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ARTICULAR AND EPIPHYSEAL CARTILAGE: ITS FORMATION, MAINTENANCE AND REPAIR

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Cover image: Reconstructed of articular cartilage from the femur of a 2-month old mouse, visualized by PTA-enhanced microCT.

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ARTICULAR AND EPIPHYSEAL CARTILAGE: ITS FORMATION, MAINTENANCE AND REPAIR

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To My Beloved Family

献予我挚爱的家人

ABSTRACT

Long bones develop via a series of ordered processes, initiated from mesenchymal stem cell condensation, cartilage anlagen formation, followed by central cell hypertrophy and vascular invasion and the finalized by the formation of primary and secondary ossification centers (SOC), where the latter is also called endochondral ossification. As long bone develops, it can be distinguished in to several morphologically distinct parts: the main shaft of a long bone, called diaphysis; a narrow disc of growth plate, providing a continuous supply of chondrocytes for longitudinal growth; a thin layer of articular cartilage at the ends of epiphysis, supporting joint movement, and a SOC, sandwiched between the two pieces of cartilage.

Evolutionary analysis revealed that growth plate first appeared as an individual organ in amniotes due to the formation of SOCs, therefore we hypothesized SOCs might be evolved to meet the mechanical demands faced by bones growing under weight-bearing conditions. Combination of mathematical modelling and physical and biological validations demonstrated that SOC significantly improved the stiffness of the epiphyseal structure; meanwhile it decreased normal shear and stresses within the growth plate, allowing chondrocytes of the growth plate to stand a six-fold higher load before undergoing apoptosis. In addition, hypertrophic cells were more sensitive to loadings than cells from proliferating zone right above them **(Paper I)**.

Growth plates provide a continuous supply of cells for childhood longitudinal growth; however, its growth mechanism is still unclear. In the second paper, we aimed to understand the growth model of the growth plate and its maintenance. We demonstrate that a depletion manner of chondro-progenitor occurs during the fetal and neonatal stages; whereas after the formation of SOC, the chondro-progenitors obtain the capacity for self-renewal, generating large and stable monoclonal columns. The hedgehog and mammalian target of rapamycin complex 1 (mTORC1) signaling pathways regulate this stem cell pool **(Paper II)**.

Articular cartilage has a poor capacity to self-repair due to its particular structure. Therefore, the existence of chondro-progenitors in the articular cartilage superficial zone has been attracted more attentions. Here, we further characterized these superficial cells *in vivo* **(Paper III)** and explored their capacity to form hyaline cartilage *in vitro* **(Paper IV)**. We showed that superficial cells proliferate more slowly than the underlying chondrocytes. Moreover, they divide symmetrically to self-renew and differentiate symmetrically and asymmetrically into underlying chondrocytes. Furthermore, the progenies of superficial cells fully substitute fetal chondrocytes during early postnatal life **(Paper III)**. In monolayer and 3D *in vitro* culture, we found that exogenous Jagged1 (a Notch signaling against) had the most capacity to facilitate cell expansion while sacrificing their chondrogenic potential. Conversely, XAV (a Notch signaling antagonist) preserved the chondrogenic potential. In addition, the dedifferentiation might be via Jagged1/Notch3 signaling pathway **(Paper IV)**.

Collectively, we first show that the evolution of epiphyseal cartilage into a separate organ allows epiphyseal chondrocytes to withstand the high mechanical stress placed on them by the terrestrial environment. Secondly, the stem cell niche forms coinciding with the formation of the secondary ossification center, which provides a continuous supply of chondrocytes for postnatal bone growth. Finally, superficial cells are progenitors of articular cartilage whose progenies fully replace the fetal chondrocytes. Furthermore, the inhibition of Notch signaling preserves the chondrogenic potential of articular cartilage progenitors during monolayer expansion.

LIST OF SCIENTIFIC PAPERS

I. Xie M, Gol'din P, Herdina AN, Estefa J, Medvedeva EV, **Li L**, Newton PT, Kotova S, Shavkuta B, Saxena A, Shumate LT, Metscher B, Großschmidt K, Shigeki Nishimori, Anastasia Akovantseva, Irene Linares Arregui, Tafforeau P, Fried K, Carlström M, Simon A, Gasser C, Kronenberg HM, Bastepe M, Cooper KL, Timashev P, Sanchez S, Adameyko I, Eriksson A, Chagin AS. Secondary ossification centers evolved to make endochondral bone growth possible under the weight-bearing demands of a terrestrial environment. *Manuscript in preparation.*

II. Newton PT, **Li L**, Zhou B, Schweingruber C, Hovorakova M, Xie M, Sun X, Sandhow L, Artemov AV, Ivashkin E, Suter S, Dyachuk V, El Shahawy M, Gritli-Linde A, Bouderlique T, Petersen J, Mollbrink A, Lundeberg J, Enikolopov G, Qian H, Fried K, Kasper M, Hedlund E, Adameyko, Sävendahl L, Chagin AS. A radical switch in clonality reveals a stem cell niche in the epiphyseal growth plate. Nature. 2019 Mar;567(7747):234-238.

III. **Li L**[*], Newton PT[*], Bouderlique T, Sejnohova M, Zikmund T, Kozhemyakina E, Xie M, Krivanek J, Kaiser J, Qian H, Dyachuk V, Lassar AB, Warman ML, Barenius B, Adameyko, Chagin AS. Superficial cells are self-renewing chondrocyte progenitors, which form the articular cartilage in juvenile mice. FASEB J. 2017 Mar;31(3):1067-1084. *Equally contributing authors

IV. **Li L**, Feng XG, Sandhow L, Zhou BY, Newton PT, Chagin AS. Characterization of chondro-pellets form by chondroprogenitors of articular cartilage. *Manuscript in preparation*.

ADDITIONAL PUBLICATIONS (not included in the present thesis)

Shkhyan R, Lee S, Gullo F, **Li L**, Peleli M, Carlstrom M, Chagin AS, Banks NW, Limfat S, Liu NQ, Evseenko D. Genetic ablation of adenosine receptor A3 results in articular cartilage degeneration. J Mol Med (Berl). 2018 Oct;96(10):1049-1060.

Tong D, Lönnblom E, Yau ACY, Nandakumar KS, Liang B, Ge C, Viljanen J, **Li L**, Bãlan M, Klareskog L, Chagin AS, Gjertsson I, Kihlberg J, Zhao M, Holmdahl R. A Shared Epitope of Collagen Type XI and Type II Is Recognized by Pathogenic Antibodies in Mice and Humans with Arthritis. Front Immunol. 2018 Apr 12;9: 451.

Kaucka M, Zikmund T, Tesarova M, Gyllborg D, Hellander A, Jaros J, Kaiser J, Petersen J, Szarowska B, Newton PT, Dyachuk V, **Li L**, Qian H, Johansson AS, Mishina Y, Currie JD, Tanaka EM, Erickson A, Dudley A, Brismar H, Southam P, Coen E, Chen M, Weinstein LS, Hampl A, Arenas E, Chagin AS, Fried K, Adameyko I. Oriented clonal cell dynamics enables accurate growth and shaping of vertebrate cartilage. Elife. 2017 Apr 17;6.

Bouderlique T, Vuppalapati KK, Newton PT, **Li L**, Barenius B, Chagin AS. Targeted deletion of Atg5 in chondrocytes promotes age-related osteoarthritis. Ann Rheum Dis. 2016 Mar;75(3):627-31.

CONTENTS

LIST OF ABBREVIATIONS

ACAN	Aggrecan
ACI	Autologous chondrocytes implantation
ADAM	Metalloprotease proteins
APC	Adenomatosis polyposis coli
BrdU	5-bromo-2'-deoxyuridine
Col1	Collagen type I
Col2	Collagen type II
ColV	Collagen type V
DAPT	N-[N-(3,5- difluorophenacetyl-l-alanyl)]-S-phenylglycine t-butyl ester
Dhh	Desert Hedgehog
Dkk3	Dickkopf-3
DTA	Diphtheria toxin
Dvl	Dishevelled
E10	Embryonic day 10
ECM	Extracellular matrix
Edu	5-ethynyl-2'-deoxyuridine
Erg	Ets-related gene
Gdf5	Growth and differentiation factor 5
Gli	Glioma
GSK-3β	Glycogen synthase kinase 3β
Hh	Hedgehog
Ihh	Indian Hedgehog
LEF	Lymphoid enhancer factor
Lrp	Lipoprotein receptor-related protein
Matn1	Matrilin-1
MIB	Mind bomb
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
NECD	Extracellular protein of Notch receptors

NICD	Notch intracellular domain
OA	Osteoarthritis
Oc	Osteocalcin
Osx	Osterix
Prg4	Proteoglycan 4
Ptc	Patched
PTHrP	Parathyroid hormone-related peptide
P3	Postnatal day 3
Runx2	Runt-related transcription factor 2
Shh	Sonic Hedgehog
Smo	Smoothened
Sox9	Sex-determining region Y-Box 9
TCF	T-cell factor
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type mouse mammary tumor virus integration site family

1 INTRODUCTION

Skeleton, a rigid frame in vertebrates, serves to support soft organs and provide plenty of attachment spots for muscles, ligaments, tendons, and joints. Bone is one of the most important components of the skeleton and is generally formed via two different processes: intramembranous ossification and endochondral ossification. Intramembranous ossification is involved in the formation of clavicles and some flat bones in face and skull, which develops directly from embryonic mesenchymal cells to specific bones without forming a cartilage template. The process of endochondral ossification that underlies the formation of long bones and most of the other bones of the skeleton is rather complicated, which can be divided into three major steps: 1. formation of pre-chondrocytic mesenchymal stem cell condensation; 2. central cells of cartilage anlagen undergo hypertrophy accompanied by invasion of blood vessel, osteoclasts, osteoblasts and other types of progenitor cells; 3. primary ossification center formation in the diaphysis followed by SOC formation in the epiphysis. Both ends of the epiphysis are covered by a thin layer of cartilage, called articular cartilage. Beneath the SOC, there is a piece of narrow cartilage, called epiphyseal plate (growth plate) (Figure 1).

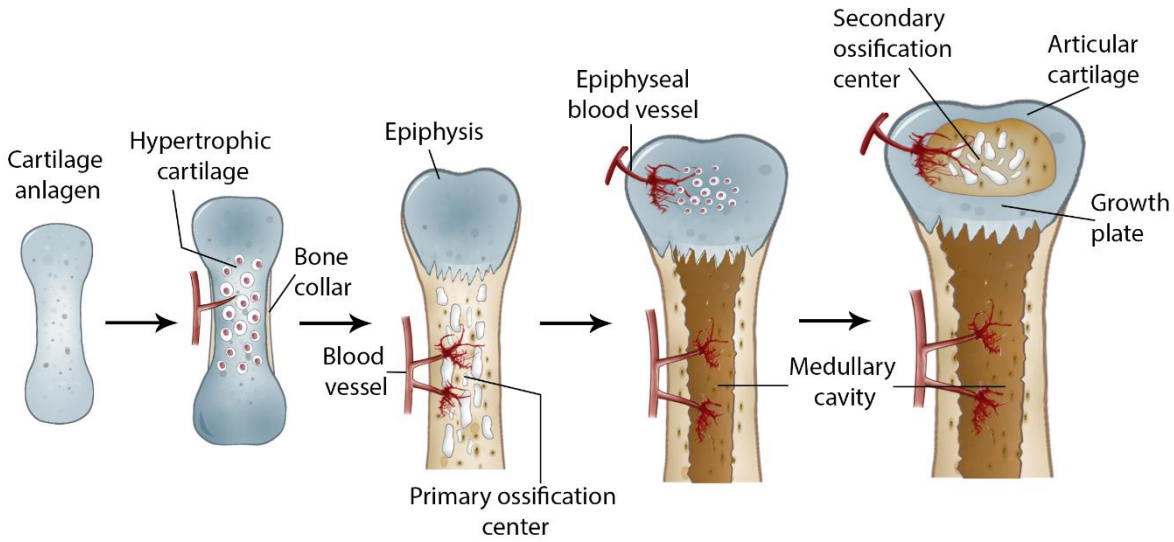

Figure 1. Endochondral ossification. Lateral plate mesoderm mesenchymal stem cells (MSCs) migrate to limb bud sites and aggregate to form pre-chondrocytic mesenchymal stem cells condensation. These cells then proliferate, differentiate into chondroprogenitors and start to produce extracellular matrix (ECM) to constitute cylindrical structures called cartilage anlagen. Later, the cells in the center of cartilage anlagen quit the cell cycle and become hypertrophy. These hypertrophic cells firstly mineralize the surrounding matrix, then through the secretion of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), hypertrophic cells induce angiogenesis from surrounding perichondrium driving the invasion of osteoblasts, osteoclasts and hematopoietic cells. The central hypertrophic cells undergo apoptosis and the middle part of cartilage anlagen is replaced by mineralized bone, which is called the primary ossification center. At the meantime, the osteoblasts in the perichondrium form bone collars surround the cartilage anlagen except for both ends. These two round ends are known as epiphysis. Soon after, secondary ossification centers are formed in the epiphysis. On top of the SOC is articular cartilage and the cartilage dics between secondary ossification and diaphysis is called growth plate.

1.1 LIMB BUD DEVELOPMENT

When mesoderm-derived mesenchymal stem cells (MSCs) aggregate to form mesenchymal condensations, they give rise to lineage-restricted chondrocytes and osteoblasts of skeletogenic elements. A delicate balance between the expression and activity of Sex-determining region Y-Box 9 (Sox9) and Runt-related transcription factor 2 (Runx2) decides chondrogenic or osteogenic cell fate. Sox9 is firstly expressed in limb bud mesenchyme at embryonic day (E) 10 and later in cells of mesenchymal condensations (1). Cells localized at the central part the mesenchymal condensation express high levels of Sox9, which regulates the downstream genes, such as collagen type II (Col2a1) and aggrecan (ACAN) to initiate the process of chondrogenesis. At E10.5, cells localized at the periphery of the condensation express low levels of Sox9, but high levels of Runx2 to upregulate expression of osteogenic gene, such like collagen type I (Col1a1), osterix (Osx) and osteocalcin (Oc), thereby promoting osteogenesis for perichondrial bone formation (2, 3).

1.2 JOINT DEVELOPMENT

As the mesenchymal condensation process proceeds, condensations at the future hind limb site acquire a "Y-shape", where the upper part turns into tibia and fibula and the lower part turns into femur (40). The first histological sign of joint formation is the establishment of a secondary remodeling area, called the interzone, which is characterized by a high density of flattened cells. The interzone comprises three layers, two chondrogenic outer layers and one intermediate layer in-between the two outer layers, containing the flattened cells (4). Molecularly, the interzone is characterized by high expression of molecular markers, like growth and differentiation factor 5 (Gdf5), wingless-type mouse mammary tumor virus integration site family (Wnt) 4 and Wnt9a (5, 6, 7) ; and low expression of Matrilin-1 (Matn1) (8) and diminished Col2 expression (9). Surgical removal of the interzone in chick wing-buds caused fusion of the joint (10). Moreover, Gdf5 mutation led to defects in skeletal joints (11). Double deletion of Wnt4 and Wnt9a resulted in formation of ectopic cartilage nodules at the site of synovial tissues and joint fusion in carpal, tarsal and limb (6). Thus, interzone cells are considered as joint progenitors during development.

Gdf5Cre; ROSA26^{lacZ} transgenic mouse tracing studies demonstrated that articular cartilage, cruciate ligament and meniscus are developed from the interzone as illustrated by the β-galactosidase-positive stainings in all these structures (12, 13). Yulia Shwartz *et al.* later proposed an influx model for joint formation based on genetic tracings of Gdf5CreER^{T2}; ROSA26^{Tomato} mice, where a continuous stream of Gdf5-positive cells was added to the joint surface at different embryonic developmental time points (Figure 2).

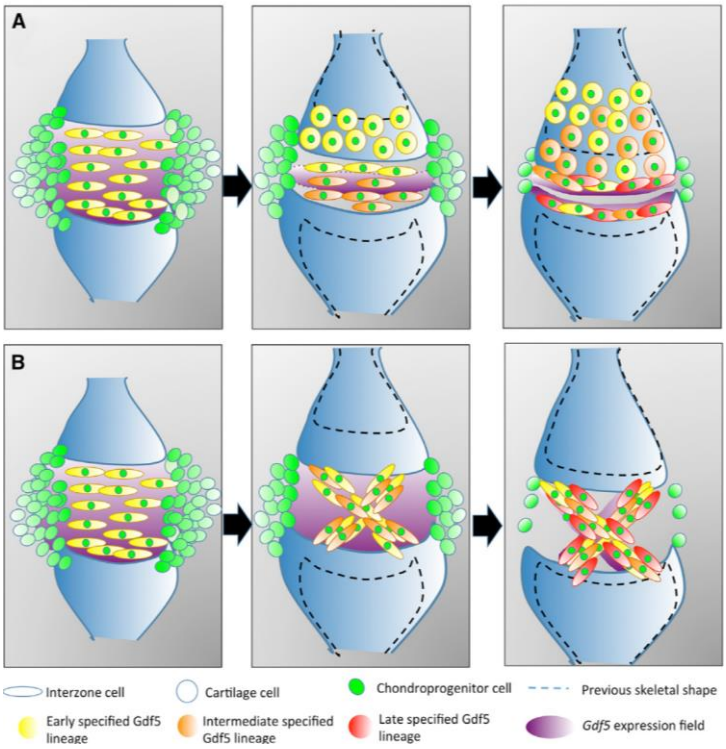

Figure 2. Schematic drawing illustrates an influx model during joint development. (A) Some early-specified GDF5 cells (yellow) in the interzone lose Gdf5 expression and contribute to the growing epiphyses. Its contribution for epiphysis growth is more than later-recruited cells (orange and red). (B) Some specified GDF5 cells continuously contribute to the intra-articular ligaments and maintain Gdf5 expression and their localization to the developing joint site.

Ultrastructural and histochemical studies suggested that cells from the outer layers of the interzone contribute to chondrocytes of the epiphysis, while cells in the intermediate layer develop into articular cartilage and menisci (14). However, other studies proposed that articular cartilage originates from the outer layers of interzone based on the same expression pattern of Col2 and collagen type V (ColV) both in outer interzone cells and mature articular chondrocytes; and the intermediate interzone cells differentiate into other synovial components (8, 9,15, 16).

Even though the mechanism is still unclear, microarray analysis of cells in the outer layers and the intermediate layer of the interzone provided us a solid platform for future extensive research (16).

1.3 ARTICULAR CARTILAGE

1.3.1 Articular cartilage structure

In new-borns, immature articular cartilage (also referred to as articular-epiphyseal cartilage) lies at the ends of long bones, occupying the entire epiphysis surface. During this period of time, SOC has not yet formed and cartilage canals that are tunnels containing blood vessels and loose connective tissue invade into the epiphysis to from vascularized perichondrium (17, 18, 19). The role of cartilage canals in development has not been fully established. Several groups showed that cartilage canals not only contribute to the formation of endochondral and perichondral bones, but also play an important role in nutrition supply and waste elimination of the articular cartilage (20, 21, 22). As the SOC forms, the volume of cartilage decreases and gradually turns into a thin layer, covering the ends of epiphysis and persists throughout the entire lifetime.

Adult articular cartilage is highly hydrated, but neither innervated nor vascularized. Morphologically, it can be divided into 3 distinct zones: superficial zone, middle zone and deep zone (Figure 3).

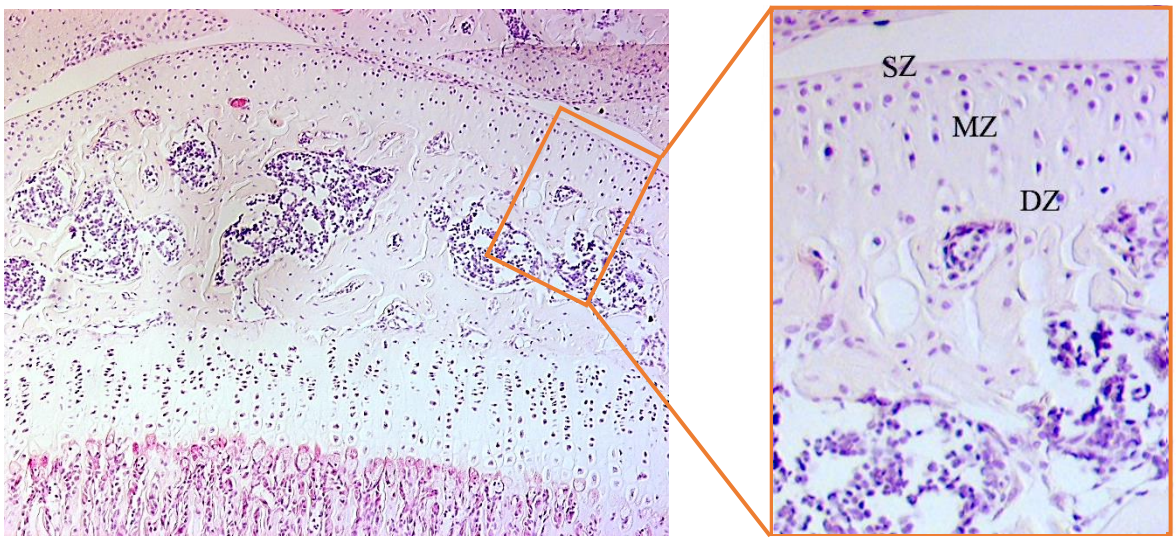

Figure 3. Structure of the adult murine articular cartilage. Hematoxylin & Eosin staining. SZ-superficial zone, MZ-middle zone, DZ-deep zone.
Images are kindly provided by Dr. Phillip Newton.

The superficial zone, which occupies approximately 10-20% of articular cartilage thickness, contains flat cells embedded by a large amount of lubricant lubricin [encoded by proteoglycan 4 (Prg4)], Tenascin-C and low levels of Col2 and ACAN (23). Previous studies have demonstrated that cells in the superficial zone behave as slowly dividing chondroprogenitors to generate underlying chondrocytes (24), however, others observed that the cells in the superficial zone divide faster than the underlying cells (25, 26). Lubricin secreted by the superficial cells protects the articular cartilage from tearing during joint movement.

The middle zone occupies the largest portion of articular cartilage, which accounts for 40-60% of cartilage volume. Compared to cells in the superficial zone, middle zone chondrocytes are bigger in size and rounder in shape. Studies employing histone H2B (H2B)-GFP Ter-On mouse model and EdU and/or BrdU retention experiments showed that the proliferative activity of chondrocytes is higher in this zone (27, 28). Functionally, the middle zone absorbs compressive forces and equally distributes the pressure to the underlying cartilage.

The deep zone is above the calcified zone accounting for about 30% of articular cartilage volume. It is characterized by the largest diameter of collagen bundles, the highest proteoglycan density and the lowest water content, all of which allow the deep zone to transmit the external forces to subchondral bones via the calcified zone. Tide mark is the marker that distinguishes the deep zone from the underlying calcified cartilage.

1.3.2 Extracellular matrix

Extracellular matrix (ECM) is a predominant composition of cartilage secreted by chondrocytes. The functional difference between elastic, hyaline and fibrocartilage is attributed to the different compositions of ECM. Hyaline cartilage is composed of collagenous (15 - 27% of wet weight) and non-collagenous proteins (4 - 7% of wet weight), interstitial water (65 - 80% of wet weight), and ions (predominantly Na^+ and Cl^-ions). On the other hand, the actual cells only account for less than 10% of the tissue wet weight in mature articular cartilage (29, 30).

1.3.2.1 Collagens

Collagens are the major fibers of ECM that contain a number of families and provide tensile strength to the cartilage. Among them, Col2 (90% of the collagen in mature cartilage, 75% of the collagen in immature cartilage) and collagen type X (ColX) are widely studied.

Col2 is the most abundant collagen in the ECM of adult articular cartilage and it plays an important role during skeletal development. Col2 is expressed during the formation of hind limb mesenchymal condensation around E10.5 in mice (31, 32). The homozygous Col2a1 mutant mice manifested a severe chondrodysplasia due to lack of endochondral bone formation (31), indicating the indispensable role of Col2 in chondrogenesis and cartilage maintenance.

ColX is a short-chain collagen that consists of three α1 (X) chains, each of which contains 3 domains: a short non-helical amino terminus (NC2), a triple helix and a non-collagenous (NC1) at the C-terminus (33). Hypertrophic chondrocytes secret ColX exclusively to the matrix and mutations of Col X showed phenotypic consequences of Schmid metaphyseal chondrodysplasia (SMCD) both in mice and human (34, 35), suggesting that ColX is essential for the distribution of matrix vesicles within the growth plate, articular cartilage and endochondral bone growth.

1.3.2.2 Non-collagenous proteins

Among a large variety of non-collagenous cartilage proteins, proteoglycans have been attracting the most attention due to their comparably high composition in cartilage. ACAN is a dominant proteoglycan component of the ECM in articular cartilage that consists of about 100 chondroitin sulfate and 30 keratin sulfate chains, enduing cartilage with a capacity to resist compressive loads (36, 37). ACAN binds to water molecules and provides the compressive strength to cartilage. ACAN homozygous mutant mice showed severe dwarfism and a cleft palate, resulting in premature death right after birth due to respiratory failure (38). In human, at least 25 pathological ACAN have been reported (39, 40, 41) and the symptoms are characterized by short stature, variable facial and skeletal features and early-onset of osteoarthritis (41, 42).

Prg4, also referred to lubricin, is specifically secreted by cells at the articular cartilage surface and synovial lining cells (43). Prg4 loss-of-function and genetic knock-out mice caused early

onset of osteoarthritis (OA) both in humans (44) and mice (43, 45). On the other hand, intra-articular injection of helper-dependent Prg4-expressing adenoviral virus protected cartilage from development of post-traumatic OA (46). Thus, Prg4 not only serves as a friction reducer, but also protects cartilage against the OA. However, Zhang *et al.* (47) found that loss of Prg4 positive cells did not cause cartilage damage. 10 days of consecutive tamoxifen administration from P21 in Prg4CreER^{T2}; ROSA26^{DTA} mice (diphtheria toxin, DTA) significantly reduced the amount of cells in the superficial zone of articular cartilage. Interestingly, mutant mice did not show obvious signs of OA. Moreover, higher proliferation rate was detected in mutant mice and the cell number slowly caught up with wild type mice at 9 months of age. There are two possible explanations: 1) with the high rate of cell proliferation, surviving superficial cells may repopulate themselves; 2) diphtheria-induced cell death may not cause physical damage to the microenvironment of the niche.

1.3.3 Growth pattern of articular cartilage

With the formation of joint cavity during embryonic development, different structures of the joint elements become gradually clear. Articular cartilage eventually becomes a thin layer of tissue with no nerves, blood vessels, or lymphatics from the formation of SOC. It is generally believed that the superficial zone plays a crucial role in articular cartilage growth by providing cartilage progenitor cells (36, 48, 49). Dowthwaite *et al.* showed that cells in the superficial zone not only possess a high affinity to serum fibronectin in vitro, but also have the capacity to differentiate into diverse skeletal elements *in vivo*. Furthermore, these cells also expressed Notch1 (50), Dkk3 (51), Tenascin-C and Ets-related gene (Erg), and MSCs markers, such as CD73, CD105 and CD34 (28).

Early studies proposed two mechanisms for articular cartilage growth: 1) appositional growth mechanism suggests that a progenitor population in the superficial zone would first give rise to cells in the middle zone (the second daughter cell), and then the daughter cells in the middle zone continue to self-divide and differentiate into the underneath chondrocytes (49); 2) the interstitial growth pattern means that after a chondrocyte with lacuna divides once or several times to form an isogenic group, newly formed chondrocytes within the isogenic group start to produce matrix and gradually separate from each other with their own lacuna. Later, Kozhemyakina *et al.* (24) also suggested an appositional growth pattern but in a different process based on Prg4GFPCreER^{T2}; ROSA26^{lacZ} lineage tracing. They found that progenies of the superficial cells, initially labelled at E17.5, could be detected throughout the entire articular cartilage at 12 months of age. However, progenies could not be detected over two-thirds of the articular cartilage (above the tidemark) even at 18 months of age, when cells of the superficial zone were labelled at 1 month of age. These observations suggest that the progenitors first generate cells in parallel and/or above and leave themselves at the bottom of the tissue. These newly generated cells then continuously give rise to new cells on top of the original cell layer to form articular cartilage in a column manner. Later, a similar genetic mouse model (52) that has the same promoter, but a different reporter was used to continue exploring the growth pattern of articular cartilage. Confetti reporter mice, carrying a

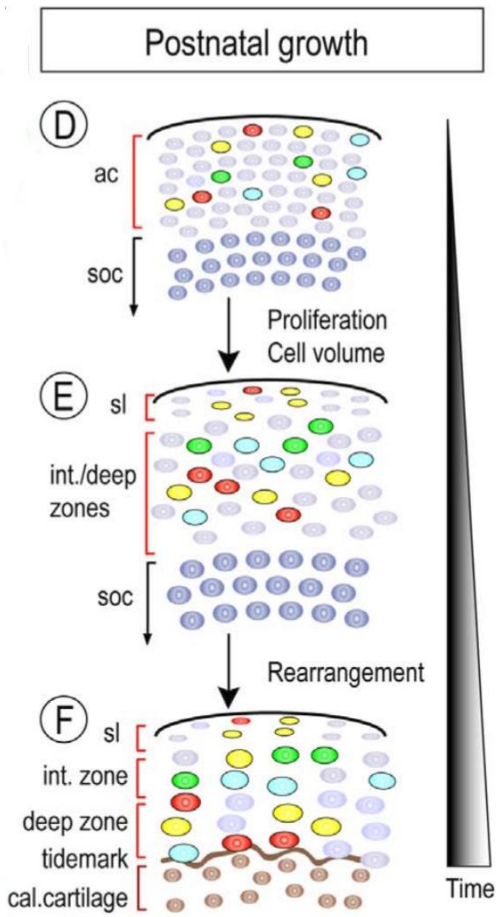

Figure 4. Growth pattern of articular cartilage in postnatal stage. ac-articular cartilage, SOC-secondary ossification center, sl-superficial layer, cal. cartilage-calcified cartilage.

Brainbow 2.1 construct (2) that randomly produces one of the four designated fluorescent proteins based on Cre recombination, were crossed with Prg4GFPCreER^{T2} mice. Taking advantage of the Confetti reporter, the model demonstrated that instead of proliferating in a vertical column manner, the progenies of superficial cells form clusters during tracings from fetal to adult stage. In addition, they also indicated that the growth mechanism of articular cartilage may not fully sustain the apposition pattern, it also relied on several combined factors, such as increase in chondrocyte volume and changes in chondrocyte distribution and rearrangement (Figure 4).

1.3.4 Chondroprogentors expansion *in vitro*

The unique structure of articular cartilage determines their poor capacity in self-repairing. Application of a tissue engineering strategy, autologous chondrocyte implantation (ACI), is widely used in cartilage repair for young adults due to its long-term effectiveness (53, 54). Primary chondrocytes are usually considered as the main source for ACI. However, one weak point of ACI is that the chondrocytes undergo progressive dedifferentiation during their expansion in monolayer cultures, which is necessary for their proliferation (55, 56). Dedifferentiated chondrocytes exhibit the same phenotypes as fibroblasts where they gradually lose expressions of several chondrogenesis-related genes, such as Col2 and ACAN and increase the expression level of Col1. Some redifferentiation methods, such as high-density cultures (57), defined growth factor additives (58, 59), physioxia culture condition (60), extracellular matrix pre-coated culture (61) and 3D culture with hydrogels (57, 60, 62) have been developed to reverse chondrocyte dedifferentiation to a certain extent, but the outcomes are still far from ideal.

As the exploration of chondroprogentors in articular cartilage surface, several groups attempted to expand chondroprogentors and/or differentiate chondroprogentors to chondrocytes *in vitro*. Rika Yasuhara *et al*. (28) isolated chondroprogentors via a fibronectin differential adhesion assay and manipulated the levels of several stimulators and inhibitors of the Wnt/β-catenin signaling pathway. They found that Wnt3a treated group kept intensive

expression levels of Prg4 and Erg with higher proliferation rate, while β-catenin deficiency in superficial cells displayed the opposite results. Moreover, when these differentially treated superficial cells were aggregated to pellets, Wnt3a treated group also formed pellets with better chondrogenic phenotype. The data suggest that Wnt/β-catenin signaling plays a crucial role in the maintenance of superficial cell phenotype and proliferation. Furthermore, Devon E. Anderson *et al.* (60) showed that pellets consisted of superficial cells had better chondrogenic phenotype when culturing under physioxic oxygen level (5%) compared to the hyperoxic oxygen level (20%), indicating that physioxia facilitates the hyaline cartilage tissue differentiation from chondroprogenitors. Taken together, these results demonstrated that chondroprogenitors in the superficial zone may represent a promising source for cartilage repair. However, given the small quantity of superficial cells in the entire articular cartilage, obtaining enough of such cells for cartilage engineering could become a problem. Previous study (63) derived superficial cells from MSCs using the co-culture method and showed that MSCs not only partially resembled the phenotype of superficial cells with high expression of Prg4, but also expressed significantly higher levels of Col2 and Sox9 compared to the control cells that were co-cultured with mid/deep zone explants. Despite the lack of assessment of superficial cell properties and function, this study opens up a new insight for zonal cartilage engineering and provides a possibility to solve the quantity limitation problem of superficial cells.

1.4 GROWTH PLATE

Similar to the articular cartilage, growth plate is also a layer of hyaline cartilage localized between the primary and secondary ossification centers, severing as a cellular source for long bone growth. In human, growth plates fuse after sexual maturity; while in mice and rats, growth plates exist for one quarter to one-third of their lifespans (49), which could be a limitation for the bone development studies with rodent models.

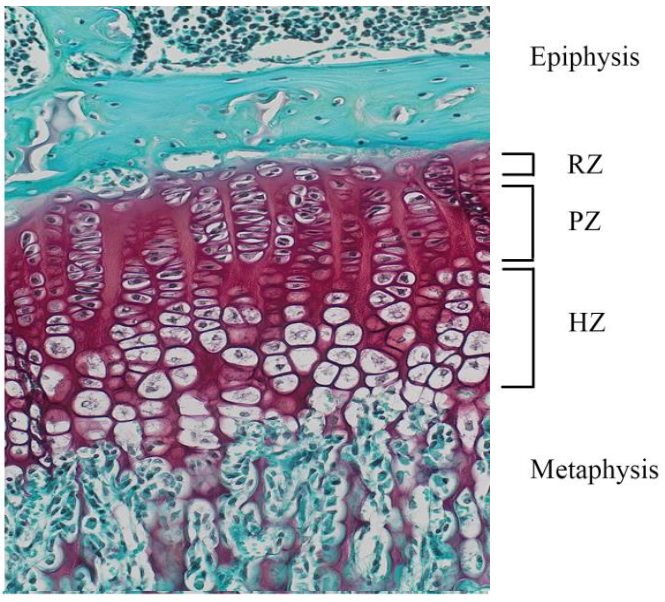

Figure 5.Structure of the murine growth plate. Safranin-O & Fast green staining. RZ-resting zone, PZ-proliferating zone, HZ-hypertrophic zone.
Images are kindly provided by Dr. Phillip Newton.

After the formation of the SOC, growth plate can be morphologically classified into three layers: resting zone, proliferating zone and deep zone (Figure 5).

The resting zone contains round cells right underneath the SOC. These cells are a unique class of skeletal stem cells that give rise to cells in the underlying zones, contribute to bone elongation (2, 36) and regulate the alignment of the proliferative clones

into columns parallel to the longitudinal axis of the bone (48). Compared to the underlying chondrocytes, slowly dividing resting zone cells distinctively express skeletal stem cell and progenitor markers and gradually gain the expression of PTHrP from as early as E17.5 (36). Postnatal lineage tracing of PTHrP mice showed that some of PTHrP-positive cells in the resting zone generated underlying cells in proliferating and hypertrophic zones as a monoclonal manner during long-term tracings. Then these PTHrP labelled hypertrophic cells transdifferentiated into osteoblasts and bone marrow stroma cells underneath the growth plate. These observations demonstrate that a type of initially unipotent cells obtains multipotency during a maturation process, emphasizing the malleable nature of the skeletal cell lineage (Figure 6). Therefore, two different functions of resting zone cells can be identified: 1. these cells generate underlying chondrocytes as stem cells and eventually differentiate into osteoblasts and bone marrow stromal cells. 2. Together with Ihh sent by pre-hypertrophic cells, PTHrP expressed by resting zone cells regulate the proliferation and differentiation of epiphyseal chondrocytes.

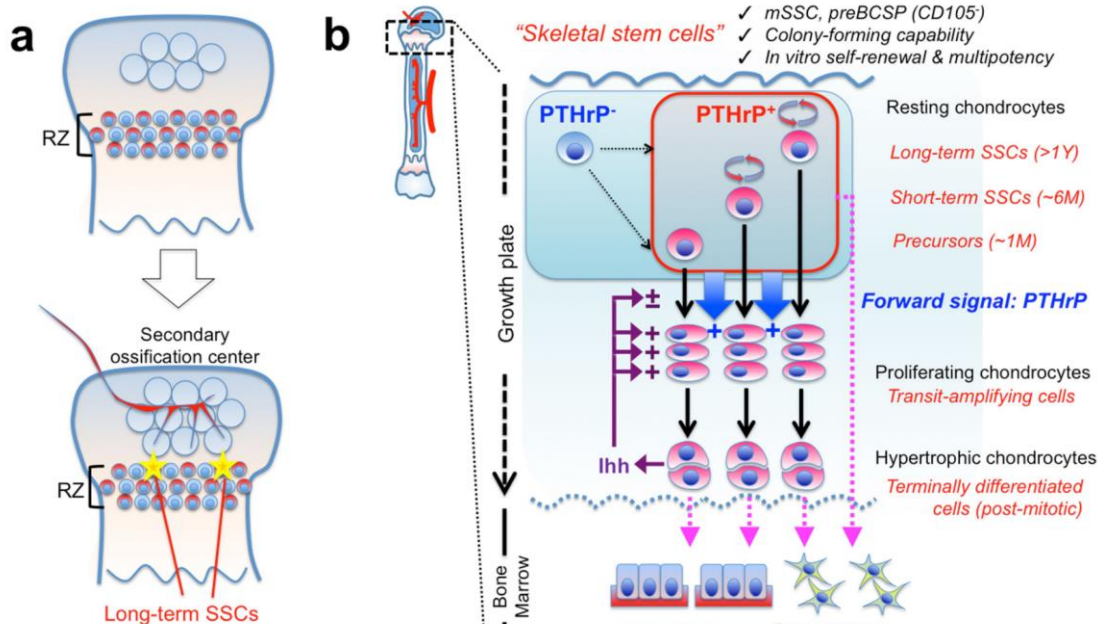

Figure 6. Schematic drawing illustrates that the resting zone of the growth plate houses a unique class of skeletal stem cells. **a**. Some of the PTHrP-positive resting zone cells obtain a long-term stem cell capacity during the late stage of SOC formation. **b**. PTHrP labelled hypertrophic cells trans-differentiate into osteoblasts and bone marrow stroma cells in bone marrow.

The proliferating zone is a matrix-rich zone enriched with Col2, ACAN, biglycan and glypican (64), which are indispensable for the structure of the growth plate ECM. Cells in the proliferating zone are compact flat chondrocytes lining up along the longitudinal axis of the bone. At a certain time, either because of limited cell divisions or regulations of local growth factors, such as PTHrP (65, 66); proliferating chondrocytes decelerate their proliferation and simultaneously differentiate into prehypertrophic chondrocytes that start to express Ihh (chapter 1, 5, 3), accompanied by an increase in cell volume.

Hypertrophic chondrocytes are terminally differentiated chondrocytes with a round and big appearance. These cells release a large amount of annexin-rich matrix vesicles that regulate their calcium uptake (67, 68). The vesicles secrete matrix metalloproteinases (MMPs) and other proteinases to mineralize the surrounding matrix. Together with the mineralization process, low oxygen tension and secretion of angiogenic growth factors from hypertrophic chondrocytes attract blood vessels from the primary spongiosum (68). Thereafter, the mineralized hypertrophic chondrocytes undergo apoptosis and leave a scaffold for new bone formation. In addition, a trans-differentiation from chondrocytes to osteoblasts and marrow stromal cells during both embryonic and postnatal endochondral bone development is introduced in above paragraph (36, 69).

Taken together, resting zone cells acquire multipotency in the postnatal stage regulating bone growth and homeostasis via giving rise to diverse cell types, including chondrocytes, osteoblasts and marrow stromal cells. Except for chondrocyte proliferation and hypertrophy, ECM production also contributes to bone elongation.

1.5 SIGNALLING PATHWAYS GOVERNING CARTILAGE GROWTH

1.5.1 Notch signaling pathway

The Notch signaling pathway is a highly conserved pathway involved in cell fate decisions, stem/progenitor cell self-renewal, proliferation, differentiation, and apoptosis; ranging from embryonic development to tissue homeostasis in adulthood (70, 71). It can be classified into two types: the canonical and the non-canonical Notch pathways.

The non-canonical Notch pathway has been detected in neural cell differentiation and brain development (72, 73), but not in chondrogenesis. The canonical Notch pathway consists of four transmembrane receptors, known as Notch1-4; and five ligands from the Jagged and the Delta protein family, known as Jagged1-2, and Delta1, 3 and 4. Activation of the canonical Notch pathway is based on cell-cell communication. When a ligand from the sending cell is ubiquitylated by Mind bomb (MIB), it binds to an extracellular Notch receptor of the recipient cell. Once the combination forms, metalloprotease proteins (ADAM) cleave the extracellular part of the Notch receptors (NECD). Next, the region within the transmembrane domain of Notch receptor is cleaved by the γ- secretase complex, causing the release of the Notch intracellular domain (NICD) into the cytoplasm, followed by its translocation to the nucleus and activation of target genes, such as HES and HEY transcription factors (74, 75, 76).

Most of Notch receptors and their ligands are widely expressed in the mesenchyme of the developing cartilage anlagen, interzone and proliferating region; and become progressively restricted to the articular surface during development (50, 77, 78). This expression pattern is associated with chondroprogenitors with stem-like properties, suggesting that Notch signaling pathway might be important for the maintenance of chondrocyte progenitors. Indeed, inhibition of Notch signaling pathway by conditional loss of RBPjk accelerated the chondrogenesis process at E12.5, while continuous activation of Notch signaling pathway

suppressed the differentiation of mesenchymal progenitors, but facilitated their proliferation (80), indicating that the Notch signaling pathway facilitates stem cell proliferation while preventing its differentiation. Moreover, some Notch receptors and their ligands are also expressed in pre-hypertrophic and hypertrophic regions of the growth plate, indicating Notch signaling is also associated with the subsequent chondrocyte differentiation (77, 78, 79). Prx1Cre; Notch1$^{-/flox}$; Notch2 $^{flox/flox}$ and Prx1Cre; γ-secretase $^{flox/flox}$ mice both showed elongated growth plate (because of hypertrophic zone expansion), a significant increase in bone mass and loss of MSCs in bone marrow, suggesting that the acceleration of osteoblast differentiation is due to the absence of normal regulation of the Notch signaling pathway (81, 82). In summary, the above studies demonstrated two main functions of the Notch signaling pathway in cartilage: 1. regulation of chondrogenic differentiation and chondrocyte proliferation in the earlier progenitor pool; 2. Mediation of chondrocyte differentiation and maturation during later chondrocyte development.

In vitro studies related to Notch signaling in early chondrogenesis are contradictory. Human MSCs in chondrogenic culture condition temporally expressed HEY1, followed by the onset of Sox9 and Col2 expression. In contrast, inhibition of Notch signaling by N-[N-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine t-butyl ester (DAPT) (83) for several days during the time of high Jagged1 expression led to chondrogenesis inhibition, indicating that Notch signaling was essential for the initiation of chondrogenesis (84). Vujovic *et al.* also obtained similar results from pellet cultures (85). However, Fujimaki *et al.* showed the opposite results from murine limb bud cells culture (86). Additionally, overexpression of Notch1 and Delta1 in chondrogenic ATDC5 cells (87) resulted in reduced expression of Sox9, Col2, and ACAN. Altogether, the effects of Notch signaling *in vitro* might relate to the cell differentiation stage, as well as the surrounding conditions.

1.5.2 Wnt signaling pathway

Wnt are secreted proteins that function as growth factors that regulate many steps of vertebrate limb development (88, 89, 90, 91). These growth factors can be classified into two major types: the canonical pathway (also called the Wnt/β-catenin signaling pathway) and the non-canonical pathways including the planar cell polarity pathway and the Wnt/calcium pathway.

β-catenin is a multi-functional protein, participating in both cell adhesion and gene expression regulation (92, 93). When the Wnt ligand is absent, β-catenin in the cytoplasm is phosphorylated by a degradation molecule complex composed of glycogen synthase kinase 3β (GSK-3β), adenomatosis polyposis coli (APC), dishevelled (Dvl), axin, CK1 and other proteins. Then, the phosphorylated β-catenin is degraded by the ubiquitin-26S proteasome pathway. When the Wnt ligands bind to the Wnt receptor complex (Frizzled receptor and lipoprotein receptor-related protein-Lrp 5/6), Dvl binds to the co-receptor LRP, disrupting the degradation complex formation. The stabilized β-catenin accumulates in the cytoplasm and translocates into the nucleus to stimulate the expression of target genes and co-transcription factors, such as T-cell factor and lymphoid enhancer factor (TCF/LEF) family (94, 95, 96).

Using Wnt/β-catenin reporter mice (TOPGAL mice and BATLacz mice), previous studies showed that Wnt/β-catenin signaling was active at early stages of development, which can be detected in the nuclei of interzone cells, pre-hypertrophic zone and periosteal, and even stronger detection in most-epiphyseal juxta-articular cells within the synovial cavities (13, 99). Conditional deletion of β-catenin under the control of Prx promoter has shown the wide expansion of Sox 9, Sox5, Sox6 and Col2 in the frontal and hind limb mesenchyme and the formation of ectopic cartilage (98, 99). During synovial joint formation, most of the joint-facing cells in β-catenin transgenic deficient mice (under the Col2 or GDF5 promoter) displayed a round shape compared to the flat shape in the wild-type mice in addition to some fusions in the wrist joints. Expression of GDF5, Prg4 and Erg was dramatically decreased at the joint surfaces (13). Moreover, overexpression of β-catenin in the early stage of limb development diminished the Sox9 expression, thereby preventing mesenchymal cells from differentiation during pre-chondrogensis (99). Thus, β-catenin-mediated canonical Wnt signaling acts as a negative regulator of chondrogenesis during the formation phases of cartilage anlagen, limb bud and synovial cavity. Apart from the influence on chondrogenesis, hypertrophy was significantly delayed in DermolCre; β-catenin conditional knockout mice (100). Moreover, Col2 specifically destabilized β-catenin in embryonic endochondral bones of mice with reduced expression of Runx2, Col1 and Osx1 and absence of osteoblast preciouses-derived Osteocalcin+ osteoblasts, indicating that Wnt/β-catenin signaling pathway not only plays an essential role in the processes of osteogenesis, but also might be needed at a later stage downstream of Osx1 during osteogenesis (101). Therefore, these studies show that Wnt/β-catenin signaling pathway is a negative regulator for chondrogenesis during early development, meanwhile, it is also a positive mediator for osteogenesis.

In addition to the regulation of Wnt/β-catenin signaling pathway during embryonic stage, it also mediates the skeleton system during postnatal life. When temporally overexpressed β-catenin in Col11-CA-β-catER transgenic mice in early postnatal stage for a week, the superficial zone became 1-2 times thicker and the overall proliferation in articular cartilage was significantly increased. However, the proteoglycan content reduced in cartilage but it restored after two weeks of the last tamoxifen injection (102, 103). More severe loss of abundant proteoglycan happened in the continuous activation of β-catenin in Col2a1CreER^{T2}; β-catenin cAct mice from the adulthood (3 or 6 months of age). Moreover, the thickness of articular cartilage was reduced and ACAN was cleaved, eventually developed into OA (103). The data indicate that the activation of Wnt/β-catenin signaling strongly irritates the matrix catabolism and protease activity. Apart from this, columns in the growth plate displayed a disorganized pattern and the growth plate was almost fused abnormally after three weeks, which might be because of the increased cell apoptosis that was confirmed by GSK3β inhibition in epiphyseal chondrocytes *in vitro* (102). Thus, Wnt/β-catenin signaling plays an essential role in the maintenance of mature articular cartilage and growth plate. Additionally, it is also a key regulator for OA development.

Taken together, the normal spatial-temporal expression pattern and expression levels of Wnt/β-catenin signaling at the embryonic and postnatal stages are required for maintenance of skeletal growth and organization of articular cartilage, growth plate, and bone homeostasis.

1.5.3 Hedgehog pathway and its feedback loop with PTHrP in cartilage development

The Hedgehog (Hh) family is a group of secreted proteins, which plays an important role in vertebrate embryonic development. When Hh is absent, Patched (Ptc) inhibits Smoothened (Smo) from entering into the plasma membrane. The glioma-associated (Gli) family proteins are then phosphorylated by CK1, PKA and GSK3 in the cytoplasma and cleaved into the repressor forms (Gli-Rs). With the presence of Hh, it binds to Ptc and releases Smo from Ptc inhibition. Therefore, Smo accumulates at the plasma membrane, Gli proteins disassociate from SUFU and will not be phosphorylated. The full-formed Gli proteins then translocate into nucleus and activate consequent pathways.

Hh contains three paralogous genes: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh). Shh is a critical signaling molecule at the early stage of embryonic development, inducing multifarious neuronal populations in the central nervous system, cell polarity regulation in early limbs and differentiation of mesenchymal cells in the limbs and spinal cord into chondrocytes (104, 105). Dhh signaling is strictly expressed in germ cells, including Sertoli cells on the testis and granulosa cells of the ovary (106). Indian hedgehog plays a major role in the endochondral ossification in mammals.

The expression of Ihh has been widely detected in many types of soft tissues, pre-hypertrophic chondrocytes, early hypertrophic chondrocytes and osteoblasts of developing endochondral bones (107, 108, 109). In human, Ihh protein expressed in growth plates reaches the highest level during early stages of puberty (110). The Ihh-mediated activation of Hh signaling functions in chondrogenesis and osteogenesis during endochondral ossification by mediating the dynamic equilibrium among chondrocyte differentiation, chondrocyte proliferation and osteoblasts formation.

Ihh and PTHrP negative feedback loop:

Parathyroid hormone-related peptide (PTHrP), an important growth factor in the regulation of chondrocyte function in epiphysis, is expressed by cells at the top of bones (pre-cartilage)(111), growth plate (resting zone cells) (36) and perichondrium. It binds to its receptors PTH/PTHrP on proliferating cells to promote proliferation and delay differentiation. When chondrocytes are far enough from the PTHrP proteins, they stop proliferating and produce Ihh (pre-hypertrophic chondrocytes and early hypertrophic chondrocytes). Then, Ihh acts back to the top of the fetal bones or growth plates to stimulate the release of more PTHrP.

The way Ihh regulating the physiologically dynamic equilibrium in skeleton might be via a PTHrP dependent or independent manner. $Ihh^{-/-}$ mice showed a significant reduction in

chondrocyte proliferation and the appearance of ectopic mature chondrocytes. The phenotype was more severe than that in PTHrP$^{-/-}$ mice and only the decreased proliferation cannot be rescued by the addition of PTHrP (112, 113). Therefore, in addition to mediating PTHrP for chondrocyte differentiation, Ihh promotes its proliferation in a PTHrP independent manner.

Additionally, Ihh is indispensable to regulate osteoblast differentiation during endochondral bone development. After differentiating into osteoblast precursors from immature mesenchymal stem cells, Runx2- and Sp7-positive osteoblast precursors further differentiate into mature osteoblasts. Mature osteoblasts secret bone matrix to participate in the formation of bone. However, Ihh$^{-/-}$ mice have less or no bone collars that express Runx2 or Bglap (115, 116). When Hh signaling activity was determined by Smo deletion in perichondrial cells, Runx2 expression and bone collar formation were entirely absent in the perichondrium (117, 118). Furthermore, the number of trabecular bones was also significantly reduced in Ihh$^{-/-}$ mice (112). The results indicate that Ihh is required for osteoblast differentiation, specifying progenitors into osteoblast precursors.

Altogether, Ihh-PTHrP signaling pathway regulates chondrocyte proliferation, endochondral ossification and osteoblast differentiation together with other signaling pathways during endochondral development.

1.5.4 Sox9

Sox9, a mammalian testis-determining factor (SRY)-related transcription factor with a high mobility-group box DNA-binding domain, is expressed by all the chondroprogenitors and chondrocytes, expect by hypertrophic chondrocytes (32, 120).

Conditional deficiency of Sox9 under the Prx promoter exhibited no detectable chondrogenic mesenchymal condensation in the early limb buds (E12.5), which lacks expression of cartilage markers, such as Col2a1 and ACAN; and replaced with ectopic muscle bundles. Moreover, digits failed to form, and outgrowth of limb buds stopped at E13.5 with increased apoptosis in these mutant mice (121). Deletion of Sox9 gene after the formation of chondrogenic mesenchymal condensation showed a spindle-shape of mesenchymal condensed Sox9$^{fl/fl}$ cells and less alcian blue stained extracellular matrix. When the Sox9 deficiency happened during postnatal stage, mutant mice had thinner articular cartilage and lost Safranin-O staining above the tidemark. The phenotype of mutant growth plate was similar to the embryonic stage, where Sox9 mutation led to a wider hypertrophic zone and shorter proliferating zone, less proliferation rate, less proteoglycans and ACAN, and disorganized columns (121, 122, 123). These data demonstrate that Sox9 plays essential roles in cartilages from the onset of chondrogenesis to adult cartilage maintenance. Moreover, the role of Sox9 in chondrocyte maintenance is to prevent cell hypertrophy. Apart from this, Sox9 also regulates the terminal differentiation of hypertrophic chondrocytes. Overexpression of Sox9 in hypertrophic cells mice led to largely inhibited cartilage resorption and endochondral ossification, leaving non-resorbed hypertrophic cartilages that lost expressions of VEGF, MMP12 and osteopontin (125).

Several studies stated that Sox9 closely interacts with the Wnt/β-catenin and Notch signaling pathways in chondrogenesis and chondrocyte differentiation: the phenotypes of overexpression of Sox9, β-catenin deletion and RBPjk deficiency were similar; the phenotypes of Sox9 deletion and overexpression of β-catenin and RBPjk were also similar in limb development, indicating that chondrogenesis is controlled by interactions between Sox9 and the canonical Wnt and Notch signaling pathways (124, 126).

Altogether, Sox9 has been described as an indispensable regulator for chondrocyte differentiation and homeostasis in embryonic and postnatal cartilages, even though its expression level is age dependent.

2 MATERIAL AND METHODS

2.1 TRANSGENIC MICE

Except for those involving AFM (**Paper 1**), all animal experiments were permitted by Ethical Committee on Animal Experiments (Stockholm North Committee/Norra Djurförsöksetiska Nämnden) and conducted according to the Swedish Animal Agency's Provisions and Guidelines for Animal Experimentation recommendations. Animal experiments involving AFM were pre-approved by the Ethics Committee of the Sechenov First State Moscow Medical University (Moscow, Russia).

MOUSE STRAINS	REFERENCE	USED IN PAPER
Col2-creER^{T}	Nakamura et al., 2006	II,III
Gli1-creER^{T2}	Ahn et al., 2004	II
H2B-GFP Tet-On	Tumbar et al., 2004	II,III
Prg4-GFP- CreER^{T2}	Kozhemyakina et al., 2015	III
Raptor-floxed mice	Sengupta et al., 2010	II
Rosa26R-Confetti	Snippert et al., 2010	II,III
Shh- GFP stain	Harfe et al., 2004	II
Tsc1-floxed mice	Kwiatkowski et al., 2002	II
Rosa-tdTomato	Madisen et al., 2010	II
Col2-Cre:Sik3-FL/FL	Sasagawa et al., 2012	I
Prx-Cre:Gsα^{R201H}	Karaca et al., 2018	I

Table 1. Mouse strains used in this thesis.

2.2 ANTIBODY LIST

ANTIBODY	COMPANY	USED IN PAPER
Acetylated tubulin	Sigma, T6793	II
Alexa Fluor 647/488	The Jackson Laboratory	I,II,III, IV
CD105	BioLegend, clone MJ7/18	II
CD29	BioLegend, clone ebioHMB1-1	II
CD39	BioLegend, 143807	II
CD44	BioLegend, clone IM7	II
CD54	BioLegend, clone YN1/1.7.4	II
CD73	BioLegend, clone TY/11.8	II,III
CD90	BioLegend, clone Ox-7	II
Cleaved caspase 3	Cell Signaling Technology, 9662	I,II
Col1	Sigma, clone COL-1	IV
Col2	provided by Rikard Holmdahl (KI)	II
Col2	Invitrogen, SK2473911B	II
Col2	Thermofisher, MA5-12789	IV
GFP	Abcam, ab6662	II
Ki67	Invitrogen, MA5-14520	I,II,III
MEF2C	Sigma, HPA005533	II
Notch 1	Santa Cruz Biotechnology, sc-6014	III
PAR3	Millipore, 07330	II

Phospho-histone H3	Millipore, Billerica, 04-817	II,III
PKC ζ	Santa Cruz Biotechnology, sc-1778	II,III
pS6	Cell Signaling Technology, 4858	II
Runx2	Abcam, ab23981	IV
SCA1	BD Bioscience, clone E13-161.7	II
Sox9	Sigma-Aldrich, HPA001758	II,III
survivin	Santa Cruz Biotechnology, sc-17779	III

Table 2. Antibodies used in this thesis.

2.3 METHOD LIST

Detailed information regarding the methods below, please go for the methods of paper I, II, III and IV.

- Sample collection and preparation (Paper I, II,III and IV)
- Immunohistochemistry (Paper I, II,III and IV)
- Histology staining (Paper I, II,III and IV)
- In Situ hybridization (Paper I and II)
- Calcein–xylenol double labeling (Paper III)
- Triple S-phase labelling (EdU, CldU and IdU) protocol (Paper II and III)
- TUNEL staining (Paper I and III)
- Flow Cytometry (Paper II and IV)
- Magnetic-activated cell sorting (Paper IV)
- Phosphotungstic acid–enhanced micro–computed tomography (Paper I and III)
- Laser-capture microdissection (Paper II)
- Atomic Force Microscopy (Paper I)
- Nanoindentation (Paper I)
- Finite Element Analysis (Paper I)
- cDNA library preparation and sequencing (Paper II)
- LCM -seq data analysis (Paper II)
- Cell/tissue culture (Paper I, II and IV)
- Real-time polymerase chain reaction (Paper I and IV)
- Statistical analysis (Paper I, II,III and IV)

3 SHORT SUMMARY AND DISCUSSION

3.1 PAPER I

Evolutionary analysis revealed that the epiphyseal growth plate first appeared as an individual organ in amniotes due to the formation of SOC. We therefore hypothesized that the existence of SOC might meet the mechanical demands faced by bones growing under weight-bearing conditions. By analyzing in several species of animals, finite element analysis (FEA), Instron ElectroPuls E1000 test instrument, genetic mouse models, atomic force microscopy (AFM) and other experimental approaches we showed the potential association between SOC and mechanical demands.

- To experimentally verify whether SOC has the potential to protect growth plate from mechanical impact, we selected tibia bones from 30-day-old mice (with an SOC (SOC+)) and 10-day-old rats (without an SOC (SOC-)) based on the similarity of their size, shape and mechanical properties of cartilage. We showed that chondrocytes of growth plate from SOC- bones were more sensitive to loads, which around 80% chondrocytes died with vertical or angle 1.5N load. In comparison, SOC+ bones significantly protected growth plate chondrocytes compared with SOC- bones. Taken together, our results indicated that the SOC significantly improves the stiffness of the entire epiphyseal structure, thereby protecting the epiphysis from weight-bearing conditions.

- With only a quarter as stiff as the proliferating zone cells, we highlighted that hypertrophic cells are more sensitive to loadings than cells from proliferating zone, suggesting that SOCs protect growth plate chondrocytes, especial hypertrophic chondrocytes from apoptosis induced by mechanical loads.

- We found that the mechanical loading triggered caspase-dependent apoptosis of epiphyseal chondrocytes, probably via the Yes-associated protein 1 (YAP)-p73 signaling pathway.

In conclusion, combining of mathematical modelling and physical and biological experiments we show that the SOC reduces normal stresses and shear within the growth plate, allowing growth plate chondrocytes to withstand a six-fold higher load before undergoing apoptosis via the YAP-p73 pathway. Moreover, the hypertrophic chondrocytes were the most sensitive to mechanical stress due to their least mechanical stiffness. Our results suggest that the evolution of epiphyseal cartilage into a separate organ allows epiphyseal chondrocytes to withstand the high mechanical stress placed on them by the terrestrial environment.

3.2 PAPER II

Growth plates are crucial for normal bone growth. It is generally believed that chondro-progenitors within the growth plate provide a sufficient cell input and the consumption of these progenitors eventually leads to the fusion of the growth plate, thereby ceasing the longitudinal growth. However, this pattern has never been experimentally proven. In this study, we applied state of the art techniques including clonal genetic tracing combined with functional perturbations to explore the growth pattern of epiphyseal cartilage in postnatal life and the underlying mechanisms of growth plate stem cell niche in postnatal life.

- Taking advantage of the Confetti reporter mice, we demonstrated that two different growth patterns of epiphyseal cartilage occus in neonatal/fetal and postnatal stages: short clones (multiclonal) that build on top of one another are constantly consumed (deletion), resulting in the consumption of round cells; whereas after around 1 month of age, the progenitors in the resting zone generate long and stable monoclonal columns. Furthermore, the progenitors that generate large, stable clones in mature mice can be labelled as early as E14.5.

- We verified the stem cell properties of resting zone cells *in vitro* and found that these progenitors divide symmetricly to renew their own population and asymmetricly to generate underlying proliferating chondrocytes *in vivo*.

- We also found two types of progenitors existing in the resting zone. Some progenitors in the resting zone underwent self-renewal and proliferation even after five months of tracing, some Confetti-labelled cells stayed alone and remain in the resting zone. This phenomenon of two populations of stem cells with distinct proliferation activity also exists in the stem cell niches of hair follicles (127, 128), bone marrow (129) and intestine (130, 131).

- We examined if the formation of the SOC influences the microenvironment/ stem cell niche. Not unexpectedly, the inhibition of SOC formation with axitinib delayed the shift from multiclonal to monoclonal, supporting the possibility that the formation of SOC alters the microenvironment to provide a stem cell niche.

- We identified the Shh protein expressed by mesenchymal progenitor cells (MPC), MSCs, endothelial and hematopoietic cells existed in the SOC. Together with Ihh, hedgehog signaling regulates the maintenance of growth plate chondro-progenitors proliferation, but does not disturb their stem cell identity.

- We discovered that mTORC1 pathway interferes the balance between asymmetrical and symmetrical cell division within the stem cell niche.

In summary, we show the existence of a stem cell niche in the postnatal growth plate. It facilitates chondro-progenitors to self-renew and to contribute to bone elongation. The maintenance of these self-renewal of chondro-progenitors is regulated by Hh and mTORC1 signaling pathways (Figure 7).

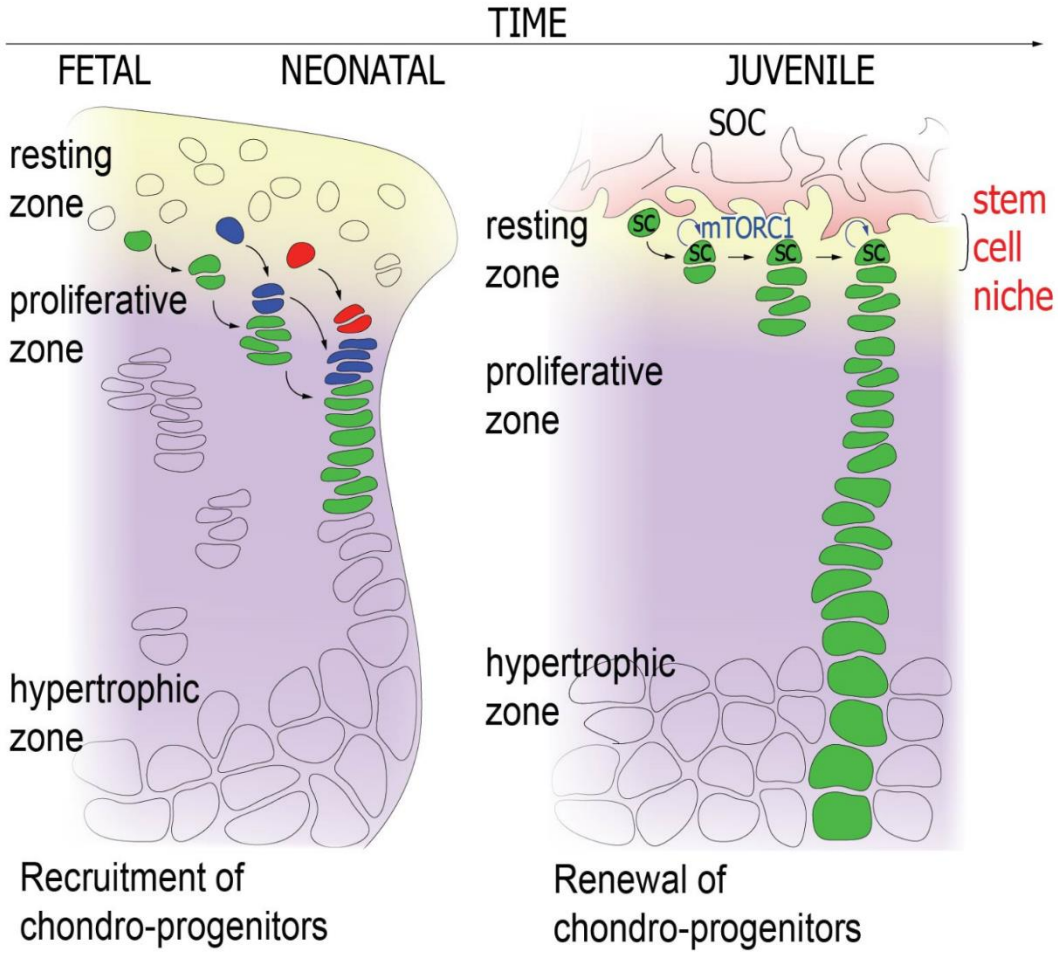

Figure 7. Conceptualization of Paper II showing two different growth patterns of epiphyseal plate before (left) and after (right) formation of the SOC. SOC- secondary ossification center, SC- stem cell.

Illustrations are from Newton and Li et al., 2019.

3.3 PAPER III

It has been proposed that superficial cells of articular cartilage are chondro-progenitors in consideration of the capacity of forming colonies *in vitro* and differentiating into other skeletal elements *in vivo* transplantation. Further evidences of chondro-progenitor nature were acquired from Kozhemyakina *et al.* (24) and Decker *et al*. (52) with genetic tracing of multiple transgenic mouse models. In our study, we further explored these superficial cells and growth pattern of articular cartilage in postnatal life.

- Using transgenic mice and pulse-chase labeling experiments, we described that cells in the superficial zone are slowly dividing cells. Moreover, superficial cells generate underlying chondrocytes as a cluster-manner.

- We discovered that superficial cells undergo both symmetrical and asymmetrical cell division. They maintain their own population by symmetrical division and give rise to the underlying chondrocytes in the articular cartilage through asymmetrical division and symmetrical differentiation, which is also described as an adult stem cell behavior in other tissues such as resting zone cells in the growth plate (132), germline stem cells (133), intestinal crypts stem cells (130), and hair follicles (134, 135).

- Combining with Col2CreER; $ROSA26^{Confetti}$ and $Prg4CreER^{T2}$; $ROSA26^{Confetti}$ lineage tracing together, we demonstrated that superficial cells almost fully replace the fetal chondrocytes during early postnatal life.

- By applying the Micro CT analysis on articular cartilage of different ages, we provided a detailed description of the reshaping during articular cartilage development.

- We also found that Col2-labelled cells were within epiphyseal bone along the bone surface or inside bones, indicating articular chondrocyte trans-differentiate during early postnatal development. The same growth pattern also exists in the growth plate where the hypertrophic cells trans-differentiate to osteoblast and bone marrow stroma cells. Furthermore, two populations can be separated by size in the deep zone chondrocytes, which might be relative with cells trans-differentiation.

In summary, we suggest that the superficial cells with lower proliferation rate are progenitors of underlying chondrocytes, they achieve self-renew through symmetrical division and give rise to underlying chondrocytes by asymmetrical division and symmetrical differentiation. Moreover, Prg4-labelled cells replace fetal chondrocytes during early postnatal growth and we propose an influx-efflux model of cartilage formation.

3.4 PAPER IV

Since articular cartilage is a highly hydrated, avascular and aneural tissue, it has limited capacity to spontaneously repair injures. The application of autologous chondrocytes implantation (ACI) increases interest in cartilage repair for young adults due to the long-term functionality. Primary chondrocytes are a common cell source for ACI, but the weakness of primary chondrocytes was well described before (chapter 1.3.4). To overcome that, we explored whether CD73 positive chondro-progenitors could serve as a remarkable source of cells for cartilage repair.

- We showed that active Notch signaling pathway trigger the highest proliferation rate in all groups, while it also caused the entire loss of chondrogenic capacity during monolayer culture. The same observation has also been found in adipocytes when Notch signaling was active, they underwent dedifferentiation and metabolic dysfunction (136). In contrast, the inhibiton of Notch signaling largly differentiated/redifferentated cells into chondrogenic phenotype.

- We demonstrated that Jagged1 likely acts via Notch3 receptor to activate Notch signaling pathway during chondro-progenitors monolayer culture causing cell dedifferentation.

- We suggested that Wnt/β-catenin might be a positive regulator for the maintance of chondro-progenitors. Activation of Wnt/β-catenin signaling resulted in an increase in the number of chondro-progenitors compared with the inhibition group. Moreover, the inhibition of Wnt/β-catenin signaling by XAV in chondro-progenitors monolayer culture prevented the identity of progenitor cells by losing typical superficial cells' markers, which is in accordance with conditionally genetic deletion of β-catenin in the earlier postnatal stage.

- We found that even fibronectin and FGF2 largly enhanced the cell proliferation during chondro-progenitors monolayer culture, none of them could maintain the progenitor property.

Summarily, we preliminary conclude that chondro-progenitors are a promising cell source for cartilage engineering. Jagged1/Notch3 signaling promotes cell proliferation but dedifferentiates chondro-progenitors in monolayer culture. Inhibition of Notch signaling helps reduce cell dedifferentiation in monolayer culture and promote chondrogenic differentiation in 3D culture.

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5 REFERENCES

1. Akiyama, H. *et al.* Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc. Natl. Acad. Sci.* **102**, 14665–14670 (2005).

2. Kronenberg, H. M. Developmental regulation of the growth plate. *Nature* **423**, (2003).

3. Drissi, H. *et al.* Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. *J. Cell. Physiol.* **184**, 341–350 (2000).

4. Mitrovic, D. R. Development of the metatarsophalangeal joint of the chick embryo: Morphological, ultrastructural and histochemical studies. *Am. J. Anat.* **150**, 333–347 (1977).

5. Shwartz, Y., Viukov, S., Krief, S. & Zelzer, E. Joint Development Involves a Continuous Influx of Gdf5-Positive Cells. *Cell Rep.* **15**, 2577–2587 (2016).

6. Später, D., Hill, T. P., Gruber, M. & Hartmann, C. Role of canonical Wnt-signalling in joint formation. *Eur. Cells Mater.* **12**, 71–80 (2006).

7. Khan, I. M. *et al.* The Development of Synovial Joints. *Curr. Top. Dev. Biol.* **79**, 1–36 (2007).

8. Hyde, G. *et al.* Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. *Dev. Biol.* **304**, 825–833 (2007).

9. Hyde, G., Boot-Handford, R. P. & Wallis, G. A. Col2a1 lineage tracing reveals that the meniscus of the knee joint has a complex cellular origin. *J. Anat.* **213**, 531–538 (2008).

10. Holder, N. An experimental investigation into the early development of the chick elbow joint. *J. Embryol. Exp. Morphol.* **39**, 115–27 (1977).

11. Storm, E. E. & Kingsley, D. M. GDF5 coordinates bone and joint formation during digit development. *Dev. Biol.* **209**, 11–27 (1999).

12. Rountree, R. B. *et al.* BMP receptor signaling is required for postnatal maintenance of articular cartilage. *PLoS Biol.* **2**, (2004).

13. Koyama, E. *et al.* A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev. Biol.* **316**, 62–73 (2008).

14. Ito, M. M. & Kida, M. Y. Morphological and biochemical re-evaluation of the process of cavitation in the rat knee joint: Cellular and cell strata alterations in the interzone. *J. Anat.* **197**, 659–679 (2000).

15. Bland, Y. S. & Ashhurst, D. E. Development and ageing of the articular cartilage of the rabbit knee joint: Distribution of the fibrillar collagens. *Anat. Embryol. (Berl).* **194**, 607–619 (1996).

16. Jenner, F. *et al.* Differential Gene Expression of the Intermediate and Outer Interzone Layers of Developing Articular Cartilage in Murine Embryos. *Stem Cells Dev.* **23**, 1883–1898 (2014).

17. Doschak, M. R. *et al.* Angiogenesis in the distal femoral chondroepiphysis of the rabbit during development of the secondary centre of ossification. *J. Anat.* **203**, 223–

233 (2003).

18. Ganey, T. M., Ogden, J. A., Sasse, J., Neame, P. J. & Hilbelink, D. R. Basement membrane composition of cartilage canals during development and ossification of the epiphysis. *Anat. Rec.* **241**, 425–437 (1995).

19. Wilsman, N. J. & Van Sickle, D. C. Cartilage canals, their morphology and distribution. *Anat. Rec.* **173**, 79–93 (1972).

20. Blumer, M. J. F. *et al.* The role of cartilage canals in endochondral and perichondral bone formation: are there similarities between these two processes? *J. Anat.* **206**, 359–372 (2005).

21. Haines, R. W. Cartilage Canals. *J. Anat.* **68**, 45–64 (1933).

22. Chandraraj, S. & Briggs, C. A. Role of cartilage canals in osteogenesis and growth of the vertebral centra. *J. Anat* **158**, 121–136 (1988).

23. Mallein-Gerin, F., Kosher, R. A., Upholt, W. B. & Tanzer, M. L. Temporal and spatial analysis of cartilage proteoglycan core protein gene expression during limb development by in situ hybridization. *Dev. Biol.* **126**, 337–345 (1988).

24. Kozhemyakina, E. *et al.* Identification of a Prg4-expressing articular cartilage progenitor cell population in mice. *Arthritis Rheumatol.* **67**, 1261–1273 (2015).

25. Henry, B. Y. Localization of tritiated thymidine in articular cartilage of rabbits: III. Mature articular cartilage. *J Bone Jt. Surg* **45**, 529–540 (1963).

26. Ray, A., Singh, P. N. P., Sohaskey, M. L., Harland, R. M. & Bandyopadhyay, A. Precise spatial restriction of BMP signaling is essential for articular cartilage differentiation. *Development* **142**, 1169–1179 (2015).

27. Hunziker, E. B., Kapfinger, E. & Geiss, J. The structural architecture of adult mammalian articular cartilage evolves by a synchronized process of tissue resorption and neoformation during postnatal development. *Osteoarthr. Cartil.* **15**, 403–413 (2007).

28. Yasuhara, R. *et al.* Roles of β-catenin signaling in phenotypic expression and proliferation of articular cartilage superficial zone cells. *Lab. Investig.* **91**, 1739–1752 (2011).

29. Jackson, A. & Gu, W. Transport Properties of Cartilaginous Tissues. *Curr. Rheumatol. Rev.* **5**, 40–50 (2009).

30. Luo, Y. *et al.* The minor collagens in articular cartilage. *Protein Cell* **8**, 560–572 (2017).

31. S.-W., L. *et al.* Transgenic mice with targeted inactivation of the Col2a1 gene for collagen II develop a skeleton with membranous and periosteal bone but no endochondral bone. *Genes Dev.* **9**, 2821–2830 (1995).

32. Ng, L. J. *et al.* SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev. Biol.* **183**, 108–121 (1997).

33. Shen, G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. *Orthod. Craniofacial Res.* **8**, 11–17 (2005).

34. Kwan, K. M. *et al.* Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: Implications for function. *J. Cell Biol.* **136**, 459–471 (1997).

35. Poole, A. R. Immunoelectron microscopic studies of type X collagen in endochondral ossification. *J. Cell Biol.* **109**, 2547–2554 (2004).

36. Mizuhashi, K. *et al.* Resting zone of the growth plate houses a unique class of skeletal stem cells. *Nature* **563**, 254–258 (2018).

37. Aspberg, A. The Different Roles of Aggrecan Interaction Domains. *J. Histochem. Cytochem.* **60**, 987–996 (2012).

38. Watanabe, H., Nakata, K., Kimata, K., Nakanishi, I. & Yamada, Y. Dwarfism and age-associated spinal degeneration of heterozygote cmd mice defective in aggrecan. *Proc. Natl. Acad. Sci.* **94**, 6943–6947 (2002).

39. Steen, M. Vander *et al.* ACAN gene mutations in short children born SGA and response to growth hormone treatment. *J. Clin. Endocrinol. Metab.* **102**, 1458–1467 (2017).

40. Stattin, E. L. *et al.* A Missense Mutation in the Aggrecan C-type Lectin Domain Disrupts Extracellular Matrix Interactions and Causes Dominant Familial Osteochondritis Dissecans. *Am. J. Hum. Genet.* **86**, 126–137 (2010).

41. Gkourogianni, A. *et al.* Clinical characterization of patients with autosomal dominant short stature due to aggrecan mutations. *J. Clin. Endocrinol. Metab.* **102**, 460–469 (2017).

42. Anderson, I. J., Tsipouras, P., Scher, C., Ramesar, R. S. & Robert, W. Spondyloepiphyseal dysplasia, mild autosomal dominant type is not due to primary defects of type II collagen. *Am. J. Med. Genet.* **37**, 272–276 (1990).

43. Rhee, D. K. *et al.* The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth Find the latest version : The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. *J Clin Invest.* **115**, 622–631 (2005).

44. Marcelino, J. *et al.* CACP , encoding a secreted proteoglycan , is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. **23**, 319–322 (1999).

45. Coles, J. M. *et al.* Loss of Cartilage Structure , Stiffness , and Frictional Properties in Mice Lacking PRG4. **62**, 1666–1674 (2010).

46. Ruan, M. *et al.* Proteoglycan 4 expression protects against the development of osteoarthritis. *PLoS One* **5**, (2013).

47. Zhang, M. *et al.* Induced superficial chondrocyte death reduces catabolic cartilage damage in murine posttraumatic osteoarthritis. *J. Clin. Invest.* **126**, 2893–2902 (2016).

48. Abad, V. *et al.* The Role of the Resting Zone in Growth Plate Chondrogenesis. *Endocrinology* **143**, 1851–1857 (2002).

49. Kilborn, S. H., Trudel, G. & Uhthoff, H. Review of growth plate closure compared with age at sexual maturity and lifespan in laboratory animals. *Contemp. Top. Lab. Anim. Sci.* **41**, 21–6 (2002).

50. Dowthwaite, G. P. The surface of articular cartilage contains a progenitor cell

population. *J. Cell Sci.* **117**, 889–897 (2004).

51. Kaul, R. *et al.* The Effect of Altered Loading on Mandibular Condylar Cartilage. *PLoS One* **11**, e0160121 (2016).

52. Decker, R. S. *et al.* Cell origin, volume and arrangement are drivers of articular cartilage formation, morphogenesis and response to injury in mouse limbs. *Dev. Biol.* **426**, 56–68 (2017).

53. Brittberg, M. *et al.* Treatment of Deep Cartilage Defects in the Knee with Autologous Chondrocyte Transplantation. *N. Engl. J. Med.* **331**, 889–895 (1994).

54. Drobnic, M. *et al.* The outcome of autologous chondrocyte transplantation treatment of cartilage lesions in the knee. *Cell Mol Biol Lett* **7**, 361–363 (2002).

55. MÜLLER, P., MARK, H. V. O. N. D. E. R., GAUSS, V. & MARK, K. V. O. N. D. E. R. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* **267**, 531–532 (1977).

56. Minegishi, Y., Hosokawa, K. & Tsumaki, N. Time-lapse observation of the dedifferentiation process in mouse chondrocytes using chondrocyte-specific reporters. *Osteoarthr. Cartil.* **21**, 1968–1975 (2013).

57. Schulze-Tanzil, G. *et al.* Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. *Cell Tissue Res.* **308**, 371–379 (2002).

58. Goldberg, A. J., Lee, D. A., Bader, D. L. & Bentley, G. Culture in a tgf-β-containing medium enhances the re- expression of a chondrocytic phenotype in passaged human chondrocytes in pellet culture. *J. Bone Joint Surg. Br.* **87-B**, 128–134 (2005).

59. Yaeger, P. C., Masi, L., Ortiz, J. L. B. De, Tubo, R. & Mcpherson, J. M. Synergistic Action of Transforming Growth Factor- b and Insulin-like Growth Factor-I Induces Expression of Type II Collagen and Aggrecan Genes in Adult Human Articular Chondrocytes. **325**, 318–325 (1997).

60. Anderson, D. E., Markway, B. D., Weekes, K. J., McCarthy, H. E. & Johnstone, B. Physioxia Promotes the Articular Chondrocyte-Like Phenotype in Human Chondroprogenitor-Derived Self-Organized Tissue. *Tissue Eng. Part A* **24**, 264–274 (2018).

61. Gunja, N. J. & Athanasiou, K. A. Passage and reversal effects on gene expression of bovine meniscal fibrochondrocytes. *Arthritis Res. Ther.* **9**, 1–12 (2007).

62. Barlič, A., Drobnič, M., Maličev, E. & Kregar-Velikonja, N. Quantitative analysis of gene expression in human articular chondrocytes assigned for autologous implantation. *J. Orthop. Res.* **26**, 847–853 (2008).

63. Coates, E. E. & Fisher, J. P. Engineering Superficial Zone Chondrocytes from Mesenchymal Stem Cells. *Tissue Eng. Part C Methods* **20**, 630–640 (2013).

64. Van Der Eerden, B. C. J., Karperien, M. & Wit, J. M. Systemic and Local Regulation of the Growth Plate. *Endocr. Rev.* **24**, 782–801 (2003).

65. Gafni, R. I. *et al.* Catch-up growth is associated with delayed se- nescence of the growth plate in rabbits. **50**, 618–623 (2001).

66. Minina, E. *et al.* Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates

chondrocyte proliferation and hypertrophic differentiation. **3**, 439–449 (2002).

67. Wang, W., Xu, J. & Kirsch, T. Annexin-mediated Ca2+ influx regulates growth plate chondrocyte maturation and apoptosis. **278**, 3762–3769 (2003).

68. Schipani, E. *et al.* Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. 2865–2876 (2001). doi:10.1101/gad.934301.ticular

69. Zhou, X. *et al.* Chondrocytes Transdifferentiate into Osteoblasts in Endochondral Bone during Development, Postnatal Growth and Fracture Healing in Mice. *PLoS Genet.* **10**, (2014).

70. Artavanis-tsakonas, S., Rand, M. D. & Lake, R. J. Notching up another pathway. *Science (80-.).* **284**, 770–777 (1999).

71. Brennan, K. & Gardner, P. Notching up another pathway. *Bioessays* **24**, 405–410 (2002).

72. Hu, Q. *et al.* F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. *Cell* **115**, 163–175 (2003).

73. Brosnan, C. F. & John, G. R. Revisiting Notch in remyelination of multiple sclerosis lesions. *J. Clin. Invest.* **119**, 6–9 (2009).

74. Iso, T., Kedes, L. & Hamamori, Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol.* **255**, 237–255 (2003).

75. Mumm, J. S. *et al.* A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Mol. Cell* **5**, 197–206 (2000).

76. Schroeter, E. H., Kisslinger, J. A. & Kopan, R. Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382–386 (1998).

77. Williams, R., Nelson, L., Dowthwaite, G. P., Evans, D. J. R. & Archer, C. W. Notch receptor and Notch ligand expression in developing avian cartilage. *J. Anat.* **215**, 159–169 (2009).

78. Hayes, A. J., Dowthwaite, G. P., Webster, S. V. & Archer, C. W. The distribution of Notch receptors and their ligands during articular cartilage development. *J. Anat.* **202**, 495–502 (2003).

79. Watanabe, N. *et al.* Suppression of differentiation and proliferation of early chondrogenic cells by Notch. *J. Bone Miner. Metab.* **21**, 344–352 (2003).

80. Dong, Y. *et al.* RBPj -dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. *Development* **137**, 1461–1471 (2010).

81. Crowe, R., Zikherman, J. & Niswander, L. Delta-1 negatively regulates the transition from prehypertrophic to hypertrophic chondrocytes during cartilage formation. **998**, 987–998 (1999).

82. Hilton, M. J. *et al.* Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. **14**, 306–314 (2008).

83. Dovey, H. F. *et al.* Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* 173–181 (2001).

84. Oldershaw, R. A. & Hardingham, T. E. Notch signaling during chondrogenesis of human bone marrow stem cells. *Bone* **46**, 286–293 (2010).

85. Vujovic, S., Henderson, S. R., Flanagan, A. M. & Clements, M. O. Inhibition of γ-secretases alters both proliferation and differentiation of mesenchymal stem cells. *Cell Prolif.* **40**, 185–195 (2007).

86. Fujimaki, R., Toyama, Y. & Hozumi, N. Involvement of Notch signaling in initiation of prechondrogenic condensation and nodule formation in limb bud micromass cultures. 191–192 (2006). doi:10.1007/s00774-005-0671-y

87. Atsumi, T., Ikawa, Y., Miwa, Y. & Kimata, K. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. *Cell Differ. Dev.* **30**, 109–116 (1990).

88. Cheng, P. *et al.* Chondrogenic Differentiation Of Bone Marrow-derived Mesenchymal Stem Cells Regulated By Wnt / beta-catenin Signaling Pathway.

89. Narcisi, R. *et al.* Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. *Stem Cell Reports* **4**, 459–472 (2015).

90. Yang, Y. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. *Development* **130**, 1003–1015 (2003).

91. Witte, F., Dokas, J., Neuendorf, F., Mundlos, S. & Stricker, S. Comprehensive expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation. *Gene Expr. Patterns* **9**, 215–223 (2009).

92. Willert, K. & Nusse, R. β-catenin: A key mediator of Wnt signaling. *Curr. Opin. Genet. Dev.* **8**, 95–102 (1998).

93. Ben-Ze'Ev, A. & Geiger, B. Differential molecular interactions of β-catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Biol.* **10**, 629–639 (1998).

94. Usami, Y., Gunawardena, A. T., Iwamoto, M. & Enomoto-Iwamoto, M. Wnt signaling in cartilage development and diseases: Lessons from animal studies. *Lab. Investig.* **96**, 186–196 (2016).

95. B. TACIAK, I. PRUSZYNSKA, L. KIRAGA, M. BIALASEK, M. K. Wnt Signaling Pathway in Development and Cancer. *J. Physiol. Pharmacol.* **69**, 185–196 (2018).

96. Kobayashi, Y., Maeda, K. & Takahashi, N. Roles of Wnt signaling in bone formation and resorption. *Jpn. Dent. Sci. Rev.* **44**, 76–82 (2008).

97. Hartmann, C. & Tabin, C. J. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. *Development* **127**, 3141–59 (2000).

98. Guo, X. *et al.* Wnt/β-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev.* **18**, 2404 (2004).

99. Hill, T. P., Später, D., Makoto M. Taketo, Birchmeier, W. & Hartmann, C. Canonical Wnt/β-Catenin Signaling Prevents Osteoblasts from Differentiating into Chondrocytes. *Developmental Cell* **8**, (2005).

100. Hu, H. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Development* **132**, 49–60 (2004).

101. Rodda, S. J. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* **133**, 3231–3244 (2006).

102. Yuasa, T. *et al.* Transient activation of Wnt/β-catenin signaling induces abnormal growth plate closure and articular cartilage thickening in postnatal mice. *Am. J. Pathol.* **175**, 1993–2003 (2009).

103. Zhu, M. *et al.* Activation of β-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult β-catenin conditional activation mice. *J. Bone Miner. Res.* **24**, 12–21 (2009).

104. Tickle, C. & Towers, M. Sonic Hedgehog Signaling in Limb Development. *Front. Cell Dev. Biol.* **5**, 1–19 (2017).

105. Schilling, T. F., Concordet, J. & Ingham, P. W. Regulation of Left – Right Asymmetries in the Zebrafish by Shh and BMP4. *Dev. Biol.* **287**, 277–287 (1999).

106. Murtaugh, L. C., Chyung, J. H. & Lassar, A. B. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* 225–237 (1999).

107. Bitgood, M. J. & Mcmahon, A. P. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **138**, 126–138 (1995).

108. Factor-, G. Expression of Indian hedgehog in osteoblasts and its posttranscriptional regulation by transforming growth factor-beta. *Endocrinology.* **138**, 1972–1978 (1997).

109. Murakami, S. & Noda, M. Expression of Indian Hedgehog During Fracture Healing in Adult Rat Femora. *Calcif Tissue Int* **66**, 272–276 (2000).

110. Kindblom, J. M., Nilsson, O., Hurme, T. & Ohlsson, C. Expression and localization of Indian hedgehog (Ihh) and parathyroid hormone related protein (PTHrP) in the human growth plate during pubertal development. *J Endocrinol* 1–6 (2002).

111. Kobayashi, T. *et al.* Indian hedgehog stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP. *J Clin Invest* **115**, (2005).

112. Rutenberg, J. B., Johnson, R. L., Vortkamp, A. & St-Jacques, B. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Dev. Biol.* **209**, 239–253 (1999).

113. Vortkamp, A. *et al.* Regulation of rate of cartilage differentiation by Indian Hedgehog and PTH-related protein. *Science* **273**, 613–622 (1996).

114. Long, F., Zhang, X. M., Karp, S., Yang, Y. & McMahon, A. P. Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. *Development* **128**, 5099–108 (2001).

115. Ronenberg, H. E. K. The parathyroid hormone ¯parathyroid hormone-related peptide

receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. *Proc Natl Acad Sci U S A* **95**, 13030–13035 (1998).

116. Chung, U., Schipani, E., Mcmahon, A. P. & Kronenberg, H. M. Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J Clin Invest.* **107**, 295–304 (2001).

117. Mak, K. K., Chen, M.-H., Day, T. F., Chuang, P.-T. & Yingzi Yang. Wnt/β-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation. *Development* **133**, 3695–3707 (2006).

118. Long, F. Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development* **131**, 1309–1318 (2004).

119. Shuang, F. *et al.* Indian Hedgehog signaling pathway members are associated with magnetic resonance imaging manifestations and pathological scores in lumbar facet joint osteoarthritis. *Sci Rep* 1–7 (2015). doi:10.1038/srep10290

120. Zhao, Q., Eberspaecher, H., Lefebvre, V. & De Crombrugghe, B. Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev. Dyn.* **209**, 377–386 (1997).

121. Pan, Y., Wong, E. A., Dibner, J. J., Vázquez-Añón, M. & Webb Jr., K. E. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *J. Nutr.* **132**, 2813–2828 (2002).

122. Henry, S. P., Liang, S., Akdemir, K. C. & De Crombrugghe, B. The postnatal role of Sox9 in cartilage. *J. Bone Miner. Res.* **27**, 2511–2525 (2012).

123. Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R. & De Crombrugghe, B. Sox9 is required for cartilage formation. *Nat. Genet.* **22**, 85–89 (1999).

124. Akiyama, H. *et al.* Interactions between Sox9 and β-catenin control chondrocyte differentiation. *Genes Dev.* 1072–1087 (2004). doi:10.1101/gad.1171104.Wagner

125. Hattori, T. *et al.* SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. *Development* **137**, 901–911 (2010).

126. Kormish, J. D., Sinner, D. & Zorn, A. M. Interactions between SOX factors and Wnt/β-catenin signaling in development and disease. *Dev. Dyn.* **239**, 56–68 (2010).

127. Jacob, T., Lo, P., Linnarsson, S., Kasper, M. & Linnarsson, S. Single-Cell Transcriptomics Reveals that Differentiation and Spatial Signatures Shape Epidermal and Hair Follicle Heterogeneity Article Single-Cell Transcriptomics Reveals that Differentiation and Spatial Signatures Shape Epidermal and Hair Follicle Heter. *cell Syst.* 221–237 (2016). doi:10.1016/j.cels.2016.08.010

128. Rompolas, P. *et al.* SpatiotempRompolas, P., Mesa, K. R., Kawaguchi, K., Park, S., Klein, A. M., & Greco, V. (2016). Spatiotemporal coordination of stem cell commitment during epidermal homeostasis. Science, 352(6292), 0–4.oral coordination of stem cell commitment during epid. *Science (80-.).* **352**, 0–4 (2016).

129. Suda, T., Takubo, K. & Semenza, G. L. Metabolic Regulation of Hematopoietic Stem Cells in the Hypoxic Niche. *Stem Cell* **9**, 298–310 (2011).

130. Snippert, H. J. *et al.* Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* **143**, 134–144 (2010).

131. Clevers, H. The Intestinal Crypt , A Prototype Stem Cell Compartment. *Cell* **154**, 274–284 (2013).

132. Newton, P. T. *et al.* A radical switch in clonality reveals the formation of a stem cell niche in the epiphyseal growth plate. doi:10.1038/s41586-019-0989-6

133. Klein, A. M., Nakagawa, T., Ichikawa, R., Yoshida, S. & Simons, B. D. Mouse Germ Line Stem Cells Undergo Rapid and Stochastic Turnover. *Stem Cell* **7**, 214–224 (2010).

134. Klein, A. M. *et al.* A single type of progenitor cell maintains normal epidermis. *Nature* **446**, (2007).

135. Klein, A. M., Simons, B. D., Jones, P. H. & Doupe, D. P. The Ordered Architecture of Murine Ear Epidermis Is Maintained by Progenitor Cells with Random Fate. *Dev Cell.* 317–323 (2010). doi:10.1016/j.devcel.2009.12.016

136. Bi, P. *et al.* Notch activation drives adipocyte dedifferentiation and tumorigenic transformation in mice. *J. Exp. Med.* **213**, 2019–2037 (2016).