bind to the promoter regions of and cooperatively induce expression of *Col2a1* and *Acan* genes (Lefebvre, Li & de Crombrugghe 1998, Lefebvre, Behringer & de Crombrugghe 2001, Han, Lefebvre 2008). This Sox transcription factor trio is called a master regulator (Sox trio) of chondrogenesis due to its prominent role (Ikeda et al. 2004).

2.4.2 Extracellular signals

Conditional inactivation of both bone morphogenetic protein receptor 1a (*Bmpr1a*) *(Col2a1-Cre)* and *Bmpr1b (-/-)* in early chondrocytes induced severe chondrodysplasia indicating that Bmp signaling is important in chondrogenesis after activation of *Col2a1* gene. Expression of Sox trio was suppressed in prechondrocytic condensations compared to wild type (WT), indicating that Bmp signaling is required for Sox5, Sox6 and Sox9 expression (Yoon et al. 2005). *In vitro* analyses have also demonstrated that Bmp signaling induces Sox9 expression to maintain chondrogenic potential of mesenchymal cells, and Sox trio expression to commence and maintain differentiation of chondrocytes (Zehentner, Dony & Burtscher 1999, Chimal-Monroy et al. 2003). Other extracellular signaling factors important in chondrocytic differentiation are retinoic signaling, which suppresses Bmp signaling in mesenchymal cells, and Wnt signaling action through β-catenin, which also is a suppressor of chondrogenic differentiation (Long, Ornitz 2013).

2.5 Endochondral bone formation

Differentiated chondrocytes form cartilage primordia, which expands through proliferation (Fig. 1B). In the mid-shaft of the primordia, chondrocytes exit cell cycle and become hypertrophic (which happens at E14.5 in mouse tibia) and osteoblasts surrounding the mid-shaft of primordia form periosteal bone collar (Fig. 1C). Hypertrophic chondrocytes further differentiate to terminal hypertrophic chondrocytes and undergo apoptosis. Blood vessels invade to terminal hypertrophic cartilage, and precursors of osteoclasts and osteoblasts arrive from the bone collar and begin to form primary ossification center (POC) using cartilage model as mechanical template (Fig. 1D). In long bones, secondary ossification center (SOC) emerges in the epiphyses forming cartilaginous growth plate (GP) between primary and secondary ossification centers (Fig. 1E). In mouse tibia, SOC formation begins at postnatal day (P) 7. Longitudinal bone growth depends on GP. In mature bone, permanent articular cartilage covers both ends. (Fig. 1F) (Mackie, Tatarczuch & Mirams 2011, Long, Ornitz 2013).

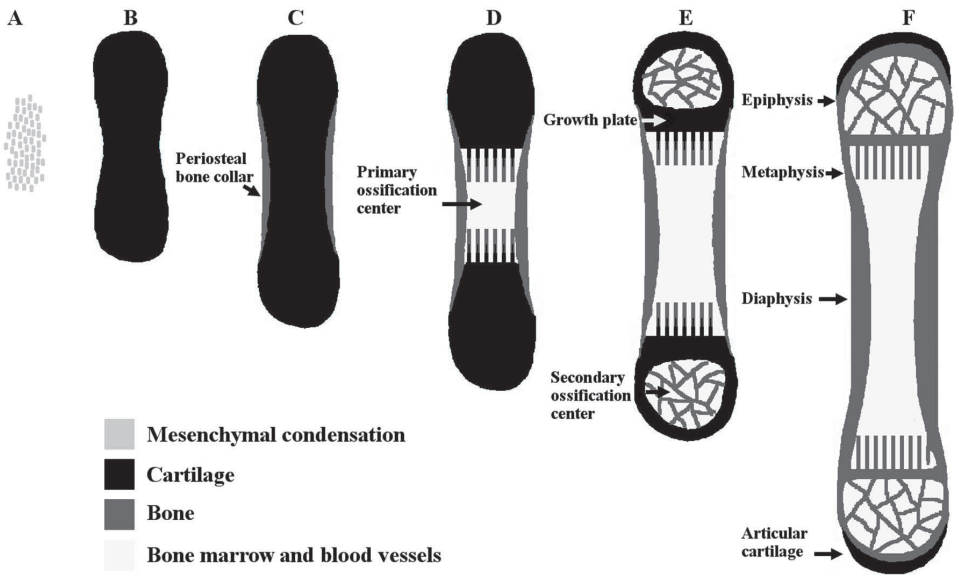

Figure 1. Overview of development of long bone through chondrogenesis and endochondral ossification. Precartilaginous condensation (A). Cartilage primordia (B). Periosteal bone collar is formed in the mid-shaft of cartilage primordia (C). Blood vessels from bone collar bring osteoblasts to terminal hypertrophic cartilage, which give rise to formation of expanding primary ossification center (D). SOCs appear to the epiphyseal ends of long bones and cartilaginous GP remains between POC and SOC (E). Epiphyseal articular cartilage covers the ends of the mature long bones (F). Figure modified from E J Mackie et al. J Endocrinol 2011;211:109-121

2.5.1 Growth plate zones

In embryonic chondroepiphysis and postnatal GP, chondrocytes undergo progressive differentiation, which can be observed as organized differentiation zones with characteristic cell morphology and gene expression profile (Fig. 2). Resting and columnar zone chondroblasts are proliferative and express abundantly early cartilage matrix genes like *Col2a1* and *Acan* (Sandell, Sugai & Trippel 1994). However, cell morphology and organization varies in these zones: resting zone chondroblasts are roundish when columnar are flattened and arranged into columns parallel to bone axis (Michigami 2013). In the prehypertrophic phase, chondroblasts stop proliferation (become chondrocytes), express still early cartilage matrix genes but are specifically marked by expression of parathyroid hormone-related peptide receptor 1 (*Pthr1*) and indian hedgehog (*Ihh*) genes (MacLean, Kronenberg 2005). When prehypertrophic chondrocytes differentiate into hypertrophic, dramatic change in gene expression and cell volume takes place. Expression of early cartilage matrix genes cease and collagen, type X, alpha I chain (*Col10a1*) expression emerges (Lefebvre, Smits 2005). Hypertrophic chondrocytes differentiate to terminal chondrocytes expressing matrix metalloproteinases (*Mmp*) 13 and 9, and then undergo apoptosis (Stickens et al. 2004). During endochondral ossification, matrix remodeling through Mmp13 and Mmp9 activity is rate limiting process for chondrocyte apoptosis, vascular invasion and osteoblast recruitment, which are prerequisite for POC and following

spongious bone formation (Stickens et al. 2004). Spongious bone is marked by expression of collagen, type I, alpha I chain (*Col1a1*) (Green et al. 2015).

In addition to systemic hormones and transcriptional regulators, paracrine signaling molecules are important regulators of differentiation and organization of chondrocytes in GP affecting endochondral ossification. Mutations in genes of these secreted proteins or in their cellular signaling pathways are linked to defects in chondrocyte differentiation and skeletal dysplasias causing, e.g., dwarfism (Bonafe et al. 2015).

2.5.2 Extracellular signals regulating growth plate development

Parathyroid hormone related peptide

Parathyroid hormone related peptide (Pthrp) is a paracrine factor, whose mRNA is expressed in periarticular chondrocytes and adjacent perichondrium in embryonic GP (Hilton, Tu & Long 2007, Lee et al. 1996). Instead, in postnatal GP expression was observed in stem cells and prehypertrophic chondrocytes (van der Eerden et al. 2000). Pthrp is regulated by Ihh. Pthrp acts through the Pthr1, and is crucial for normal bone growth (Fig. 2).

Pthr1 is mainly produced by prehypertrophic chondrocytes, i.e., chondrocytes undergoing change from the proliferative to post-proliferative state, but it is also expressed in low degree in proliferative chondrocytes (Fig. 2) (MacLean et al. 2004, MacLean, Kronenberg 2005, Vortkamp et al. 1996, Lee et al. 1996).

Pthrp-deficient mice die after birth probably from asphyxia and have widespread abnormalities in endochondral ossification and dwarfism. Histological examination of GP revealed a diminution of chondrocyte proliferation and premature hypertrophy of chondrocytes (Karaplis et al. 1994). In addition, irregular columnar arrangement was observed, and cell clusters of chondrocytes having similarities with proliferative chondrocytes were scattered among hypertrophic chondrocytes (Karaplis et al. 1994, Amizuka et al. 1994).

In line, *Pthrp* overexpression in chondrocytes resulted in delayed chondrocyte hypertrophy and endochondral ossification. Endochondral skeleton was completely cartilaginous at birth and dwarfism was demonstrated. Interestingly, hypertrophic differentiation and ossification was more delayed in medial part of the GP than in lateral one (Weir et al. 1996).

Pthr1-deficient mice exhibited accelerated hypertrophic differentiation that was rescued by constitutively active *Pthr1* targeted to GP (Lanske et al. 1996, Schipani et al. 1997). Postnatal inactivation of *Pthr1* in mouse cartilage resulted in accelerated chondrocyte differentiation and premature closure of GP, demonstrating that Pthrp signaling hindered proliferative chondrocyte differentiation during postnatal development like during embryonic development. In addition, abnormal apoptosis was observed that might contribute to GP phenotype (Hirai et al. 2011). Similarly, inactivating mutations in Pthr1 cause Blomstrand chondrodysplasia in humans with symptoms, such as advanced skeletal maturation, shortened long bones and increased bone density (Jobert et al.

1998, Karperien et al. 1999). Constitutively active Pthr1 causes Jansens chondrodysplasia in humans (Schipani et al. 1996).

These results suggest that Pthrp is an inhibitor of the program leading to chondrocyte differentiation. It regulates the length of the columnar region by allowing maintenance of the proliferation of columnar chondrocytes and suppressing their differentiation into postmitotic hypertrophic chondrocytes in fetal and perinatal GPs (Hirai et al. 2011). The gradient model suggests that Pthrp, expressed in periarticular chondrocytes in embryonic chondroepiphysis and resting chondrocytes of GP after formation of SOC, forms a gradient over proliferative zone, and that concentration of Pthrp is an essential regulator of onset of prehypertrophic differentiation of chondrocytes in GP (Chen et al. 2008b).

Pthrp maintains the capability of proliferative zone chondrocytes to proliferate and delay their further differentiation via signalling through Pthr1 (Chung et al. 1998). Pthr1 signal activates G_s protein (Guo et al. 2002, Kronenberg 2006). This activated G_s protein further activates adenylate cyclase and give rise to a release of cyclic adenosine monophosphate (cAMP) that activates protein kinase A (PKA). PKA phosphorylation of Sox9 contributes to differentiation delay, as phosphorylated Sox9 induces target genes more efficiently than its unfosforylated form (Huang et al. 2001). Pthr1 receptor activation also leads to suppression of cell cycle inhibitor P57, which contributes to the maintainance of proliferative zone chondrocytes in proliferative state (MacLean et al. 2004).

Indian hedgehog

Ihh is a member of the hedgehog (HH) family that in mammals contain also sonic hedgehog (Shh) and desert hedgehog (Dhh) genes. HH family proteins are conserved from invertebrates to humans and have essential roles in development. The HH signal is transduced through patched (Ptc1) receptor that activates a multi-pass transmembrane protein smoothened (Smo). Smo ultimately controls the processing and nuclear translocation of the Gli transcription factors (Gli1 to -3) that regulate the expression of target genes (Robbins, Fei & Riobo 2012, Wu et al. 2017). In GP, *Ihh* is expressed and secreted by prehypertrophic cells and the *Ihh* transcript is considered as a marker of prehypertrophic zone together with the transcript of *Pthr1* (MacLean, Kronenberg 2005).

In GPs of $Ihh^{-/-}$ mice, premature hypertrophy and considerably reduced chondrocyte proliferation is detected. Additionally, *Pthrp* expression was absent and GP resembled phenotype of *Pthrp*-deficient mouse (St-Jacques, Hammerschmidt & McMahon 1999, Lanske et al. 1996, Chung et al. 1998). This demonstrates that Pthrp induced by Ihh is indispensable for inhibiting onset of hypertrophic differentiation of chondrocytes and, thereby, for a proper thickness of proliferative zone in GP (Karp et al. 2000). Ihh induces Pthrp expression directly on periarticular chondrocytes (Hilton, Tu & Long 2007) by antagonizing Gli3 repressor in these cells (Hilton et al. 2005) (Fig. 2). In addition, Ihh signals directly to the proliferative chondrocytes in embryonic GP. Genetic manipulation of *Smo* revealed that direct Ihh signalling on proliferative chondrocytes is needed to maintain their proliferation

(Long et al. 2001). Reduced proliferation of GP proliferative chondrocytes was independent of Pthrp signalling in *Ihh*$^{-/-}$ mice that expressed constitutively active mutant of Pthr1 (Karp et al. 2000) (Fig. 2). Direct Ihh signalling to periarticular chondrocytes stimulates their conversion to flat columnar chondrocytes (Kobayashi et al. 2005b). These findings suggest that Ihh controls maturation and proliferation of chondrocytes: Chondrocyte hypertrophy is negatively regulated by Pthrp-dependent pathway and conversion to columnar chondrocyte and chondrocyte proliferation is positively regulated by a Pthrp-independent pathway (Fig. 2).

Ihh-Pthrp negative feedback loop model summarizes interaction of Ihh and Pthrp signalling pathways that are important regulators of initiation of chondrocyte hypertrophic differentiation and proliferation in GP. Ihh, expressed exclusively by prehypertrophic cells, forms diffusion gradient and stimulates Pthrp synthesis in periarticular chondrocytes and proliferation of the proliferative chondrocytes. Pthrp diffuses into the GP and keeps cells in proliferative state. Pthrp concentration is directly proportional to the distance of expression site. When the Pthrp concentration drops below a critical level, cells stop proliferating, begin to express Ihh and hypertrophy is initiated (Fig. 2) (Kronenberg 2003, Vortkamp et al. 1996). ECM heparan and chondroitin sulfate PGs are important in proper formation of Ihh gradient and, thereby, Ihh signalling in GP (Cortes, Baria & Schwartz 2009, Koziel et al. 2004).

Bone morphogenetic proteins

Bmps are growth factors belonging to the Tgfβ superfamily. Bmps transduce their signals through type I and II serine/threonine kinase receptors. During the binding of Bmp, type II receptor kinase phosphorylates type I receptors. Based on similarities in structure and function, type I receptors are divided into three groups: BmprI, activin receptor-like kinase 1 (Alk-1) and Tβr-1 group. BmprIa/Alk-3 and Bmpr1b/Alk-6 from BmprI group and activin receptor type I (ActrI/Alk2) from Alk-1 group are important in transduction of Bmp signal and significant also in chondrogenesis. Activated type I receptors phosphorylate, and so activate, intracellular receptor-regulated Smad proteins, including R-Smad1, R-Smad5 and R-Smad8. The activated R-Smads recruit and bind Smad4, and these Smad complexes translocate to the nucleus to regulate transcription of target genes (Miyazono, Kamiya & Morikawa 2010, Massague, Gomis 2006, Yoon et al. 2005, Rigueur et al. 2015). In addition to canonical Smad dependent pathway, Bmps also signal via non-Smad pathways like e.g., via Tgfβ-activated kinase 1 (Tak1). Tak1 activates p38 mitogen activated protein kinase (Mapk) (Moustakas, Heldin 2005, Miyazono, Kamiya & Morikawa 2010, Gao et al. 2013).

Analysis of mRNA expression of paracrine agonists and antagonists of Bmp signaling in seven-day old rat GP suggested Bmp signaling gradient across the GP: Bmp signaling inhibitors, like gremlin, chordin and *Bmp3,* are expressed primarily in resting chondrocytes and agonists, like *Bmp2* and *Bmp6,* expressed principally in hypertrophic zone (Nilsson et al. 2007). In E16.5 mouse proximal femur, both BmprIa and -b proteins are distributed throughout GP, although there is regional difference in intensity. BmprIa expression is focused to the prehypertrophic and

hypertrophic zones, while the highest levels of BmprIb are observed near the epiphyseal surface of the resting zone (Yoon et al. 2006). Actr1/Alk2 is expressed in proliferative and hypertrophic zones (Rigueur et al. 2015). In E16.5 mice proximal femur, the quantity of activated R-Smad 1, 5 and 8 proteins are increased from early proliferative cartilage to prehypertrophic zone of proximal femur, and then faded towards terminal hypertrophy. This suggests that proliferative and prehypertrophic chondrocytes are the most important targets of canonical Bmp signaling, and that canonical Bmp signaling is important in transition of proliferative chondrocytes to hypertrophic ones.

In *Bmpr1a* and *Bmpr1b* double knockout (KO) (*Bmpr1a*$^{fx/fx}$*;Col2Cre; Bmpr1b*$^{-/-}$) mice, GPs do not form and chondrogenesis remains mainly at prechondrogenic stage (Yoon et al. 2005). Overexpression of Bmp antagonist noggin gene in cartilage under *Col11a2* promoter also revealed that Bmp signal is crucial for cartilage development. In embryonic mice, metacarpal cartilage was hypoplastic and hypertrophic chondrocytes were absent, suggesting importance of Bmp signaling in chondrocyte maturation (Fig. 2) (Tsumaki et al. 2002). Cartilage-specific *Bmpr1a* KO (*Bmpr1a*$^{fx/fx}$*;Col2Cre*) mice die at birth due to a respiratory failure caused by skeletal effects, but GPs are formed (Yoon et al. 2005, Yoon et al. 2006). In these embryonic mice, the resting zone of GPs of distal femur does not differ from WT but proliferative zone is shorter and thinner, indicating a failure in differentiation from resting to proliferative cartilage. Defects were also observed in hypertrophic and terminal hypertrophic differentiation, demonstrated by increased terminal hypertrophic marker Mmp13 expression (Fig. 2). In proliferative zone, decrease in proliferation was detected and in resting and proliferative zones increase in apoptosis was demonstrated (Yoon et al. 2006). Defects in terminal hypertrophic differentiation were not observed in *Bmpr1b*$^{-/-}$ mice, indicating that *Bmpr1a* has more prominent role in hypertrophic zone (Yi et al. 2000, Yoon et al. 2006). Cartilage-specific overexpression of B*mpr1a* caused a shortening of the columnar layer of proliferating chondrocytes and up-regulation of maturation markers, suggesting acceleration of differentiation of proliferating chondrocytes toward hypertrophic chondrocytes (Kobayashi et al. 2005a).

In line with *Bmpr1* KO models and transgenic mouse models, cartilage-specific *Bmp2* KO demonstrated defects in chondrocyte proliferation and differentiation. In embryonic mice, hypertrophic differentiation and POC formation were delayed at least partly via reduction in runt-related transcription factor 2 expression. In addition, proliferative cartilage zone thickness was decreased and columnar structure disturbed (Shu et al. 2011). Combined cartilage-specific loss of function mutation of transcription factors *Smad 1* and *5* caused several defects in chondrocyte proliferation and proliferative and especially hypertrophic differentiation and demonstrated that *Smad1* and *Smad5* are key regulators of Bmp canonical signaling in the growth plate. Triple KO *Smad1*, *5* and *8* demonstrated almost similar phenotype indicating that *Smads 5* and *8* are more important than *Smad 8* in cartilage (Retting et al. 2009).

Bmp signaling is required to express and maintain *Sox trio* expression during chondrogenesis and maintenance of chondrocyte phenotype (Retting et al. 2009, Yoon et al. 2005, Tsumaki et al. 2002). *In vitro*, Bmp2 stimulated *Sox9* gene expression and *Sox9* promoter activity, and this effect was regulated by both canonical and noncanonical Bmp signal transduction (Gao et al. 2013).

Fibroblast growth factors

Fgf ligand family is divided into three groups: Canonical Fgfs (Fgf1-10, 16-18, 20, 22), endocrine Fgfs (Fgf15/19, 21, 23) and intracellular Fgfs (Fgf11-14). Canonical Fgfs are paracrine factors, and canonical and endocrine Fgfs bind and activate Fgf receptors (Fgfrs). Fgfrs are transmembrane receptor tyrosine kinases and there are four different types (Fgfr1-4). GAGs, especially HS, are needed for canonical Fgf-Fgfr interaction. Canonical Fgf signalling and Fgfr1 and 3 are important in GP development and homeostasis (Degnin, Laederich & Horton 2010, Sterner et al. 2013, Ornitz, Marie 2015).

In GP, Fgfr3 is highly expressed in proliferating and prehypertrophic chondrocytes and Fgfr1 in hypertrophic chondrocytes (Ornitz, Marie 2002, Lazarus et al. 2007, Karolak, Yang & Elefteriou 2015). Fgfr2 is expressed at low level in resting zone, but it does not have a nonredundant role in chondrogenesis as Fgfr3 and Fgfr1 have (Yu et al. 2003).

Fgfr3 in GP is a crucial regulator of endochondral ossification. Achondroplasia, the most common form of dwarfism in humans is a result of gain-of-function mutations in *Fgfr3*. In postnatal epiphysis of mouse with chondrocyte-specific overexpression of activated Fgfr3, chondrocyte proliferation and differentiation were inhibited and Ihh signalling pathway and Bmp4 expression were down-regulated (Naski et al. 1998). Instead, inactivation of Fgfr3 causes opposite effects with increased endochondral bone growth: Chondrocyte proliferation and Ihh signalling are increased and proliferative and hypertrophic zones are lengthened in epiphyseal GP (Deng et al. 1996, Naski et al. 1998, Eswarakumar, Schlessinger 2007) (Fig. 2). Fgfr3 signalling inhibits differentiation of chondrocytes to prehypertrophic and hypertrophic throughout the embryonic and postnatal development. Also, proliferation during late embryonic stages and postnatal development is inhibited by Fgfr3 signalling, but enhanced during early embryonic development (Iwata et al. 2000).

Conditional deletion of *Fgfr1* in chondrocytes (*Col2a1*-Cre) results in a transient increase in hypertrophic zone during late embryonic stage and early postnatal development. Osteopontin that is expressed in late hypertrophic zone and osteoblasts, was downregulated, indicating delayed hypertrophic chondrocyte or osteoblast differentiation. Expression of *Mmp9* was also downregulated, which may account for phenotype (Jacob et al. 2006, Karolak, Yang & Elefteriou 2015) (Fig. 2). Ablation of *Mmp9* and *Mmp13* has been reported to cause thickening in hypertrophic zone delaying exit of chondrocytes from GP (Stickens et al. 2004, Wu et al. 2002).

Fgfr3 signalling in chondrocytes is mediated at least by Stat, Akt and Erk1/2 Mapk pathways (Sahni et al. 2001, Murakami et al. 2004, Priore, Dailey & Basilico 2006). Ablation of Stat1 can rescue suppression of chondrocyte

proliferation caused by activating mutations in Fgfr3 or overexpression of Fgf2 *in vivo* (Sahni et al. 2001, Murakami et al. 2004). Fgfr3 signalling dephosphorylates Akt to give rise to reduced chondrocyte proliferation (Priore, Dailey & Basilico 2006). However, Stat1 deficiency did not rescue impaired chondrocyte differentiation that is mediated by activation of Erk1/2 Mapk pathways (Murakami et al. 2004).

In hypertrophic and articular chondrocytes, Fgfr1 signalling is suggested to be mediated through Ras-Raf-Mek-Erk1/2 pathway with neurofibromin as an essential regulator (Karolak, Yang & Elefteriou 2015, Yan, Chen & Im 2012). Fgfr1 signaling mediates Mmp9 and 13 expression in terminal hypertrophic chondrocytes and articular chondrocytes (Jacob et al. 2006, Karolak, Yang & Elefteriou 2015, Ono et al. 2013, Yan, Chen & Im 2012). In articular cartilage, Fgf2 signal is mediated through Fgfr1 and it promotes catabolism (Yan, Chen & Im 2012).

Interaction of canonical Fgfs with tyrosine kinase receptors is enhanced by coreceptors that are mainly HS but also dermatan sulfate (DS) and CS PGs (Shimokawa et al. 2011, Sterner et al. 2013). Always HS, DS and CS are attached covalently to core protein forming a PG. Also, the core protein can function as coreceptor for Fgf-Fgfr interaction (Goretzki et al. 1999, Ozerdem, Stallcup 2004). These PGs can be located in cell surface (transmembrane or glycerophosphatidylinositide (GPI) anchored) or be soluble ECM components (Roughley 2006, Filmus, Capurro & Rast 2008, Couchman 2010). In addition to coreceptor function, membrane and ECM PGs regulate diffusion gradients of Fgfs in tissues through their interaction (Makarenkova et al. 2009).

Fgfs 1, 2, 6, 7, 9, 18, 21 and *22* are expressed in perichondrium. *Fgfs 2, 7, 18* and *22* are also expressed in GP (Lazarus et al. 2007). Fgf 9 and 18 are suggested to be principal ligands for Fgfr3 (Liu et al. 2002, Ohbayashi et al. 2002, Hung et al. 2007) (Fig. 2). *Fgf 9*- and *18*-deficient mice phenotype remind phenotype of *Fgfr3*-deficient mice with increased proliferation, differentiation and Ihh signalling in GP in late embryonic phase (Liu et al. 2002, Ohbayashi et al. 2002, Hung et al. 2007). Also at an early embryonic stage the deficiency of *Fgf9* and *18* resembled phenotype of *Fgfr3*-deficient mice with decreased proliferation, and also the differentiation of chondrocytes was decreased (Hung et al. 2007, Liu et al. 2007). *Fgf2*-deficient mice have no distinct phenotype in GP structure, while bone formation and mass was decreased (Montero et al. 2000). In AC, Fgf2 is mediator of catabolic activities (Yan et al. 2011).

Interaction of Bmp, Fgf and Ihh signaling in growth plate

Bmp signaling functions as an antagonist to Fgf signaling. Balance of Bmp and Fgf signaling is important in chondrocyte progression through GP. Bmp signalling maintains chondrocyte proliferation and delays hypertrophic differentiation and terminal differentiation of hypertrophic chondrocytes when Fgf signaling acts as an antagonist. Both Bmp and Fgf signaling pathways act upstream of Ihh signaling (Minina et al. 2002) (Fig. 2).

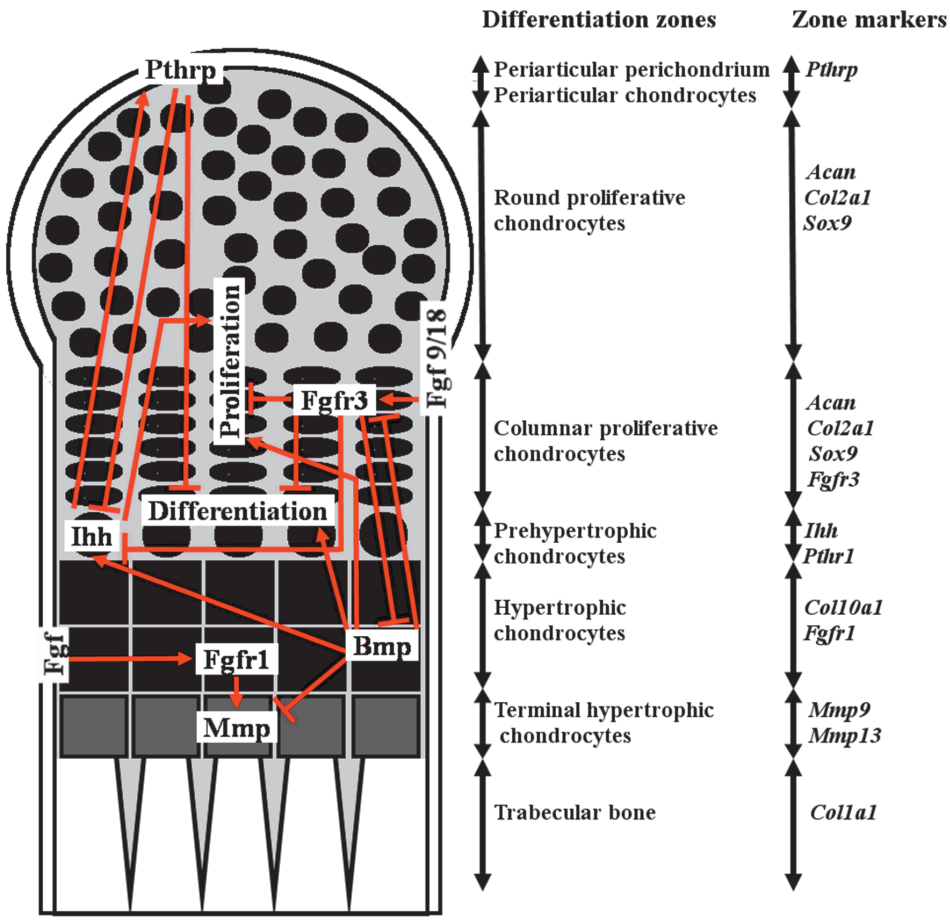

Figure 2. Marker genes of chondrocyte differentiation zones and essential paracrine signals in growth plate. In mouse chondroepiphysis at late embryonic stage (E16.5-E19.5), distinct chondrocyte differentiation zones are visible, but SOC is not yet formed. Each chondrocyte differentiation zone has specific gene expression profile. Fgfr3 mediates Fgf signal in proliferative chondrocytes and its activation inhibit proliferation, hypertrophic differentiation, Ihh expression and Bmp4 synthesis. Fgfr1 mediates Fgf signal in hypertrophic zone, and its activation increases Mmp expression and promotes terminal differentiation. Perichondrially expressed Fgf9 and 18 are probable ligands for Fgfr3. Bmp signaling counteracts Fgf signaling. Bmp signal promotes chondrocyte proliferation and hypertrophic differentiation in proliferative chondrocytes, Ihh expression in prehypertrophic chondrocytes and inhibits Fgf signaling in proliferative zone and Mmp expression in terminal hypertrophic chondrocytes. Ihh and Pthrp negative feedback mechanism regulates chondrocyte proliferation and differentiation. Ihh is expressed in prehypertrophic chondrocytes and it induces directly proliferation in columnar chondrocytes and Pthrp expression in periarticular perichondrium and chondrocytes. Ihh-induced Pthrp inhibits Ihh expression and is necessary for keeping columnar chondrocytes proliferative. Figure modified from Long and Ornitz Cold Spring Harb Perspect Biol 2013;5:a008334.

Mice with activating mutations in *Fgfr3* and mice lacking *Fgfr3* (*Fgfr3*$^{-/-}$) show that Fgfr3 signalling suppresses expression of Bmp4 and Ihh, suggesting that influence of Fgfr3 on growth and differentiation of chondrocyte is mediated through these pathways (Naski et al. 1998, Chen et al. 2001). In line with this,

chondrocyte-specific *Bmpr1a*-deficiency rescued phenotype of *Fgfr3*$^{-/-}$ mice by reducing chondrocyte differentiation. This can be explained by the fact that Fgfr3 facilitate Bmpr1a degradation through Smurf-1 mediated ubiquitination pathway and that Fgfr3-induced Bmpr1a degradation is important in Fgfr3 associated skeletal diseases (Qi et al. 2014).

Immunohistochemistry revealed increased signal of activated Stat1 and Stat5a in proliferative and hypertrophic chondrocytes and increased signal of Erk ½ Mapk in periarticular zone of cartilage specific *Bmpr1a* KO; *Bmpr1B*$^{+/-}$ mice, indicating increased Fgf signaling. In addition, Fgfr1 expression in hypertrophic zone was increased and it was expanded to proliferative zone (Yoon et al. 2006). These observations suggest that Bmp signaling suppresses Fgf signaling in GP.

Bmp signaling promotes Ihh expression in GP (Fig. 2): Ihh expression was decreased in chondrocyte-specific *Bmpr1a*-deficient mice GP compared to WT and in *Smad1/5* cartilage-specific double KO, Ihh expression and Pthr1 were undetectable (Yoon et al. 2006, Retting et al. 2009).

2.5.3 Secondary ossification center formation

Endochondral ossification processes in POC and SOC are principally the same: Mineralization of hypertrophic cartilage matrix and elimination of terminal hypertrophic chondrocytes are accompanied with matrix mineralization, angiogenesis and bone formation by osteogenic cells. However, there are differences: SOC formation is initiated by formation of cartilage canals, which occurs before hypertrophy and mineralization of the cartilage and bone collar is absent in SOC (Blumer, Longato & Fritsch 2008). Moreover, secondary ossification occurs via ossification of articular growth cartilage where spatial organization of chondrocytes and calcification and morphology of osteocytic cells are different compared to ossification of metaphyseal GP (Delgado-Martos et al. 2013, Byers, Brown 2006). In addition, SOC formation associates with radial expansion of ends of bones when POC is responsible for longtitudinal growth of bones (Mackie et al. 2008, Rivas, Shapiro 2002).

SOC formation process is divided into quiescent and reactive angiogenesis phases occurring in long bones of mice at P5-7 and P8-10 onwards, respectively. Cartilage canal formation initiates during quiescent angiogenesis and degradation of cartilage matrix by Mmps 9, 13 and 14 are central in this process according to expression and KO mouse studies. Unlike quiescent angiogenesis, reactive angiogenesis is dependent from angiogenic factors like vascular endothelial growth factor (Vegf). Chondrocyte hypertrophy and apoptosis, vascularization of hypertrophic cartilage and bone formation is taking place in epiphysis during reactive angiogenesis. During secondary ossification, cartilage canals are important for supply of nutrients and osteogenic cells for SOC and elimination of waste (Blumer et al. 2007, Blumer, Longato & Fritsch 2008).

2.5.4 Extracellular signals in secondary ossification center development

Thyroid hormones (THs) thyroxine and triiodothyronine are essential for initiation and maintenance of SOC formation. Peak levels of circulating THs are measured

during initiation of SOC formation (van der Heide, Ende-Visser 1980, Zoetis et al. 2003). THs influence directly to chondrocytes of epiphyseal cartilage promoting chondrocyte differentiation to matrix producing osteoblasts. THs stimulate Ihh and Osterix (Osx) expression in chondrocytes at P7, which are required for their differentiation into type X collagen and osteocalcin producing osteoblasts at P10. Also, Mmp 13 and 14 expression is promoted by THs (Xing et al. 2014).

Wnt family contains several structurally related secreted signaling proteins. Wnts interact, e.g., with plasma membrane receptor frizzled (FZD) and coreceptors as low-density lipoprotein receptor-related proteins 5 and 6. Activation of receptors stabilizes cytoplasmic β-catenin, which enters into nucleus and acts as a coactivator of lymphoid enhancer factor/T-cell factor (Lef/Tcf) family of transcription factors, which activates the transcription of downstream target genes (Usami et al. 2016). β-catenin is upregulated in prehypertrophic chondrocytes of SOC and cartilage specific gain of function mutation of *β-catenin* promote premature cartilage canal, SOC and POC formation. This β-catenin overexpression induces formation of vascularization via induction of Bmp2 expression, which further induces expression of Ihh and Mmp 9, 13 and 14 and cartilage hypertrophy (Dao et al. 2012).

Basic fibroblastic growth factor (b-Fgf/Fgf2) promotes angiogenesis and SOC formation in rabbit chondroepiphysis. Fgf2 and its receptors were localized to chondrocytes associated with cartilage canals (Melton, Clarke & Roach 2006, Leach, Sokol & McMurtry 1997). Fgf2 is reported to induce expression of Vegf1 in chondrosarcoma cells via Fgfr1 (Tzeng et al. 2015).

2.6 Proteoglycans

2.6.1 Classification of proteoglycans

PGs are molecules containing a core protein and one or more covalently attached GAG side chain(s), which can be HS, CS, DS and keratan sulfate (KS). Depending on the PG, the number and type of GAGs can vary and certain PGs can be part-time PGs so functioning with or without GAGs (Couchman, Pataki 2012). Mammalian PGs can be divided into four classes according to location: intracellular, cell surface, pericellular and extracellular (Iozzo, Schaefer 2015). The extracellular PGs is the major group containing 25 members. It can be divided into structural subgroups as lecticans (hyaluronan (HA) interacting proteoglycans) including e.g. Acan, small leucine-rich PGs (SLRP) including e.g. biglycan (Bgn) and decorin (Dcn) and SPARC/osteonectin CWCV and Kazal-like domain PGs (SPOCK) (Iozzo, Schaefer 2015). Second largest class is membrane PGs containing 15 members. Membrane PGs can be divided into two structural subgroups such as transmembrane proteoglycans including e.g. syndecans (Sdc) 1-4 and GPI-anchored PGs including glypicans (Gpc) 1-6 (Iozzo, Schaefer 2015, Couchman 2010, Dwivedi, Lam & Powell 2013).

2.6.2 Proteoglycans are an important part of cartilage extracellular matrix

Cartilage ECM consists of collagens and noncollagenous proteins and PGs. The major protein in cartilage ECM is fibril forming type II collagen. Other collagens specific to cartilage ECM are fibril forming type XI collagen and fibril associated type IX collagen, which is bound to fibrils of type II and XI collagens. Type IX collagen regulate fibril formation and interactions between fibrils and other ECM molecules. It is also classified as proteoglycan due to attached CS chain. Type X collagen marks hypertrophic cartilage (Eyre, Weis & Wu 2006).

Abundant noncollagenous proteins in cartilage ECM are, e.g., matrilins (Mtn) and cartilage oligomeric matrix protein (Comp). Matrilins 1 and 3 are abundant in cartilage. They are associated to type II, IX and XI collagens and noncollagenous proteins like Acan, Comp, Bgn and Dcn and participate to ECM assembly connecting collagen network to other ECM molecules (Klatt et al. 2011). Comp is pentameric protein with five identical subunits. It is able to bind collagen molecules, bring them close to each other and so facilitate collagen fibril formation (Heinegard 2009).

Abundant PGs in cartilage ECM are Acan, PG4/lubricin and SLRPs including Dcn, Bgn, fibromodulin (Fmod) and lumican (Lum). In cartilage, Acan form aggregates in cooperation with hyaluronan and link protein. Each aggregate consists of HA GAG chain to which up to 100 Acan molecules are bound. An important function of Acan is to provide high anionic charge density to form osmotic properties to hold water in tissue. CS chains attached to Acan play a key role in this function (Kiani et al. 2002). CS chains of Acan are also suggested to participate formation of morphogen gradients in GP (Cortes, Baria & Schwartz 2009). The common feature of SLRPs is leucine-rich repeats in conserved locations (Iozzo 1997). Via these leucine-rich repeats SLRPs bind to fibril-forming collagens and regulate fibril formation and enable interaction of fibrils with ECM (McEwan et al. 2006, Kalamajski, Oldberg 2010). GAG chains of SLRPs can participate to collagen fibril formation, but also interact with extracellular signal molecules, and potentially participate diffusion of signal molecules in ECM (Lord, Whitelock 2013, Heinegard 2009). Bgn and Dcn are CS/DS PGs. Fmod and Lum are KS PGs containing several sulfated tyrosine residues in N-terminus. In Fmod this anionic N-terminal domain is able to bind growth factors, cytokines and Mmp13 (Tillgren et al. 2009). Premature osteoarthritis is observed in *Fmod*/*Bgn* double-deficient mice (Wadhwa et al. 2005).

A large number of enzymes are associated to synthesis of GAGs. Mutations in these enzymes cause wide range of diseases, in which defects in skeleton and connective tissue are characteristic, demonstrating that GAG side chains of PGs have an important role in skeletogenesis. However, defects can also occur in other tissues (Sasarman et al. 2016). GAGs, such as CS, HS and HA, are necessary for cartilage homeostasis. Gene trap mutation in chondroitin 4 sulfotransferase 1 (*C4st1*) gene caused imbalance in chondroitin sulfation, which leaded severe chondrodysplasia with upregulation of Tgfβ signalling and downregulation of Bmp signalling in mouse GP (Kluppel et al. 2005). Missense mutation in

phosphoadenosine phosphosulfate synthetase (*Papss2*) gene caused preferential undersulfation of CS in mouse cartilage with normal HS sulfation. Therefore, this *Papss2* mutated (brachymorphic) mouse was used as model for CS undersulfation (Cortes, Baria & Schwartz 2009, Kurima et al. 1998). In these mice, chondrodysplasia was observed in postnatal GP. Restricted Ihh diffusion and chondrocyte proliferation was detected (Cortes, Baria & Schwartz 2009). Exostosin1 (Ext1) enzyme is necessary for HS synthesis. Hypomorphic mutation in *Ext1* resulted also alterations in GP. Interestingly, Ihh diffusion was enhanced, indicating that HS inhibit Ihh diffusion and, thus, have an opposite role than CS (Koziel et al. 2004). HA is non-sulphated GAG, which is not attached to core protein. It is synthetized by hyaluronan synthases (Has) in plasma membrane. Conditional deletion of Has2 in limb bud mesenchyme demonstrated that HA is necessary for normal progression of chondrocyte maturation (Matsumoto et al. 2009).

2.6.3 Membrane proteoglycans

So far 9 genes coding for transmembrane PGs are known. Four of them are syndecans (*Sdc* 1-4) and the others are chondroitin sulfate PG 4 (*Cspg4*), Cd44 antigen (*Cd44*), neuropilin 1 (*Nrp1*), betaglycan (*Tgfbr3*), phosphacan (*Ptprz1*) (Couchman 2010, Iozzo, Schaefer 2015) (Table 1). In addition, there is HS PG family glypicans (*Gpc* 1-6) anchored to plasmamembrane with GPI (Filmus, Capurro & Rast 2008, Dwivedi, Lam & Powell 2013). Primary sequences of these membrane PGs are not related to each other excluding members of syndecan and Gpc families. So, the uniformity in this group of membrane PGs is based on the ability of core proteins to be attached with GAG chain and binding to plasma membrane. Even in syndecans only transmembrane and intracellular domains are significantly similar, while ectodomains are different (Filmus, Capurro & Rast 2008, Couchman 2010, Couchman et al. 2015).

All known transmembrane PGs are type I membrane PGs. Syndecans are full-time proteoglycans substituted with HS chains in their N-terminus. Syndecans 1 and 3 may contain also CS chains. The Cspg4 is also full-time PG containing one CS chain. Remainder part are part-time PGs appearing both as PG and glycoprotein form. Cd44 can contain both CS and HS. Neuropilin 1 and betaglycan may contain one GAG that can be either CS or HS (Couchman 2010, Iozzo, Schaefer 2015) (Table 1).

Ectodomain interaction with extracellular signaling factors and matrix molecules can take place by particularly HS, but also DS and CS side chains and PG core protein (Yan, Lin 2009, Sterner et al. 2013, Ozerdem, Stallcup 2004). All known membrane PGs act as coreceptors to enhance or inhibit interaction between extracellular signal molecule and its high affinity receptor and contribute to their signal transduction (Couchman 2010, Dwivedi, Lam & Powell 2013, Pacifici et al. 2005, Pap, Bertrand 2013). Transmembrane PGs can function also adhesion receptors and in cell signaling through cytoplasmic domain often to the actin cytoskeleton (Afratis et al. 2017). Many transmembrane PGs can be shed usually

by Mmps when Gpcs are shed by phospholipases. Ectodomain shedding (enzymatic release of intact extra cellular domain (ECD)) is important regulatory mechanism changing cell surface receptor function and yielding soluble PGs that can function as paracrine or autocrine effectors, or competitive inhibitors (Manon-Jensen, Itoh & Couchman 2010, Matsuo, Kimura-Yoshida 2014). In addition to coreceptor function, these membrane PGs also bind and sequester growth factors contributing their gradients in tissue and so acting in growth factor-dependent cell regulation (Yan, Lin 2009, Mythreye, Blobe 2009, Fuerer, Habib & Nusse 2010). Of transmembrane PGs Cd44, Cspg4 and all four syndecans, are expressed in skeletal tissues, but no one is specific to cartilage. In addition, all Gpcs are expressed in skeletal tissues.

Table 1. Transmembrane proteoglycans (Couchman 2010, Iozzo, Schaefer 2015).

Name	Gene symbol	GAG
Syndecan 1	*Sdc1*	Several HS and CS
Syndecan 2	*Sdc2*	Several HS
Syndecan 3	*Sdc3*	Several HS and CS
Syndecan 4	*Sdc4*	Several HS
Chondroitin sulfate PG 4	*Cspg4*	CS
Cd44 antigen	*Cd44*	HS and CS
Neuropilin 1	*Nrp1*	HS or CS
Betaglycan	*Tgfbr3*	HS or CS
Phosphacan	*Ptprz1*	Several CS

2.6.4 Membrane proteoglycans and skeletal diseases

Gpc6 and *Gpc3* genes are associated to human skeletal diseases (Dwivedi, Lam & Powell 2013). *Gpc3* mutations causing dysfunction or loss-of-function of the gene, are associated to Simpson-Golabi-Behmel syndrome (SGBS) in humans, which is characterized by, for instance pre- and postnatal overgrowth and skeletal defects (Pilia et al. 1996). *Gpc3*-deficient mouse phenotype is reminiscent of symptoms of SBGS. Skeletal phenotype included overgrowth (increase in body weight and size), increased thickness of hypertrophic zone, delay in cartilage replacement by bone and trabecular bone ossification (Viviano et al. 2005, Chiao et al. 2002). In GP, *Gpc3* is expressed in proliferative and prehypertrophic zones (Viviano et al. 2005). Overgrowth of *Gpc3* KO mice compared to WT is associated partly to increased Ihh signaling in cartilage including GPs (Capurro, Li & Filmus 2009). Gpc3 compete with Ihh receptor Ptc1. Interaction of Ihh and Gpc3 induce endocytosis and degradation of the complex decreasing Ihh available for binding to Ptc1 (Capurro et al. 2008).

Gpc6 loss-of-function mutations are predicted to associate omodysplasia in humans, which is characterized by e.g. severe short stature and shortened limbs (Campos-Xavier et al. 2009). Histological analyses are contradictory, but it is probable that Gpc6 promote chondrocyte proliferation in GP (Borochowitz et al. 1998, Dwivedi, Lam & Powell 2013). Gpc6 is predicted to promote chondrocyte proliferation by promoting Wnt5a signalling via direct interaction (Dwivedi, Lam

& Powell 2013). In mouse GP, Gpc6 is upregulated in resting and proliferative zones colocalizing with Wnt5a (Andrade et al. 2007, Campos-Xavier et al. 2009). However, research data of skeletal phenotype of *Gpc6* KO mouse is not available (Dwivedi, Lam & Powell 2013).

3. AIMS OF THE STUDY

Understanding of molecular biology of chondrogenesis is necessary for development of treatments for GSDs and defects and diseases of AC, like OA. Gene expression determines specific properties of each tissue. Therefore, it is likely that tissue-specific genes have a special role in the formation of structural and functional properties of tissue. The purpose of this study was to identify a novel gene important in cartilage and bone homeostasis. Criteria for selection of potential gene candidate were high, cartilage-specific expression during mouse limb development and structural conservation throughout species. Selected gene (*Snorc*) was further studied to define more closely its expression, tissue distribution, interaction partners and role in cartilage and bone homeostasis. The specific aims of the study were to:

1. Select a novel, unidentified transcript potentially important in chondrogenesis using following criteria:
   1) the transcript is expressed intensely during embryonic mouse limb development in genome wide microarray experiment.
   2) It is expressed specifically in cartilage according to gene expression information data bases, and the gene and the predicted protein are conserved in vertebrates.

2. Verify cartilage-specific expression of the transcript and localize the gene and protein expression in the skeletal tissues of developing and adult mouse.

3. Study functional domains and posttranslational modifications in predicted mouse protein and its vertebrate homologues.

4. Study the interaction with growth factors and extracellular matrix components.

5. Analyse the role in skeletal tissues during development and adulthood using two *Snorc*-deficient mouse models.

4. MATERIALS AND METHODS

4.1 Screening of novel cartilage genes (I)

Mesenchymal condensations of limb buds and developing cartilage tissues of knee joints were microdissected from embryonic mouse hind limbs during E9.5 to E20.5. Microarray analysis was used to study expression profiles of the mRNAs genome widely. Available microarray data was searched for the novel genes expressed intensely during chondrogenesis. Ensembl and National Center for Biotechnology Information (NCBI) databases were used to find out cartilage specificity, genomic structure, homologues and paralogues of the genes of interest. Potential gene candidates were selected, and predicted proteins coded by selected genes were analyzed *in silico*. Primarily functional domains and posttranslational modifications were searched for these predicted proteins. Promising Riken transcript 3110079O15Rik was selected for further studies, and its expression, structure and function was studied in more detail in laboratory. It was named *Snorc* (small novel rich in cartilage) based on its novelty and specific and intense expression in developing cartilage.

4.2 Generation of *Snorc*-deficient mice (II)

An embryonic stem cell clone containing one of the *Snorc* alleles targeted by a promoter driven "knockout first" EUCOMM/KOMP-CSD allele (3110079O15Rik$^{tm1a(EUCOMM)Hmgu}$) was obtained from the International Knockout Consortium (Skarnes et al. 2011). This 3110079O15Rik$^{tm1a(EUCOMM)Hmgu}$ allele is called *Snorc*^{a} in this paper (II, Fig. 1). Correct targeting was confirmed using long-range polymerase chain reaction (PCR) over the 5´ and 3´ homology arms using SequalPrep™ Long PCR Kit (Life Technologies), providing the expected fragments of 6.37 kbp (5'- homology arm) and 5.74 kbp (3'- homology arm), and by sequencing the ends of these PCR products. For 5´arm verification by PCR we used primers F1 and R1 and for 3' arm verification we applied the primers F2 and R2 (II, Fig. 1, Supplementary Fig 1A and Supplementary Table I). The ES cells were then used for blastocyst injection by applying standard techniques. The resulting three chimeric males were bred with C57BL/6NCrl females to acquire *Snorc*$^{a/+}$ mice that were further bred with each other to get homozygous *Snorc*-deficient mice (*Snorc*$^{a/a}$). Germline transmission of *Snorc*^{a} allele and genotypes of further generations were analyzed using PCR with the primers F3 and R3 for the *Snorc*^{a} allele and F4 and R4 for the *Snorc*$^{+}$ allele (II, Fig. 1, Supplementary Table I). Disruption of mRNA expression in *Snorc*^{a} allele is based on the utilization of a novel splice acceptor, engrailed-2 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 (II, fig. 1; Supplementary Fig. 1 C).

In addition, by taking the advantage of the presence of loxP sites at the introns of the targeted allele we generated a second *Snorc* mutant mouse model 3110079O15Rik $^{tm1- b(EUCOMM)Hmgu}$ (*Snorc*$^{b/b}$) with loss of exons 2 and 3 of the

Snorc gene (II, Fig. 1) (Testa et al. 2004). For this purpose, male *Snorc*$^{a/a}$ mice were bred with transgenic female mice expressing Cre-recombinase in the mature oocytes, allowing recombination to occur in zygote prior the 2-cell stage (Sakai, Miyazaki 1997). Occurrence of preferred site-specific recombination in the offspring, and further genotyping of mouse produced was carried out by PCR using primers F4 and R4 for WT allele, and primers F7 and R7 for *Snorc*^{b} allele (II, Supplementary Fig. 1 B, Supplementary Table I). In addition to these PCRs, right deletion was ensured using PCRs with several additional primer pairs (data not shown).

4.3 Genotyping and gender determination (II)

Genonomic DNA was isolated from pieces of ear of young pubs or tail of terminated animals. Tissue was digested at 55°C for 6 hours – overnight with 0.2 mg/ml Proteinase K (Thermo Fisher Scientific), in 100 mM Tris-HCL, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl. Undigested material was removed by centrifugation and the DNA was precipitated in 50% isopropanol and dissolved in sterile water. Mouse genotypes were determined using primers F3 and R3 (*Snorc*^{a} allele), F7 and R7 (*Snorc*^{b} allele) and F4 and R4 (*Snorc* WT allele). For gender determination, a fragment of male specific Sry were amplified using DyNAzyme II (Thermo) DNA polymerase (II, Fig. 1, Supplementary Table 1) (McClive, Sinclair 2001). PCR reactions were carried out using DyNAzyme II (Thermo Fisher Scientific).

4.4 Experimental animals (I, II)

The mice were bred in the Central Animal Laboratory of University of Turku in standardized environmental conditions. Soy-free SDS RM3 (Special Diet Service, Whitman, Essex, UK) and tap water were available ad libitum.

In study I, the animal maintenance and the use of animal material were accepted by the national committee for animal welfare (Project licence KEK/1908-2010). Tissues of C57Bl/6xDBA mice or C57Bl/6N mice (Saamanen et al. 2000, Uusitalo et al. 2001) were dissected under stereomicroscope for immunohistochemical and mRNA expression studies. In study II, study plan for *Snorc*-deficient mouse phenotype characterization was accepted by the National Animal Experiment Board ELLA (project license ESAVI-2010-04857/Ym-23). *Snorc*-deficient mice were crossbred with C57Bl/6NCrl background and *Snorc*$^{b/b}$, *Snorc*$^{a/a}$ and WT mice used in this study were collected from heterozygous matings.

4.5 Gene expression analyses (I, II)

Gene expression analysis methods, performed analyses and tissues used in the analyses are summarized in Table 2.

Expression of 40 000 transcripts, containing 6000 unannotated expressed sequence tags (EST), during development of mouse limb was analyzed using Mouse Genome 430 2.0 array (Affymetrix). Analysis was performed at the Finnish DNA Microarray Centre in Turku, Finland. Using Affymetrix GCOS software and mean scaled to 500 data was preprocessed and normalized. Inforsense KDE software was used to obtain the expression profiles of at least four-fold upregulated genes after E12.5.

Total RNA used in Affymetrix array was extracted in 4 M guanidine isothiocyanate solution and isolated by CsCl buoyant density gradient centrifucation (Saamanen et al. 2000). For other analyses, total RNA of tissues and cell cultures was extracted using TRIsure (Bioline) reagent following the instructions of the manufacturer.

Ten µg aliquots of total RNA were subjected for Northern hybridization. Full-length mouse *Snorc* cDNA clone (accession number BX_518642) was from German Resource Center for Genome Research (RZPD). Mouse cDNA probe for *Comp* was a 242 bp fragment cloned in pGEMT Easy vector (Promega) (Salminen et al. 2000). Released inserts were labeled by α^{32}P-dCTP using Prime-a-Gene Labeling System (Promega, Madison, Wi). Probe for 28S rRNA was labelled by Nick Translation kit (Roche Diagnostics). After high stringency washes, the bound radioactivity was detected and quantified on a Bio-imaging analyzer BAS-5000 (Fuji).

For *in situ* hybridization, embryos were fixed in 4% paraformaldehyde, embedded in paraffin and serially sectioned (5 µm). A 381 bp 5' cDNA fragment of mouse *Snorc* was PCR-amplified using a full-length cDNA clone BX_518642 (RZPD, Germany) as a template, and cloned into pCR II-TOPO (Invitrogen) (I: Table 1). Antisense and sense 35S- UTP-labeled cRNA probes were synthesized by *in vitro* transcription with SP6 and T7 RNA polymerases (Promega, Madison, Wi), respectively. Radioactive *in situ* hybridization procedures were carried out as previously described (Takatalo et al. 2008).

To verify ablation of *Snorc* expression in *Snorc*-deficient mouse by RT-PCR, total RNA was treated with DNase I (Amplification Grade, Invitrogen) and reverse-transcribed to cDNA using Maxima H Minus Reverse Transcriptase (Thermo) in the presence of RNaseOut ribonuclease inhibitor (Invitrogen). RT-PCR was performed using DyNAzyme II DNA polymerase (Thermo). hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) was analyzed as a housekeeping gene (II, supplementary fig. 1 C). Used primers are listed in Table 3.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses using mouse tissues were performed as follows: Total RNA from mice tissues was treated with DNase I (Amplification Grade, Invitrogen). Complementary DNA was synthesized by DyNAmo cDNA Synthesis Kit for quatitative polymerase chain reaction (qPCR) (Thermo) and PCR analysis was performed using DyNAmo Flash SYBR Green qPCR kit (Thermo). Beta-actin

(*Actb)* or peptidylprolyl isomerase A (*Ppia*) was used for normalization. Analyzed genes and their primer sequences are shown in Table 3.

For qRT-PCR analysis of *Snorc* and cartilage marker genes in limb bud culture, RevertAid H minus first strand cDNA synthesis kit (Fermentas) was used for synthetization of the cDNA. qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems). Expression was normalized to *Actb*. Relative expression levels and fold induction of each target gene were calculated using a comparative C_T method [$(1/(2^{\Delta C}{}_T))$ formula; $\Delta C_T = C_T$ target - C_T reference] (Harkness et al. 2009). Primers of analyzed genes are listed in Table 4.

Table 2. Summary of gene expression analysis methods and analyses and tissues or cell cultures used in these analyses.

Method	Analysis	Mouse tissue/cell culture	Age/time point
Microarray	*Snorc* expression during embryonic limb development	Limb buds, mesenchymal condensations	E9.5-E11.5
		Knee epiphyseal cartilage	E12.5-E20
Northern blot	*Snorc* tissue distribution	Several different mouse tissues	P4
	Snorc expression during mouse life span	Knee epiphyses	E12.5 to 20-day-old
		Proximal tibial cartilages	40-day- to 10- month-old
	Effect of Bmp2 on *Snorc* induction	Fracture callus	4, 7 and 10 days after induced fracture
In situ hybridization	*Snorc* mRNA distribution	Embryo	E16.5, E18.5
RT-PCR	Verification of silencing of *Snorc* expression in *Snorc*-deficient mice	Knee epiphysis	P5
qRT-PCR	Snorc expression	Prostate, mammary gland, cartilage	P5, P22, 2 m, 4 m
	Expression of cartilage marker genes in *Snorc*-deficient and WT mice	Knee epiphysis	P10
	Expression of proliferation marker topoisomerase II A (*Top2a*) in *Snorc*-deficient and WT mice	Knee epiphysis	P10, P22
	Bmp2 effect on expression of *Snorc* and cartilage marker genes	Limb bud cell culture	0, 1, 5, 10 and 15 days after culture

Table 3. Primers used to verify silencing of *Snorc* expression in *Snorc*$^{a/a}$ and *Snorc*$^{b/b}$ mouse cartilage by RT-PCR and to analyze gene expression in various tissues of mouse by qRT-PCR.

mRNA	Forward primer	Reverse primer
Acan	CCCGGTACCCTACAGAGACA	ACAGTGACCCTGGAACTTAG
Actb	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG
Col2a1	GCTGGAAAACCTGGTGACGA	GCCTGGGTAACCTCTGTGAC
Col10a1	CATCTCCCAGCACCAGAATCTA	CAAGTGGGCCCTTTATGCCT
Hprt1	GCTGGTGAAAAGGACCTC	CACAGGACTAGAACACCTGC
Ihh	CGTGCATTGCTCTGTCAAGT	CTCGATGACCTGGAAAGCTC
Mmp9	TAGATCATTCCAGCGTGCCG	GCCTTGGGTCAGGCTTAGAG
Mmp13	CTTCTGGCACACGCTTTTCC	TGGCTTTTGCCAGTGTAGGT
Ppia	CATCCTAAAGCATACAGGTCCTG	TCCATGGCTTCCACAATGTT
Sox9	GCCACGGAACAGACTCACAT	AGATTGCCCAGAGTGCTCG
Snorc, exons 1-2 (F5-R5)	AGCCGCCTAGTCCTCTGTGT	AAATTCCTGGTTGTGGCTTG
Snorc exons 2-3 (F6-R6)	AAGCCACAACCAGGAATTTG	GCTTCAAGAAGCGGAAAACTT
Top2A	CAACTGGAACATATACTGCTCCG	GGGTCCCTTTGTTTGTTATCAGC

Table 4. Primers used to analyze gene expression in mouse limb bud cultures using qRT-PCR.

mRNA	Forward primer	Reverse primer
Actb	TTCCTTCTTGGGTATGGAAT	GAGCAATGATCTTGATCTTC
Acan	CCCGGTACCCTACAGAGACA	ACAGTGACCCTGGAACTTGG
Col2a1	ACATGTCAGCCTTTGCTGGC	CATGGTCTCTCCAAACCAGA
Comp	GTTCAGCGGACCCACCCACG	GCATCTCCGATGCCGTCCCG
Sox9	CGACTACGCTGACCATCAGA	AGACTGGTTGTTCCCAGTGC
Snorc	CTGCTCATCTCTGGGGTCC	GATCACGATGGCTGCAATG

4.6 Detection of *Snorc* promoter activity (II)

Snorc promoter activity was studied by comparing activity of *Snorc* reporter gene LacZ expression in mice carrying *Snorc*^{a} or *Snorc*^{b} and WT allele using X-gal staining (Zhang et al. 2007). Stained tissues were decalcified in 5% formic acid, embedded in paraffin, cut into 5 μm sections and counterstained with eosin.

4.7 Primary antibodies (I, II)

For immunohistochemical detection of Snorc, polyclonal rabbit antibodies against two peptide sequences including amino acids 38–52 and 71–86 of the ectodomain (MedProbe, Norway) was raised. Other primary antibodies used were rabbit anti proliferating cell nuclear antigen (PCNA) and goat anti-Bmp-2 (Santa Cruz Biotechnology Inc., Ca, USA), mouse anti-type II collagen (Clone 6B3, Chemicon International Inc), rabbit anti-mouse type IIA procollagen (MoIIA) (Salminen, Vuorio & Saamanen 2001) and rat anti-HA (epitope 12CA; Zymed).

4.8 Histochemical and immunohistochemical studies (I, II)

Fixed samples (4% paraformalin) were decalcified in 10% Na_2-EDTA, 0.1 M phosphate buffer, pH 7.0, embedded in paraffin, cut into 5 µm sections and stained using hematoxylin-eosin (HE) or used for immunohistochemistry after epitope retrieval by digestion with 1 mg/ml hyaluronidase 1040 U/mg (type IV-S from bovine testes, Sigma-Aldrich) or 0.5 U/ml chondroitinase ABC (Sigma-Aldrich) or Ficin (Zymed) followed by inactivation of endogenous peroxidase by 3% H_2O_2 in methanol (Heinonen et al. 2011). Rabbit polyclonal antibodies were detected using MACH2 detection system with Warp Red chromogen (Histolab) for Snorc or Histostain Plus Broad Spectrum kit with DAB-Plus Substrate (Invitrogen) for PCNA and type IIA procollagen. Goat polyclonal antibody was detected with Vectastain Goat ABC with DAB detection (Vector laboratories). Mouse monoclonal antibodies were detected using Mouse Links and Label (BioGenex) (Salminen, Vuorio & Saamanen 2001). Hematoxylin counterstaining was performed using Meyers Hematoxylin (Histolab).

4.9 *In silico* analyses (I)

Full-length homologous transcripts of *Snorc* were identified using Ensembl and NCBI HomoloGene, RefSeq mRNA and dbEST databases, and protein precursors of homologs were aligned using M-coffee multiple sequence alignment software (Moretti et al. 2007). The following softwares or databases were used to predict functional features or domains in protein precursor homologs: cleaved endoplasmic reticulum (ER) signal peptide: SignalP 3.0, transmembrane sequence: TMHMM 2.0, O-glycosylation sites: NetOGlyc and YinOYang 1.2, serine and threonine residues prone to phosphorylation: NetPhos and ScanProsite; GAG attachment: The Eucaryotic Linear Motif Resource for Functional Sites (ELM) database.

4.10 Analysis of hind limb long bone morphology (II)

E20 and P10 whole skeletons were stained with Alcian Blue for cartilage and Alizarin Red S (Sigma-Aldrich) for bone (Kimmel, Trammell 1981). Hind limbs were imaged using Olympus SZ61 stereomicroscope and DeltaPix Invenio digital camera and software and morphology of long bones was analyzed using ImageJ (NIH, Bethesda, MD, USA) (II, Supplementary Fig. 2) (Schneider, Rasband & Eliceiri 2012). P22 femur and tibia and 2 months of age (m) and 4 m tibias were X-ray imaged (Faxitron, AZ; USA), and their morphology was analyzed using ImageJ (II, Supplementary Fig. 2 and Fig. 3.). Lengths of 2 m and 4 m femurs were analyzed during peripheral quantitative tomography (pQCT) (paragraph 4.19). X-ray images and ImageJ were used for analysis of proximal subchondral bone area of 2 m and 4 m tibia (Fig 3.).

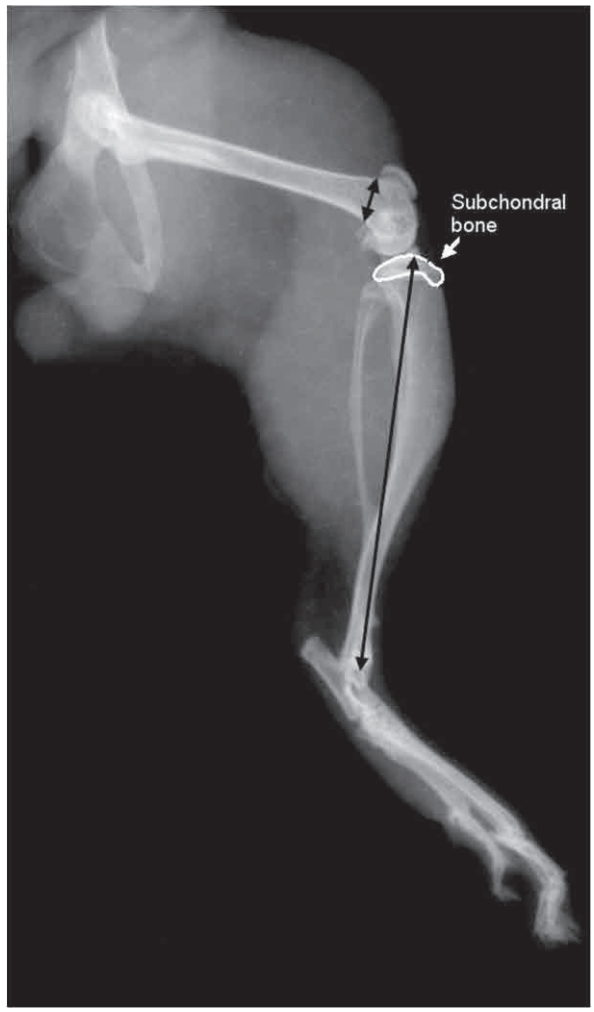

Figure 3. Morphological analysis of tibia and pQCT analysis of femur of adult (2 m and 4 m) *Snorc*$^{a/a}$ and WT mouse. Line for tibia total length measurement and area for proximal tibial subchondral bone measurement is depicted in 4 m male WT mouse hind limb. In addition, position of cross-section in femur distal metaphysis used in pQCT analysis is marked using two-headed arrows.

4.11 Histomorphometric analysis (II)

Sagittal tissue sections (from the middle of tibial epiphysis of P1 and P10 mice and from the middle of tibial lateral condyle of P22 mice) and frontal tissue sections (from the middle of tibial lateral condyle of 2 m mice) were selected for HE staining using serially sectioned paraffin-embedded blocks to get the equal anatomical level of parallel samples. Histomorphometrical analysis was performed using ImageJ. Average zone thicknesses were analyzed. For P1, P10 and P22 sections analysis included one medial and two marginal measurement lines per zone (II, Supplementary Fig. 3B) and for 2 m sections four measurement lines with equal distance covering certain area in lateral part of the GP (Fig 4.).

Average cell density of the GP zones of the proximal tibia was analyzed by calculating the average cell number in 10000 μm^2 squares, which covered measured area. For P1, P10 and P22 sections, squares covered the entire zone. Proliferative and hypertrophic zones were measured separately (II, Supplementary Fig. 3A). For 2 m sections, 7 squares covered certain area of lateral GP (Fig. 4.).

In addition, total number of cells and zone thickness were analyzed separately in marginal and medial parts of proliferative and hypertrophic zones of P10 sections (II, Supplementary Fig. 3B).

The percentage of proliferating cells was calculated by analyzing the number of PCNA-positive cells per number of total cells.

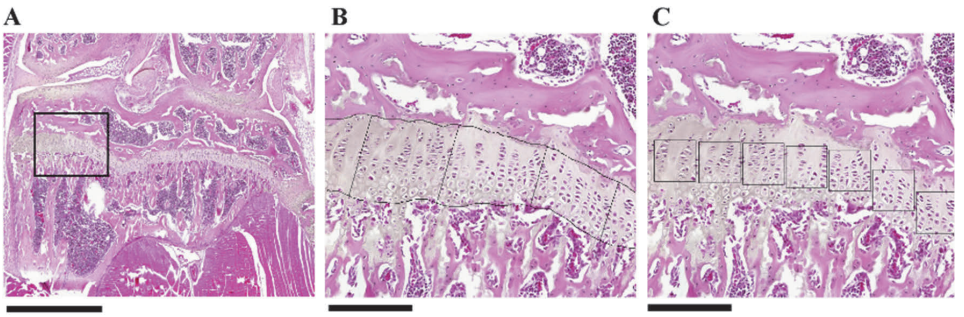

Figure 4. Measurement of thickness and cell density of adult mouse growth plate. HE-stained frontal section of proximal tibia of 2 m WT mouse. Square marks location of magnified pictures (A). GP thickness was analyzed by calculation of average length of four separate measurement lines, evenly distributed inside certain area in lateral half of the section (B). GP cell density was analyzed by calculating average cell number of 7 squares (10 000 μm^2 in size) covering the certain area of GP in lateral half of section (C). Scale bars are 1000 μm in A and 200 μm in B and C.

4.12 Preparation of recombinant Snorc (I, II)

To produce recombinant Snorc for analysis of GAG attachment, human *Snorc* cDNA open reading frame with C-terminal HA-tag was cloned into pcDNA3.1/Hygro(+) vector (Invitrogen) to produce pcDNA3.1/SNORC-HA plasmid (I, Table I). pcDNA3.1/SNORC-HA or pcDNA3.1/Hygro(+) vector were transfected in Cos7 cells (700,000 cells/Ø10 cm Petri dish) using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's instructions and cultured for 2 days in DMEM, 10 % fetal bovine serum (FBS) (Promocell), 50 U/ml penicillin + 50 mg/ml streptomycin (Gibco) (Sundvall et al. 2008). Cells were extracted with 4 M GuHCl, 50 mM sodium acetate, pH 5.8 (Saamanen et al. 1989), containing 1 × Complete protease inhibitor (Gibco). Extract was precipitated in 75% ethanol, 2.5% sodium acetate at 20 °C, and dissolved in 25 mM Tris pH 8.0 + 1 × Complete protease inhibitor.

For slot blot and proliferation assays, Snorc-ectodomain (ECD) recombinant was expressed episomally. The coding sequence of the Snorc-ECD including amino acids 25–92 (AEGPQEPDPTLWNEPIELPSGEGPLESTSHNQEFAVSG PPFPTSAPAPEDSTPPARVDQDGGSLGPGA) and C-terminally fused His and

myc tags were cloned into a modified pCEP-Pu vector in frame with an N-terminal BM-40 signal peptide. HEK293EBNA cells (Invitrogen, routinely authenticated and tested for contaminations) were then transfected for stable expression of expression vector. Episomally expressed Snorc-ECD(His)6myc was collected from serum-free culture medium (Dulbecco's modified Eagle's medium (DMEM)/Ham's F12; PAA) and purified using affinity chromatography on nickel-nitriloacetic acid agarose (Qiagen). Using SDS-PAGE and Western blotting with antibody against the myc-tag (Sigma-Aldrich), purity, integrity, and size of the purified protein were analyzed. The sequence of the purified peptide was analyzed using N-terminal sequencing (Applied Biosystems).

4.13 Chondroitinase ABC digestion (I, II)

Recombinant Snorc-ECD and Snorc were digested by Chondroitinase ABC (Seikagaku, Japan and Sigma-Aldrich respectively), according to the manufacturer's instructions. Subsequently, proteins were separated by SDS-PAGE. Purity of Snorc-ECD was checked using Coomassie Blue staining (II, Supplementary Fig. 4).

4.14 Slot blot analysis (II)

Recombinant proteins Fgf2 (Sigma-Aldrich), platelet derived growth factor (Pdgf) (Immunotools, Germany), Vegf (Immunotools), Wnt inhibitory factor 1 (Wif-1) produced in mouse (Surmann-Schmitt et al. 2009a) and fibronectin, supplied by Klaus von der Mark (Kuhl et al. 1986), were dotted onto a nitrocellulose membrane, blocked using 2% bovine serum albumin (BSA) in tris-buffered saline (TBS) for 1 h and incubated for 1 h with Snorc-ECD-his-myc (2 µg/ml in TBS) at RT. The membrane was washed vigorously and blocked again using BSA. Then the membrane was incubated overnight with a rabbit anti-myc antibody (Sigma–Aldrich) to observe bound Snorc-ECD-His-myc. This bound rabbit anti-myc antibody was detected by incubation with a horseradish peroxidase (HRP) conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories) followed by ECL-based chemiluminescence detection using 2.5 mM Luminol, 0.4 mM p-coumaric acid, 100 mM Tris–HCl, pH 8,5, 0.01% H_2O_2.

4.15 Proliferation assay (II)

Proliferation of C3H10T1/2 cells was studied by a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as earlier reported (Surmann-Schmitt et al. 2009b). In brief, 625 cells were seeded in each well in triplicates in 96-well format. 100 µl medium (DMEM, 10% FCS) with or without of 50 ng/ml Fgf-2 and/or 200 ng/ml Snorc-ECD was used. 20 µl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)) was inserted to the wells after 3 days at 37°C. Then, cells were incubated for 2 h at 37 C, lysed in 100 µl dimethyl sulfoxide DMSO, and formazan-specific absorption at 550 nm was

measured using a means of viable cell counts. Reference absorbance was measured at 670 nm.

4.16 Adenovirus mediated Bmp2 transfer (I, II)

Messenger RNA of mouse fracture callus tissue with adenovirus mediated Bmp2 gene and control transfection were obtained from a previously described study (Uusitalo et al. 2001), where a standardized fracture was induced into tibial bone, and adenoviral vectors RAdBmp2 or RAdLacZ were injected into the location of fracture. Expression of mRNAs of *Snorc* and *Comp* and *28S* rRNA was studied in samples collected at 5^{th}, 7^{th} and 14^{th} day after the generation of fracture using Northern hybridization, as described above.

4.17 Limb bud micromass culture (I)

Mouse limb bud cells were isolated from E11.5 embryos as earlier described (Mello, Tuan 1999), suspended to a density of 2×10^7 cells/ml in DMEM/F12, 10 % FBS (PAA, Pasching), plated in 10 ml of media and incubated for 2 h at 37°C. After this 500 µl of medium (D-MEM/F12, 10% FBS, 50 mg/ml ascorbic acid, 10 nM -glycerophosphate (Sigma-Aldrich) was gently added. To study the regulation by Bmp signaling, medium was supplemented with recombinant Bmp2 (200 ng/ml; Peprotech) or noggin (300 ng/ml, R&D). Medium was changed every other day. Cultures were terminated by fixation with 4% paraformalin for 15 min or subjected to RNA isolation.

4.18 Microscopic imaging (I, II)

In situ hybridization reactions were imaged using virtual microscope (Dot Slide, Olympus). Images of promoter activity X-gal stainings, histochemical and immunohistochemical stainings were taken using Digital microscopy by P-250 FLASH pannoramic slide scanner was used for imaging (3DHistec, Hungary) or Zeiss AxioImager M1 microscope.

4.19 Peripheral quantitative tomography

Collected right femurs were stored in 70% ethanol 4°C before analysis. Measurements and analyses were performed using Norland Stratec XCT research m device with software version 5.4 (Stratec GmbH, Pforzheim, Germany). Slice thickness in all computed tomography was 0.5 mm and a voxel size 0.07 mm. The distal end of femur was used as an anatomical marker. The bone cross-sectional area was imaged at 15 % + 0.6 mm of femur length proximal to this anatomical point. Location of measurement site of cross-sectional images in distal metaphysis is depicted in fig. 3. Volymic bone mineral density (mg/cm3), bone cross-sectional area (mm^2), bone mineral content (mg), cortical bone thickness (mm), endosteal (mm) and periosteal circumference (mm) were recorded as given by the pQCT software. Voxels outside the bone (soft tissue) with lower attenuation coefficients

than the threshold 250 mg/cm^3 were removed within the region of interest. Threshold value 500 mg/cm^3 was used in the measurements of trabecular bone and 710 mg/cm^3 for cortical bone. Calculations of cortical thickness and endosteal and periosteal cicumferences were made using the ring model supplied by the software.

4.20 Statistical analyses (I, II)

Data analysis for two-group comparisons was performed using two-tailed Student's T-test and Microsoft Excel (Microsoft, WA, USA). One way ANOVA and Tukey's Multiple Comparison Test was used in proliferation assay. Values are presented as mean and 95% confidence interval. Adobe Photoshop CS6 (Adobe) and Gnu Image Manipulation Program was used for assembly of microscopic images and graphs.

5. RESULTS

5.1 Snorc is a small type I single-pass transmembrane chondroitin or dermatan sulfate proteoglycan (I)

Predicted Snorc protein precursor (NP_082749.1) length is 121 AA and it contains a putative cleaved ER signal peptide, lumenal/extracellular (L/EC) domain, transmembrane domain and short intracellular domain with topology that N-terminus is lumenal/extracellular and C-terminus cytoplasmic. Conserved functional domains or paralogues were not found. Orthologs were detected from 23 species from all vertebrate classes, but not from invertebrates. The transmembrane domain and cytoplasmic tail were the most conserved regions (I: Fig. 2 A and B).

There were nine threonine/serine residues susceptible to O-linked N-acetylgalactosamine (O-GalNAc) modification in the L/EC domain. The L/EC domain contained also GAG binding consensus sequence ELPSGEG. Recombinant Snorc treatment using chondroitinase ABC verified CS or DS attachment to Snorc core protein (I: Fig. 2 B and C). Putative protein kinase A phosphorylation site, which also was a putative O-linked N-acetyl-glucosamine (O-GlcNAc) modification site was detected in the cytoplasmic tail (I: Fig. 2 B).

5.2 *Snorc* mRNA expression is highly enriched in cartilage (I, II)

Snorc mRNA (NM_028473.1) expression was studied in 19 tissues of P4 mouse using Northern blot assay and in sagittal sections of E16.5 and E18.5 mice using *in situ* hybridization (I: Fig. 4 A, B, C, D, E, F, G, H). Moreover, E13.5 and E16.5 whole-mount stained *Snorc*$^{a/+}$ embryos were used to analyze *Snorc* promoter activity. *Snorc*^{a} allele contain LacZ reporter gene under *Snorc* promoter allowing monitoring of promoter activity by X-gal staining (II: Fig. 2 A). These studies demonstrated highly cartilage-specific *Snorc* expression. *Snorc* expression was detected in hyaline cartilage including cartilage anlage and structures with permanent cartilage, like nasal and tracheal cartilage. *Snorc* expression was not observed in ear, indicating that it is not expressed in elastic cartilage.

Internet databases containing gene tissue distribution data gave clues that prostate and mammary gland may express *Snorc* to some extent. Hence, we studied *Snorc* mRNA expression in these tissues. Compared to epiphysis of P5 *Snorc* $^{a/a}$ mice, *Snorc* expression was 25-fold higher (P=0.002, N=3) in ventral prostate of 4 m WT mouse and 17-fold (P= 0.049, N=3) and 42-fold (P=0.02, N=3) in mammary gland of P22 and 2 m WT mouse respectively. However, *Snorc* expression was 922-fold (P=0.0008, N=3) higher in the knee epiphysis of P5 WT mouse and 154-fold (P=0.007, N=3) and 141-fold (P=0.0001, N=3) higher in AC of P22 and 2 m WT mice respectively. This demonstrates that *Snorc* expression is minor in ventral prostate and mammary gland compared to cartilage samples, especially compared to epiphyseal cartilage of developing mouse. Interestingly, *Snorc* expression restricted to ventral prostate and was not detected in other prostate lobes (Fig. 5 A).

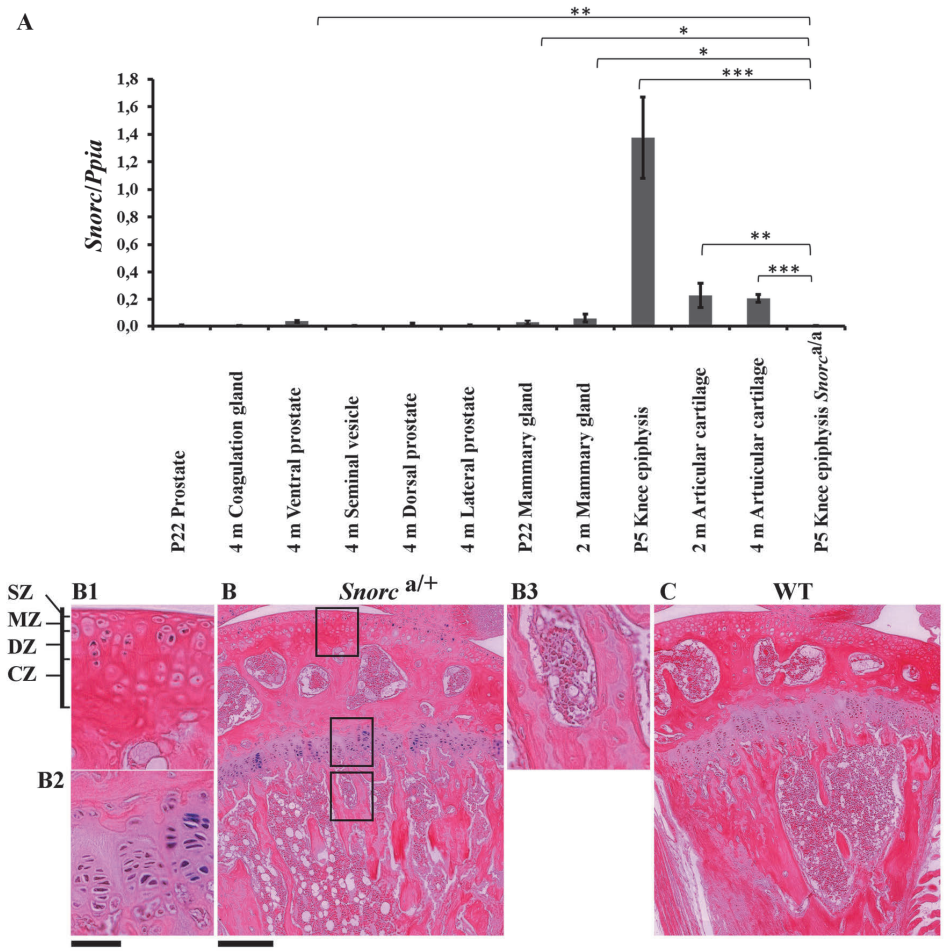

Figure 5. Quantitation of *Snorc* expression in prostate, mammary gland and cartilage and *Snorc* promoter activity in adult epiphyseal cartilage. A. Quantitation of *Snorc* mRNA expression in developing prostate, adult prostate lobes, developing and adult mammary gland, developing postnatal epiphysis and adult articular cartilage using qRT-PCR. Expression was compared to knee epiphysis of P5 $Snorc^{a/a}$ mouse (N=3) and significantly higher expression levels are shown as follows: *=P<0.05, **=P<0.01, ***=P<0.001. Expression of *Ppia* was used for normalization. X-gal stained sagittal sections of proximal tibial epiphysis of 6 m old female heterozygous $Snorc^{a/+}$ (B) and 3 m WT $Snorc^{+/+}$ (C) mouse. Higher magnifications of AC (B1), GP (B2) and trabecular bone (B3) are shown. SZ=superficial zone, MZ=medial zone, DZ=deep zone, CZ=calcified zone. Scale bars are 200 µm in epiphyses (B and C) and 50 µm for magnifications (B1, B2 and B3). X-gal staining enabled visualization of *Snorc* promoter activity via promoter driven LacZ reporter gene of $Snorc^{a}$ allele.

5.3 *Snorc* mRNA is expressed in epiphyseal cartilage throughout mouse life span (I, II)

In mouse knee epiphyseal cartilage, *Snorc* expression was detected from E12.5 forward using Northern blot assay and microarray approach (I: Fig. 1 and 4 J). Expression intensity peaked at P5 and lasted detectable at least until 10 months of

age. Expression profile was highly similar to *Comp* that is a cartilage-specific gene and a marker of proliferative and prehypertrophic cartilage (I: Fig. 4 J).

In situ hybridization revealed that *Snorc* mRNA expression was strongest in proliferative and prehypertrophic zones in embryonic mice distal femoral epiphysis (I: Fig. 4 D, E H I). Instead, in sagittal sections of proximal tibial epiphysis of P22 *Snorc*$^{a/+}$ mouse, the most intense *Snorc* promoter activity was demonstrated in prehypertrophic/hypertrophic chondrocytes surrounding the secondary ossification center and a lower activity was seen in the GP. (II: Fig. 2B). In proximal tibial epiphysis of adult *Snorc*$^{a/+}$ mice *Snorc* promoter activity was still detected in GP and AC. In AC, activity was concentrated in the middle and deep zones (Fig. 5B and C). It is noteworthy, that *Snorc* mRNA expression was not detected in bone in any stages of development.

5.4 Snorc protein was detected in epiphyseal and articular cartilage but also in extracellular space of calcified cartilage and trabecular bone (I, II).

Snorc distribution was analyzed using a combination of two affinity-purified rabbit polyclonal antibodies. These antibodies were raised against two different synthetic peptides whose amino acid sequences were part of the Snorc L/EC domain.

In the sections of proximal tibia of E18.5 and P5 mouse, immunohistochemical staining revealed highest Snorc expression in the proliferating and prehypertrophic zones. Intracellular and pericellular ECM staining was demonstrated throughout the chondroepiphysis, excluding the hypertrophic zone, where only ECM staining was detected (I: Fig. 5A). In the sections of P10 mouse proximal tibia, *Snorc* protein was detected mainly in lower proliferating and prehypertrophic/hypertrophic regions. Immunoreactivity was considerably more intense in pericellular ECM than inside the cell. ECM staining was also demonstrated in primary spongiosa. In secondary ossification center Snorc was seen around cartilage canals. No immunoreactivity was detected in a section of *Snorc*$^{b/b}$ mice proximal tibia, which was used as a negative control, indicating that antibody was specific to Snorc (II: Fig. 2C).

In the adult mice, Snorc immunoreactivity was still observed in AC, GP and trabecular bone in histological sections of proximal tibia. In AC immunoreactivity was detected primarily in uncalcified cartilage (I: Fig. 5B).

5.5 Bmp2 upregulated *Snorc* expression during *in vivo* and *in vitro* chondrogenesis (I)

An *in vivo* mouse closed fracture healing model with adenovirus mediated expression of Bmp2 was used to investigate *Snorc* expression during fracture healing and influence of Bmp2 on *Snorc* expression. *Snorc* and cartilage-specific *Comp* mRNA expression reached the highest level in callus at day 7. At the same time *Snorc* expression was significantly elevated in *Bmp2*-transfected callus compared to control. Bmp2 did not affect to *Comp* expression (I: Fig. 7A).

Limb bud micromass culture model also indicated the effect of Bmp2 on *Snorc* expression. During the chondrocyte proliferative phase, Bmp2 enhanced *Snorc* expression the most but enhancement was observed also during the prehypertrophic and hypertrophic phase. During the proliferative phase, Bmp2 induced also *Sox9*, *Acan* and *Col2a1* expression. Gene expression values are averages from pooled micromass cultures from two separate experiments (I: Fig. 7B).

5.6 Snorc core protein had glycosaminoglycan independent affinity to Fgf2 (II)

Snorc-ECD interaction with several growth factors and matrix components was studied using slot blot analysis. Snorc-ECD had affinity to Fgf2, but not to Pdgf, Vegf, or Wif-1 (II: Fig. 6A). Growth factors Bmp2 and connective tissue growth factor (Ctgf) and ECM proteins fibronectin and type I and II collagens had not either affinity to Snorc (not shown).

Deletion of CS or DS chains from Snorc-ECD using Chondroitinase ABC digestion did not disturb Fgf2 affinity to Snorc-ECD, demonstrating that Snorc core protein is responsible for the affinity with Fgf2 (II: Fig. 6B).

5.7 Fgf2-dependent stimulation of cell growth was inhibited by Snorc-ECD (II)

Fgf2 and/or Snorc-ECD effect on proliferation of C3H10T1/2 embryonic fibroblasts was investigated by measuring cell number after 3 days of culture using a colorimetric assay. Fgf2 stimulated proliferation of C3H10T1/2 cells, but not Snorc-ECD. However, Fgf2-dependent stimulation of proliferation was blocked by Snorc-ECD (II: Fig. 6C).

5.8 *Snorc*-deficiency affected secondary ossification and growth plate thickness in postnatal tibial epiphysis (II)

Alcian blue/alizarin red staining was used to analyze morphology of E20 and P10 hind limbs and X-ray images to study morphology of P22, 2 m and 4 m hind limbs. Proximal tibial SOCs (mineralized part) were smaller both in the male (47%, P = 0.005) and female (46%, P = 0.046) *Snorc*$^{b/b}$ mice at P10 compared to WT. Proximal tibial SOCs were also smaller in males of P22 *Snorc*$^{b/b}$ mice (−9%, P = 0.03) compared with the WT. Furthermore, in females, a similar indicative trend of reduced SOC size, was perceived (P = 0.066) (II: Fig. 3 Supplementary Tables III and IV and Supplementary Fig. 2). Still in 2 m male *Snorc*$^{a/a}$ mice, subchondral bone area of proximal tibia was reduced (7%) being close to statistical significance (P=0.077, N=9). However, in 4 m male *Snorc*$^{a/a}$ mice this trend was not anymore demonstrated (Table 5).

Proximal tibial GPs were thicker in P10 (18%, P = 0.041) and P22 (17%, P = 0.03) male and P22 female (24%, P = 0.002) *Snorc*$^{b/b}$ mice compared to WT (II: Fig. 3 Supplementary Tables III and IV and Supplementary Fig. 2).

Length and width of total and mineralized part of tibia and femur of P10 and P22 *Snorc*$^{b/b}$ mice did not vary significantly from WT mice (II: Fig. 3 and Supplementary Tables III and IV and Supplementary Fig. 2). Also, length of tibia and femur were not altered in 2 m and 4 m male *Snorc*$^{a/a}$ mice compared to WT (Table 6).

Table 5. Area (mm^2) of subchondral bone of proximal tibia of 2 m and 4 m male *Snorc*$^{a/a}$ and WT mice.

	***Snorc*$^{+/+}$**		***Snorc*$^{a/a}$**			**Change**
	Mean ± SD	**N**	**Mean ± SD**	**N**	**P-value$^{\#}$**	**(%)**
2 m	2.02 ± 0.11	9	1.87 ± 0.22	13	0.077	-7.38
4 m	1.91 ± 0.24	10	1.94 ± 0.09	6	0.82	1.29

$^{\#}$Two-tailed Student's T-test.

Table 6. Length (mm) of tibia and femur of 2 m and 4 m *Snorc*$^{a/a}$ and WT male mice.

	***Snorc*$^{+/+}$**		***Snorc*$^{a/a}$**			**Change**
	Mean ± SD	**N**	**Mean ± SD**	**N**	**P-value$^{\#}$**	**(%)**
Tibia						
2 m	17.34 ± 0.60	9	17.17 ± 0.47	14	0.45	-0.99
4 m	18.03 ± 0.30	10	17.86 ± 0.21	7	0.21	-0.96
Femur						
2 m	14.25 ± 0.5	10	14.49 ± 0.24	13	0.1407	1.7
4 m	15.29 ± 0.31	14	15.10 ± 0.28	12	0.1096	-1.3

$^{\#}$Two-tailed Student's T-test.

5.9 Trabecular bone cross-sectional area and endosteal and periosteal perimeter were increased in adult *Snorc*$^{a/a}$ mice

To analyze if *Snorc*-deficiency has an impact on adult femur geometry and mineral density, right femurs of 2 m and 4 m male mice were analyzed using pQCT (N=10 for 2 m and 12 for 4 m mice). Analysis of distal femoral metaphysis (15% of total length from distal end of the bone, Fig. 3) revealed 4.5% (P=0.003) and 4.6% (P<0.001) increase of periosteal perimeter, 7.3% (P<0.001) and 5.7% (P=0.0009) increase of endosteal perimeter, 9.2% (P=0.0035) and 9.3% (P<0.001) increase of total bone cross sectional area (Tt.CSA), 21% (P<0.001) and 9.3% (P=0.02) increase of trabecular bone cross-sectional area (Tb.CSA) and 18.5% (P=0.001) and 22% (P<0.001) increase of trabecular bone mineral content (Tb.BMC) in male 2 m and 4 m *Snorc*$^{a/a}$ mice respectively compared to WT (Tables 7 and 8).

Consistent increase of Tt.CSA in 2 m and 4 m *Snorc*$^{a/a}$ distal femoral metaphysis compared to WT is due to increase of Tb.CSA (Tables 7 and 8). In addition, consistent increase of Tb.BMC in 2 m and 4 m *Snorc*$^{a/a}$ mice compared to WT seem to be more due to increase of Tb.CSA than increase of trabecular bone mineral density (Tb.BMD) because Tb.BMD was increased only in 4 m *Snorc*$^{a/a}$ mouse compared to WT (Tables 7 and 8).

Table 7. pQCT variables of 2 m male distal femoral metaphysis.

	***Snorc*$^{+/+}$**		***Snorc*$^{a/a}$**			
	Mean ± SD	**N**	**Mean ± SD**	**N**	**P-value$^{\#}$**	**Change (%)**
Trabecular bone						
Mineral density (mg/cm^3)	261.7 ± 20.4	10	257.7 ± 23.9	13	0.6773	-1.5
Cross-sectional area (mm^2)	2.41 ± 0.17	10	2.91 ± 0.27	13	0.00004	20.7***
Mineral content (mg/ml)	0.63 ± 0.07	10	0.75 ± 0.08	13	0.0011	18.5**
Cortical bone						
Mineral density (mg/cm^3)	887.4 ± 28.7	10	872.1 ± 32.9	13	0.2578	-1.7
Cross-sectional area (mm^2)	0.72 ± 0.15	10	0.57 ± 0.16	13	0.0387	-19.8*
Mineral content (mg/ml)	0.64 ± 0.15	10	0.50 ± 0.15	13	0.0483	-21.0*
Total bone						
Mineral density (mg/cm^3)	432 ± 35.9	10	395 ± 37.6	13	0.0243	-8.7*
Cross-sectional area (mm^2)	4.13 ± 0.26	10	4.51 ± 0.29	13	0.0035	9.2**
Mineral content (mg/ml)	1.79 ± 0.23	10	1.78 ± 0.19	13	0.8815	-0.7
Cortical thickness (µm)	103.7 ± 21.8	10	78.8 ± 22.5	13	0.0146	-24*
Periosteal perimeter (mm)	7.2 ± 0.22	10	7.53 ± 0.24	13	0.0033	4.5**
Endosteal perimeter (mm)	6.55 ± 0.21	10	7.03 ± 0.30	13	0.0003	7.3***

$^{\#}$Two-tailed Student's T-test. *=P<0.05, **= P<0.01, ***=P<0.001.

Table 8. pQCT variables of 4 m male distal femoral metaphysis.

	***Snorc*$^{+/+}$**		***Snorc*$^{a/a}$**			
	Mean ± SD	**N**	**Mean ± SD**	**N**	**P-value$^{\#}$**	**Change (%)**
Trabecular bone						
Mineral density (mg/cm^3)	238.7 ± 37.2	14	268.2 ± 26.9	12	0.0314	12.4*
Cross-sectional area (mm^2)	2.57 ± 0.18	14	2.81± 0.28	12	0.0155	9.3*
Mineral content (mg/ml)	0.61 ± 0.10	14	0.75 ± 0.06	12	0.0006	21.8***
Cortical bone						
Mineral density (mg/cm^3)	927.3 ± 23.3	14	923.9 ± 16.7	12	0.6762	-0.4
Cross-sectional area (mm^2)	0.87± 0.13	14	0.86 ± 0.11	12	0.9861	-0.1
Mineral content (mg/ml)	0.80 ± 0.14	14	0.80 ± 0.11	12	0.9429	-0.4
Total bone						
Mineral density (mg/cm^3)	432.2 ± 44.1	14	444.3 ± 37.7	12	0.4629	2.8
Cross-sectional area (mm^2)	4.26 ± 0.25	14	4.65± 0.28	12	0.0008	9.3***
Mineral content (mg/ml)	1.84 ± 0.24	14	2.07 ± 0.19	12	0.0161	12.1*
Cortical thickness (µm)	124.9 ± 19.1	14	119.2 ± 15.9	12	0.4218	-4.6
Periosteal perimeter (mm)	7.31 ± 0.22	14	7.64 ± 0.23	12	0.0008	4.6***
Endosteal perimeter (mm)	6.53 ± 0.23	14	6.9 ± 0.27	12	0.0009	5.7***

$^{\#}$Two-tailed Student's T-test. *=P<0.05, **= P<0,01, ***=P<0.001.

5.10 Zone thickness was altered, extra cellular matrix increased and chondrocytes rounded in postnatal *Snorc*$^{b/b}$ mice growth plate (II).

The average thickness and cell density of differentiation zones in the sagittal sections of proximal tibial GP were analysed. At P10, proliferative zone thickness was increased by 24% (P = 0.005) in *Snorc*$^{b/b}$ mice compared to WT, while the hypertrophic zone was not altered. Instead, at P22 both proliferative and hypertrophic zone thicknesses were increased (48%, P<0.0001) and (41%, P=0.0003), respectively in *Snorc*$^{b/b}$ mice. Average cell density was decreased in proliferative regions at both P10 (−11%, P = 0.047) and P22 (−19%, P=0.002) of *Snorc*$^{b/b}$ mice compared to WT, but not in hypertrophic regions (II: Fig. 4 A, C and 5B)

Because zone thicknesses and cell densities varied between medial and marginal regions in proximal tibial sections of P10 *Snorc*$^{b/b}$ mouse compared to WT, these regions were measured separately. Proliferative zone thickness was increased particularly in medial (31%, P = 0.003) but also in marginal region (21%, P = 0.08) in *Snorc*$^{b/b}$ mice. Instead, in hypertrophic zone the thickness was increased only in the medial (26%, P = 0.004), but decreased in the marginal region (−13%, P = 0.01) in *Snorc*$^{b/b}$ mice getting hypertrophic zone to look like V-shaped in *Snorc*$^{b/b}$ mice. Cell density was decreased only in medial region of proliferative zone (−20%, P = 0.03) of *Snorc*$^{b/b}$ mice. Change in total cell number between WT and *Snorc*$^{b/b}$ mice was not detected in any regions. Instead, area of the medial region was increased in both proliferative (42%, P = 0.002) and hypertrophic (17%, P = 0.011) regions of *Snorc*$^{b/b}$ mice showing that the expansion of medial region of proliferative zone of *Snorc*$^{b/b}$ mice is due to increased ECM (II: Table II). In the proliferative zone columns of proximal tibia of P10 and P22, *Snorc*$^{b/b}$ mice chondrocytes were rounded in comparison to WT (II: Fig. 4B). At P1, no differences were observed between the genotypes in the thickness or cell density (not shown), indicating that *Snorc* has a role in postnatal, but not prenatal GP homeostasis.

5.11 Growth plate of adult *Snorc*$^{a/a}$ mouse was hypocellular

GP thickness and cell density were analyzed using HE-stained frontal sections. GP thickness was not altered in proximal tibia of 2 m old *Snorc*$^{a/a}$ mice compared to WT (Fig. 6A, B and E). Instead, density of chondrocytes was reduced 18 % in 2 m old *Snorc*$^{a/a}$ mice GP compared to WT (p=0.048, n=4) (Fig. 6A, A1, B, B1 and F). Immunohistochemical staining of sagittal sections of the proximal tibial GP using antibody against type II collagen demonstrated increased matrix area in GP of 2 m old *Snorc*$^{a/a}$ mice compared to WT (Fig. 6 C and D).

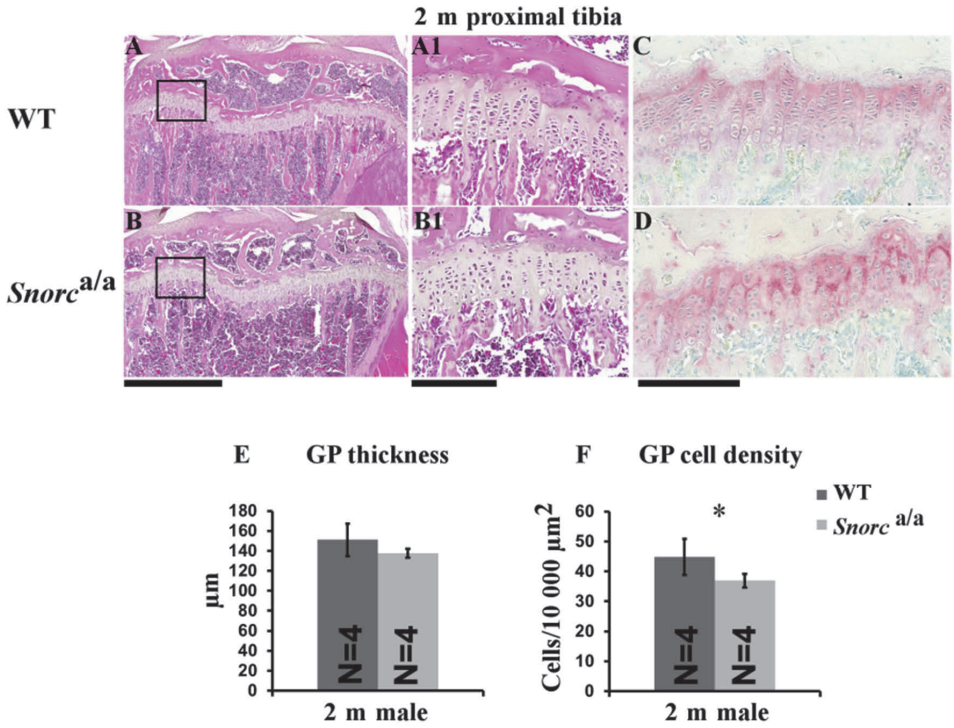

Figure 6. Growth plate of adult *Snorc*$^{a/a}$ mouse. Representative hematoxylin-eosin (HE) stained frontal sections proximal tibias of 2 m old *Snorc*$^{+/+}$ (A) and *Snorc*$^{a/a}$ (B) mice. Higher magnifications of GP cartilage are marked A1 and B1 respectively. Representative immunohistochemical staining of frontal sections of proximal tibias of 2 m old *Snorc*$^{+/+}$ (C) and *Snorc*$^{a/a}$ (D) mice using antibody against type II collagen. Scale bars for A and B are 1 mm, and 200 µm for their magnifications in A1 and B1, and 200 µm for C and D. Thickness (E) and cell density (F) of proximal tibial GP of 2 m old *Snorc*$^{+/+}$ and *Snorc*$^{a/a}$ mice.

5.12 Expression of *Col10a1* and *Ihh* was increased and *Mmp13* decreased in P10 *Snorc*$^{b/b}$ mice proximal tibial epiphysis (II)

Gene expression in knee epiphyses of P10 WT and *Snorc*$^{b/b}$ mice was investigated using qRT-PCR. Expression of *Sox9, Acan* and *Col2a1*, that describe chondrocyte proliferative phase in GP, were not changed. Instead *Col10a1*, marker of hypertrophic phase, expression was increased by 87% (P = 0.009). *Ihh* expression was also increased (60%, P = 0.007) and terminal hypertrophic marker *Mmp13* expression was decreased (−30%, P = 0.032) in *Snorc*$^{b/b}$ mice. On the other hand, a decreasing trend was observed in *Mmp9* expression, but it did not reach statistical significance. (II: Fig. 5A)

6. DISCUSSION

6.1 *Snorc* mRNA expression is cartilage-specific and commence simultaneously with early cartilage genes

Northern blot, qRT-PCR and whole embryo *in situ* hybridization and promoter activity studies revealed that *Snorc* expression is cartilage-specific, excluding minor expression in ventral prostate and mammary gland. Especially high expression was observed in cartilage anlage during development. Genome wide DNA microarray analysis of developing embryonic mouse hind limb and Northern blot analysis of embryonic and postnatal knee epiphysis established the appearance of *Snorc* expression at E12.5, indicating that *Snorc* codes a protein of differentiated cartilage. At this developmental stage in developing hind limb, differentiating chondroblasts begin also to produce and secrete early cartilage matrix proteins, such as Comp and major structural cartilage molecules Acan and type II, IX and XI collagens, which are markers of differentiated cartilage (Goldring, Tsuchimochi & Ijiri 2006, Lefebvre, Smits 2005). After the initiation at E12.5, the *Snorc* gene expression was detected until at least 10 months of age in mouse knee epiphysis.

6.2 *Snorc* mRNA expression is more similar with early cartilage matrix genes than hypertrophic cartilage genes.

During development and adulthood, *Snorc* mRNA expression was detected primarily in proliferative and prehypertrophic zones in metaphyseal GP. The expression, thus, is similar to the expression of early cartilage matrix genes, e.g., *Acan* and *Col2a1* (Lefebvre, Smits 2005). However, minor *Snorc* expression in hypertrophic phase was also observed, especially during postnatal development. In AC of 2 m *Snorc*$^{+/a}$ mice, *Snorc* promoter activity was detected in chondrocytes of MZ and DZ, but not in the CZ and SZ. MZ and DZ of AC are equivalent to proliferative cartilage of GP and CZ to hypertrophic zone (Lefebvre, Smits 2005). Cell culture studies also associated *Snorc* mRNA to proliferative phase together with early cartilage matrix genes (Takacs et al. 2013). In epiphyseal and metaphyseal trabecular bone *Snorc* mRNA was not detected. These observations suggest that *Snorc* is regulated similarly to genes expressed by proliferative and prehypertrophic cartilage, such as *Acan*, *Comp* and *Col2a1*, and the expression is different from genes of hypertrophic cartilage, such as *Col10a1* (Lefebvre, Smits 2005).

6.3 Bmp2 may induce *Snorc* expression via *Sox* trio

In this study, we observed that Bmp2 induced *Snorc* expression in fracture healing callus during cartilage phase *in vivo* and in limb bud micromass culture. *In vitro*, Bmp2 induced also chondrogenic markers like *Sox9, Col2a1* and *Acan*. Noggin, an inhibitor of Bmp signalling, repressed expression of these genes in this

micromass culture. Bmp signalling is required for *Sox* trio expression and is necessary for commence and maintain chondrocyte differentiation (Yoon et al. 2005, Zehentner, Dony & Burtscher 1999, Chimal-Monroy et al. 2003). *Sox* trio genes, the master regulators of chondorogenesis, cooperatively and directly bind to the promoters of important cartilage genes like *Col2a1* and *Acan*, and promote their expression (Ikeda et al. 2004, Lefebvre, Li & de Crombrugghe 1998, Lefebvre, Behringer & de Crombrugghe 2001, Han, Lefebvre 2008, Oh et al. 2010). In the present study, we observed that *Snorc* expression is similar with expression of the Sox trio-regulated genes *Sox9*, *Acan* and *Col2a1* during chondrogenesis. *Snorc* gene 5′-flanking region contains also potential *Sox9* and *Sox5/6* binding sites, suggesting that Bmp2 induces also *Snorc* expression via the *Sox* trio.

6.4 Snorc is a type I single-pass transmembrane proteoglycan interacting with Fgf2

The *Snorc* gene contains three exons and encodes a predicted protein with 121 amino acids containing an ER signal peptide, a L/EC domain, a transmembrane domain and a small intracellular domain in C-terminus. The protein is conserved throughout vertebrates, and the transmembrane and intracellular domains are the most conserved parts of the orthologues. Between mouse and human, the transmembrane domain and cytoplasmic tail are 100 % identical, while the whole protein is 86% identical. Interestingly, several serine/threonine residues predicted to be prone to O-GalNAc glycosylation were highly conserved in Snorc suggesting a functional role for the glycosylation. One of those conserved serines in Snorc L/ECD domain was within the putative conserved GAG attachment sequence (ELPSGEG) similar to that of in Acan at the CS attachment sites (Rodriguez et al. 2006). This suggested GAG attachment instead of O-GalNAc glycosylation and a possibility that Snorc is a PG.

Snorc recombinant protein was produced in mammalian cell line (Cos7). Chondroitinase ABC digestion of the recombinant Snorc revealed CS or DS attachment to the core protein, demonstrating that Snorc is a PG. In addition to Snorc, at least 10 other genes encoding transmembrane PG core proteins are known thus far, including *Sdc1-4*, *Cspg4*, *Cd44*, *Tgfbr3*, *Nrp1* and *Ptprz1* (Couchman 2010, Iozzo, Schaefer 2015). In addition, there is HS PG family named Gpcs, which are associated to membrane with GPI anchor and contain 6 members (Filmus, Capurro & Rast 2008). All membrane PGs are reported to have coreceptor function: they can affect interaction of signalling molecule and its specific high affinity receptor or matrix ligand and its adhesion receptor. This suggests that also Snorc may have these properties (Couchman 2010, Choi et al. 2010, Pap, Bertrand 2013).

In order to find evidence for the possible coreceptor function of Snorc, its interaction partners were searched. Interestingly, interaction of Snorc and Fgf2 was demonstrated and the interaction was independent of GAG attached to Snorc, even

though especially GAG is the interaction site in many PGs (Mythreye, Blobe 2009). However, similar to Snorc, Cspg4 interacts with Fgf2 independent of GAG, and Cspg4 is required for Fgf2 induced angiogenesis, e.g., in corneal angiogenesis model (Ozerdem, Stallcup 2004, Goretzki et al. 1999). Additionally, soluble Snorc-ECD inhibited Fgf2-dependent proliferation of C3H10T1/2 cells, suggesting that Snorc-ECD is able to bind Fgf2 and influence on its signalling function. These results indicated that also Snorc may have coreceptor function affecting on Fgf signalling.

6.5 *In vivo Snorc*-deficiency affects secondary ossification

In the knee epiphyses of postnatal mice, *Snorc* promoter activity localized to prehypertrophic chondrocytes around the forming mineralized SOC. The pericellular appearance of the Snorc protein localized to chondrocytes that were adjacent to cartilage canals and forming SOC. In line with the localization of the promoter activity and protein, a smaller mineralized SOC size in proximal tibias of P10 and P22 *Snorc*-deficient mice was observed in comparison to WT.

In mammals, SOC formation is divided into quiescent angiogenesis and reactive angiogenesis occurring in mouse long bones at P5-7 and P8-10 onwards, respectively. During quiescent angiogenesis, cartilage canal development is accompanied with degradation of ECM by Mmps without the involvement of chondrocyte hypertrophy. Instead, during reactive angiogenesis chondrocytes undergo hypertrophy, terminally differentiated cartilage is vascularized, and ossification occurs in a process that is dependent from angiogenic factors (Blumer, Longato & Fritsch 2008). In *Snorc*-deficient mice, smaller mineralized SOC size was detected during reactive angiogenesis phase. However, this study does not exclude possibility whether abnormalities occurred already during quiescent angiogenesis.

Fgf2 localizes to the chondrocytes adjacent to the SOC, and promote the formation of cartilage canals and angiogenesis (Leach, Sokol & McMurtry 1997, Melton, Clarke & Roach 2006). This, together with observed Snorc interaction with Fgf2, raises a question whether *Snorc*-deficiency disturbs Fgf signalling, and thereby, contributes to impaired SOC development. In addition to Fgf signalling, there are many other signalling pathways participating to SOC development, including Ihh, Wnt and Bmp pathways (Xing et al. 2014, Chen et al. 2008a, Dao et al. 2012). In this study, we demonstrated that *Ihh* was upregulated in P10 knee epiphysis of *Snorc*-deficient mice, and Bmp2 induced *Snorc* expression in fracture healing model during cartilage phase, indicating an interaction of Snorc with several pathways essential for SOC formation.

In skeletally mature mice (2 m males), the decrease in subchondral bone size was still close to significant in *Snorc*-deficient mice compared to WT. However, in 4 m male this indicative trend was no more observed, suggesting that disturbed secondary ossification during postnatal development does not affect the final size of subchondral bone of adult *Snorc*-deficient mice. Maybe the role of *Snorc* in

secondary ossification is reduced alongside with reduction of endochondral ossification, and observed defects in SOC formation during postnatal development can be compensated in adult *Snorc*$^{a/a}$ mice by other genes.

6.6 Molecular mechanisms contributing to the growth plate phenotype of *Snorc*-deficient mouse.

Snorc immunoreactivity was observed in embryonic chondroepiphysis and postnatal AC and GP. In the sections of P10 mouse proximal tibia, Snorc protein was detected primarily in lower proliferating and prehypertrophic/hypertrophic regions. Signal was significantly more intense in pericellular ECM than inside the cell, and ECM immunoreactivity was also established in primary spongiosa. In GP of P10 *Snorc*-deficient mice, proliferative zone was thicker in medial and lateral regions, but hypertrophic zone only in medial regions causing V-shape appearance for hypertrophic zone. Total cell number was not changed so ECM was dilated in these zones. Observed upregulation of *Col10a1* in P10 mice knee epiphyses was in accordance with increased thickness of the hypertrophic zone. Several observations were made, which give reason to conclude molecular mechanisms that may induce the GP phenotype of *Snorc*-deficient mice.

6.6.1 Mmp13 downregulation

Mmp13 downregulation was demonstrated in the knee epiphyses of P10 *Snorc*-deficient mice. Terminal hypertrophic chondrocytes express *Mmp13* and *Mmp9*. Mmp13 cleaves fibrillar collagens into ¼ and ¾ fragments, and they are further digested by gelatinases, such as Mmp9 (Cieplak, Strongin 2017). During endochondral ossification, Mmp13 and 9 are required for matrix modification, which is necessary for chondrocyte apoptosis, vascular invasion and osteoblast recruitment. These in turn are required for POC formation, followed by trabecular bone formation (Stickens et al. 2004). *Mmp13*-deficiency causes remarkable thickening of hypertrophic zone by delaying exit of chondrocytes from GP during postnatal development until age of 12 weeks (Stickens et al. 2004, Wu et al. 2002). In *Snorc*-deficient mice, increase in hypertrophic zone thickness was milder and major increase in GP thickness took place in the proliferative zone suggesting that also other mechanisms are behind *Snorc*-deficient mouse phenotype.

6.6.2 Ihh upregulation

Ihh upregulation was demonstrated in knee epiphyses of P10 *Snorc*-deficient mice compared to WT. In GP, *Ihh* is expressed and secreted by prehypertrophic cells, and transcript is considered as a marker of prehypertrophic zone (MacLean, Kronenberg 2005). Ihh is central in maintaining the proliferative zone by regulating Pthrp expression and is needed also for proliferation and proper arrangement of the columnar proliferating chondrocytes (St-Jacques, Hammerschmidt & McMahon 1999, Karp et al. 2000, Maeda et al. 2010). Therefore, upregulation of *Ihh* expression may partially contribute to expansion of proliferative zone and disturbed columnar organization of chondrocytes.

6.6.3 Changes in Mmp13 and Ihh expression may be due to disturbed Fgf signalling in Snorc-deficient mouse?

Observed downregulation of *Mmp13* and upregulation *Ihh* in knee epiphysis of postnatal *Snorc*-deficient mouse raised a question if *Snorc*-deficiency inhibits Fgf signaling in GP, especially since Fgf2 binds to Snorc. Fgf signaling induces expression of *Mmp13* in chondrocytes via Fgfr1 that is the essential Fgfr in GP hypertrophic zone (Nishida et al. 2011). Chondrocyte-specific deletion of *Fgfr1* results in a transient thickening in hypertrophic zone and a postponement in terminal chondrocyte differentiation and endochondral ossification in mouse GP, without influence on the chondrocyte proliferation (Jacob et al. 2006, Karolak, Yang & Elefteriou 2015). During late embryonic stages and postnatal development, Fgfr3 signalling is an important inhibitor of chondrocyte proliferation and differentiation to prehypertrophic and hypertrophic chondrocytes (Iwata et al. 2000). Fgfr3 signaling inhibits *Ihh* expression and reduces thickness of proliferative and hypertrophic zones of GP (Deng et al. 1996).

6.6.4 Snorc may be a cell-matrix receptor or coreceptor?

In addition to increase in GP zone thickness with reduced cell density, the chondrocyte morphology and organization was disturbed in the proliferative zone at P10 and P22 in *Snorc*-deficient mice compared to WT. This raised a question whether Snorc has a role as a coreceptor for cell matrix receptor, such as integrins, or could it be a cell-matrix receptor itself, similar to some known transmembrane PGs, such as syndecans (Couchman 2010, Choi et al. 2010). *Snorc* deficiency may disturb ligand-receptor interaction and cause the phenotype. In GP, proliferative chondrocytes are organized into columns and flattened. After mitosis, daughter chondrocytes are semi-circular, locating next to each other perpendicular to the long axis of the bone, inside the lacuna. Then these chondrocytes become flattened again and glide on top of each other to form organized columns (Dodds 1930). Integrins are important in this process. Cartilage specific inactivation of 1-integrin changed cell morphology from flattened to more circular and disturbed column arrangement in proliferative zone indicating that integrins are essential cell-matrix receptors affecting cell morphology in this process (Aszodi et al. 2003). Deficiency of important integrin ligand, type IX collagen, can also disturb chondrocyte morphology: Circular chondrocyte morphology with hypocellular center in proliferative GP was observed in collagen, type 9, alpha 1 chain -deficient mice (Kapyla et al. 2004, Blumbach et al. 2008).

6.6.5 Cytoplasmic protein kinase A phosphorylation site

Pthrp sustains the ability of proliferative zone chondrocytes to be proliferative and inhibits their further differentiation. Pthrp signals directly to proliferative chondrocytes via Pthr1 (Chung et al. 1998), activating G_s protein. The G_s protein then activates adenylate cyclase giving rise to the release of cAMP that activates PKA, which mediates Pthrp effects in chondrocytes (Guo et al. 2002, Kronenberg 2006). Pthrp signal-activated PKA can phosphorylate Sox9, which contributes to the delay of proliferative chondrocyte differentiation (Huang et al. 2001). Pthr1

signal gives rise also to the suppression of cell cycle inhibitor P57, which maintains the proliferative zone chondrocytes in proliferative state (MacLean et al. 2004). Snorc protein was localized, e.g., in proliferative and prehypertrophic regions, where Pthr1 is also expressed. Intracellular domain of Snorc contains a putative PKA phosphorylation site, which is a Yin-Yang site, meaning that it is also a putative O-GlcNAc glycosylation site. Phosphorylation and glycosylation events are mutually exclusive in this site. This raises a question whether Snorc participates in Pthrp signalling pathway through its potential Yin-Yang site and regulates the chondrocyte differentiation this way.

6.7 Are changes of metaphyseal bone of adult *Snorc*-deficient mice derived from cartilage or bone?

Using pQCT, anabolic phenotype was demonstrated in distal femoral metaphyseal bone of *Snorc*-deficient mouse including, e.g., increased peri- and endosteal perimeters and trabecular bone cross sectional area.

Observed changes in Tb.CSA and periosteal and endosteal perimeters in *Snorc*$^{a/a}$ mice femoral metaphysis can be due to changes in GP during development. Trabecular bone is built around cartilage model, so alterations in this model have an effect on forming bone (Pechak, Kujawa & Caplan 1986). In this study, it was observed that the proximal tibial GP cell density in columnar proliferating zone was reduced and matrix portion was seemingly increased in the age of P10 and P22 in *Snorc*-deficient mice proximal tibial epiphysis compared to WT. So, increased cartilage matrix may also cause increased Tb.CSA observed in *Snorc*-deficient mice.

It is possible that reduced Mmp13 activity in terminal hypertrophic chondrocytes may disturb cartilage ECM degradation in terminal chondrocytes and trabecular bone, and cause increased Tb.CSA observed in *Snorc*-deficient mice compared to WT. Proliferative and medial hypertrophic zones at P10 and proliferative and hypertrphic zones at P22 were thicker and *Mmp13* expression was reduced in proximal tibial GPs of *Snorc*-deficient mice compared to WT. In *Mmp13*-deficient mice, increased hypertrophic differentiation in GP of developing mouse and increased trabecular bone in adults was detected due to improper ECM degradation during cartilage terminal differentiation (Stickens et al. 2004). Interestingly, Mmp13 is induced by Fgf2 at least in AC (Nishida et al. 2011, Krejci et al. 2005).

Snorc mRNA expression was restricted strictly to cartilage during endochondral ossification. Instead, immunoreactivity against Snorc-ECD was detected in lower proliferating and prehypertrophic/hypertrophic regions of GP cartilage primarily in ECM, but also in primary spongiosa exclusively in ECM. This suggest that *Snorc* is expressed in chondrocytes, but PG is released to ECM of cartilage and trabecular bone, e.g., via ectodomain shedding or chondrocyte apoptosis. This raised a question whether Snorc or its ECD has a function in

metaphyseal trabecular bone, affecting, e.g., on osteocyte differentiation, matrix production or bone remodelling during development.

Interaction of Snorc-ECD with Fgf2 was demonstrated in this study. Interestingly, Fgf2 is an important regulator of bone mass and is expressed by periosteal cells and osteoblasts (Sabbieti et al. 1999). *Fgf2*$^{-/-}$ (4.5 and 8 months old) mice demonstrated decreased trabecular bone volume and bone formation rates compared to WT (Montero et al. 2000). Again, in transgenic mice overexpressing secreted (18 kD) Fgf2 isoform in osteoblastic lineage increased bone volume, trabecular thickness and cortical bone area without dwarfism was observed compared to WT (Xiao et al. 2009). These findings were suggested to be explained at least partially by modulation of Wnt signalling pathway via Fgfr2 mediated signalling, causing increased proliferation and differentiation of osteoblasts (Xiao et al. 2009). In addition, endogenous Fgf2 is needed for maximal response of Bmp2 in bone (Naganawa et al. 2008). Since anabolic bone phenotype was observed in *Snorc*$^{a/a}$ mice metaphyseal bone, Snorc may inhibit of Fgf signalling via Fgfr2 in metaphyseal osteoblasts.

Main Fgf receptors in bone are Fgfr1 and Fgfr2 (Ornitz, Marie 2015). Mice with conditional inactivation of *Fgfr2* in chondrocyte and osteoblastic lineage using Dermo1 promoter-mediated Cre had postnatal dwarfism, reduced bone mineral density and less trabecular bone compared to WT mice. Proliferation of osteoprogenitors and the anabolic function of mature osteoblasts were severely affected (Yu et al. 2003). Instead, in mice with conditional inactivation of *Fgfr1* in osteoprogenitors or differentiated osteoblasts bone mass was increased in adults including increased trabecular bone compared to WT. Increased trabecular bone resulted from increased matrix deposition (Jacob et al. 2006). Putting these together, *Snorc*-deficiency may have changed balance between Fgfr1 and Fgfr2 signalling in osteoblasts so that Fgfr2 signalling diminished in relation to Fgfr1 signalling.

7. SUMMARY AND CONCLUSIONS

In this study, a novel cartilage specific transcript, which is highly conserved in vertebrates was identified using genome wide microarray approach. Based on *in silico* studies, this transcript encodes a transmembrane protein with the topology C-terminus intracellular. N-terminal part includes a predicted ER signal peptide, and lumenal/extracellular domain contains several conserved serine and threonine residues prone to O-GalNAc glycosylation. One of those serines locates inside GAG binding consensus sequence, ELPSGEG. Enzymatic digestion studies verified CS or DS GAG attachment to Snorc, indicating that Snorc is a type I single-pass transmembrane PG.

Snorc mRNA expression was limited precisely to cartilage during endochondral ossification, while immunoreactivity against Snorc-ECD was observed also in ECM of trabecular bone. In GP cartilage, the immunoreactivity was detected in lower proliferating, prehypertrophic and hypertrophic zones principally in ECM. This suggests that *Snorc* is expressed in chondrocytes, while the protein is released to ECM, e.g., via ectodomain shedding or chondrocyte apoptosis.

Snorc expression was induced by Bmp2 in cartilage, indicating that it is a novel target for the Bmp2-regulated chondrogenesis. Bmp2 is necessary for chondrogenesis promoting it via Sox trio. Similarities in expression between *Snorc* and genes regulated directly by Sox trio raised a question whether *Snorc* is also regulated directly by Sox trio. This needs to be further studied.

Interaction of Snorc and Fgf2 was demonstrated, and it was shown to be independent of GAG. Moreover, soluble Snorc-ECD inhibited Fgf2-dependent proliferation of C3H10T1/2 cells, suggesting that Snorc-ECD can bind Fgf2 and influence its signalling. These results, with the knowledge that coreceptor function is very common in membrane PGs, suggest that Snorc present with coreceptor function affect Fgf signalling.

Studies using *Snorc*-deficient mouse revealed that *Snorc* has a role in regulation of chondrocyte maturation in GP and SOC, and trabecular bone formation during postnatal development. *Snorc*-deficiency resulted in decreased SOC size in proximal tibial epiphysis. Expression of Snorc also emphasized to cartilage surrounding cartilage canals and mineralized SOC. Induction of SOC formation by Fgf2 and interaction of Snorc and Fgf2 raised a question if disturbed Fgf signalling impaired SOC formation in *Snorc*-deficient mice. However, there are also several other signalling pathways important for SOC formation, including Ihh and Bmp. *Ihh* upregulation in P10 knee epiphysis of *Snorc*-deficient mice and induction of *Snorc* expression by Bmp2 during cartilage maturation was observed. In adult *Snorc*-deficient mice, size of subchondral bone was not altered, indicating that developmental disturbation of SOC formation did not decrease subchondral bone size permanently.

In *Snorc*-deficient mice during postnatal development, proliferative and hypertrophic zones in GP were thicker, especially in the medial part. Reduced cell density due to enlarged ECM volume and abnormal rounded proliferative chondrocyte morphology was also detected. Potential molecular mechanisms contributing to the GP phenotype may be associated, e.g., to observed *Mmp13* downregulation and *Ihh* upregulation in P10 *Snorc*-deficient mouse epiphysis. Mmp13 is necessary for ECM modification in terminal hypertrophic chondrocytes and its deficiency causes hypertrophic zone thickening. Ihh is an important regulator of proliferative zone thickness. Mmp13 is up-regulated and Ihh down-regulated by Fgf signalling in GP, suggesting that Snorc may inhibit Fgf signalling in GP, and so influence on *Mmp13* and *Ihh* expression. Deficiency of matrix receptors like integrins and their ligands are associated to altered chondrocyte morphology in GP suggesting that Snorc may act as matrix receptor or coreceptor for such receptor.

Alterations in femoral metaphyseal bone, as increased trabecular and Tt.CSA area and increased mineral content, were demonstrated in adult 2 m and 4 m *Snorc* deficient mouse. These alterations may be due to alterations in GP or direct effect of Snorc PG on osteocytes in metaphyseal bone.

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9. REFERENCES

Afratis, N.A., Nikitovic, D., Multhaupt, H.A., Theocharis, A.D., Couchman, J.R. & Karamanos, N.K. 2017, "Syndecans - key regulators of cell signaling and biological functions", *The FEBS journal,* vol. 284, no. 1, pp. 27-41.

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A. & de Crombrugghe, B. 2002, "The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6", *Genes & development,* vol. 16, no. 21, pp. 2813-2828.

Amizuka, N., Warshawsky, H., Henderson, J.E., Goltzman, D. & Karaplis, A.C. 1994, "Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation", *The Journal of cell biology,* vol. 126, no. 6, pp. 1611-1623.

Andrade, A.C., Nilsson, O., Barnes, K.M. & Baron, J. 2007, "Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation", *Bone,* vol. 40, no. 5, pp. 1361-1369.

Aszodi, A., Hunziker, E.B., Brakebusch, C. & Fassler, R. 2003, "Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis", *Genes & development,* vol. 17, no. 19, pp. 2465-2479.

Bell, D.M., Leung, K.K., Wheatley, S.C., Ng, L.J., Zhou, S., Ling, K.W., Sham, M.H., Koopman, P., Tam, P.P. & Cheah, K.S. 1997, "SOX9 directly regulates the type-II collagen gene", *Nature genetics,* vol. 16, no. 2, pp. 174-178.

Bi, W., Huang, W., Whitworth, D.J., Deng, J.M., Zhang, Z., Behringer, R.R. & de Crombrugghe, B. 2001, "Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 98, no. 12, pp. 6698-6703.

Blumbach, K., Niehoff, A., Paulsson, M. & Zaucke, F. 2008, "Ablation of collagen IX and COMP disrupts epiphyseal cartilage architecture", *Matrix biology,* vol. 27, no. 4, pp. 306-318.

Blumer, M.J., Longato, S. & Fritsch, H. 2008, "Structure, formation and role of cartilage canals in the developing bone", *Annals of Anatomy,* vol. 190, no. 4, pp. 305-315.

Blumer, M.J., Longato, S., Schwarzer, C. & Fritsch, H. 2007, "Bone development in the femoral epiphysis of mice: the role of cartilage canals and the fate of resting chondrocytes", *Developmental dynamics,* vol. 236, no. 8, pp. 2077-2088.

Bonafe, L., Cormier-Daire, V., Hall, C., Lachman, R., Mortier, G., Mundlos, S., Nishimura, G., Sangiorgi, L., Savarirayan, R., Sillence, D., Spranger, J., Superti-Furga, A., Warman, M. & Unger, S. 2015, "Nosology and classification of genetic skeletal disorders: 2015 revision", *American journal of medical genetics.Part A,* vol. 167A, no. 12, pp. 2869-2892.

Borochowitz, Z., Sabo, E., Misselevitch, I. & Boss, J.H. 1998, "Autosomal-recessive omodysplasia: prenatal diagnosis and histomorphometric assessment of the physeal plates of the long bones", *American Journal of medical genetics,* vol. 76, no. 3, pp. 238-244.

Bridgewater, L.C., Lefebvre, V. & de Crombrugghe, B. 1998, "Chondrocyte-specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer", *The Journal of biological chemistry,* vol. 273, no. 24, pp. 14998-15006.

Briggs, M.D., Bell, P.A., Wright, M.J. & Pirog, K.A. 2015, "New therapeutic targets in rare genetic skeletal diseases", *Expert opinion on orphan drugs,* vol. 3, no. 10, pp. 1137-1154.

Byers, P.D. & Brown, R.A. 2006, "Cell columns in articular cartilage physes questioned: a review", *Osteoarthritis and cartilage,* vol. 14, no. 1, pp. 3-12.

Campos-Xavier, A.B., Martinet, D., Bateman, J., Belluoccio, D., Rowley, L., Tan, T.Y., Baxova, A., Gustavson, K.H., Borochowitz, Z.U., Innes, A.M., Unger, S., Beckmann, J.S., Mittaz, L., Ballhausen, D., Superti-Furga, A., Savarirayan, R. & Bonafe, L. 2009, "Mutations in the heparan-sulfate proteoglycan glypican 6 (GPC6) impair endochondral ossification and cause recessive omodysplasia", *American Journal of Human Genetics,* vol. 84, no. 6, pp. 760-770.

Capurro, M.I., Li, F. & Filmus, J. 2009, "Overgrowth of a mouse model of Simpson-Golabi-Behmel syndrome is partly mediated by Indian hedgehog", *EMBO reports,* vol. 10, no. 8, pp. 901-907.

Capurro, M.I., Xu, P., Shi, W., Li, F., Jia, A. & Filmus, J. 2008, "Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding", *Developmental cell,* vol. 14, no. 5, pp. 700-711.

Chen, L., Li, C., Qiao, W., Xu, X. & Deng, C. 2001, "A Ser(365)-->Cys mutation of fibroblast growth factor receptor 3 in mouse downregulates Ihh/PTHrP signals and causes severe achondroplasia", *Human molecular genetics,* vol. 10, no. 5, pp. 457-465.

Chen, M., Zhu, M., Awad, H., Li, T.F., Sheu, T.J., Boyce, B.F., Chen, D. & O'Keefe, R.J. 2008a, "Inhibition of beta-catenin signaling causes defects in postnatal cartilage development", *Journal of cell science,* vol. 121, no. Pt 9, pp. 1455-1465.

Chen, X., Macica, C.M., Nasiri, A. & Broadus, A.E. 2008b, "Regulation of articular chondrocyte proliferation and differentiation by indian hedgehog and parathyroid hormone-related protein in mice", *Arthritis and rheumatism,* vol. 58, no. 12, pp. 3788-3797.

Chiao, E., Fisher, P., Crisponi, L., Deiana, M., Dragatsis, I., Schlessinger, D., Pilia, G. & Efstratiadis, A. 2002, "Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signaling", *Developmental biology,* vol. 243, no. 1, pp. 185-206.

Chimal-Monroy, J., Rodriguez-Leon, J., Montero, J.A., Ganan, Y., Macias, D., Merino, R. & Hurle, J.M. 2003, "Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling", *Developmental biology,* vol. 257, no. 2, pp. 292-301.

Choi, Y., Chung, H., Jung, H., Couchman, J.R. & Oh, E.S. 2010, "Syndecans as cell surface receptors: Unique structure equates with functional diversity", *Matrix biology,* vol. 30, no. 2, pp. 93-99.

Chung, U.I., Lanske, B., Lee, K., Li, E. & Kronenberg, H. 1998, "The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 95, no. 22, pp. 13030-13035.

Cieplak, P. & Strongin, A.Y. 2017, "Matrix metalloproteinases - From the cleavage data to the prediction tools and beyond", *Biochimica et biophysica acta,* vol. 1864, no. 11, pp. 1952-1963.

Cortes, M., Baria, A.T. & Schwartz, N.B. 2009, "Sulfation of chondroitin sulfate proteoglycans is necessary for proper Indian hedgehog signaling in the developing growth plate", *Development,* vol. 136, no. 10, pp. 1697-1706.

Couchman, J.R. 2010, "Transmembrane signaling proteoglycans", *Annual Review of cell and developmental biology,* vol. 26, pp. 89-114.

Couchman, J.R., Gopal, S., Lim, H.C., Norgaard, S. & Multhaupt, H.A. 2015, "Fell-Muir Lecture: Syndecans: from peripheral coreceptors to mainstream regulators of cell behaviour", *International Journal of experimental pathology,* vol. 96, no. 1, pp. 1-10.

Couchman, J.R. & Pataki, C.A. 2012, "An introduction to proteoglycans and their localization", *The Journal of histochemistry and cytochemistry,* vol. 60, no. 12, pp. 885-897.

Couly, G.F., Coltey, P.M. & Le Douarin, N.M. 1993, "The triple origin of skull in higher vertebrates: a study in quail-chick chimeras", *Development,* vol. 117, no. 2, pp. 409-429.

Dao, D.Y., Jonason, J.H., Zhang, Y., Hsu, W., Chen, D., Hilton, M.J. & O'Keefe, R.J. 2012, "Cartilage-specific beta-catenin signaling regulates chondrocyte maturation, generation of ossification centers, and perichondrial bone formation during skeletal development", *Journal of bone and mineral research,* vol. 27, no. 8, pp. 1680-1694.

Degnin, C.R., Laederich, M.B. & Horton, W.A. 2010, "FGFs in endochondral skeletal development", *Journal of cellular biochemistry,* vol. 110, no. 5, pp. 1046-1057.

Delgado-Martos, M.J., Touza Fernandez, A., Canillas, F., Quintana-Villamandos, B., Santos del Riego, S., Delgado-Martos, E., Martos-Rodriguez, A. & Delgado-Baeza, E. 2013, "Does the epiphyseal cartilage of the long bones have one or two ossification fronts?", *Medical hypotheses,* vol. 81, no. 4, pp. 695-700.

DeLise, A.M. & Tuan, R.S. 2002, "Alterations in the spatiotemporal expression pattern and function of N-cadherin inhibit cellular condensation and chondrogenesis of limb mesenchymal cells in vitro", *Journal of cellular biochemistry,* vol. 87, no. 3, pp. 342-359.

Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A. & Leder, P. 1996, "Fibroblast growth factor receptor 3 is a negative regulator of bone growth", *Cell,* vol. 84, no. 6, pp. 911-921.

Dodds, G.S. 1930, "Row formation and other types of arrangement of cartilage cells in endochondral ossification", *The Anatomical Record,* vol. 46, no. 4, pp. 385-399.

Dwivedi, P.P., Lam, N. & Powell, B.C. 2013, "Boning up on glypicans--opportunities for new insights into bone biology", *Cell biochemistry and function,* vol. 31, no. 2, pp. 91-114.

Eames, B.F., Allen, N., Young, J., Kaplan, A., Helms, J.A. & Schneider, R.A. 2007, "Skeletogenesis in the swell shark Cephaloscyllium ventriosum", *Journal of anatomy,* vol. 210, no. 5, pp. 542-554.

Eswarakumar, V.P. & Schlessinger, J. 2007, "Skeletal overgrowth is mediated by deficiency in a specific isoform of fibroblast growth factor receptor 3", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 10, pp. 3937-3942.

Eyre, D.R., Weis, M.A. & Wu, J.J. 2006, "Articular cartilage collagen: an irreplaceable framework?", *European cells & materials,* vol. 12, pp. 57-63.

Filmus, J., Capurro, M. & Rast, J. 2008, "Glypicans", *Genome biology,* vol. 9, no. 5, pp. 224-2008-9-5-224.

Fuerer, C., Habib, S.J. & Nusse, R. 2010, "A study on the interactions between heparan sulfate proteoglycans and Wnt proteins", *Developmental dynamics :* vol. 239, no. 1, pp. 184-190.

Funari, V.A., Day, A., Krakow, D., Cohn, Z.A., Chen, Z., Nelson, S.F. & Cohn, D.H. 2007, "Cartilage-selective genes identified in genome-scale analysis of non-cartilage and cartilage gene expression", *BMC genomics,* vol. 8, pp. 165.

Gao, L., Sheu, T.J., Dong, Y., Hoak, D.M., Zuscik, M.J., Schwarz, E.M., Hilton, M.J., O'Keefe, R.J. & Jonason, J.H. 2013, "TAK1 regulates SOX9 expression in chondrocytes and is essential for postnatal development of the growth plate and articular cartilages", *Journal of cell science,* vol. 126, no. Pt 24, pp. 5704-5713.

Goldring, M.B., Tsuchimochi, K. & Ijiri, K. 2006, "The control of chondrogenesis", *Journal of cellular biochemistry,* vol. 97, no. 1, pp. 33-44.

Goretzki, L., Burg, M.A., Grako, K.A. & Stallcup, W.B. 1999, "High-affinity binding of basic fibroblast growth factor and platelet-derived growth factor-AA to the core protein of the NG2 proteoglycan", *The Journal of biological chemistry,* vol. 274, no. 24, pp. 16831-16837.

Green, J.D., Tollemar, V., Dougherty, M., Yan, Z., Yin, L., Ye, J., Collier, Z., Mohammed, M.K., Haydon, R.C., Luu, H.H., Kang, R., Lee, M.J., Ho, S.H., He, T.C., Shi, L.L. & Athiviraham, A. 2015, "Multifaceted signaling regulators of chondrogenesis: Implications in cartilage regeneration and tissue engineering", *Genes & diseases,* vol. 2, no. 4, pp. 307-327.

Guo, J., Chung, U.I., Kondo, H., Bringhurst, F.R. & Kronenberg, H.M. 2002, "The PTH/PTHrP receptor can delay chondrocyte hypertrophy in vivo without activating phospholipase C", *Developmental cell,* vol. 3, no. 2, pp. 183-194.

Hall, B.K. & Miyake, T. 2000, "All for one and one for all: condensations and the initiation of skeletal development", *BioEssays : news and reviews in molecular, cellular and developmental biology,* vol. 22, no. 2, pp. 138-147.

Han, Y. & Lefebvre, V. 2008, "L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer", *Molecular and cellular biology,* vol. 28, no. 16, pp. 4999-5013.

Harkness, L., Taipaleenmäki, H., Mahmood, A., Frandsen, U., Säämänen, A.M., Kassem, M. & Abdallah, B.M. 2009, "Isolation and differentiation of chondrocytic cells derived from human embryonic stem cells using dlk1/FA1 as a novel surface marker", *Stem cell reviews,* vol. 5, no. 4, pp. 353-368.

Heinegård, D. 2009, "Proteoglycans and more--from molecules to biology", *International journal of experimental pathology,* vol. 90, no. 6, pp. 575-586.

Heinonen, J., Taipaleenmäki, H., Roering, P., Takatalo, M., Harkness, L., Sandholm, J.,

Uusitalo-Järvinen, H., Kassem, M., Kiviranta, I., Laitala-Leinonen, T. & Säämänen, A.M. 2011, "Snorc is a novel cartilage specific small membrane proteoglycan expressed in differentiating and articular chondrocytes", *Osteoarthritis and cartilage,* vol. 19, no. 8, pp. 1026-1035.

Hermanns, P., Unger, S., Rossi, A., Perez-Aytes, A., Cortina, H., Bonafe, L., Boccone, L., Setzu, V., Dutoit, M., Sangiorgi, L., Pecora, F., Reicherter, K., Nishimura, G., Spranger, J., Zabel, B. & Superti-Furga, A. 2008, "Congenital joint dislocations caused by carbohydrate sulfotransferase 3 deficiency in recessive Larsen syndrome and humero-spinal dysostosis", *American Journal of Human Genetics,* vol. 82, no. 6, pp. 1368-1374.

Hilton, M.J., Tu, X., Cook, J., Hu, H. & Long, F. 2005, "Ihh controls cartilage development by antagonizing Gli3, but requires additional effectors to regulate osteoblast and vascular development", *Development,* vol. 132, no. 19, pp. 4339-4351.

Hilton, M.J., Tu, X. & Long, F. 2007, "Tamoxifen-inducible gene deletion reveals a distinct cell type associated with trabecular bone, and direct regulation of PTHrP expression and chondrocyte morphology by Ihh in growth region cartilage", *Developmental biology,* vol. 308, no. 1, pp. 93-105.

Hirai, T., Chagin, A.S., Kobayashi, T., Mackem, S. & Kronenberg, H.M. 2011, "Parathyroid hormone/parathyroid hormone-related protein receptor signaling is required for maintenance of the growth plate in postnatal life", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 108, no. 1, pp. 191-196.

Huang, W., Chung, U.I., Kronenberg, H.M. & de Crombrugghe, B. 2001, "The chondrogenic transcription factor Sox9 is a target of signaling by the parathyroid hormone-related peptide in the growth plate of endochondral bones", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 98, no. 1, pp. 160-165.

Hung, I.H., Yu, K., Lavine, K.J. & Ornitz, D.M. 2007, "FGF9 regulates early hypertrophic chondrocyte differentiation and skeletal vascularization in the developing stylopod", *Developmental biology,* vol. 307, no. 2, pp. 300-313.

Ikeda, T., Kamekura, S., Mabuchi, A., Kou, I., Seki, S., Takato, T., Nakamura, K., Kawaguchi, H., Ikegawa, S. & Chung, U.I. 2004, "The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage", *Arthritis and rheumatism,* vol. 50, no. 11, pp. 3561-3573.

Iozzo, R.V. 1997, "The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth", *Critical reviews in biochemistry and molecular biology,* vol. 32, no. 2, pp. 141-174.

Iozzo, R.V. & Schaefer, L. 2015, "Proteoglycan form and function: A comprehensive nomenclature of proteoglycans", *Matrix biology,* vol. 42, pp. 11-55.

Iwata, T., Chen, L., Li, C., Ovchinnikov, D.A., Behringer, R.R., Francomano, C.A. & Deng, C.X. 2000, "A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos", *Human molecular genetics,* vol. 9, no. 11, pp. 1603-1613.

Jacob, A.L., Smith, C., Partanen, J. & Ornitz, D.M. 2006, "Fibroblast growth factor receptor 1 signaling in the osteo-chondrogenic cell lineage regulates sequential steps of osteoblast maturation", *Developmental biology,* vol. 296, no. 2, pp. 315-328.

Jobert, A.S., Zhang, P., Couvineau, A., Bonaventure, J., Roume, J., Le Merrer, M. & Silve, C. 1998, "Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia", *The Journal of clinical investigation,* vol. 102, no. 1, pp. 34-40.

Kalamajski, S. & Oldberg, A. 2010, "The role of small leucine-rich proteoglycans in collagen fibrillogenesis", *Matrix biology,* vol. 29, no. 4, pp. 248-253.

Käpyla, J., Jäälinoja, J., Tulla, M., Ylöstalo, J., Nissinen, L., Viitasalo, T., Vehviläinen, P., Marjomäki, V., Nykvist, P., Säämänen, A.M., Farndale, R.W., Birk, D.E., Ala-Kokko, L. & Heino, J. 2004, "The fibril-associated collagen IX provides a novel mechanism for cell adhesion to cartilaginous matrix", *The Journal of biological chemistry,* vol. 279, no. 49, pp. 51677-51687.

Karaplis, A.C., Luz, A., Glowacki, J., Bronson, R.T., Tybulewicz, V.L., Kronenberg, H.M. &

Mulligan, R.C. 1994, "Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene", *Genes & development,* vol. 8, no. 3, pp. 277-289.

Karolak, M.R., Yang, X. & Elefteriou, F. 2015, "FGFR1 signaling in hypertrophic chondrocytes is attenuated by the Ras-GAP neurofibromin during endochondral bone formation", *Human molecular genetics,* vol. 24, no. 9, pp. 2552-2564.

Karp, S.J., Schipani, E., St-Jacques, B., Hunzelman, J., Kronenberg, H. & McMahon, A.P. 2000, "Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways", *Development,* vol. 127, no. 3, pp. 543-548.

Karperien, M., van der Harten, H.J., van Schooten, R., Farih-Sips, H., den Hollander, N.S., Kneppers, S.L., Nijweide, P., Papapoulos, S.E. & Lowik, C.W. 1999, "A frame-shift mutation in the type I parathyroid hormone (PTH)/PTH-related peptide receptor causing Blomstrand lethal osteochondrodysplasia", *The Journal of clinical endocrinology and metabolism,* vol. 84, no. 10, pp. 3713-3720.

Kawasaki, K. & Weiss, K.M. 2006, "Evolutionary genetics of vertebrate tissue mineralization: the origin and evolution of the secretory calcium-binding phosphoprotein family", *Journal of experimental zoology.Part B, Molecular and developmental evolution,* vol. 306, no. 3, pp. 295-316.

Kiani, C., Chen, L., Wu, Y.J., Yee, A.J. & Yang, B.B. 2002, "Structure and function of aggrecan", *Cell research,* vol. 12, no. 1, pp. 19-32.

Kimmel, C.A. & Trammell, C. 1981, "A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals", *Stain technology,* vol. 56, no. 5, pp. 271-273.

Klatt, A.R., Becker, A.K., Neacsu, C.D., Paulsson, M. & Wagener, R. 2011, "The matrilins: modulators of extracellular matrix assembly", *The international journal of biochemistry & cell biology,* vol. 43, no. 3, pp. 320-330.

Kluppel, M., Wight, T.N., Chan, C., Hinek, A. & Wrana, J.L. 2005, "Maintenance of chondroitin sulfation balance by chondroitin-4-sulfotransferase 1 is required for chondrocyte development and growth factor signaling during cartilage morphogenesis", *Development (Cambridge, England),* vol. 132, no. 17, pp. 3989-4003.

Kobayashi, T., Lyons, K.M., McMahon, A.P. & Kronenberg, H.M. 2005a, "BMP signaling stimulates cellular differentiation at multiple steps during cartilage development", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 50, pp. 18023-18027.

Kobayashi, T., Soegiarto, D.W., Yang, Y., Lanske, B., Schipani, E., McMahon, A.P. & Kronenberg, H.M. 2005b, "Indian hedgehog stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP", *The Journal of clinical investigation,* vol. 115, no. 7, pp. 1734-1742.

Koziel, L., Kunath, M., Kelly, O.G. & Vortkamp, A. 2004, "Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification", *Developmental cell,* vol. 6, no. 6, pp. 801-813.

Krejci, P., Masri, B., Fontaine, V., Mekikian, P.B., Weis, M., Prats, H. & Wilcox, W.R. 2005, "Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis", *Journal of cell science,* vol. 118, no. Pt 21, pp. 5089-5100.

Kronenberg, H.M. 2006, "PTHrP and skeletal development", *Annals of the New York Academy of Sciences,* vol. 1068, pp. 1-13.

Kronenberg, H.M. 2003, "Developmental regulation of the growth plate", *Nature,* vol. 423, no. 6937, pp. 332-336.

Kuhl, U., Ocalan, M., Timpl, R. & von der Mark, K. 1986, "Role of laminin and fibronectin in selecting myogenic versus fibrogenic cells from skeletal muscle cells in vitro", *Developmental biology,* vol. 117, no. 2, pp. 628-635.

Kurima, K., Warman, M.L., Krishnan, S., Domowicz, M., Krueger, R.C.,Jr, Deyrup, A. & Schwartz, N.B. 1998, "A member of a family of sulfate-activating enzymes causes murine brachymorphism", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 95, no. 15, pp. 8681-8685.

Lanske, B., Karaplis, A.C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L.H., Ho, C., Mulligan, R.C., Abou-Samra,

A.B., Juppner, H., Segre, G.V. & Kronenberg, H.M. 1996, "PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth", *Science,* vol. 273, no. 5275, pp. 663-666.

Lazarus, J.E., Hegde, A., Andrade, A.C., Nilsson, O. & Baron, J. 2007, "Fibroblast growth factor expression in the postnatal growth plate", *Bone,* vol. 40, no. 3, pp. 577-586.

Leach, R.M.,Jr, Sokol, C. & McMurtry, J.P. 1997, "Immunolocalization of basic fibroblast growth factor in porcine epiphyseal growth plate", *Domestic animal endocrinology,* vol. 14, no. 2, pp. 129-132.

Lee, K., Lanske, B., Karaplis, A.C., Deeds, J.D., Kohno, H., Nissenson, R.A., Kronenberg, H.M. & Segre, G.V. 1996, "Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development", *Endocrinology,* vol. 137, no. 11, pp. 5109-5118.

Lefebvre, V., Behringer, R.R. & de Crombrugghe, B. 2001, "L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway", *Osteoarthritis and cartilage,* vol. 9 Suppl A, pp. S69-75.

Lefebvre, V., Li, P. & de Crombrugghe, B. 1998, "A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene", *The EMBO journal,* vol. 17, no. 19, pp. 5718-5733.

Lefebvre, V. & Smits, P. 2005, "Transcriptional control of chondrocyte fate and differentiation", *Birth defects research.Part C, Embryo today : reviews,* vol. 75, no. 3, pp. 200-212.

Liu, C.F., Samsa, W.E., Zhou, G. & Lefebvre, V. 2017, "Transcriptional control of chondrocyte specification and differentiation", *Seminars in cell & developmental biology,* vol. 62, pp. 34-49.

Liu, Z., Lavine, K.J., Hung, I.H. & Ornitz, D.M. 2007, "FGF18 is required for early chondrocyte proliferation, hypertrophy and vascular invasion of the growth plate", *Developmental biology,* vol. 302, no. 1, pp. 80-91.

Liu, Z., Xu, J., Colvin, J.S. & Ornitz, D.M. 2002, "Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18", *Genes & development,* vol. 16, no. 7, pp. 859-869.

Long, F. & Ornitz, D.M. 2013, "Development of the endochondral skeleton", *Cold Spring Harbor perspectives in biology,* vol. 5, no. 1, pp. a008334.

Long, F., Zhang, X.M., Karp, S., Yang, Y. & McMahon, A.P. 2001, "Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation", *Development,* vol. 128, no. 24, pp. 5099-5108.

Lord, M.S. & Whitelock, J.M. 2013, "Recombinant production of proteoglycans and their bioactive domains", *The FEBS journal,* vol. 280, no. 10, pp. 2490-2510.

Mackie, E.J., Ahmed, Y.A., Tatarczuch, L., Chen, K.S. & Mirams, M. 2008, "Endochondral ossification: how cartilage is converted into bone in the developing skeleton", *The international journal of biochemistry & cell biology,* vol. 40, no. 1, pp. 46-62.

Mackie, E.J., Tatarczuch, L. & Mirams, M. 2011, "The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification", *The Journal of endocrinology,* vol. 211, no. 2, pp. 109-121.

MacLean, H.E., Guo, J., Knight, M.C., Zhang, P., Cobrinik, D. & Kronenberg, H.M. 2004, "The cyclin-dependent kinase inhibitor p57(Kip2) mediates proliferative actions of PTHrP in chondrocytes", *The Journal of clinical investigation,* vol. 113, no. 9, pp. 1334-1343.

MacLean, H.E. & Kronenberg, H.M. 2005, "Localization of Indian hedgehog and PTH/PTHrP receptor expression in relation to chondrocyte proliferation during mouse bone development", *Development, growth & differentiation,* vol. 47, no. 2, pp. 59-63.

Maeda, Y., Schipani, E., Densmore, M.J. & Lanske, B. 2010, "Partial rescue of postnatal growth plate abnormalities in Ihh mutants by expression of a constitutively active PTH/PTHrP receptor", *Bone,* vol. 46, no. 2, pp. 472-478.

Makarenkova, H.P., Hoffman, M.P., Beenken, A., Eliseenkova, A.V., Meech, R., Tsau, C., Patel, V.N., Lang, R.A. & Mohammadi, M. 2009, "Differential interactions of FGFs with heparan sulfate control gradient formation and branching morphogenesis", *Science signaling,* vol. 2, no. 88, pp. ra55.

Manon-Jensen, T., Itoh, Y. & Couchman, J.R. 2010, "Proteoglycans in health and disease: the multiple roles of syndecan shedding", *The FEBS journal,* vol. 277, no. 19, pp. 3876-3889.

Mariani, F.V. & Martin, G.R. 2003, "Deciphering skeletal patterning: clues from the limb", *Nature,* vol. 423, no. 6937, pp. 319-325.

Massague, J. & Gomis, R.R. 2006, "The logic of TGFbeta signaling", *FEBS letters,* vol. 580, no. 12, pp. 2811-2820.

Matsumoto, K., Li, Y., Jakuba, C., Sugiyama, Y., Sayo, T., Okuno, M., Dealy, C.N., Toole, B.P., Takeda, J., Yamaguchi, Y. & Kosher, R.A. 2009, "Conditional inactivation of Has2 reveals a crucial role for hyaluronan in skeletal growth, patterning, chondrocyte maturation and joint formation in the developing limb", *Development,* vol. 136, no. 16, pp. 2825-2835.

Matsuo, I. & Kimura-Yoshida, C. 2014, "Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis", *Philosophical transactions of the Royal Society of London.Series B, Biological sciences,* vol. 369, no. 1657, pp. 10.1098/rstb.2013.0545.

McClive, P.J. & Sinclair, A.H. 2001, "Rapid DNA extraction and PCR-sexing of mouse embryos", *Molecular reproduction and development,* vol. 60, no. 2, pp. 225-226.

McEwan, P.A., Scott, P.G., Bishop, P.N. & Bella, J. 2006, "Structural correlations in the family of small leucine-rich repeat proteins and proteoglycans", *Journal of structural biology,* vol. 155, no. 2, pp. 294-305.

Melton, J.T., Clarke, N.M. & Roach, H.I. 2006, "Matrix metalloproteinase-9 induces the formation of cartilage canals in the chondroepiphysis of the neonatal rabbit", *The Journal of bone and joint surgery.American volume,* vol. 88 Suppl 3, pp. 155-161.

Mello, M.A. & Tuan, R.S. 1999, "High density micromass cultures of embryonic limb bud mesenchymal cells: an in vitro model of endochondral skeletal development", *In Vitro Cellular & Developmental Biology - Animal,* vol. 35, no. 5, pp. 262-269.

Memon, A.R. & Quinlan, J.F. 2012, "Surgical treatment of articular cartilage defects in the knee: are we winning?", *Advances in orthopedics,* vol. 2012, pp. 528423.

Michigami, T. 2013, "Regulatory mechanisms for the development of growth plate cartilage", *Cellular and molecular life sciences,* vol. 70, no. 22, pp. 4213-4221.

Minina, E., Kreschel, C., Naski, M.C., Ornitz, D.M. & Vortkamp, A. 2002, "Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation", *Developmental cell,* vol. 3, no. 3, pp. 439-449.

Miyazono, K., Kamiya, Y. & Morikawa, M. 2010, "Bone morphogenetic protein receptors and signal transduction", *Journal of biochemistry,* vol. 147, no. 1, pp. 35-51.

Montero, A., Okada, Y., Tomita, M., Ito, M., Tsurukami, H., Nakamura, T., Doetschman, T., Coffin, J.D. & Hurley, M.M. 2000, "Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation", *The Journal of clinical investigation,* vol. 105, no. 8, pp. 1085-1093.

Moretti, S., Armougom, F., Wallace, I.M., Higgins, D.G., Jongeneel, C.V. & Notredame, C. 2007, "The M-Coffee web server: a meta-method for computing multiple sequence alignments by combining alternative alignment methods", *Nucleic acids research,* vol. 35, no. Web Server issue, pp. W645-8.

Moustakas, A. & Heldin, C.H. 2005, "Non-Smad TGF-beta signals", *Journal of cell science,* vol. 118, no. Pt 16, pp. 3573-3584.

Murakami, S., Balmes, G., McKinney, S., Zhang, Z., Givol, D. & de Crombrugghe, B. 2004, "Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype", *Genes & development,* vol. 18, no. 3, pp. 290-305.

Mythreye, K. & Blobe, G.C. 2009, "Proteoglycan signaling co-receptors: roles in cell adhesion, migration and invasion", *Cellular signalling,* vol. 21, no. 11, pp. 1548-1558.

Naganawa, T., Xiao, L., Coffin, J.D., Doetschman, T., Sabbieti, M.G., Agas, D. & Hurley, M.M. 2008, "Reduced expression and function of bone morphogenetic protein-2 in bones of Fgf2 null mice", *Journal of cellular biochemistry,* vol. 103, no. 6, pp. 1975-1988.

Naski, M.C., Colvin, J.S., Coffin, J.D. & Ornitz, D.M. 1998, "Repression of hedgehog signaling and BMP4 expression in growth plate cartilage

by fibroblast growth factor receptor 3", *Development,* vol. 125, no. 24, pp. 4977-4988.

Nilsson, O., Parker, E.A., Hegde, A., Chau, M., Barnes, K.M. & Baron, J. 2007, "Gradients in bone morphogenetic protein-related gene expression across the growth plate", *The Journal of endocrinology,* vol. 193, no. 1, pp. 75-84.

Nishida, T., Kubota, S., Aoyama, E., Janune, D., Maeda, A. & Takigawa, M. 2011, "Effect of CCN2 on FGF2-induced proliferation and MMP9 and MMP13 productions by chondrocytes", *Endocrinology,* vol. 152, no. 11, pp. 4232-4241.

Oh, C.D., Maity, S.N., Lu, J.F., Zhang, J., Liang, S., Coustry, F., de Crombrugghe, B. & Yasuda, H. 2010, "Identification of SOX9 interaction sites in the genome of chondrocytes", *PloS one,* vol. 5, no. 4, pp. e10113.

Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N. & Takada, S. 2002, "FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis", *Genes & development,* vol. 16, no. 7, pp. 870-879.

Olsen, B.R., Reginato, A.M. & Wang, W. 2000, "Bone development", *Annual review of cell and developmental biology,* vol. 16, pp. 191-220.

Ono, K., Karolak, M.R., Ndong Jde, L., Wang, W., Yang, X. & Elefteriou, F. 2013, "The ras-GTPase activity of neurofibromin restrains ERK-dependent FGFR signaling during endochondral bone formation", *Human molecular genetics,* vol. 22, no. 15, pp. 3048-3062.

Ornitz, D.M. & Marie, P.J. 2015, "Fibroblast growth factor signaling in skeletal development and disease", *Genes & development,* vol. 29, no. 14, pp. 1463-1486.

Ornitz, D.M. & Marie, P.J. 2002, "FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease", *Genes & development,* vol. 16, no. 12, pp. 1446-1465.

Ozerdem, U. & Stallcup, W.B. 2004, "Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan", *Angiogenesis,* vol. 7, no. 3, pp. 269-276.

Pacifici, M., Shimo, T., Gentili, C., Kirsch, T., Freeman, T.A., Enomoto-Iwamoto, M., Iwamoto, M. & Koyama, E. 2005, "Syndecan-3: a cell-surface heparan sulfate proteoglycan important for chondrocyte proliferation and function during limb skeletogenesis", *Journal of bone and mineral metabolism,* vol. 23, no. 3, pp. 191-199.

Pap, T. & Bertrand, J. 2013, "Syndecans in cartilage breakdown and synovial inflammation", *Nature reviews.Rheumatology,* vol. 9, no. 1, pp. 43-55.

Pechak, D.G., Kujawa, M.J. & Caplan, A.I. 1986, "Morphological and histochemical events during first bone formation in embryonic chick limbs", *Bone,* vol. 7, no. 6, pp. 441-458.

Pilia, G., Hughes-Benzie, R.M., MacKenzie, A., Baybayan, P., Chen, E.Y., Huber, R., Neri, G., Cao, A., Forabosco, A. & Schlessinger, D. 1996, "Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome", *Nature genetics,* vol. 12, no. 3, pp. 241-247.

Priore, R., Dailey, L. & Basilico, C. 2006, "Downregulation of Akt activity contributes to the growth arrest induced by FGF in chondrocytes", *Journal of cellular physiology,* vol. 207, no. 3, pp. 800-808.

Qi, H., Jin, M., Duan, Y., Du, X., Zhang, Y., Ren, F., Wang, Y., Tian, Q., Wang, X., Wang, Q., Zhu, Y., Xie, Y., Liu, C., Cao, X., Mishina, Y., Chen, D., Deng, C.X., Chang, Z. & Chen, L. 2014, "FGFR3 induces degradation of BMP type I receptor to regulate skeletal development", *Biochimica et biophysica acta,* vol. 1843, no. 7, pp. 1237-1247.

Retting, K.N., Song, B., Yoon, B.S. & Lyons, K.M. 2009, "BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation", *Development,* vol. 136, no. 7, pp. 1093-1104.

Rigueur, D., Brugger, S., Anbarchian, T., Kim, J.K., Lee, Y. & Lyons, K.M. 2015, "The type I BMP receptor ACVR1/ALK2 is required for chondrogenesis during development", *Journal of bone and mineral research,* vol. 30, no. 4, pp. 733-741.

Rivas, R. & Shapiro, F. 2002, "Structural stages in the development of the long bones and epiphyses: a study in the New Zealand white rabbit", *The Journal of bone and joint surgery.American volume,* vol. 84-A, no. 1, pp. 85-100.

Robbins, D.J., Fei, D.L. & Riobo, N.A. 2012, "The Hedgehog signal transduction network", *Science signaling,* vol. 5, no. 246, pp. re6.

Rodriguez, E., Roland, S.K., Plaas, A. & Roughley, P.J. 2006, "The glycosaminoglycan attachment regions of human aggrecan", *The Journal of biological chemistry,* vol. 281, no. 27, pp. 18444-18450.

Roughley, P.J. 2006, "The structure and function of cartilage proteoglycans", *European cells & materials,* vol. 12, pp. 92-101.

Säämänen, A.K., Salminen, H.J., Dean, P.B., De Crombrugghe, B., Vuorio, E.I. & Metsäranta, M.P. 2000, "Osteoarthritis-like lesions in transgenic mice harboring a small deletion mutation in type II collagen gene", *Osteoarthritis and cartilage,* vol. 8, no. 4, pp. 248-257.

Säämänen, A.M., Tammi, M., Kiviranta, I., Jurvelin, J. & Helminen, H.J. 1989, "Levels of chondroitin-6-sulfate and nonaggregating proteoglycans at articular cartilage contact sites in the knees of young dogs subjected to moderate running exercise", *Arthritis and rheumatism,* vol. 32, no. 10, pp. 1282-1292.

Sabbieti, M.G., Marchetti, L., Abreu, C., Montero, A., Hand, A.R., Raisz, L.G. & Hurley, M.M. 1999, "Prostaglandins regulate the expression of fibroblast growth factor-2 in bone", *Endocrinology,* vol. 140, no. 1, pp. 434-444.

Sahni, M., Raz, R., Coffin, J.D., Levy, D. & Basilico, C. 2001, "STAT1 mediates the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF2", *Development,* vol. 128, no. 11, pp. 2119-2129.

Sakai, K. & Miyazaki, J. 1997, "A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission", *Biochemical and biophysical research communications,* vol. 237, no. 2, pp. 318-324.

Salminen, H., Perala, M., Lorenzo, P., Saxne, T., Heinegard, D., Säämänen, A.M. & Vuorio, E. 2000, "Up-regulation of cartilage oligomeric matrix protein at the onset of articular cartilage degeneration in a transgenic mouse model of osteoarthritis", *Arthritis and rheumatism,* vol. 43, no. 8, pp. 1742-1748.

Salminen, H., Vuorio, E. & Säämänen, A.M. 2001, "Expression of Sox9 and type IIA procollagen during attempted repair of articular cartilage damage in a transgenic mouse model of osteoarthritis", *Arthritis and rheumatism,* vol. 44, no. 4, pp. 947-955.

Sandell, L.J., Sugai, J.V. & Trippel, S.B. 1994, "Expression of collagens I, II, X, and XI and aggrecan mRNAs by bovine growth plate chondrocytes in situ", *Journal of orthopaedic research : official publication of the Orthopaedic Research Society,* vol. 12, no. 1, pp. 1-14.

Sasarman, F., Maftei, C., Campeau, P.M., Brunel-Guitton, C., Mitchell, G.A. & Allard, P. 2016, "Biosynthesis of glycosaminoglycans: associated disorders and biochemical tests", *Journal of inherited metabolic disease,* vol. 39, no. 2, pp. 173-188.

Schipani, E., Langman, C.B., Parfitt, A.M., Jensen, G.S., Kikuchi, S., Kooh, S.W., Cole, W.G. & Juppner, H. 1996, "Constitutively activated receptors for parathyroid hormone and parathyroid hormone-related peptide in Jansen's metaphyseal chondrodysplasia", *The New England journal of medicine,* vol. 335, no. 10, pp. 708-714.

Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovacs, C.S., Lee, K., Pirro, A., Kronenberg, H.M. & Juppner, H. 1997, "Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 94, no. 25, pp. 13689-13694.

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. 2012, "NIH Image to ImageJ: 25 years of image analysis", *Nature methods,* vol. 9, no. 7, pp. 671-675.

Shimeld, S.M. & Holland, P.W. 2000, "Vertebrate innovations", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, no. 9, pp. 4449-4452.

Shimizu, H., Yokoyama, S. & Asahara, H. 2007, "Growth and differentiation of the developing limb bud from the perspective of chondrogenesis", *Development, growth & differentiation,* vol. 49, no. 6, pp. 449-454.

Shimokawa, K., Kimura-Yoshida, C., Nagai, N., Mukai, K., Matsubara, K., Watanabe, H., Matsuda, Y., Mochida, K. & Matsuo, I. 2011,

"Cell surface heparan sulfate chains regulate local reception of FGF signaling in the mouse embryo", *Developmental cell,* vol. 21, no. 2, pp. 257-272.

Shu, B., Zhang, M., Xie, R., Wang, M., Jin, H., Hou, W., Tang, D., Harris, S.E., Mishina, Y., O'Keefe, R.J., Hilton, M.J., Wang, Y. & Chen, D. 2011, "BMP2, but not BMP4, is crucial for chondrocyte proliferation and maturation during endochondral bone development", *Journal of cell science,* vol. 124, no. Pt 20, pp. 3428-3440.

Skarnes, W.C., Rosen, B., West, A.P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A.O., Thomas, M., Harrow, J., Cox, T., Jackson, D., Severin, J., Biggs, P., Fu, J., Nefedov, M., de Jong, P.J., Stewart, A.F. & Bradley, A. 2011, "A conditional knockout resource for the genome-wide study of mouse gene function", *Nature,* vol. 474, no. 7351, pp. 337-342.

Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J. M., Behringer, R.R., de Crombrugghe, B., Lefebvre, V. 2001, "The transcription factors L-Sox5 and Sox6 are essential for cartilage formation", *Developmental Cell*, vol. 1, no. 2, pp. 277-290.

Spector, T.D. & MacGregor, A.J. 2004, "Risk factors for osteoarthritis: genetics", *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society,* vol. 12 Suppl A, pp. S39-44.

Sterner, E., Meli, L., Kwon, S.J., Dordick, J.S. & Linhardt, R.J. 2013, "FGF-FGFR signaling mediated through glycosaminoglycans in microtiter plate and cell-based microarray platforms", *Biochemistry,* vol. 52, no. 50, pp. 9009-9019.

Stickens, D., Behonick, D.J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., Fosang, A.J., Schorpp-Kistner, M., Angel, P. & Werb, Z. 2004, "Altered endochondral bone development in matrix metalloproteinase 13-deficient mice", *Development,* vol. 131, no. 23, pp. 5883-5895.

St-Jacques, B., Hammerschmidt, M. & McMahon, A.P. 1999, "Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation", *Genes & development,* vol. 13, no. 16, pp. 2072-2086.

Sundvall, M., Korhonen, A., Paatero, I., Gaudio, E., Melino, G., Croce, C.M., Aqeilan, R.I. & Elenius, K. 2008, "Isoform-specific monoubiquitination, endocytosis, and degradation of alternatively spliced ErbB4 isoforms", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 105, no. 11, pp. 4162-4167.

Surmann-Schmitt, C., Widmann, N., Dietz, U., Saeger, B., Eitzinger, N., Nakamura, Y., Rattel, M., Latham, R., Hartmann, C., von der Mark, H., Schett, G., von der Mark, K. & Stock, M. 2009a, "Wif-1 is expressed at cartilage-mesenchyme interfaces and impedes Wnt3a-mediated inhibition of chondrogenesis", *Journal of cell science,* vol. 122, no. Pt 20, pp. 3627-3637.

Surmann-Schmitt, C., Widmann, N., Mallein-Gerin, F., von der Mark, K. & Stock, M. 2009b, "Stable subclones of the chondrogenic murine cell line MC615 mimic distinct stages of chondrocyte differentiation", *Journal of cellular biochemistry,* vol. 108, no. 3, pp. 589-599.

Takacs, R., Matta, C., Somogyi, C., Juhasz, T. & Zakany, R. 2013, "Comparative analysis of osteogenic/chondrogenic differentiation potential in primary limb bud-derived and C3H10T1/2 cell line-based mouse micromass cultures", *International journal of molecular sciences,* vol. 14, no. 8, pp. 16141-16167.

Takatalo, M., Jarvinen, E., Laitinen, S., Thesleff, I. & Ronnholm, R. 2008, "Expression of the novel Golgi protein GoPro49 is developmentally regulated during mesenchymal differentiation", *Developmental dynamics,* vol. 237, no. 8, pp. 2243-2255.

Testa, G., Schaft, J., van der Hoeven, F., Glaser, S., Anastassiadis, K., Zhang, Y., Hermann, T., Stremmel, W. & Stewart, A.F. 2004, "A reliable lacZ expression reporter cassette for multipurpose, knockout-first alleles", *Genesis,* vol. 38, no. 3, pp. 151-158.

Tillgren, V., Onnerfjord, P., Haglund, L. & Heinegard, D. 2009, "The tyrosine sulfate-rich domains of the LRR proteins fibromodulin and osteoadherin bind motifs of basic clusters in a variety of heparin-binding proteins, including bioactive factors", *The Journal of biological chemistry,* vol. 284, no. 42, pp. 28543-28553.

Tsumaki, N., Nakase, T., Miyaji, T., Kakiuchi, M., Kimura, T., Ochi, T. & Yoshikawa, H. 2002, "Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis", *Journal of bone and mineral research,* vol. 17, no. 5, pp. 898-906.

Tzeng, H.E., Chen, P.C., Lin, K.W., Lin, C.Y., Tsai, C.H., Han, S.M., Teng, C.L., Hwang, W.L., Wang, S.W. & Tang, C.H. 2015, "Basic fibroblast growth factor induces VEGF expression in chondrosarcoma cells and subsequently promotes endothelial progenitor cell-primed angiogenesis", *Clinical science,* vol. 129, no. 2, pp. 147-158.

Usami, Y., Gunawardena, A.T., Iwamoto, M. & Enomoto-Iwamoto, M. 2016, "Wnt signaling in cartilage development and diseases: lessons from animal studies", *Laboratory investigation,* vol. 96, no. 2, pp. 186-196.

Uusitalo, H., Hiltunen, A., Ahonen, M., Gao, T.J., Lefebvre, V., Harley, V., Kähäri, V.M. & Vuorio, E. 2001, "Accelerated up-regulation of L-Sox5, Sox6, and Sox9 by BMP-2 gene transfer during murine fracture healing", *Journal of bone and mineral research,* vol. 16, no. 10, pp. 1837-1845.

van der Eerden, B.C., Karperien, M., Gevers, E.F., Lowik, C.W. & Wit, J.M. 2000, "Expression of Indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth", *Journal of bone and mineral research,* vol. 15, no. 6, pp. 1045-1055.

van der Heide, D. & Ende-Visser, M.P. 1980, "T4, T3 and reverse T3 in the plasma of rats during the first 3 months of life", *Acta Endocrinologica,* vol. 93, no. 4, pp. 448-454.

Viviano, B.L., Silverstein, L., Pflederer, C., Paine-Saunders, S., Mills, K. & Saunders, S. 2005, "Altered hematopoiesis in glypican-3-deficient mice results in decreased osteoclast differentiation and a delay in endochondral ossification", *Developmental biology,* vol. 282, no. 1, pp. 152-162.

Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M. & Tabin, C.J. 1996, "Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein", *Science,* vol. 273, no. 5275, pp. 613-622.

Wada, H. 2010, "Origin and genetic evolution of the vertebrate skeleton", *Zoological Science,* vol. 27, no. 2, pp. 119-123.

Wadhwa, S., Embree, M., Ameye, L. & Young, M.F. 2005, "Mice deficient in biglycan and fibromodulin as a model for temporomandibular joint osteoarthritis", *Cells, tissues, organs,* vol. 181, no. 3-4, pp. 136-143.

Warman, M.L., Cormier-Daire, V., Hall, C., Krakow, D., Lachman, R., LeMerrer, M., Mortier, G., Mundlos, S., Nishimura, G., Rimoin, D.L., Robertson, S., Savarirayan, R., Sillence, D., Spranger, J., Unger, S., Zabel, B. & Superti-Furga, A. 2011, "Nosology and classification of genetic skeletal disorders: 2010 revision", *American journal of medical genetics.Part A,* vol. 155A, no. 5, pp. 943-968.

Weir, E.C., Philbrick, W.M., Amling, M., Neff, L.A., Baron, R. & Broadus, A.E. 1996, "Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 93, no. 19, pp. 10240-10245.

White, D.G., Hershey, H.P., Moss, J.J., Daniels, H., Tuan, R.S. & Bennett, V.D. 2003, "Functional analysis of fibronectin isoforms in chondrogenesis: Full-length recombinant mesenchymal fibronectin reduces spreading and promotes condensation and chondrogenesis of limb mesenchymal cells", *Differentiation; research in biological diversity,* vol. 71, no. 4-5, pp. 251-261.

Wu, C.W., Tchetina, E.V., Mwale, F., Hasty, K., Pidoux, I., Reiner, A., Chen, J., Van Wart, H.E. & Poole, A.R. 2002, "Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization", *Journal of bone and mineral research,* vol. 17, no. 4, pp. 639-651.

Wu, F., Zhang, Y., Sun, B., McMahon, A.P. & Wang, Y. 2017, "Hedgehog signaling: from basic biology to cancer therapy", *Cell chemical biology,* vol. 24, no. 3, pp. 252-280.

Xiao, L., Liu, P., Li, X., Doetschman, T., Coffin, J.D., Drissi, H. & Hurley, M.M. 2009, "Exported 18-kDa isoform of fibroblast growth factor-2 is a critical determinant of bone mass in mice", *The Journal of biological chemistry,* vol. 284, no. 5, pp. 3170-3182.

Xing, W., Cheng, S., Wergedal, J. & Mohan, S. 2014, "Epiphyseal chondrocyte secondary ossification centers require thyroid hormone activation of Indian hedgehog and osterix signaling", *Journal of bone and mineral research,* vol. 29, no. 10, pp. 2262-2275.

Yan, D., Chen, D., Cool, S.M., van Wijnen, A.J., Mikecz, K., Murphy, G. & Im, H.J. 2011, "Fibroblast growth factor receptor 1 is principally responsible for fibroblast growth factor 2-induced catabolic activities in human articular chondrocytes", *Arthritis research & therapy,* vol. 13, no. 4, pp. R130.

Yan, D., Chen, D. & Im, H.J. 2012, "Fibroblast growth factor-2 promotes catabolism via FGFR1-Ras-Raf-MEK1/2-ERK1/2 axis that coordinates with the PKCdelta pathway in human articular chondrocytes", *Journal of cellular biochemistry,* vol. 113, no. 9, pp. 2856-2865.

Yan, D. & Lin, X. 2009, "Shaping morphogen gradients by proteoglycans", *Cold Spring Harbor perspectives in biology,* vol. 1, no. 3, pp. a002493.

Yi, S.E., Daluiski, A., Pederson, R., Rosen, V. & Lyons, K.M. 2000, "The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb", *Development (Cambridge, England),* vol. 127, no. 3, pp. 621-630.

Yong, L.W. & Yu, J.K. 2016, "Tracing the evolutionary origin of vertebrate skeletal tissues: insights from cephalochordate amphioxus", *Current opinion in genetics & development,* vol. 39, pp. 55-62.

Yoon, B.S., Ovchinnikov, D.A., Yoshii, I., Mishina, Y., Behringer, R.R. & Lyons, K.M. 2005, "Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 102, no. 14, pp. 5062-5067.

Yoon, B.S., Pogue, R., Ovchinnikov, D.A., Yoshii, I., Mishina, Y., Behringer, R.R. & Lyons, K.M. 2006, "BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways", *Development,* vol. 133, no. 23, pp. 4667-4678.

Yu, K., Xu, J., Liu, Z., Sosic, D., Shao, J., Olson, E.N., Towler, D.A. & Ornitz, D.M. 2003, "Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth", *Development,* vol. 130, no. 13, pp. 3063-3074.

Zehentner, B.K., Dony, C. & Burtscher, H. 1999, "The transcription factor Sox9 is involved in BMP-2 signaling", *Journal of bone and mineral research,* vol. 14, no. 10, pp. 1734-1741.

Zhang, F.P., Domanskyi, A., Palvimo, J.J., Sariola, H., Partanen, J. & Jänne, O.A. 2007, "An adenosine triphosphatase of the sucrose nonfermenting 2 family, androgen receptor-interacting protein 4, is essential for mouse embryonic development and cell proliferation", *Molecular endocrinology,* vol. 21, no. 6, pp. 1430-1442.

Zhang, Y. & Jordan, J.M. 2010, "Epidemiology of osteoarthritis", *Clinics in geriatric medicine,* vol. 26, no. 3, pp. 355-369.

Zoetis, T., Tassinari, M.S., Bagi, C., Walthall, K. & Hurtt, M.E. 2003, "Species comparison of postnatal bone growth and development", *Birth defects research.Part B, Developmental and reproductive toxicology,* vol. 68, no. 2, pp. 86-110.

Zuniga, A. 2015, "Next generation limb development and evolution: old questions, new perspectives", *Development (Cambridge, England),* vol. 142, no. 22, pp. 3810-3820.

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