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The role of integrin α10 in the growth plate cytoarchitecture

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ABSTRACT

Integrins are heterodimeric transmembrane proteins composed of an α and ß subunit. Besides their ability to act as cell surface anchoring receptors, they also play an important role in signal transduction between cells and the extracellular matrix (ECM). Integrin α10ß1 is one of the most important collagen-binding integrins in cartilage with affinity to collagens II, IV and VI. While most α subunits are known and studied for decades, little is known about integrin α10. Knockout studies in mice provide an important and established platform to study and analyse the function of a gene and its associated protein. Mice carrying a constitutive loss of the integrin α10 gene have previously been reported to have a mild dwarfism phenotype due to a partially disrupted growth plate architecture, characterized by increased chondrocyte apoptosis and less dense collagen network in the ECM. The aim of this study was to further characterize the role of integrin α10 during cartilage and bone development by analysing and comparing growth plate cytoarchitecture of integrin α10 knockout and wildtype mice at different ages by cytoskeleton immunofluorescence staining and confocal microscopy. The normal growth plate is organized into three horizontally zones, namely the resting, the proliferative and the hypertrophic zone. In these zones, chondrocytes undergo a differentiation and maturation processes that reflects the specific transcriptional, metabolically, and morphological state of the cells. In the present work, the proliferative zone was analysed, here chondrocytes exhibit a flat shape, perpendicular orientation to the direction of the bone growth, and organization into columns parallel to the long axis of the bone. Various morphological characteristics, namely chondrocyte geometry, cell orientation, column alignment and number of chondrocytes per column were analysed at different embryonic (E15.5 and E18.5) and postnatal stages (2 weeks, 1 month and 2 months). Staining with phalloidin demonstrated an intact actin cytoskeleton, but the cell shape of the α10 integrin knockout (KO) chondrocytes showed a tendency to fail assuming the typical flat shape. At E18.5 and 2 months of age, the loss of integrin α10 resulted in a change in the number of cells per column, and in alteration of column orientation at E18.5. Fewer chondrocytes and impaired orientation were observed in most columns compared to wild type (WT) littermates. The observed rounded cell geometry with an intact actin cytoskeleton led to the hypothesis, that KO chondrocytes were impaired in their attachment to ECM components. The binding and motility of mutant and WT chondrocytes on collagen II, collagen VI, and fibronectin (FN) was analysed by time

lapse video and optical density measurements. The migration assay showed slightly more migration of integrin α10 KO chondrocytes on collagen VI compared to WT cells, while the KO chondrocytes moved less on FN than the WT cells. Nevertheless, there was no difference in the migration velocity for cells cultivated on the different substrates. Contrary, the attachment kinetic assay showed a much slower attachment of mutant chondrocytes to collagen II as well as to collagen VI. Furthermore, the adhesion assay demonstrated lower adhesion to collagen VI. Taken together, loss of integrin α10 leads to a rounded chondrocyte shape, slightly affected column formation and cell proliferation, most likely due to an impaired interaction of the KO chondrocytes with collagen II and collagen VI.

ZUSAMMENFASSUNG

Integrine sind heterodimere Transmembranproteine, die sich aus einer α- und ß-Untereinheit zusammensetzen. Neben ihrer Funktion als Zelloberflächenrezeptoren spielen sie auch eine wichtige Rolle bei Interaktionen der Zellen mit ihrer extrazellulären Matrix (EZM). Integrin α10ß1 ist eines der wichtigsten kollagenbindenden Integrine im Knorpelgewebe und interagiert unteranderem mit den Kollagenen II, IV und VI. Während die meisten der α-Untereinheiten seit vielen Jahrzehnten bekannt und gut untersucht sind, ist über Integrin α10 nur wenig erforscht. Knockout-Studien an Mäusen sind eine wichtige und etablierte Möglichkeit, die Funktion eines bestimmten Gens und des dazugehörigen Proteins zu untersuchen und zu analysieren. Bei Mäusen, die eine konstitutive Deletion des Integrin-α10-Gens tragen, konnte bereits ein milder Zwergwuchs nachgewiesen werden. Dies wird vermutlich durch eine gestörte Wachstumsfugenarchitektur, eine erhöhte Apoptose der Chondrozyten und ein aufgelockertes Kollagennetzwerk innerhalb der EZM verursacht. Das Ziel der vorliegenden Arbeit war es, die Rolle von Integrin α10 während der Knorpel- und Knochenentwicklung weiter zu charakterisieren, indem die Morphologie der Wachstumsfuge von Integrin α10-Knockout (KO) und Wildtyp (WT) Mäusen unterschiedlichen Alters mittels Immunfluoreszenzfärbung des Cytoskeletts und konfokaler Mikroskopie analysiert und verglichen wurde. Eine gesunde Wachstumsfuge kann in drei horizontal angeordnete Zonen eingeteilt werden: die ruhende, die proliferative, und der Metaphyse am nächsten, die hypertrophe Zone. In diesen Zonen durchlaufen die Chondrozyten einen Differenzierungs- und Reifungsprozess, der den spezifischen transkriptionellen, metabolischen und morphologischen Zustand der Zellen widerspiegelt. In der vorliegenden Arbeit wurde die proliferative Zone analysiert, in der die Chondrozyten normalerweise eine abgeflachte Form zeigen und sich zu Säulen stapeln, die parallel zur Längsachse des Knochens orientiert sind. Verschiedene morphologische Merkmale wie die Geometrie der Chondrozyten, die Zellorientierung, die Säulenausrichtung und die Anzahl der Chondrozyten pro Säule wurden in verschiedenen embryonalen (E15.5 und E18.5) und postnatalen Stadien (2 Wochen, 1 Monat und 2 Monate) analysiert. Die Färbung mit Phalloidin zeigte ein intaktes Aktinzytoskelett bei einer gleichzeitig runderen Zellform der KO-Chondrozyten verglichen mit dem WT. Im Alter von E18.5 (neugeboren) und 2 Monaten konnte zudem ein Einfluss auf die Anzahl der Zellen pro Säule und bei den neugeborenen Mäusen zudem ein Unterschied in der

Säulenorientierung durch den Integrin α10 Verlust nachgewiesen werden. Die meisten Säulen beinhalteten dabei weniger Chondrozyten und eine größere Varianz in der Orientierung zur Längsachse des Knochens im Vergleich zum WT. Die beobachtete abgerundete Zellform bei jedoch intakten Aktinzytoskelett führte zur Hypothese einer gestörten Adhäsion der KO-Chondrozyten an Komponenten innerhalb der EZM. Daher wurde die Adhäsion und Migration von KO- und WT-Knorpelzellen an und auf Kollagen II, VI und Fibronektin mittels Zeitraffervideos (Attachment Kinetic und Migration Assay) und optischen Dichtemessungen (Adhesion Assay) analysiert. Es konnte eine etwas vermehrte Migration der KO-Chondrozyten auf Kollagen VI, sowie eine verminderte Bewegung auf Fibronektin verglichen mit dem WT nachgewiesen werden. Trotzdem zeigte sich insgesamt kein Unterschied in den Migrationsgeschwindigkeiten auf den verschiedenen Substraten. Im Gegensatz dazu präsentierte sich eine langsamere Adhäsion der KO-Chondrozyten sowohl an Kollagen II als auch an Kollagen VI im Attachment Kinetic Assay. Unterstützt wurde diese Beobachtung durch eine gleichermaßen verringerte Adhäsion an Kollagen VI bei dem in dieser Arbeit durchgeführten Adhesion Assay. Zusammenfassend führt der Verlust von Integrin-α10 zu einer abgerundeten Chondrozytenform, sowie einer leicht beeinträchtigten Säulenbildung und Zellproliferation, höchstwahrscheinlich bedingt durch eine gestörte Adhärenz der KO-Chondrozyten an Kollagen II und Kollagen VI.

TABLE OF CONTENTS

FIGURE LEGEND

1 INTRODUCTION

1.1 CARTILAGE

1.1.1 COMPOSITION AND ORGANIZATION

In mammals there are three different types of cartilage, namely the hyaline, elastic and fibrous cartilage, which differ in their matrix composition, anatomical location, histological appearance and mechanical properties (Wachsmuth et al., 2006). Among all, the hyaline cartilage is the most abundant one and can be found in the permanent articular cartilage covering the anatomical ends of opposing bones, as well as in the trachea, rib tips and nose, in the fracture callus forming during bone healing and in the transient embryonic and growth plate cartilage, which plays an important role in long bone formation and growth (Boyan et al., 2017; Poole et al., 2001). Articular cartilage (AC) is covering diarthrodial joints in vertebrates and provides a smooth articulation due to its unique properties such as lubricated surface, elasticity, and high resistance to shear stress and compressive forces. AC is characterized by the absence of a vascular and lymphatic system and innervation which results in its poor self-renewal capacity. For a healthy musculoskeletal system, the maintenance of the cartilage architecture is indispensable. AC is composed of four zones, namely the superficial, the transition, the deep and, adjacent to the subchondral bone, the calcified zone. The different zones and regions show distinct cell shape and extracellular matrix (ECM) organization, and therefore properties. (Fox, 2009) The cellular component in articular cartilage is solely represented by tissue-specialized cells, called chondrocytes, that account for only 2% of the total tissue volume. The chondrocytes are located in cavities described as cartilage lacunae and synthetize the specialized cartilaginous ECM. (Poole et al., 2001; Stockwell, 1978)
The focus of this thesis is the growth plate cartilage which will be discussed in detail later.

1.1.2 ECM

The ECM plays a major role in maintaining the unique cartilage functions, minimizing friction of the opposing AC surfaces and withstand compressive and shear stresses during joint movement. The ECM of the hyaline cartilage is mainly composed of water, proteoglycans, collagens and non-collagenous glycoproteins (Fig.1) (Mow et al.,

1992). Its composition is influenced by age as well as by the metabolic and differentiation state of the chondrocytes, external stimuli like mechanical load, cytokines and growth factors (Hardingham & Bayliss, 1990; Stockwell, 1978).

1.1.2.1 COLLAGENS

Collagens are the most commonly found proteins in animals where they build huge fibrillar networks within the ECM of different tissues. In AC, they account for about 60% of the dry mass and are composed of three parallel polyproline type II helices (Shoulders & Raines, 2009; Fox et al., 2009). The diameter of the collagen fibrils differs in the different zones of AC reaching a maximum from 70 to 120 nm in the deep zone (Poole et al., 2001). Till now there are 28 collagens described and in cartilage primarily collagen II and some quantitatively more minor collagens such as types I, IV, V, VI, IX, X and XI are expressed (Shoulders & Raines, 2009; Fox et al., 2009). Collagen II as the major collagen in hyaline cartilage builds an extensive fibrillar network which is mostly responsible for the tensile strength within the tissue and counteracts the swelling of proteoglycans. Collagen II is interacting with other ECM molecules, primarily with collagen IX, XI and non-collagenous ECM components like proteoglycans. Collagen II, and the minor collagens types IX and XI together form the heterotypic fibrillar network within the cartilage. Type XI is a fibril-forming collagen, while collagen IX is a “fibril-associated collagen with interrupted triple-helix” (FACIT) family member. (Eyre, 2002; Eyre et al., 2008) In contrast to collagen II, collagen I is found in fibrocartilaginous tissues like menisci (Poole et al., 2001). Collagen X, as a non-fibrillar collagen and hypertrophic marker, is only detectable in the ECM surrounding hypertrophic chondrocytes, primarily in foetal cartilage and the physis (Linsenmayer et al., 1988; Linsenmayer et al., 1991; Mendler et al., 1989). Collagen VI as a member of microfibrillar collagens shows a high affinity for hyaluronan and is predominant in the pericellular matrix (PCM), probably regulating the attachment of the chondrocytes to the surroundings (Keene et al., 1988; Kielty et al., 1992). This type of collagen was also shown to regulate cartilage stiffness and chondrocyte swelling, while studies in knockout mice revealed a faster and earlier onset of osteoarthritis accompanied by a delayed secondary ossification due to a collagen VI loss (Alexopoulos et al., 2009). Furthermore collagen VI is thought to function as kind of transducer within the PCM through interactions with integrins and hyaluronan (Alexopoulos et al., 2003; Lee et al., 2000; Loeser et al., 2000). Interestingly the ECM

within the growth plate undergoes structural changes during maturation when cells move through the different zones of the growth plate. The collagen fibres are randomly orientated in the resting zone while they form longitudinal and transverse bundles within the proliferative and hypertrophic zones (for more details see “growth plate” chapter) (Eggli et al. , 1985; Prein et al., 2016). Studies with genetically modified mice showed that mutations in the genes encoding for collagen II, IX and XI result in moderate to lethal chondrodysplasia, which shows the high importance of collagens for a proper growth plate architecture and cartilage/bone development (Blumbach et al., 2008; Kuivaniemi et al., 1997; Li S. et al., 1995; Li Y. et al., 1995).

1.1.2.2 PROTEOGLYCANS

A key function of cartilage is the ability to endure and balance mechanical stress, which is primarily fulfilled by proteoglycans (PGs) that provide the matrix with a high osmotic pressure. PGs are building large negatively charged aggregates by binding cations surrounded by water, thus forming a gel-like ECM in which the collagens as fibrillar proteins are incorporated. (Yanagishita, 1993) The importance of water is obvious when considering that water represents 65-80% of the wet weight of AC (Pearle et al., 2005). PGs are composed of a protein core which is covalently connected to different numbers of glycosaminoglycans (GAG). Depending on the repeated disaccharide sequence, different types of GAGs can be distinguished namely hyaluronan, heparan sulfate (HS)/heparin, chondroitin/dermatan sulfate (CS/DS), and keratan sulfate (KS) (Mikami & Kitagawa, 2013; Schaefer, 2014; Schaefer & Schaefer, 2010). In cartilage, lecticans and the small leucine-rich repeat proteoglycans (SLRPs) are representing the biggest PG-families. Lecticans include aggrecan and versican, while SLRPs include many different proteins *e.g.* biglycan, decorin and fibromodulin. (Iozzo, 1998; Ruoslahti, 1996a) Among all PGs, aggrecan is the most prominent, comprising 90% of the whole PG content. Aggrecan forms huge aggregates together with hyaluronan and the link protein. The molecule of aggrecan is composed of about 100 CS and additional KS chains, providing 10.000 negative charges that generate water intake establishing the so called osmotic swelling pressure typical of cartilage tissue. The KS chains seem to connect to fibrillar collagens. (Hedlund et al., 1999; Heinegård, 2009) While members like aggrecan are responsible for the high osmotic pressure, which is important to endure compressive forces, the small leucine-rich repeat proteoglycan family primarily supports the assembly of the collagen network by binding and thus

crosslinking the collagen fibrils (Kalamajski & Oldberg, 2010; Rada et al., 1993; Vogel et al., 1984). Another important aspect is the proinflammatory effect of some SLRP family members like biglycan, modelling cell interactions via Toll-like receptor binding (TLR-2,4) (Schaefer et al., 2005).

1.1.2.3 NONCOLLAGENOUS GLYCOPROTEINS

Beside the major components of cartilage, collagens and PGs, the filamentous network is interconnected by various glycoproteins (or adaptor-proteins), which also play a role in regulating developmental and homeostasis processes by influencing growth factor signalling, cell-matrix interactions and immune response (Sage & Bornstein, 1991; Murphy-Ullrich & Sage, 2014). Among others, two families, namely thrombospondins and matrilins, can be distinguished in cartilage. One of the most abundant thrombospondins found in cartilage is thrombospondin 5/Cartilage Oligomeric Matrix Protein (COMP). COMP influences structural aspects like fibril formation and fibril diameter by attaching to collagens I,II and IX, but it is also responsible for the binding and controlling the availability of members of the TGF-β superfamily, thereby, modulating chondro- and osteogenesis. (Haudenschild et al., 2011; Koelling et al., 2006) Other important adaptors for the cartilaginous ECM are the members of the matrilin family. Matrilins contain two (matrilin-1,-2 and 4) or one (matrilin-3) von Willebrand factor A-like (VWA) domains, and different numbers of epidermal growth factor like domains, based on which they interact with several other matrix components like COMP and cartilage specific collagens (Mann et al., 2004). By doing so, they seem to influence cell attachment (Mann et al., 2007) and chondrogenesis (Pei et al., 2008), at least in vitro. In skeletal tissue, the most abundant matrilins are matrilin-1 and matrilin-3 (Mann et al., 2004). Another important player in the maintenance of matrix homeostasis is the adaptor-protein fibronectin (FN), a dipeptide connected by a disulphide bond, which interacts with several other ECM proteins like collagens, heparin and COMP, and contains an Arginine-Glycine-Aspartate (RGD) sequence which facilitates cell binding via integrins (Pankov & Yamada, 2002). FN is influencing cell migration, adhesion and differentiation and, interestingly, seems to be upregulated in osteoarthritic joints (Xie & Homandberg, 1993).

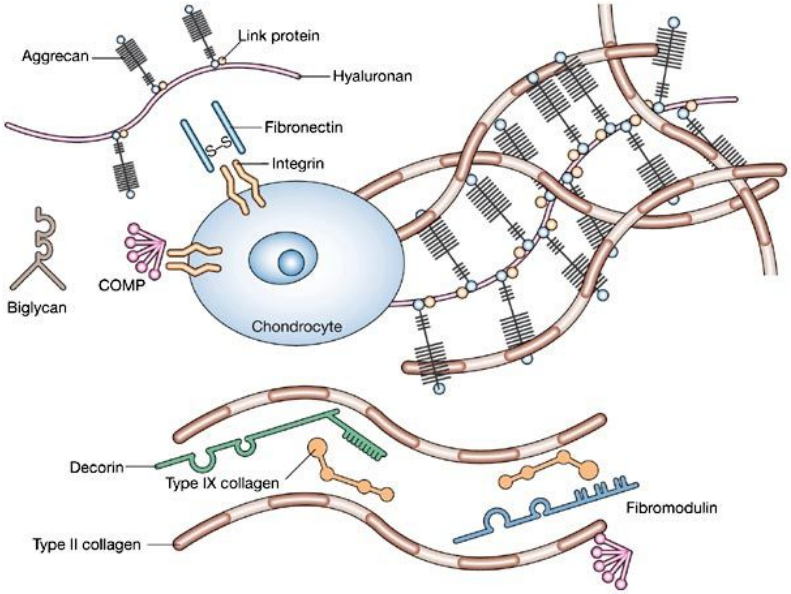

Figure 1: Scheme of the ECM within hyaline cartilage. Collagens (primarily collagen II), proteoglycans (especially aggrecan and the smaller ones: decorin, fibromodulin and biglycan) and noncollagenous glycoproteins (COMP, link protein and fibronectin) account for most of the ECM proteins. Collagens, mainly determining the tensile force of the tissue, are building a fibrillar network interconnected with adaptor proteins, in which negatively charged PGs (mainly aggrecan) are embedded, providing cartilage with high osmotic pressure that endows resistance to compressive forces. (Chen et al., 2006)[1]

1.2 SKELETAL DEVELOPMENT

The first step in the skeletal development of bones is the condensation of mesenchymal stem cells (MSC) providing the anlage of future bones. Condensed MSC either first differentiate into chondrocytes making a cartilaginous template which later on remodels into bone by a process called endochondral bone formation (or endochondral ossification), or differentiates directly into osteoblasts (intramembranous ossification) (Berendsen & Olsen, 2015; Mundlos & Olsen, 1997). Only flat bones of the skull and the clavicle form by the second process. Both ossification processes also play a role during fracture healing (Vortkamp et al., 1998).

[1] "Reprinted by permission from Springer Nature, Nature Clinical Practice Rheumatology, 2(7), "Technology Insight: adult stem cells in cartilage regeneration and tissue engineering", Chen et al., © 2006"

1.2.1 ENDOCHONDRAL OSSIFICATION

During skeletogenesis the formation of the bony elements mostly occurs by the process of endochondral ossification (Fig.2). As a first step mesenchymal cells start to migrate to the future sites of the bones, followed by a condensation and proliferation process building up a chondrogenic model of the later skeleton (Berendsen & Olsen, 2015). The aggregated cells continue proliferating and differentiate via chondroblasts into chondrocytes and start to produce a specialized and characteristic ECM containing type II collagen fibrils and aggrecan. Afterwards, the chondrocytes in the centre of the developing long bones undergo a maturation process by passing through proliferating, prehypertrophic and hypertrophic stages in a well-defined anatomical structure called the growth plate (see below). The hypertrophic chondrocytes at the metaphysis deposit collagen X instead of collagen II, modifying their surrounding ECM and partly mineralizing it. Hypertrophic cells also produce angiogenic factors which attracts blood vessels to the perichondrium surrounding the hypertrophic core. (Berendsen & Olsen, 2015; Kronenberg, 2003) The hypertrophic zone then is invaded by osteoclasts/chondroclasts and partially resorbed (Liu & Olsen, 2014). The osteoprogenitor cells differentiate into osteoblasts and start to secret osteoid, which forms the trabecular bone on the remnants of the cartilage (Mackie et al., 2008; Park et al., 2015). Interestingly, hypertrophic chondrocytes can either transdifferentiate into osteoblasts or undergo apoptosis (Park et al., 2015; Zhou et al., 2014). This process of chondrocyte maturation, hypertrophy, vascular invasion, cartilage resorption and formation of the bony spongiosa is reoccurring at the ends of the long bones (epiphysis) and the secondary ossification centres are formed (Berendsen & Olsen, 2015). Between the primary and secondary ossification centre stands the growth plate, which is responsible for longitudinal bone growth through childhood and adolescence. In human, at the end of puberty, when the primary ossification centre meets the secondary one, the entire cartilage of long bones, except the AC, is replaced by bone (Mackie et al., 2008).

Different transcription factors seem to play an important role during endochondral bone formation. The master transcription factor Sox9 is necessary for the expression of ECM components like aggrecan, collagen II and XI and differentiation of mesenchymal progenitors into chondrocytes (Bi et al., 1999; Gao et al., 2013). Furthermore, Sox9 is one of the master regulators in chondrocytes and indispensable for *Sox5* and *Sox6* expression. The three Sox genes together form the so-called Sox-trio, which

controls the expression of several cartilage specific ECM components. (Gao et al., 2013) Beside Sox9, also Runx2 and β-catenin appear to play an important role during endochondral ossification, as high β-catenin and Runx2 levels are required for a differentiation into osteoblasts (Bhattaram et al., 2014; Day et al., 2005).

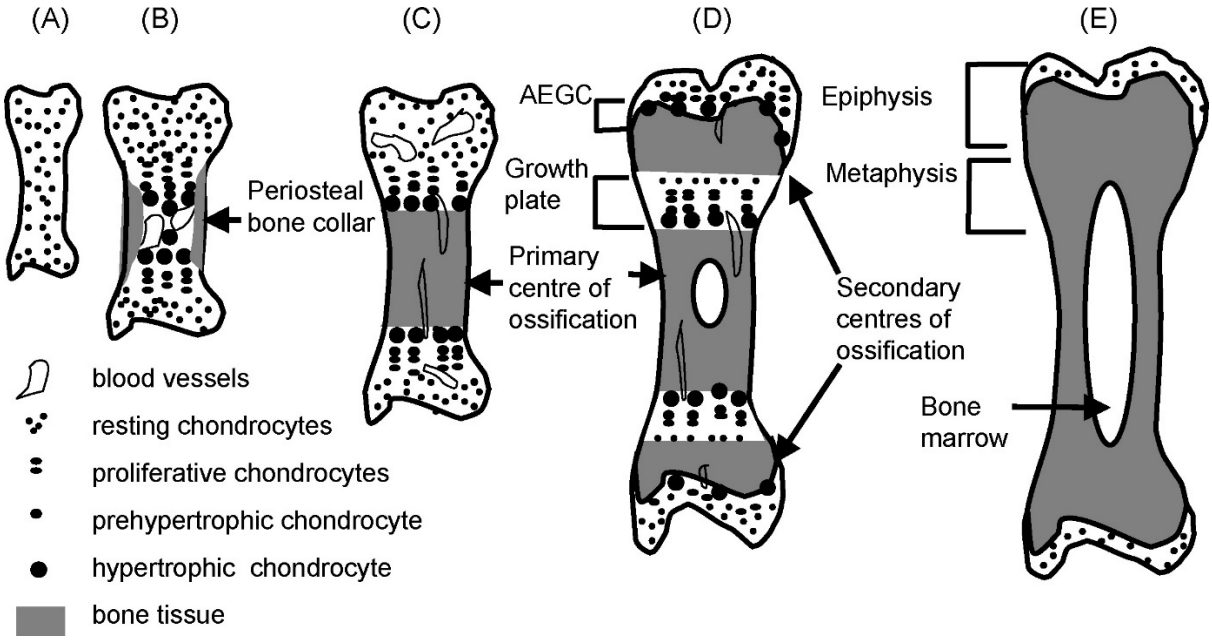

Figure 2: Endochondral bone formation. Scheme showing the processes leading to the bony replacement of the cartilage template. (A) Cartilage template. (B) Formation of the primary ossification centre (POC) including blood vessel invasion and cell hypertrophy. (C) POC is fully established, a few blood vessels are still present within the leftover cartilage. (D) The growth plate remains active between the POC and the secondary ossification centres, at both sides of the bone. At both ends the surfaces are built by articular cartilage (AC) covering the articular-epiphyseal growth cartilage (AEGC). The AEGC takes over the epiphyseal growth and shapening. (E) Scheme of a human adult bone. The growth plate has disappeared as well as the AEGC. Both sides of the long bone are covered by AC. (Mackie et al., 2008)[2]

1.2.2 INTRAMEMBRANOUS OSSIFICATION

During intramembranous ossification bones are developing directly from mesenchymal precursors, therefore, there are no cartilage intermediates. The mesenchymal cells from the neural crest migrate, proliferate and condensate to cell aggregates, followed by a differentiation into osteoblasts which directly form the bone. Parts of the neuro-

[2] "Reprinted by permission from Elsevier, The International Journal of Biochemistry and Cell Biology, 40(1), “Endochondral ossification: How cartilage is converted into bone in the developing skeleton”, Mackie et al., © 2008”

and viscerocranium, the clavicles, the pectoral girdle and also bone fractures are reconstructed by this process (Berendsen & Olsen, 2015; Franz-Odendaal, 2011).

1.3 GROWTH PLATE

1.3.1 STRUCTURE AND FUNCTION

The growth plate represents a complex anatomical structure where longitudinal and lateral growth of endochondral bones occurs. It is located between the epiphysis and the diaphysis at both sides of long bones and plays an essential role during endochondral bone formation. The anatomical location can be separated into three major interacting parts: the cartilaginous growth plate, the metaphysis, and the peripheral ossification groove of Ranvier. (Ballock & O'Keefe, 2003; Brighton, 1978)

The cartilaginous growth plate

The growth plate exists in most vertebrates during the whole life, while in humans it is replaced by bone at the end of puberty. It is divided into three horizontally arranged zones, the resting, the proliferative, and closest to the metaphysis, the hypertrophic zone (Fig.3). Sometimes also a transition area between the proliferative and the hypertrophic zone, namely the pre-hypertrophic zone, is characterized. In these zones, the chondrocytes undergo a differentiation and maturation process that reflects the specific transcriptional, metabolically and morphological state of the cells. (Farnum & Wilsman, 2011; Hunziker et al., 1987; Hunziker, 1994; Wilsman et al., 1996) Chondrocytes located in the reserve zone are small, have an isotropic shape and exhibit a very slow proliferation rate (Candela et al., 2014; Ohlsson et al., 1992). Since a slow proliferation is known to be one attribute for stem cells, chondrocytes of the resting zone are thought to serve as a "germ" region with stem-cell like precursors for cells in the adjacent proliferative zone (Abad et al., 2002). In the proliferative zone, the chondrocytes flatten and orient themselves perpendicular to the long axis of the bone. This orientation relative to the bone/tissue axis is also referred as tissue/planar cell polarity (PCP) (Wang & Nathans, 2007). The chondrocytes begin to stack into small columns of two or three cells, which also vertically divide the proliferative zone. These columns are separated from each other by longitudinally aligned septa within the ECM, while the cells in one column are divided by horizontally arranged septa. (Prein et al., 2016; Schenk et al., 1967; Shibata et al., 1997) The longitudinal septa are thought to

contribute to the bone growth by directing the cell-division and arrangement of chondrocytes within the columns due to their greater stiffness than transverse septa. The difference in the stiffness of the ECM could be due to its distinct composition of collagens and proteoglycans. (Prein et al., 2016) Beside morphological changes, several metabolic alterations occur in the proliferative zone, mainly reflected in changes of the secreted ECM and different expression of cell receptors. Thus, the chondrocytes in the prehypertrophic zone start to secret collagen X and express regulating proteins like Indian hedgehog (Ihh) (Bovée et al., 2010; St-Jacques et al., 1999; Vortkamp et al., 1996). The closer the chondrocytes are located towards the hypertrophic zone, the more they mature, increase in size and more they switch from collagen II and aggrecan expression towards collagen X deposition (Alini et al., 1992; Schmid & Linsenmayer, 1985). In the zone of provisional mineralization, near the bony metaphysis, chondrocytes undergo apoptosis, autophagic cell death or transdifferentiate into osteoblasts (Mackie et al., 2008; Yang et al., 2014; Zhou et al., 2014). The expression of matrix metalloproteinase 13 (Mmp13) in the hypertrophic chondrocytes initiates the degradation of the cartilage matrix, whereas the expression of vascular endothelial growth factor (VEGF) attracts blood vessels into the cartilage, which bring endothelial cells, osteoblast progenitors and chondroclasts that remodel the cartilage ECM and build trabecular bone on the remaining cartilage matrix (Ortega et al., 2004; Stickens et al., 2004).

The Metaphysis

The metaphysis, where primary ossification occurs, is a tiny zone between the diaphysis and the cartilaginous growth plate at the level of vascular invasion (Ballock & O'Keefe, 2003; Brighton, 1978). It consists of two different regions: the primary and the secondary spongiosa. The primary spongiosa is adjacent to the hypertrophic zone where the vascular invasion begins, hypertrophic chondrocytes undergo apoptosis or are transdifferentiating into osteoprogenitors, and the ECM of longitudinal septa separating the chondrocyte-containing columns start to calcify and are partially degraded. The modified longitudinal septa are used as a scaffold for building primary trabeculae by osteoblasts. In the zone of secondary spongiosa, those septa are continuously replaced by lamellar trabecular bone. (Aszódi, 2016; Park et al., 2015; Shapiro et al., 2005; Zhou et al., 2014)

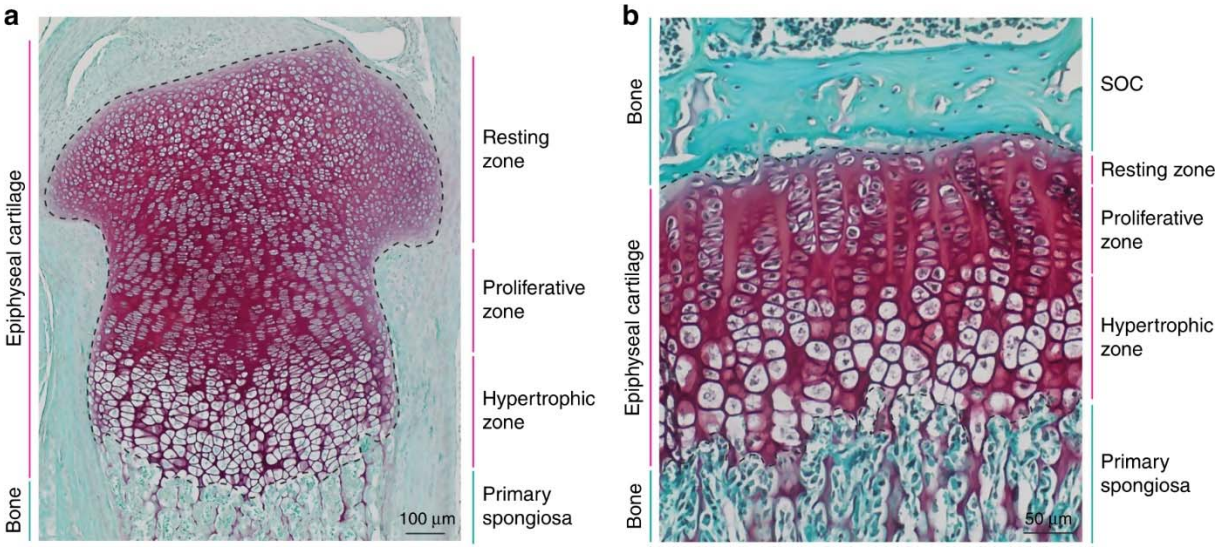

Figure 3: Histological images of the growth plate. Sections from 3 days old (a) and 30 days old (b) mouse tibia stained with Safranin O (red: cartilage) and Fast Green (green: bone and connective tissue). At 30 days the formation of the secondary ossification centre (SOC) already occurred. The growth plate or epiphyseal cartilage can be divided into three different zones, namely the resting, proliferative and hypertrophic zone. Moving down from the upper resting zone to the hypertrophic zone adjacent to the metaphysis, chondrocytes maturate, arrange into columns, flatten and orientate, and are finally partially mineralized, undergo apoptosis and osteoblasts start to form the primary spongiosa. (Chagin & Newton, 2020) [3]

The ossification groove of Ranvier

The cartilaginous growth plate is encircled by a fibro-chondro-osseous structure containing the ring of LaCroix and the groove of Ranvier (Brighton, 1978). The ring of LaCroix is surrounding part of the groove of Ranvier and ends in the periosteum of the metaphysis. Both structures contain stem cell-like chondrogenic progenitor cells, which serve as a pool of stem cells for the articular and growth plate cartilage. It is believed that those slow-cycling stem cell-like cells migrate from the perichondral ring towards the growth plate and articular cartilage. Beside the slow cell cycle, those cells also show some mesenchymal progenitor cell markers and seem to contribute to the outward growth of the bone. The ring of LaCroix also gives stability to the adjacent cartilage-bone junction. (Candela, 2014; Fenichel et al., 2006; Karlsson et al., 2009) The groove of Ranvier and the ring of LaCroix appear to play a rather important role, as surgical removal or injuries of these structures, such as Salter-Harris fractures, lead to impaired bone growth (Ilharreborde et al., 2006; Rodríguez et al., 1985). Also,

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integrin α10, which will be discussed later, was found to be expressed in the groove of Ranvier (Camper et al., 2001).

1.3.2 DIFFERENTIATION OF CHRONDROCYTES WITHIN THE GROWTH PLATE

The chondrocytes of the cartilaginous growth plate undergo a characteristic differentiation process accompanied by oriented cell division (Fig.4). In the proliferative zone, chondrocyte division follows two rules first described by Hertwig in 1893. The rules state that (1) the mitotic spindle is formed in the direction of the long axis of a cell and that (2) “the plane of division always cuts the axis of the spindle perpendicularly” (Hertwig, 1893). In contrast to most mammalian cells, which round up during mitosis, chondrocytes remain flatten. This morphogenetic feature contributes to a specific column formation and elongation observed in the proliferative zone, which follows the Dodds model: the discoid, medio-laterally (ML) oriented chondrocytes divide perpendicular to the proximal-distal (PD) axis of the bone, meaning that the plane of division is orientated in the same direction as the columns. The resulting semi-circular daughter cells are oriented perpendicular to the columnar axis and they rotate back within the column via cell-matrix-mediated interactions, thus contributing to column elongation and longitudinal bone growth (Dodds, 1930). This rearrangement and rotation of the daughter cells may be caused by specialized ECM biomechanics, characterized by stiffer longitudinal septa (LS) between the columns compared to the softer transverse septa (TS), which are separating the cells in one column. This difference in the stiffness may enable the daughter chondrocytes to stay in the column and force them to rearrange towards the softer intracolumnar regions. Furthermore, the stiffness of the TS may contributes to the flattened shape of the chondrocytes. (Prein et al., 2016) Intercalation movements of the postmitotic chondrocytes, which contribute to column formation, may also rely on cadherin/catenin complex interactions between the two daughter cells (Romereim et al., 2014). As transmembrane adhesion proteins cadherins are known to play a role in cell-cell interactions, thus influencing cell movements and shape. They form stable complexes in association with catenins, which in turn interact directly with the actin cytoskeleton of the cell. (Nelson, 2008) Those complexes have been described in growth plates chondrocytes (Sampson et al., 2007). Live imaging of chondrocyte division within the growth plate has led to the hypothesis that cadherin/catenin and thus direct cell-cell interactions may influence cell

behaviour and intercalation movements of postmitotic chondrocytes (Romereim et al., 2014). Another parameter affecting the polarity within the growth plate is the primary cilium. Primary cilia are composed of an axoneme rooted in a basal body and covered by a specialized plasma membrane. In general, they are considered to function as mechano- and chemo-sensors (Farnum & Wilsman, 2011; Haycraft & Serra, 2008). In cartilage, they influence hedgehog (Hh) and Wnt signalling pathways, thus contributing to proper chondrocyte polarity and growth plate architecture (Chang & Serra, 2013). Resting chondrocytes in the growth plate show a random distribution of the primary cilia, instead they are located parallel to the bone axis on cells within the proliferating and hypertrophic zone. This may also indicate the importance of primary cilia in chondrocyte maturation and rearrangements in the growth plate. (Ascenzi et al., 2007) Various studies with genetically modified mice lacking components of a normal primary cilia, such as *Ift88 or Kif3a*, showed disturbed bone growth, an impaired chondrocyte shape and growth plate arrangement (Haycraft et al., 2007; Kolpakova-Hart et al., 2007). In addition, also mechanical stimuli, particularly locomotion, were shown to influence growth plate architecture and bone growth (Heisenberg & Bellaïche, 2013; Shea et al., 2015).

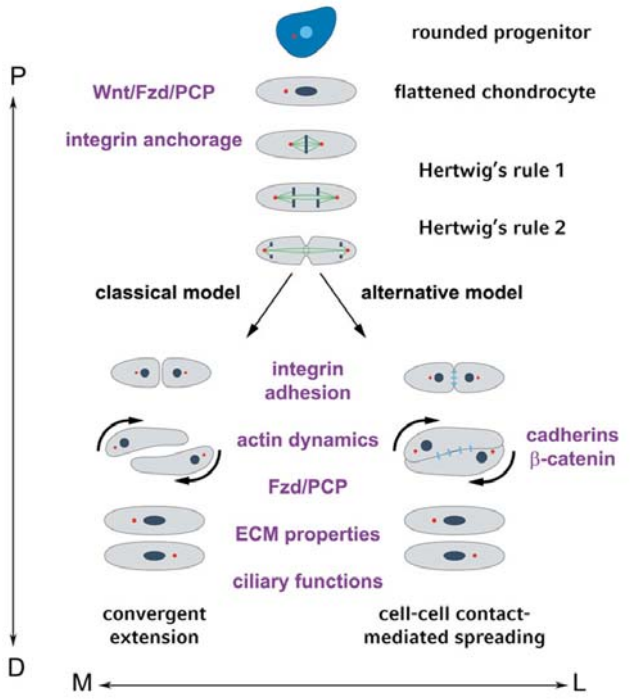

Figure 4: Models of column formation. Chondrocyte progenitors have a round shape and start to flatten in the proliferative zone after the initial cell division. This flattening and establishment of the mitotic spindle occurs perpendicular to the long, proximo-distal (PD) axis of the bone. The cells in the proliferative zone divide perpendicular to the spindle and in plane parallel to the PD-axis of the bone (Hertwig's rules). There are two different hypotheses how the PD growth takes place. In the convergent extension model, cells undergo a medio-lateral rearrangement mediated via cell-matrix interactions which contributes to PD column growth. In the second model, cells intercalate back into the column via cell-cell contact-mediated sliding movement. In purple, regulatory mechanisms influencing cell polarity and chondrocyte rearrangement are indicated. (Aszódi, 2016)[4]

1.3.3 SIGNALLING DURING GROWTH PLATE DEVELOPMENT

Longitudal bone growth and remodelling is highly dependent on extracellular signalling. One important regulatory system is the Indian hedgehog/Parathyroid hormone related peptide (Ihh/PTHrP) axis, which acts as a negative feedback-loop. Ihh is secreted by embryogenic prechondrocytes and prehypertrophic chondrocytes within the growth plate and induces PTHrP expression near the developing articular surface and perichondrium. PTHrP diffuses back to the growth plate and slows down the chondrocyte proliferation and maturation by inhibiting mitotic exit and prehypertrophic differentiation. (St-Jacques et al., 1999; Vortkamp et al., 1998, 1996) Ihh signalling directly enhances proliferation and influences osteoblasts differentiation within the perichondrium. Ihh upregulates the transcription of *Runx2* in perichondral progenitors,

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which promotes the osteoblastic differentiation program. (Rodda & McMahon, 2006; St-Jacques et al., 1999) Another important molecule for the proper formation of cartilage are bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily and are required for the expression of *Sox9* after the condensation phase of skeletogenic progenitors. BMPs and TGF-β seem to induce chondrogenic and osteogenic differentiation via different Smad- and MAPK (mitogen-activated protein kinase) - pathways. (Tapp et al., 2009; Yoon & Lyons, 2004)

In the process of endochondral bone formation FGF (fibroblast growth factor) and the Wnt signalling pathways play pivotal roles. FGF receptors (Fgfr), in cartilage primarily Fgfr1-3, show a distinct distribution between proliferating and hypertrophic chondrocytes of the growth plate (Ornitz & Marie, 2015). Fgfr3 signalling seems to enhance chondrocyte proliferation in early embryogenesis while inhibiting chondrogenesis afterwards. Fgfr1 and Fgfr2 primarily influence osteoblastogenesis. (Deng et al., 1996; Ornitz & Marie, 2015)

Wnt signalling plays an important role during chondrogenesis through both the non-canonical pathway and the canonical pathway. The non-canonical pathway is β-catenin independent and diverges into the Wnt/calcium cascade and the planar-cell polarity (PCP) pathway (Bradley & Drissi, 2010). The PCP cascade may contribute to cell polarity, oriented cell division and the polar organisation of the chondrocytes within the growth plate. Chondrocyte division and orientation appears to be influenced through the non-canonical frizzled (Fzd) signalling. (Bradley & Drissi, 2010; Li & Dudley, 2009) The β-catenin dependent canonical pathway modulates osteo-chondroprogenitor differentiation at the condensation sites and also regulates osteoblastic trans-differentiation (Kolpakova & Olsen, 2005).

1.4 INTEGRINS

1.4.1 STRUCTURE AND FUNCTION

Integrins are type I transmembrane proteins composed of non-covalently bound α and β subunits. 18 α and 8 β subunits combine to form the 24 different integrin heterodimers known in mammals (Hynes, 2002). About 50% of the α subunits contain the so called inserted (I)-domain, which is responsible for ligand binding through a metal ion-dependent adhesion site (MIDAS) (Moser et al., 2009; Plow et al., 2000). Another

opportunity for ligand binding is the EF-hand-like domain of most α subunits (Plow et al., 2000). Ligand binding depends on the availability of divalent cations like Ca^{2+} and Mg^{2+} (Tuckwell et al., 1992). Integrin heterodimers can be separated into different groups characterized by their ligand-binding profile (Fig.5) (Barczyk et al., 2010). They have large extracellular amino-acid chains which play a role in cell communication and adhesion by interacting with different cell adhesion proteins like intercellular adhesion molecule-1 (ICAM) and vascular cell adhesion molecule-1 (VCAM) for leukocyte-specific receptors (Ley et al., 2007), and multiple elements within the ECM like collagens, laminins and RGD motif-containing proteins such as fibronectin and vitronectin (Humphries et al., 2006; Ruoslahti, 1996b). The α and β subunits together form a head for ligand binding (Zhu et al., 2008). Compared to the extracellular domain, the cytoplasmic part or “tail” is relatively short. The two parts are connected by a single transmembrane domain (TM). (Hynes, 2002) Integrins form a bridge from the extracellular space towards the actin cytoskeleton (Fig.6) through interaction with focal adhesion proteins, and can act as signal transducers in both directions, which is referred to as inside-out and outside-in signalling (Hynes, 1992). As signal transducers they influence cell differentiation, proliferation, adhesion, migration, shape and gene expression through different signal-cascades and conformational changes. The ability of outside-in and inside-out signalling is based on different focal adhesion molecules such as talin, vinculin, paxillin and kindlin. In particular, the binding of talin is thought to be a widespread final step of activation. (DePasquale & Izzard, 1987; Izzard, 1988; Larjava et al., 2008; Rogalski et al., 2000; Rognoni et al., 2016; Schaller, 2001; Tadokoro et al., 2003) Binding of focal adhesion partners to the cytoplasmic β subunit leads to the phosphorylation and separation of the α and β tails. The resulting integrin activation causes conformational changes from a bent to an extended configuration within the extracellular domains leading to higher ligand affinity (inside-out signalling). (Ginsberg et al., 2005; Takada et al., 2007) The focal adhesion proteins interact with a couple of catalytic enzymes like tyrosine kinases, phosphatases, serine/threonine kinases and modulators of small GTPases, which are essential for conformational changes of the associated integrins (Lo, 2006; Mitra et al., 2005). Particularly, integrin-linked kinase (ILK) and focal-adhesion kinase (FAK) play an important role in mediating inside-out and outside-in signalling (Honda et al., 2009; Michael et al., 2009). Outside-in signalling relies on binding of extracellular ligands, mainly collagens, laminins and glycoproteins such as fibronectin. In addition to the separation of the α and β subunits,

integrin clustering is also necessary for an activation of outside-in signalling (Hynes, 2002; Miyamoto et al., 1995). One scenario of outside-in signalling is the sensing of mechanical stress and its conversion into a biochemical signal (mechanotransduction). Low lateral forces generated by shear stress play an important role by accelerating the formation of a high-affinity state of the integrin ligand-head complex. (Alon & Dustin, 2007; Anthis & Campbell, 2011) Mechanical stimuli have also been shown to lead to hyperpolarization of chondrocytes, mediated among others by integrins (Wright et al., 1997). Besides, mechanical forces influence signalling important for the formation of adhesion complexes (Wolfenson et al., 2009). In cartilage, integrin α5β1 is known to interact with ion channels and interleukin-4 in response to mechanical stimuli (Chen et al., 2013; Millward-Sadler et al., 2000). Mechanical stress in cartilage activates, in addition to growth factors and cytokines, MAP kinases, which eventually leads to matrix degradation. These interactions also appear to play important roles during inflammatory processes such as in osteoarthritis. (Loeser et al., 2014) Another possibility of outside-in signalling is the activation of integrins by ECM components such as degraded FN fragments and the transduction of this signal into the activation of metalloproteases, cytokines and chemokines which can enhance the inflammatory reaction and thus, for example, joint destruction (Forsyth et al., 2002; Pulai et al., 2005).

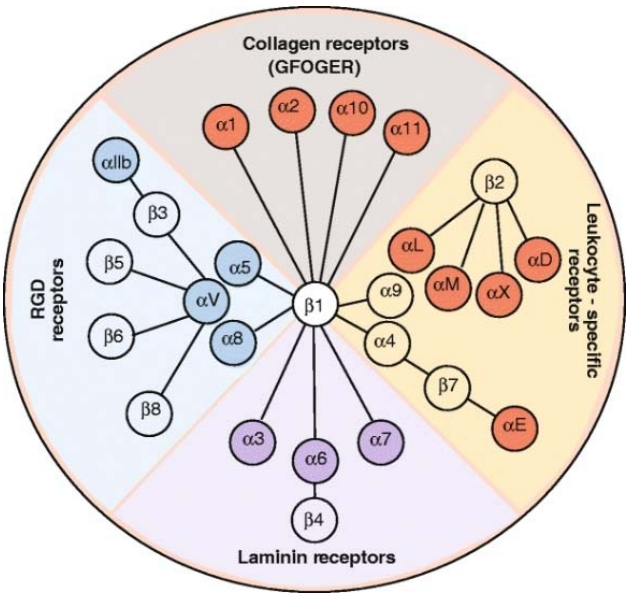

Figure 5: **The integrin family.** In vertebrates there exist 24 heterodimers, formed by the combination of 18 α and 8 β subunits, that bind different ligands such as collagens, laminins, RGD-containing proteins and leukocyte-specific proteins. (Barczyk et al., 2010)[5]

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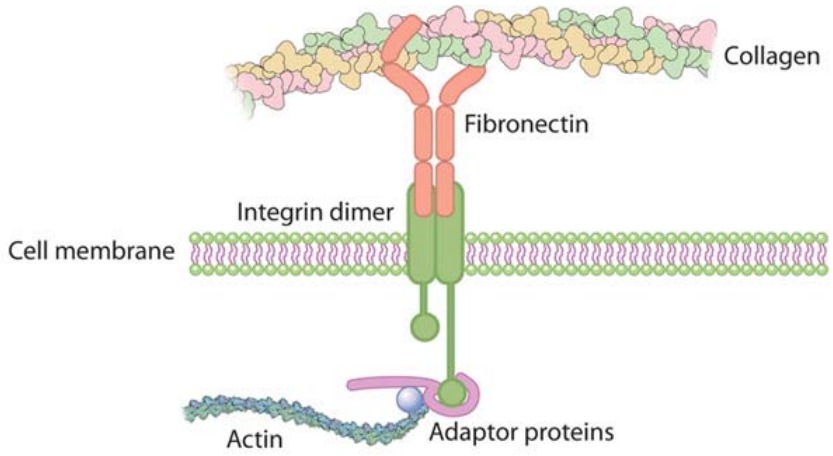

Figure 6: Integrin heterodimer. The integrin heterodimer is composed of a non-covalently bound α and β subunit (green). Integrins have an extracellular, transmembrane and cytoplasmic domain. They connect to components of the cytoskeleton (like Actin) through adaptor proteins on the cytoplasmic side and to *e.g.* collagen via RGD containing glycoproteins like fibronectin from the extracellular side. (Nature Education, 2010)[6]

1.4.2 INTEGRINS IN CARTILAGE

Chondrogenesis and thus endochondral bone formation is highly dependent on cell-matrix and a few cell-cell interactions (Bouvard et al., 2001; Solursh et al., 1982). Interactions with the matrix rely primarily on β1 and αv integrins, which therefore not only contribute to proper cartilage function but also play a role during cartilage and bone development (Loeser, 2014). In cartilage the most abundant integrins are α1β1, α2β1 and α10β1, which bind to collagens; α5β1, αvβ3 and αvβ5 bind among others to fibronectin and α6β1 connects to laminin (Loeser, 2000; Raducanu et al., 2009). Antibody perturbation studies have been performed against various integrin subunits like α1, α2, β1 and α5β1, showing that these integrins play an important role during chondrocyte survival, hypertrophic differentiation and joint/bone formation (Garciadiego-Cázares et al., 2004; Hirsch et al., 1997; Shakibaei, 1998). Most of α subunits found in cartilage associate with integrin β1, suggesting that β1 integrin plays a major role during growth plate development and cartilage maintenance. The chondrocyte-specific deletion of the gene encoding integrin β1 leads to perinatal lethality due to severe chondrodysplasia in mice. (Aszódi et al., 2003; Bouvard et al., 2001) The mice showed a severe phenotype, including growth plate destruction caused by a rounded cell shape and consequent impaired column formation, ultimately

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leading to an impaired bone formation (Aszódi et al., 2003). Further studies revealed functions of integrin β1 in cell proliferation, apoptosis, cartilage architecture and in successful cytokinesis via the small GTPase Rab21 (Aszódi et al., 2003, Pellinen et al., 2008; Raducanu, 2009). Overall, β1 integrins play a pivotal role in cartilage development, maintenance and metabolism.

1.5 INTEGRIN α10

The α10 integrin forms a heterodimer with the β1 subunit, and α10β1 integrin is thought to be the most important collagen II-binding integrin in adult and developing cartilage. In mice, it is first expressed in chondrogenic condensations at embryonic day 11.5 (E11.5) and can bind to collagen IX, XI, and VI in addition to collagen II. (Lundgren-Åkerlund & Aszódi, 2014) The I-domain of integrin α10, like other collagen-binding α-subunits, is connected to specific sequences, GFOGER and GLOGER, within the triple helices of fibrillar collagens (Lundgren-Åkerlund & Aszódi, 2014; Siljander et al., 2004). The most similarity between the collagen-binding integrins is mainly confined to the ligand binding I-domain (Gullberg & Lundgren-Åkerlund, 2002; Tulla et al., 2001). Integrin α10 is expressed by chondrocytes of hyaline cartilage within the trachea and bronchi, the vertebral column, the articular cartilage, the growth plate of long bones as well as in parts of the heart valves, the ossification groove of Ranvier and fibrous tissues of the musculoskeletal system (Camper et al., 2001). Interestingly, integrin α10 has also been found in malignant melanoma cells, where it appears to contribute to cell migration (Wenke et al., 2007). Although α10β1 integrin shares a high structural similarity with other I-domain-containing and collagen binding integrins such as α1β1, α2β1 and α11β1 (Camper et al., 1998; Lehnert et al., 1999), its distribution within the tissue is quite distinct. Furthermore, integrin α10 is an important marker for chondrogenic differentiation. It has been shown that upregulation of α10 expression in MSCs can be achieved by FGF-2 treatment and this correlates with a better chondrogenic differentiation as well as improved adhesion of MSCs to osteochondral defects, and better immunomodulatory capacities. (Delco et al., 2020; Uvebrant et al., 2019; Varas et al., 2007) In addition, integrin α10 supports the synthesis of collagen II and aggrecan during MSC differentiation, both known markers for a healthy cartilage metabolism (Varas et al., 2007). At the gene level 90% homology between human (*ITGA10*) and mouse (*Itga10*) was observed (Bengtsson et al., 2001). A genetic

mutation in the canine integrin α10 gene was found in Norwegian Elkhound and Karelian Bear dogs with short-limbed dwarfism, indicating the importance of an intact *Itga10* for proper cartilage and skeletal development (Kyöstilä et al., 2013). The integrin α10 constitutive knockout mouse displays mild growth retardation and partly mimics, but in a much milder form, the previously described cartilage-specific β1 knockout phenotype (Bengtsson et al., 2005). A comparison of the observed phenotypes and thus the known features of the consequences of integrin α10 loss compared to the loss of β1 integrins were analysed by Lundgren-Akerlund and Aszódi, 2014 (Lundgren-Åkerlund & Aszódi, 2014).

1.6 THE MOUSE AS AN ANIMAL MODEL

Sharing 85% identity in protein-coding genes with humans, the mouse is a relatively easy handling, low costing, fast developing model organism with short generation times; perfect to examine many biological processes and the origin of disorders and diseases (Mouse Genome Sequencing Consortium, 2002). Furthermore, the whole mouse genome has been sequenced. Particularly, genetically modified transgenic (gain of function) or knock-out (loss of function) mice are common tools for specific analysis of the function of a gene and its encoded protein (Capecchi, 1989). Nowadays, the mouse strains used are practically homozygous at all chromosomal loci thanks to decades of crossing with sibling mice, which increases the reproducibility of studies (Nguyen & Tian, 2008; Peters et al., 2007) and allows good comparability between different working groups, making the mouse an ideal animal model for genetic studies. Concerning the practical handling, it is useful to note for the present study that the whole knee joint can be viewed on one slide (Gregory et al., 2012).

1.7 CLINICAL RELEVANCE

1.7.1 DISORDERS OF SKELETAL DEVELOPMENT

Formation and growth of the long bones is based on an orchestrated process of chondrocyte proliferation, maturation, transdifferentiation and apoptosis, accompanied with specific ECM synthesis, complex molecular interactions and signalling. In particular, genetic disorders summarized under the generic term of skeletal dysplasia

or osteochondrodysplasia interfere with the growth plate architecture and function and, consequently, endochondral ossification and skeletal development in all the above-mentioned aspects. Even if individual skeletal dysplasia is quite rare, together they have an incidence of 1 per 5000 births (Krakow & Rimoin, 2010; Orioli et al., 1986). Skeletal dysplasia can be separated into 42 different groups, ranging from severe forms leading to perinatal lethality to milder diseases with an onset just in adulthood. They are characterized by different attributes like the molecular pattern, radiological findings or clinical observations. (Bonafe et al., 2015) Genetic sequencing studies of affected individuals have already detected some mutations leading to skeletal dysplasia (Bonafe et al., 2015; Warman et al., 2010). Most of the genes identified encode for components within the cartilaginous ECM, such as aggrecan and collagen II. Mutations in genes encoding collagens lead to different types of classified collagenopathies. Not only the collagen fibrils themselves, but also their proper assembly is affected. (Bonafe et al., 2015; Jobling et al., 2014) Of all skeletal dysplasia, achondrodysplasia is the most common in humans, affecting 1 in 25000-30000 newborns (Pauli, 2019). In addition to milder forms such as hypochondrodysplasia, a distinction is made between the more severe achondrodysplasia and the more fatal thanatophoric dysplasia (TD) (Vajo et al., 2000). All these forms share a mutation of the FGF3 receptor gene, which leads to short-limb dwarfism (Rousseau et al., 1994). This phenotype is caused by inhibition of chondrocyte differentiation and maturation within the growth plate (Murakami et al., 2004). Patients with achondrodysplasia have a higher mortality compared to the healthy population, especially in childhood, where mortality is mainly due to cervico-medullary compression (Ho et al., 2004). Current treatment options range from physical therapies to surgical interventions (Ho et al., 2004), including growth hormone treatments, which aim to antagonise the effects of the mutated and so overactive FGF3 receptor (Hertel et al., 2005; Horton, 2006). Other members of the group of skeletal dysplasia are pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED). They appear to affect cell proliferation and apoptosis during endochondral ossification by endoplasmic reticulum (ER) stress and lead to short stature, arthralgia and osteoarthritis. (Briggs et al., 2015)

Another disease affecting the growth plate architecture primarily in animals is osteochondrosis, which has been shown to impair chondrocyte maturation and results in an accumulation of hypertrophic chondrocytes which do not organize into columns (Wardale & Duance, 1994). Interestingly, chronic diseases like Crohn’s disease,

rheumatoid arthritis, renal failure and cystic fibrosis in childhood also appear to affect growth plate architecture by reducing chondrocyte proliferation and enhancing apoptosis, leading to growth retardation primarily due to inflammation and consequently high glucocorticoid levels, metabolic acidosis and reduced caloric intake (De Luca, 2006).

In order to better understand skeletal dysplasia, other pathologies associated with impaired skeletal development and also common degenerative joint diseases like osteoarthritis and thus improve treatment or prevention options, it is essential to gain knowledge of the molecules involved in endochondral bone and thus growth plate formation. This are among others integrins, which play key roles in cartilage/bone formation and maintenance.

2 AIM AND MILESTONES OF THE THESIS

Integrin α10, a relatively newly discovered member of the integrin family, appears to have a role in cartilage development, maintenance and bone growth. Little is known, however, about the role of this integrin subunit in growth plate morphogenesis. Knock-out mice provide an ideal platform for further investigation and closer analysis of specific effects and functions of integrin α10. Approaches to analyse these functions should focus on three major aspects of endochondral bone formation, namely chondrocyte differentiation, proliferation/apoptosis and cartilage cytoarchitecture. The present thesis focuses on the effect of α10 integrin on the cytoarchitecture of the growth plate.

The following specific aims were defined:

1. Immunohistochemical examination of wildtype and integrin α10 knock-out growth plates of the tibia at different ages (E15.5, E18.5, 2 weeks, 1 month, 2 months).

2. Quantitative measurements of chondrocytes and columns within the proliferative zone, including shape index, column alignment and cell orientation relative to the longitudinal axis of the bone, and determination of the average cell content per column.

3. Analysis of the effects of a loss of integrin α10 on the adhesion and migration of chondrocytes on collagen II, collagen VI and fibronectin by time lapse video microscopy and optical density measurements.

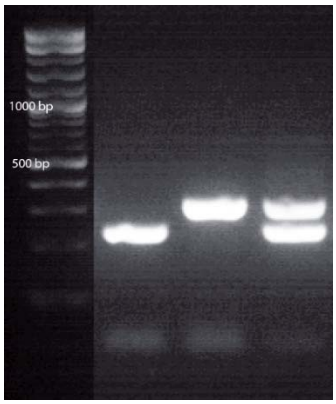
3 MATERIALS AND METHODS

3.1 EXPERIMENTAL SETUP

To analyse the influence of integrin α10 on the cytoarchitecture of the growth plate, mice carrying a constitutive deletion of the α10 integrin gene were euthanized at an age of E15.5, E18.5, 2 weeks, 1 month and 2 months by cervical dislocation. For each time point two to four wild type and integrin α10 knockout mice were used, tissue dissection was done post-mortem. Integrin KO mice were generated by the European Conditional Mouse Mutagenesis Program (EUCOMM). All animals were kept according to the regulation of the Government of Upper Bavaria as described in the animal application: TVA ROB-55.2-2532.Vet_02-16-15. Animals were kept in individually ventilated cages (IVC Sealsafe, Tecniplast, USA) under SPF I conditions. The autoclavable cages contained HEPA-filters for air supply and were of a size of 365x207x140 mm (LxWxH). A maximum of five animals were put together in one cage. Animals were kept on a 12/12 hours light/dark cycle and water and food ad libitum. Temperature was set at 20-22°C and humidity at 45-55%. Each cage is equipped with a small house rendering as shelter as well as material for bedding, gnawing and nesting, which was changed once in a week. Breeding, maintenance and scarification of animals were performed by an authorized person. The author of this Thesis only performed post-mortem analysis.

3.2 MOUSE GENOTYPING

For genotyping the genomic DNA was isolated by lysing the tip of a mouse tail for 10 minutes at 63°C in in 30 µl QE (quick extraction) buffer (Qiagen). The enzyme within the buffer was neutralized at 95°C for 5 minutes. The probes were placed on ice and directly used for a PCR reaction (PCR machine PEQSTAR 2x (PEQLAB)). The PCR products were analysed on a 2% agarose gel by electrophoresis (PEQLAB) in 1x TAE solution (dilution from 50x TAE solution with dH_2O: 242 g Tris, 57.1 ml acetic acid, 100 ml 0.5 M EDTA pH 8.0, fill up with dH_2O to 1 l, pH to 8.3) with 0.07 µl/ml ethidium bromide on the UV imager (Vilber Lourmat). A PCR fragment of 226 bp indicated a wildtype, 302 bp a knockout and two bands a heterozygous mouse (Fig.7). As a reference, the GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific) was used. The PCR program and the used primer are listed in Tables 1 and 2.



marker WT KO Het

Figure 7: Genotyping of WT and integrin α10 KO mice. An upper band indicates a knockout (KO, 302 bp), a lower band a wildtype (WT, 226 bp) and two bands a heterozygote (het) mouse.

Table 1: **PCR reaction mix and program for genotyping.**

PCR reaction mix		**PCR program for genotyping**		
10 x buffer with $MgCl_2$	2 µl	95°C	5´	
dNTPs	0.4 µl			
Primer 1	0.1 µl	95°C	30´	10 cycles
		63°C	30´(-1°C/cycle)	
Primer 2	0.1 µl	72°C	45´	
Primer 3	0.1 µl			
Taq polymerase	0.25 µl			
		95°C	30´	35 cycles
H_2O	16.15 µl	53°C	30´	
Extracted gDNA	1,0 µl	72°C	30´	
		72°C	5´	
		8°C forever		

Table 2: Nucleotide sequences and the related information of the integrin α10 genotyping. (EUCOMM: https://www.mousephenotype.org/about-impc/about-ikmc/eucomm/).

Target gene	Primer (3' to 5')	Annealing temp.	Reference
Itga10-3'arm (Primer 1)	CAGGTCATGTAGCCTCCCCAGAGG	53°	EUCOMM
LAR3_Itga10 (Primer 2)	CAACGGGTTCTTCTGTTAGTCC	53°	EUCOMM
Itga10-5'arm (Primer 3)	GTTCGTGGAGGAAGGCTAGGTTGC	53°	EUCOMM

3.3 TISSUE PROCESSING

3.3.1 FIXATION AND DECALCIFICATION

The knee joints of the hindlimbs were deskinned, dissected and briefly rinsed in sterile phosphate buffered saline (PBS: 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, 14,4 g $Na_2HPO_4x2H_2O$, 1 l dH_20, pH 7.4). Samples were fixed overnight at 4°C in pre-cooled 4% paraformaldehyde (PFA, Merck, Germany) in HANK's modified medium (137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM BGTA, 5 mM PIPES, 5.5 mM Glucose, 4 mM $NaHCO_3$, 10 mM MES /1 l dH_2O, pH 6.5) or in PBS under constant agitation. The next day, PFA samples were washed in PBS three times for 10 minutes. For better cutting and staining conditions, the hindlimbs of the 2 weeks, 1 month and 2-months-old mice were decalcified using the chelating agent EDTA (20% EDTA in PBS, pH 8.0, Carl Roth, Germany) for 2 to 4 weeks under constant shaking. The solution was renewed two times per week.

3.3.2 EMBEDDING

Before embedding the decalcified samples, they were incubated overnight in 20% sucrose in HANK's medium. The next day they were washed three times for 10 minutes in PBS under shaking. Samples were embedded in TissueTek (Sakura TissueTek O.C.T.™ Compound containing) using plastic base mould disposable cassettes (Leica,

Germany) and frozen placed on a copper plate on dry ice. Afterwards they were stored at -20°C wrapped in parafilm (PM-996, Bemis).

3.3.3 SECTIONING

Cyrosectioning of the knee joints was performed using a Cryotome Microm HM500 (Fisher, Walldorf, Germany). Probes were cut in a sagittal orientation either at 10 µm (E15.5) or 20 µm sections, collected on positively charged SuperFrost glass slides (Thermo Scientific, USA) and stored at -20°C until further use.

3.4 FLUORESCENCE STAINING OF THE ACTIN CYTOSKELETON

To analyse the actin filament network a labelling by phalloidin was used. Frozen slides were taken from -20°C to room temperature and washed twice in PBS for 5 minutes. To enhance staining conditions samples were treated for 60 minutes at 37°C with hyaluronidase (2 mg/ml, pH 5.0; Sigma-Aldrich, USA) diluted in PBS. Afterwards sections were again rinsed 2x 5 minutes in PBS, followed by an incubation at room temperature for 2 hours with Alexa Fluor 488 phalloidin (Dilution: 1:400 in PBS). After staining with phalloidin, samples were washed 2x 5 minutes in PBS and then incubated for nuclear counterstaining using 4', 6-diamidino-2-phenylindole (DAPI) (Dilution: 1:10000 in dH_2O) at room temperature for 5 minutes. Another washing procedure with PBS twice for 5 minutes was performed, then the samples were mounted with Fluoroshield (Sigma) anti-fading mounting media. During all procedures samples were kept protected from light.

3.5 ANALYSIS OF THE CELLS AND COLUMNS IN THE GROWTH PLATE

All measurements were performed in the central two-thirds of the growth plate of the tibia using only cells located in the proliferative zone which was distinguished by flattened chondrocytes located in columns. For the E18.5, 2 weeks, 1 month and 2-months-old mice the slides were divided into sagittal and parasagittal regions, taking the crucial ligaments and menisci as a reference. As the results did not show a huge difference between sagittal and parasagittal regions primarily within the KO growth plates, the measurements were later combined. The number of WT and KO mice for

the different ages was: E15.5: 2 KO, 4 WT; E18.5: 4 KO, 4 WT; 2 weeks: 3 KO, 3 WT; 1 month: 3 WT, 3 KO; 2 months: 2 WT, 4 KO. Per animal 4-5 slides were stained, per slide 30-50 cells were measured. As a reference for the orientation of a cell the proximal-distal axis of the tibia was taken. To describe the cell shape the shape index (long axis/short axis) was used, showing the more flatten or round shape of a cell by a number getting closer to 1. An orientation of 90° compared to the PD-axis was considered as an ideally orientated chondrocyte. Also, for the orientation of one column to the PD-axis of the bone was taken as reference, indicating an angle of 0° as ideally orientated. In addition, the number of cells per column was counted, taking only columns which could be clearly identified into consideration. Per slide 15-50 columns were analysed, depending on counting conditions which were influenced by partly disrupted growth plates due to a partly incomplete decalcification process. All measurements were performed using ImageJ (https://imagej.nih.gov/ij/). The shape index and the cell or column orientation was calculated by the program. The reference line for the longitude or transverse axis of the bone for measuring the cell or column orientation was determined by eye (Figs. 8 and 9). Also, the cells per column were counted manually.

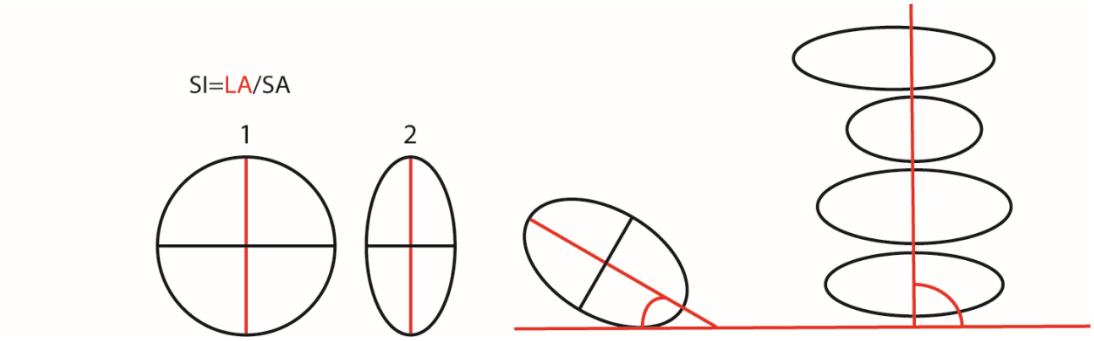


Figure 8: Scheme of cell and column measurements. On the left, the shape index (SI) is shown, which was calculated by dividing the cell long axis (LA) by the short axis (SA) of the cell. An SI of 1 indicates a round shape and of >1 an elongated cell shape. On the right, the orientation of the cell and column relative to the longitudinal growth axis (horizontal red line) of the bone is illustrated.

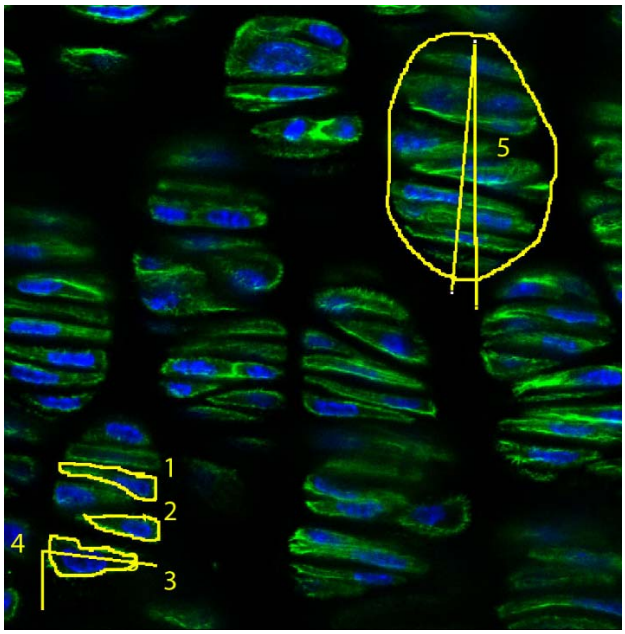


Figure 9: Column and cell measurements. All measurements marked with yellow lines, were done by manually using ImageJ. The orientation of the cells (4) and columns (5) was calculated by the software, always referring to the longitudinal, proximodistal (PD) axis of the bone. The shape index (1-3) was calculated by ImageJ.

3.6 PRIMARY CHONDROCYTE ISOLATION

For the isolation of primary chondrocytes from the rib cages, mice were euthanized at E18.5/E19.5 and briefly disinfected with 70% ethanol. Mice were genotyped and divided into integrin α10 KO and WT, deskinned and the rib cages isolated using sterile surgical forceps and scissors. Isolated rib cages were placed in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 Ham 1:1 (DMEM/F12) (Thermo Fisher Scientific, USA) media and adherent tissues were removed. After cleaning, the ribs were dissected from the rib cages and incubated at 37°C for 30 minutes in DMEM/F12 containing 2 mg/ml collagenase type II (Worthington, USA). During the incubation the perichondrium was partially digested and afterwards carefully removed using fine forceps under a stereo microscope. The remaining cartilage was again incubated in fresh DMEM/F12 media containing 2 mg/ml collagenase type II for 3 h at 37°C. After the enzymatic digestion, the cells were separated by pipetting the suspension up and down and then centrifuged at 500 g for 5 minutes. The supernatant was discarded, and the remaining cell pellet was resuspended in fresh DMEM/F12 media supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% Penicillin/Streptomycin (Biochrom, UK). Primary chondrocytes were counted and plated as passage 0.

3.7 DETERMINATION OF THE CELL NUMBER

For counting cells, 10 µl of resuspended cell suspension was injected into a Neubauer chamber (Brand, Germany). To determine the total number of cells per millilitre, cells were counted in all four quadrants (A, B, C and D) divided by 4 and multiplied by 10^4: cells/ml = [(A+B+C+D)/4] *10^4.

3.8 PRIMARY CHONDROCYTE CULTURE CONDITIONS

Chondrocytes were cultured in DMEM/F12 enriched with 10% FBS and 1% Penicillin/Streptomycin if not otherwise indicated. Cells were grown in monolayer under constant conditions in a humidified incubator at 37°C and 5% CO_2. Cells were maintained at a maximum of 50% confluence or used directly for other experiments.

3.9 COATING OF CULTURE PLATES

For the cell attachment, adhesion and migration assays and for staining of primary chondrocytes, different coatings were used. Therefore, the wells of culture plates (6-well (Cellstar® ,greiner bio-one) or 96-well (Thermo Scientific, Denmark) were either coated with 10 µg/ml collagen II (Sigma, C9301), collagen VI (Corning, USA, 354246) fibronectin (Millipore, FC010), 10% FBS (positive control) or 1% BSA (control for nonspecific binding) (Carl Roth, Germany) for 1 hour at 37°C. Afterwards the coating solution was replaced by 1% BSA for 1 h at 37°C to block non-specific binding sites. Before using the plate, it was rinsed with PBS.

3.10 INVESTIGATION OF CELL MIGRATION AND REAL TIME ATTACHMENT BY TIME LAPSE VIDEO MICROSCOPY

For the time-lapse analysis of cell migration and attachment 10^4 primary wild type or mutant chondrocytes were seeded in each well of a 6-well culture plate. E18.5/E19.5 chondrocytes were isolated like described above using 3-4 KO and WT mice which were afterwards pooled together. The wells were either coated with 10 µg/ml collagen II, collagen VI or fibronectin as described above. BSA was used to block the wells and as a background control. The cells were cultured in DMEM/F12 supplemented with 2%

FBS and 1% Penicillin/Streptomycin. The 6-well plate was transferred into a biochamber for Axio Observer (Carl Zeiss, Germany). The system included an environmental control chamber with heating unit and CO_2 controller (Pecon, Germany), and an automated XY stage controller (Proscan, Canada). AxioCam MRc (Carl Zeiss, Germany) and AxioVision Rel. 4.8. (Carl Zeiss, Germany) were used for automatically image acquisition at every 5 (real time attachment) or 15 (migration assay) minutes. Images were taken with a 10x UPLANFL Ph1/0.30 objective in bright field mode and focused on the bottom of the wells. The cell adhesion was observed for 24-48 h, followed by a migration analysis up to 96 h. For cell attachment the time point of the first visible protrusion of the chondrocytes was taken. Images were analysed for mean and maximum velocity using the MTrackJ Plugin of ImageJ (https://imagescience.org/meijering/software/ mtrackj/) (Fig.10). Due to a slow migration of primary cells in general, only every 4th picture during the migration assay was taken into count. The plotting of the migration of the last 24 h was done with SigmaPlot 12.0 software. For each condition (*e.g.* WT cultured on fibronectin) 3 videos were taken and analysed. To represent the migration into a polar plot, all cells that reached the last 96 h and did not migrate out of the microscopic field were plotted (45-50 cells per video). Cells that divided during the assay were tracked as two new cells. Cells that did not move and later died were not analysed. The cell attachment was analysed using the Zen microscope software (Zeiss). The assays were performed twice.

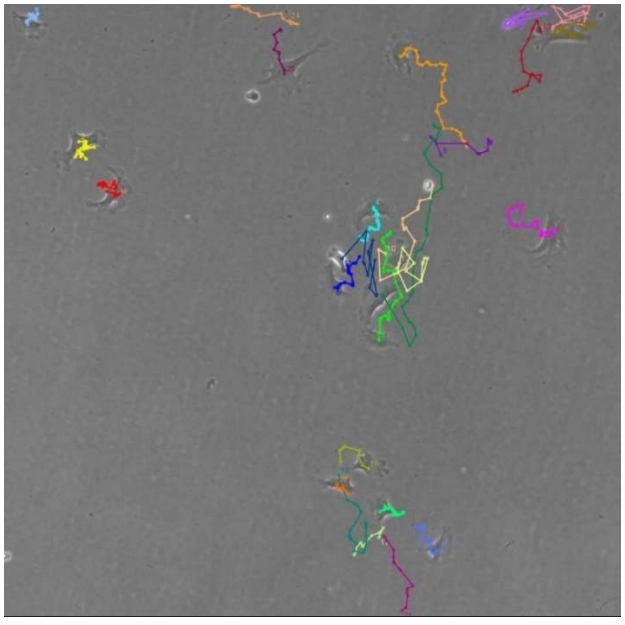


Figure 10: Tracking of the cell migration. Tracking was done by the ImageJ plugin MTrackJ. The coloured lines show the movements of the chondrocytes over 24 h.

3.11 CELL ADHESION ASSAY

For classical cell-binding adhesion assay, 96-well culture plates were coated with 10 µg/ml collagen II, collagen VI and fibronectin (R&D, USA), or with BSA for negative control experiments. In each well, 10^5 cells were seeded using serum-free medium (DMEM/F12). E18.5/E19.5 chondrocytes were isolated and pooled like described above using 3 KO and WT mice. The cells were allowed to attach for 1 h at 37°C and afterwards were washed twice with PBS to remove the unbound chondrocytes. To fix the adhered cells, they were incubated with 100 µl 96% ethanol (Merck, Germany) for 10 minutes and then stained with 100 µl 0.1% crystal violet/dH_2O (Sigma-Aldrich) for 30 minutes at RT. After washing away the excess of crystal violet with dH_2O, the cells were lysed using 100 µl 0.1% Triton X-100/dH_2O (Sigma). The absorbance at 550 nm, which should be proportional to the number of adherent cells, was measured using a microplate reader (Thermo Scientific, USA). Concentrations were calculated against a standard curve with known concentrations. The experiment was performed twice in three technical parallels per condition.

3.12 IMMUNOFLUORESCENCE STAINING OF PRIMARY CHONDROCYTES

To determine differences in the shape and actin cytoskeleton organization of wild type and mutant chondrocytes, the cells were cultivated on different substrates and sequentially stained with the monoclonal XIN9 antibody against integrin α10, phalloidin and DAPI (Table 3). For staining, freshly isolated primary wild type and mutant chondrocytes were seeded on a 8-well chamber slide (Thermo Scientific, USA) at a density of 10^4 per well. The chambers were coated with either 10 µg/ml collagen II, collagen VI or fibronectin like mentioned above. Cells were grown in DMEM/F12 media supplemented with 2% FBS and 1% Penicillin/Streptomycin for at least two or three days at 37°C in a primary cell incubator. Staining was performed twice with or without the Vector M.O.M.™ Immunodetection Kit (Vector Laboratories, USA), which allows the use of mouse primary antibodies on mouse sections by reducing background staining of endogenous mouse IgG. First, the chondrocytes were briefly washed with PBS and then blocked for 1 h in M.O.M.™ Mouse IgG Blocking Reagent. After another washing step (2x2 minutes in PBS) the cells were incubated for 5 minutes in 12,5% M.O.M.™ diluent. The diluent was removed from the chambers and replaced by the

primary antibody XIN9 (2 µg/ml, diluted in Diluent). After 30 minutes of incubation, the cells were washed 2x2 minutes with PBS. This step was followed by a 10-minute incubation with the M.O.M.™ biotinylated anti-mouse IgG reagent. Another washing was applied (2x2 minutes PBS) followed by a 5-minute incubation with Fluoroscein Avidin DCS. Chambers were washed another time with PBS and then incubated with precooled 4% PFA for 10 minutes. After washing again 2x3 minutes with PBS, cells were incubated with phalloidin 546 for 1 h (1:400 in PBS). Before performing the nuclear counterstaining with 4', 6-diamidino-2-phenylindole/dH_20 (1:10000) (DAPI) (Invitrogen) the cells were washed another time. Excess of DAPI was washed away by rinsing twice for 3 minutes with PBS. Finally, the chamber slides were mounted with Fluoroshield (Sigma) anti-fading mounting media. Negative controls for antibodies were carried out on the same slide by omitting the primary antibody.

Table 3: **List of antibodies used for immunofluorescence studies.**

Target	Type	Company Catalogue number	Dilution in PBS/dH_2O
Primary antibodies			
Anti-Itgα10 (XIN9)	monoclonal mouse	Xintela AB, Lund Sweden	2 µg/ml
Secondary antibodies			
Anti-Mouse IgG (H+L)	Donkey Alexa Fluor 488	Life Technologies A-21202	1:1000
Dyes			
Phalloidin 488		Life Technologies A-12379	1:400
Phalloidin 546		Life Technologies A-22283	1:400
DAPI		Invitrogen	1:10000 (dH_2O)

3.13 CONFOCAL MICROSCOPY

Immunofluorescence images were taken at a resolution of 1024x1024 pixels with a confocal microscope from Leica Microsystems (Leica TCS SP8 X). Two sequential scans between lines at 405 nm for DAPI and 499 nm for phalloidin using the Hybrid Detector (HyD) and the 40x oil objective (HC PL APO 40x/1.30 OIL CS2) or the 20x glycerol objective (HC PL APO 20x/0.75 IMM CORR CS2) were performed. As scanning speed 400 Hz and a line average of 2 was used. The z-axis always covered the whole 20 µm (10 µm for the E15.5) of the sample slide. No bidirectional scanning was done. The zoom was differently adapted for each age, represented by an own scale bar within the pictures. Other parameters like the line accumulation, frame average and frame accumulation were set to 1. The whole growth plate, or at least the middle part for the older stages (2 weeks, 1 month, 2 months), was recorded and later analysed.

3.14 STATISTICS

All data and statistical tests were analysed using the GraphPad Prism 8 software (GraphPad Software, USA). In general, all quantitative data were acquired from two to four different mice with 4-5 slides per mouse, each experiment was done twice and performed in triplicates. Bar charts show mean values with standard deviations. For testing statistical significances concerning the growth plate cytoarchitecture, the data was first analysed for a normality distribution. If this was reached an unpaired Student’s t-test was used considering a p-value of 0.05 as statistically significant. Otherwise, the Mann-Whitney-U-Test was applied with a significant p-value from 0.05.

3.15 COMPUTER PROGRAMS

For evaluating quantitative data and for graphical illustration, Microsoft Excel 2016 (Microsoft, USA) and GraphPad Prism 8 software (GraphPad Software, USA) were used. Figures were generated with Adobe Photoshop CS6 and Illustrator CS6 (Adobe System, USA). Time lapse images were analysed with the software ImageJ 1.52g (https://imagej.nih.gov/ij/,USA) and AxioVision LE (Carl Zeiss, Germany). The

bibliography was managed with Mendeley 1.19.2 (Mendeley Ltd, USA). For plotting migrating cells into a graph, SigmaPlot12 software (SigmaPlot) was used.

4 RESULTS

4.1 MORPHOLOGICAL CHANGES WITHIN THE GROWTH PLATE

To analyse the morphological changes within the growth plate upon α10 integrin deficiency, the tibia of mice at the ages of E15.5, E18.5, 2 weeks, 1 month and 2 months were used. For anatomical reasons, the growth plate in the tibia aligns straighter and is easier to examine than in the femur. For each time point, 2 to 4 WT and integrin α10 KO mice with 4 to 5 slides per mouse were analysed. The measurements of the shape index (SI), which is represented by the ratio of the long and short axis of the cell revealed a significant difference between the WT and KO proliferative chondrocytes at all age stages (Fig.11-15). The cells of the KO mice were less elongated, reaching mean SI values between 2.3 and 3.3, while the SI of the WT varied between 3.3 and 5.4. Normal WT chondrocytes gradually flatten and lengthen up between E15.5 and E18.5 (WT mean SI: E15.5: 3.6+/-0.9 vs E18.5: 5.4+/-2.1), whereas they show slight rounding between E18.5 and 2 months (WT SI E18.5: 5.4+/-2.1vs 2 months: 3.9+/-1.3). In the KO growth plate, such a lengthening and rounding between the different time points were not as pronounced after analysing the confocal images (Fig.11A-15A). Interestingly the actin cytoskeleton did not seem to be affected by the integrin loss, as phalloidin staining demonstrates its intact cortical organisation below the plasma membrane at all investigated stages (Fig.11A-15A).

4.1.1 E15.5 STAGE

Analysing the confocal microscopic images of tibial growth plates at E15.5, there was not a significant difference found in number of cells/column, the orientation of the chondrocytes and the orientation of the columns compared to the PD-axis in between genotypes. Not all columns were already fully developed, therefore also cell-clusters were examined. These cell clusters seemed to be distinctive trait of the KO as significantly more cell clusters and less columns were found in the KO growth plate compared with WT ($p < 0.05$) (Fig.11G). The column formation just initiated in both WT and KO showing 2-7 cells/column (Fig.11B). For the orientation of the columns, which ideally should be around 0° compared to the long axis of the bone, a mean value around 15° was observed in the WT as well as in the KO (WT 15°±11° vs KO 15°±14°) (Fig.11C). Likewise, orientation of the chondrocyte´s long axis relative to the PD axis

did not show a significant difference with values around 87° (WT 88°±13° vs KO 86°±12°) (Fig.11E). On the other hand, the SI was significantly affected by the integrin α10 loss with mean values of 3.6±0.9 for the WT and 2.5±0.6 for the KO group (p < 0.01) (Fig.11D).

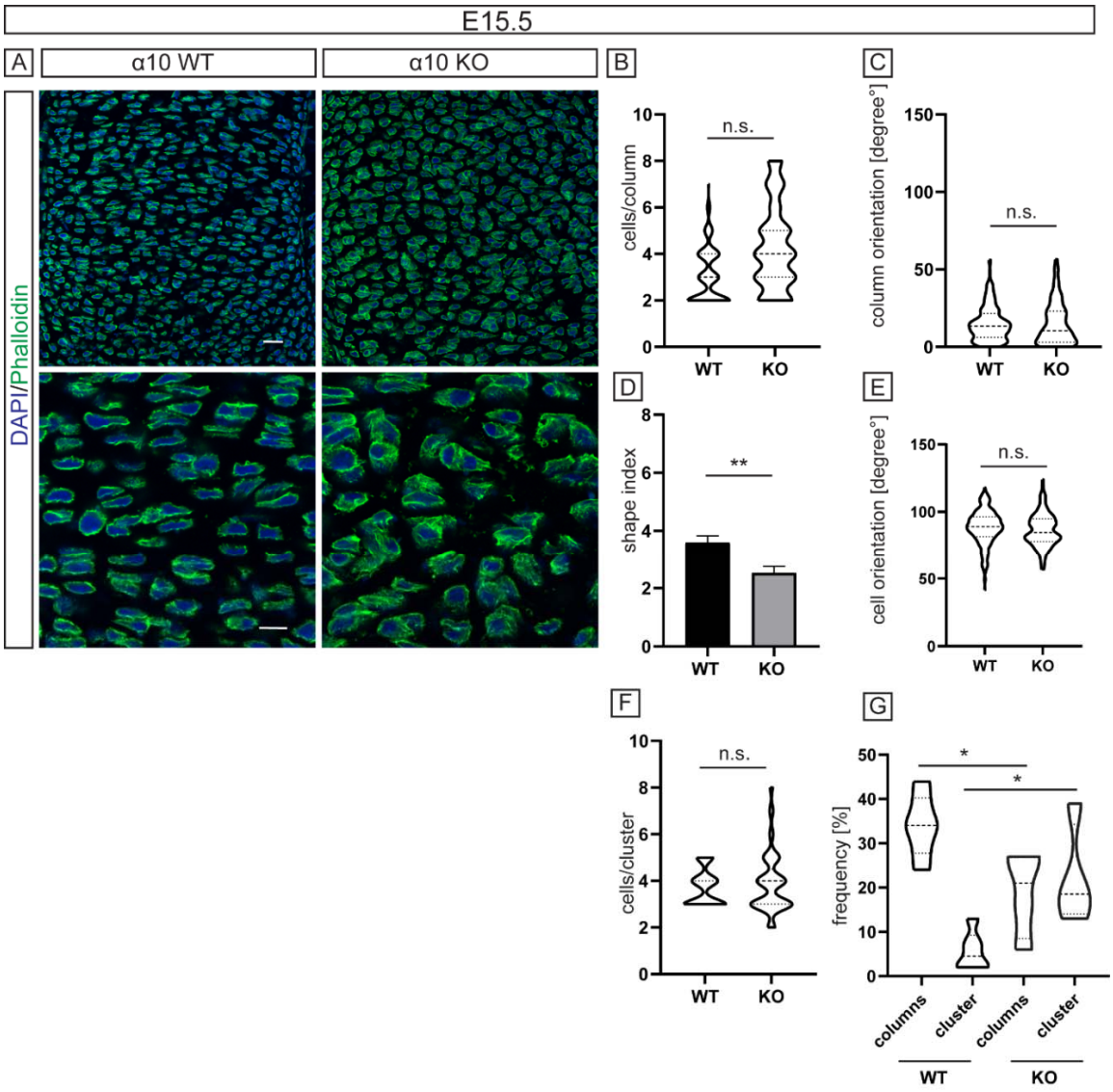


Figure 11: Morphological consequences of the loss of integrin α10 on the tibial growth plate at E15.5. Pictures and measurements from 2-4 independent integrin α10 KO and WT mice. (A) Confocal microscopy images showing the columns in the proliferative zone. In green the actin cytoskeleton is stained by phalloidin, in blue the nucleus by DAPI. The scale bar indicates 20 µm on the upper and 10 µm on the lower pictures. (B) Violin plot of the number of cells per column. (C) Violin plot of the column orientation in degree (°) compared to the PD-axis of the tibia. (D) Shape index of the chondrocytes within the proliferative zone. The error bars represent the standard deviation. (E) Violin plot of the cell orientation in degree (°) referred to the PD-axis of the tibia. (F and G) Analysis of clusters typically seen at E15.5, including number of cells per each cluster (F) and frequency of either cluster or formed columns (G). In all violin plots, the middle thick dashed line represents the median, while the lower and the upper dashed lines represent the first quartile (25%) and the third quartile (75%), respectively. N:2-4; * p < 0.05; ** p < 0.01; n.s.–statistically not significant.

4.1.2 E18.5 STAGE

While the orientation of the cells and columns did not show any significant differences for the E15.5 mice, the column orientation was impaired in the KO growth plate at E18.5. The KO columns were oriented with a mean value of 22°+/-16° compared to 14°+/-9° for the WT ($p < 0.001$) (Fig.12C). The cell orientation relative to the long axis of the bone, in contrast, was not significantly affected (WT 88°+/-14° vs KO 86°+/-11°) (Fig.12E). However, the number of cells per column was significantly different between the genotypes as WT showed mean values of 5.9+/-2.4 cells/column compared to 3.6+/-1.5 cells/columns for the KO ($p < 0.0001$) (Fig.12B). Similar to E15.5, the SI differed significantly within the KO chondrocytes compared to the WT (WT: 5.4+/-2.1 vs KO: 3.1+/-1) ($p < 0.0001$) (Fig.12D). Taken together, the growth plate architecture represented by chondrocyte geometry and the length and orientation of the columns was apparently affected by the loss of integrin α10 during embryogenesis (Fig.12).

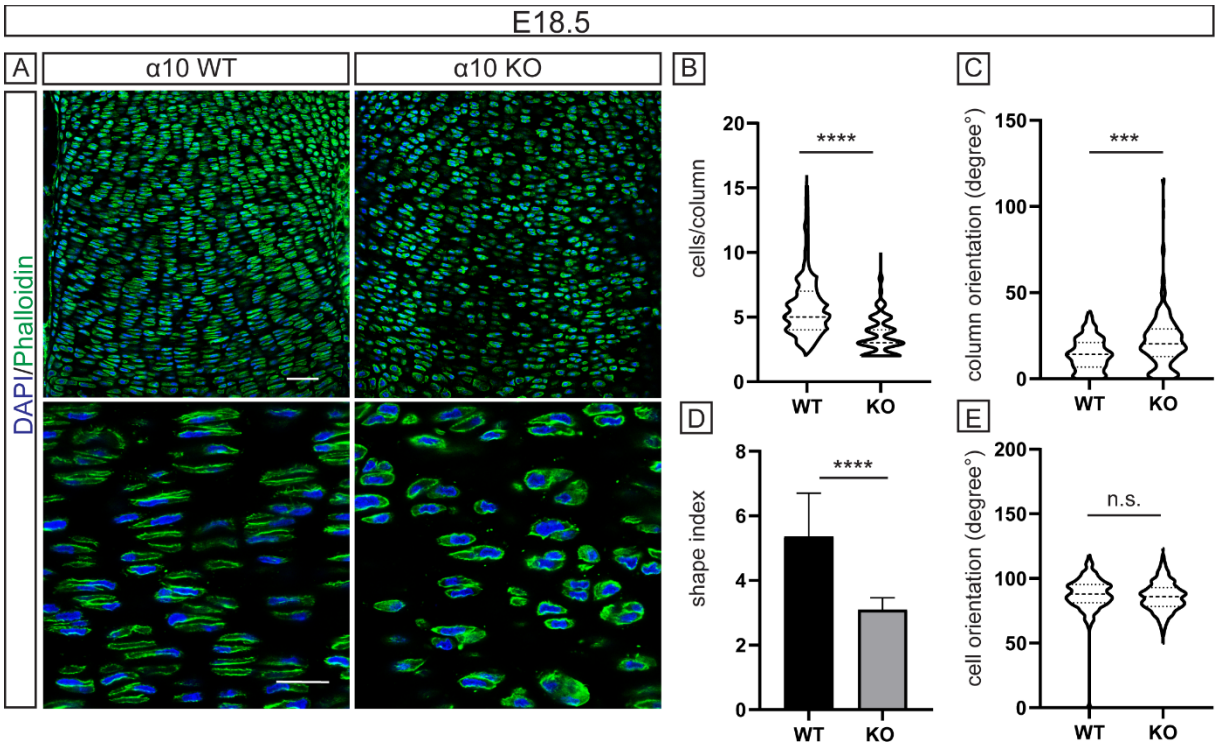


Figure 12: Morphological consequences of the loss of integrin α10 on the tibial growth plate at E18.5. Representative pictures and measurements from four independent integrin α10 KO and WT mice. (A) Confocal microscopy images showing the columns in the proliferative zone. In green the actin cytoskeleton is stained by phalloidin, in blue the nucleus by DAPI. The scale bar indicates 50 µm on the upper and 20 µm on the lower pictures. (B) Violin plot of the number of cells/column. (C) Violin plot of the column orientation in degree (°) compared to the PD-axis of the tibia. (D) Shape index of the chondrocytes within the proliferative zone. The bars represent the standard deviation. (E) Violin plot of the cell orientation in degree (°) referred to the PD-axis of the tibia. In all violin plots, the middle thick dashed line represents the median, while the lower and the upper dashed lines represent the first quartile (25%) and the third quartile (75%), respectively. N:4, n:4-5; **** $p < 0.0001$; *** $p < 0.001$; n.s.–statistically not significant.

4.1.3 2 WEEKS OLD STAGE

Despite the moderately impaired cytoarchitecture of the growth plate of the KO mice at E18.5, the measurements at 2-week-old mice revealed no significant differences in all analysed parameters apart from the SI (mean SI WT: 4.6+/-1.2 vs KO: 3.3+/-0.9) (Fig.13C). The mean values for the cells/columns varied around 10 for the WT as well as for the KO growth plates, while the WT showed slightly higher values (WT: 11.5+/-4 vs KO: 10+/-3.2 cells/column) (Fig.13B). The orientation of the cells fluctuated around 90° (WT: 87°+/-10° vs KO: 87°+/-13°) (Fig.13E), and the columns were also normally arranged represented by a mean orientation of 13°+/-9° in the WT and 14°+/-9° in the KO (Fig.13C).

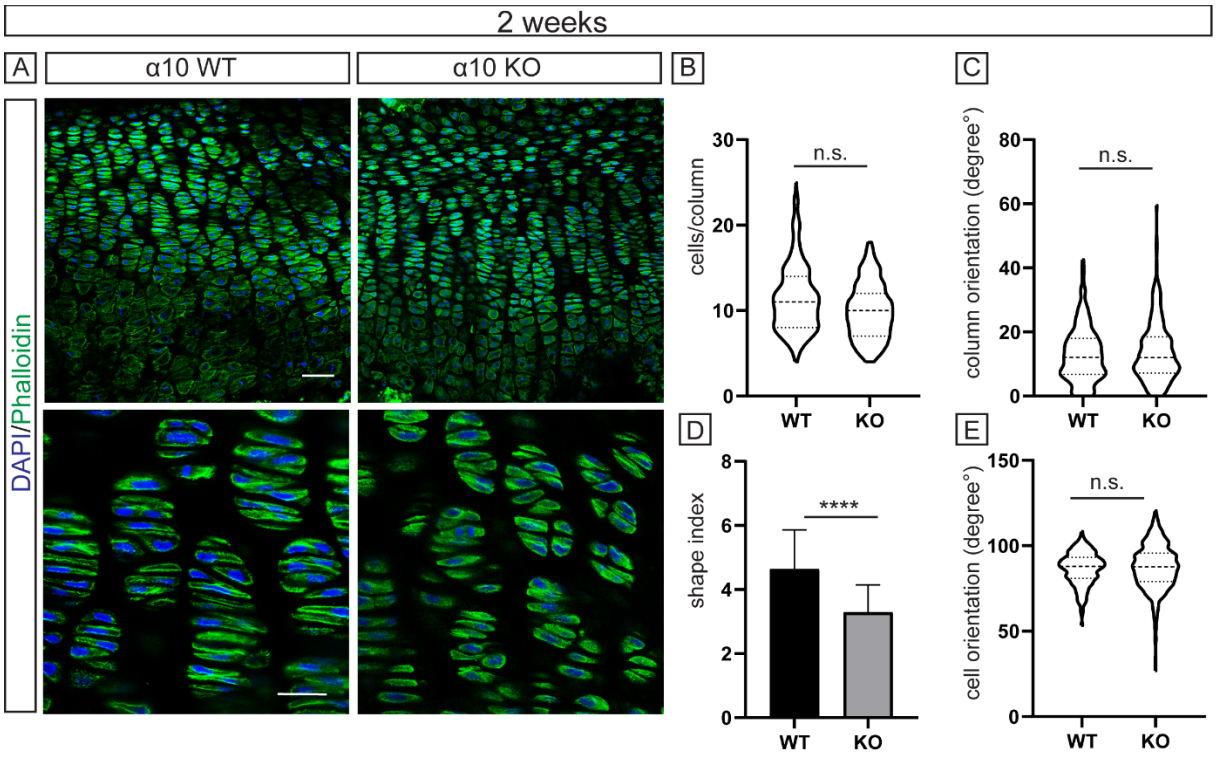


Figure 13: Morphological consequences of the loss of integrin α10 on the tibial growth plate at 2 weeks of age. Pictures and measurements from three independent integrin α10 KO and WT mice. (A) Confocal microscopy images showing the columns in the proliferative zone. In green the actin cytoskeleton is stained by phalloidin, in blue the nucleus by DAPI. The scale bars indicate 50 µm in the upper and 20 µm in the lower pictures. (B) Violin plot of the number of cells/column. (C) Violin plot of the column orientation in degree (°) compared to the PD-axis of the tibia. (D) Shape index of the chondrocytes within the proliferative zone. The bars represent the standard deviation. (E) Violin plot of the cell orientation in degree (°) referred to the PD-axis of the tibia. In all violin plots, the middle thick dashed line represents the median, while the lower and the upper dashed lines represent the first quartile (25%) and the third quartile (75%), respectively. N:3; n:4-5; **** $p < 0.0001$; n.s.–statistically not significant.

4.1.4 1 MONTH OLD STAGE

The examination of the 1-month-old mice proved again a significant difference concerning the SI. Column alignment to the PD-axis of the bone was slightly but not significantly influenced in the KO growth plate (WT: 5°+/-4° vs KO: 7°+/-5 (Fig.14C). Like for all other age stages, the cells oriented perpendicular to the long axis of the bone (WT:89°+/-13° vs KO: 92°+/-20°) (Fig.14E). The significantly different SI reached mean values of 3.3+/-0.9 for the WT and 2.3+/-0.6 for the KO (Fig.14D). Concerning the cell content per column, surprisingly the KO growth plates showed slightly but not significantly higher values compared to the WT (WT: 13.6+/-3 vs KO: 16.5+/-4).

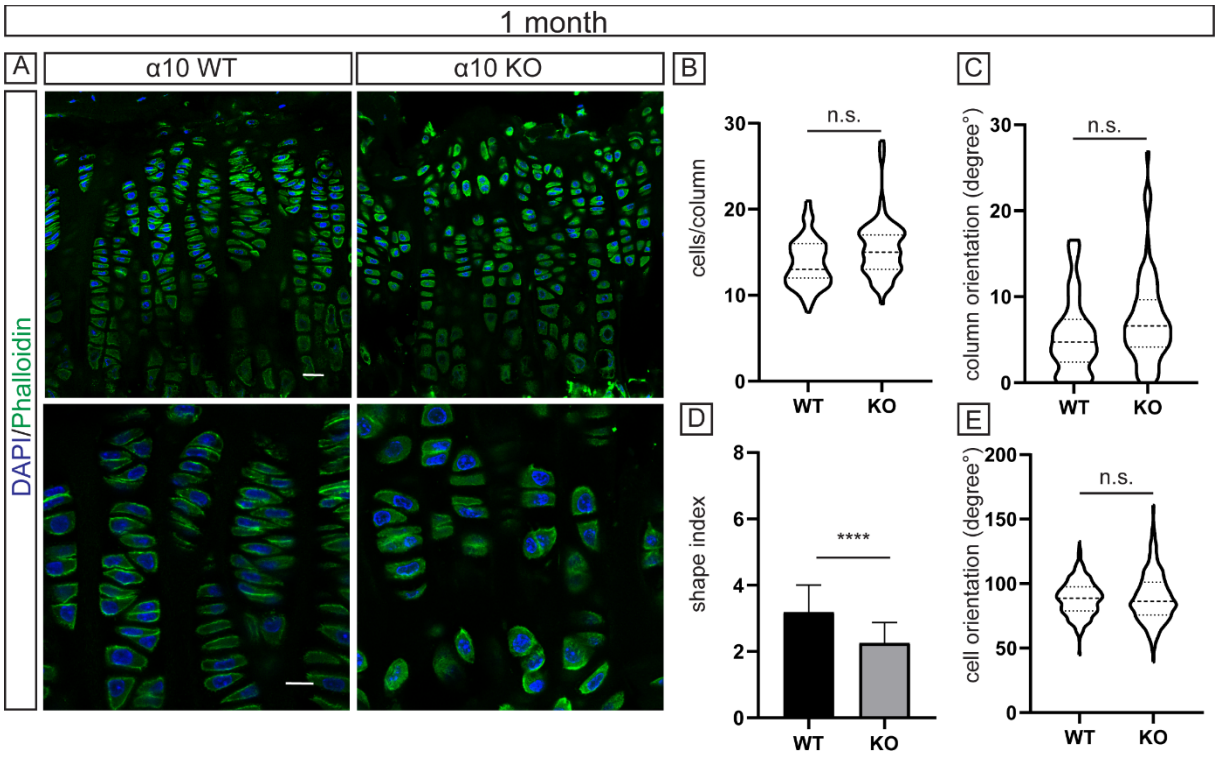


Figure 14: Morphological consequences of the loss of integrin α10 on the tibial growth plate at 1 month of age. Pictures and measurements from three independent integrin α10 KO and WT mice. (A) Confocal microscopy images showing the columns in the proliferative zone. In green the actin cytoskeleton is stained by phalloidin, in blue the nucleus by DAPI. The scale bars indicate 20 µm in the upper and 10 µm in the lower pictures. (B) Violin plot of the number of cells/column. (C) Violin plot of the column orientation in degree (°) compared to the PD-axis of the tibia. (D) Shape index of the chondrocytes within the proliferative zone. The bar represents the standard deviation. (E) In all violin plots, the middle thick dashed line represents the median, while the lower and the upper dashed lines represent the first quartile (25%) and the third quartile (75%), respectively. N:3; n:4-5; **** $p < 0.0001$; n.s.–statistically not significant.

4.1.5 2 MONTHS OLD STAGE

At 2 months-old, mice displayed an affected SI (WT: 3.9+/-1.3 vs KO: 2.5+/-0.6) (Fig.15D) and also impaired column formation represented by less chondrocytes within one column (WT: 9.3+/-3 vs KO: 6+/-1.8 cells/column) (Fig.15B). In contrast, the orientation of the cells and the columns was not significantly affected as showed by values around 8° for the column orientation (WT: 7°+/-6° vs KO: 9°+/-8°) (Fig.15C) and 90° for the chondrocyte orientation (WT: 90°+/-13° vs KO: 91°+/-16°) (Fig.15E).

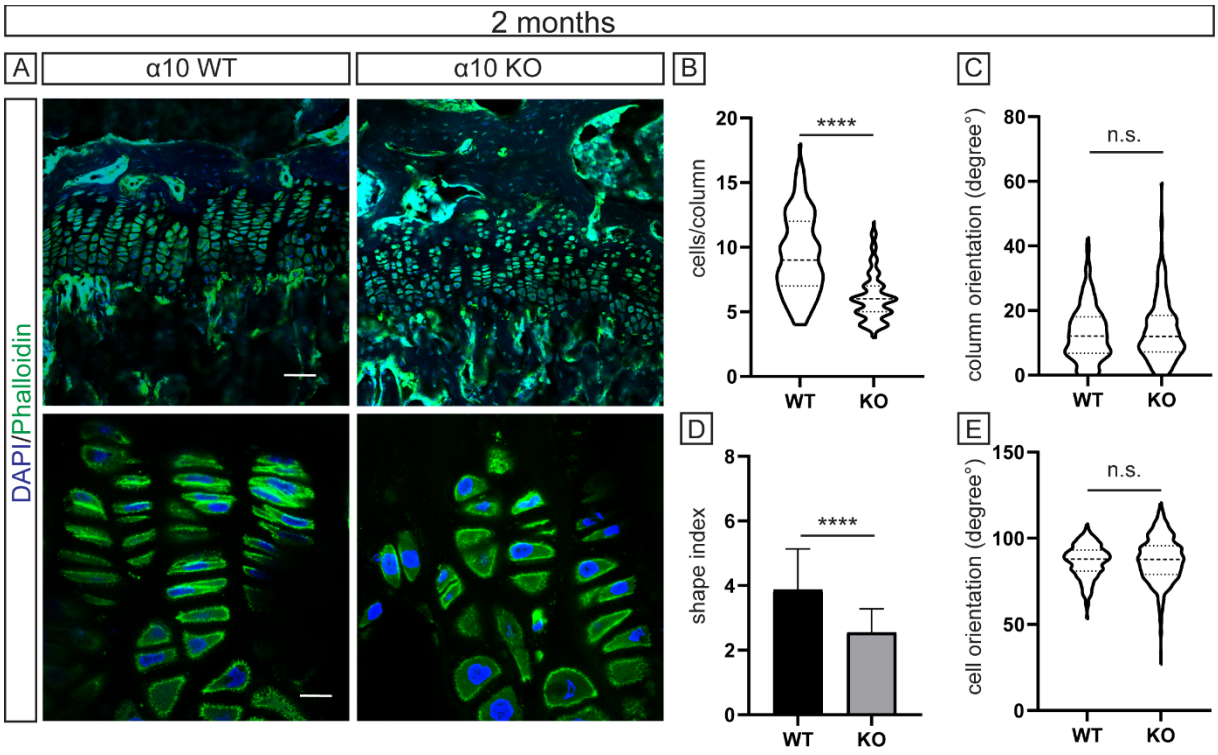


Figure 15: **Morphological consequences of the loss of integrin α10 on the tibial growth plate at 2 months of age.** Pictures and measurements from 2-4 independent integrin α10 KO and WT mice. (A) Confocal microscopy images showing the columns in the proliferative zone. In green the actin cytoskeleton is stained by phalloidin, in blue the nucleus by DAPI. The scale bar indicates 50 µm in the upper and 10 µm in the lower pictures. (B) Violin plot of the number of cells/column. (C) Violin plot of the column orientation in degree (°) compared to the PD-axis of the tibia. (D) Shape index of the chondrocytes within the proliferative zone. The bars represent the standard deviation. (E) In all violin plots, the middle thick dashed line represents the median, while the lower and the upper dashed lines represent the first quartile (25%) and the third quartile (75%), respectively. N:2-4; n:4-5; **** $p < 0.0001$; n.s.–statistically not significant.

4.1.6 DISTRIBUTION OF CELL NUMBERS PER COLUMN AT THE DIFFERENT DEVELOPMENTAL STAGES

Figure 16 shows the distribution of cell numbers per column throughout the maturation phases of the growth plate. In general, the number of chondrocytes in the proliferative zone columns showed an increase from ages E15.5 to 1 month in both genotypes. The maximum of cells/column was reached at 2 weeks and kept constant till the age of 1 month with columns containing 20-25 chondrocytes. At the age of 2 months the length of the columns seemed to decrease again, reaching maximum values of 17 cells (Fig.16). At E15.5, the KO mice exhibited significantly more cell clusters than columns compared to wild type where the columns were dominated. Like mentioned before, the frequency of the cell content per column only presented significant differences at the

ages of E18.5 and 2 months with clear reduction of chondrocytes in individual columns of the KO mice.

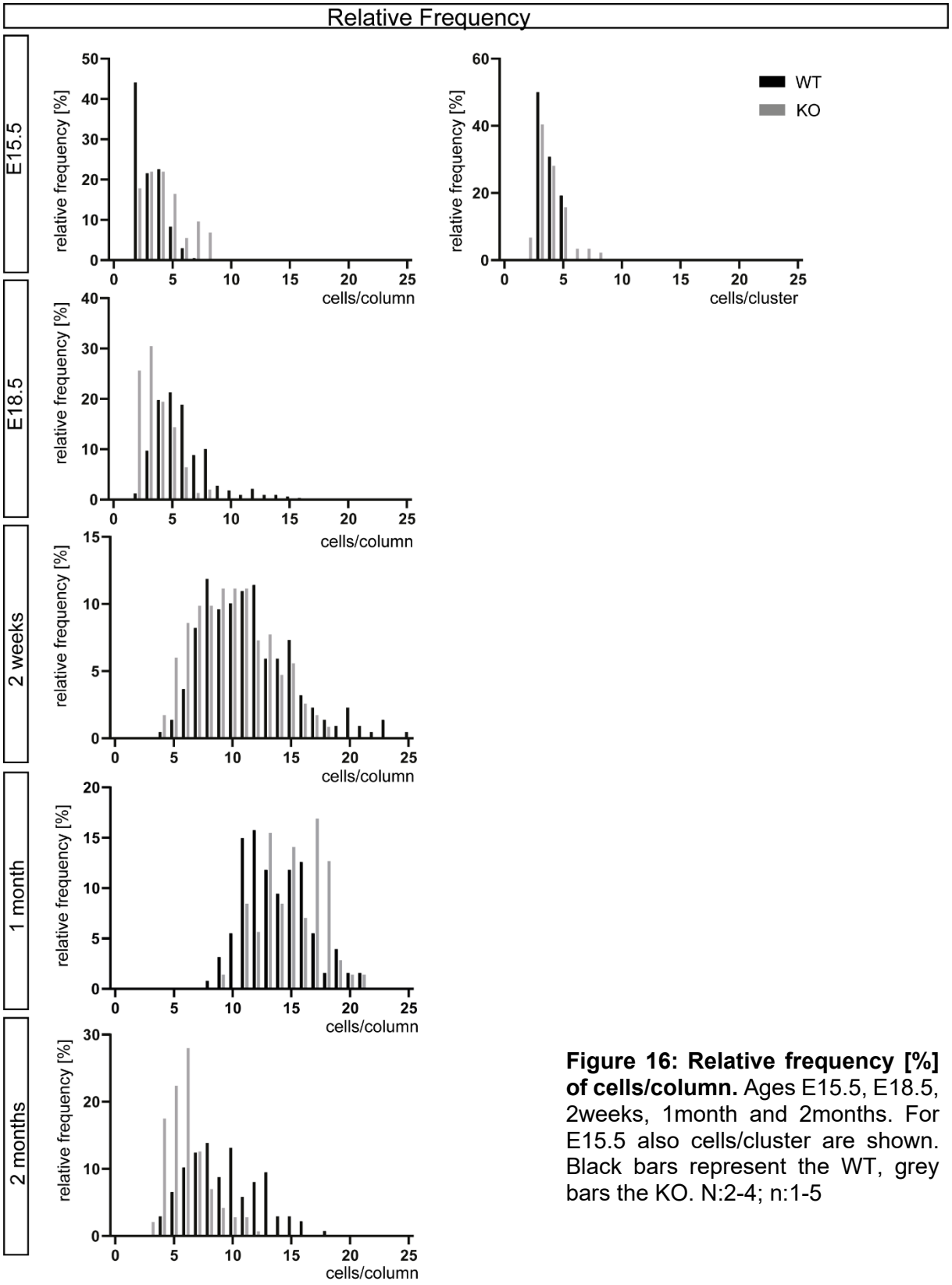


Figure 16: Relative frequency [%] of cells/column. Ages E15.5, E18.5, 2weeks, 1month and 2months. For E15.5 also cells/cluster are shown. Black bars represent the WT, grey bars the KO. N:2-4; n:1-5

4.2 INFLUENCE OF INTEGRIN α10 ON RANDOM CELL MIGRATION

Integrins are known to connect the cytoskeleton of a cell to its ECM microenvironment, containing primarily collagens, laminins and RGD peptide-containing glycoproteins, and mediate chemical and mechanical signals across the plasma membrane (Barczyk et al., 2010). The increased tendency for a more rounded, or less elongated, chondrocyte geometry in the KO growth plate at all investigated stages led to the hypothesis that the loss of integrin α10 could lead to an altered adhesion of chondrocytes to the ECM. In order to dissect the role of integrin alpha 10 in adhesion and migration, we performed adhesion, attachment and migration assays. Chondrocytes were isolated from the rib cages of WT and integrin α10 KO E18.5 or E19.5 mice (N=3-4). Cells were cultivated on different substrates, namely collagen II (Col II), collagen VI (Col VI) and fibronectin (FN). Col II is a known binding partner of integrin α10 but also Col VI was shown to interact with this integrin subunit (Tulla et al., 2001). The RGD peptide containing fibronectin binds to many different kinds of integrins, predominantly to α5β1 (Pankov & Yamada, 2002). The cells were observed by time lapse video over 96 h. In the first 24-48 h, most cells attached and spread on the surface forming stable adhesion, and then they started to slowly migrate. Due to this slow attachment/adhesion process of chondrocytes, only the last 24 h of the time lapse videos were afterwards analysed for random migration and migration velocity. The experiment was done twice with independent chondrocytes and 3 videos per condition (*e.g.* KO on FN) were recorded. Mapping the migration on a polar plot revealed slightly more migration of integrin α10 KO chondrocytes on Col VI compared to WT cells, while the KO chondrocytes moved less on FN than the WT cells. Interestingly, the cells move the most on FN and the least on Col II (Fig.17A). The mean and maximum velocity did not show a significant difference between WT and KO cells on all substrates. The maximum velocity reached values around 0.3 µm/min on Col II (WT: 0.31+/-0.18 µm/min vs KO: 0.27+/-0.17 µm/min), 0.4 µm/min on Col VI (WT: 0.41+/-0.25 µm/min vs KO: 0.43+/-0.25 µm/min) and 0.5 µm/min on FN (WT: 0.52+/-0.33 µm/min vs KO: 0.43+/-0.29 µm/min) (Fig.17B). The mean velocity, in contrast, showed lower values with velocities around 0.09 µm/min on Col II (WT: 0.09+/-0.07 µm/min vs KO: 0.08+/-0.07 µm/min), 0.15 µm/min on Col VI (WT: 0.13+/-0.1 µm/min vs KO: 0.17+/-0.1 µm/min) and 0.16 µm/min on FN (WT: 0.19+/-0.14 µm/min vs KO: 0.14+/-0.11 µm/min) (Fig.17C).

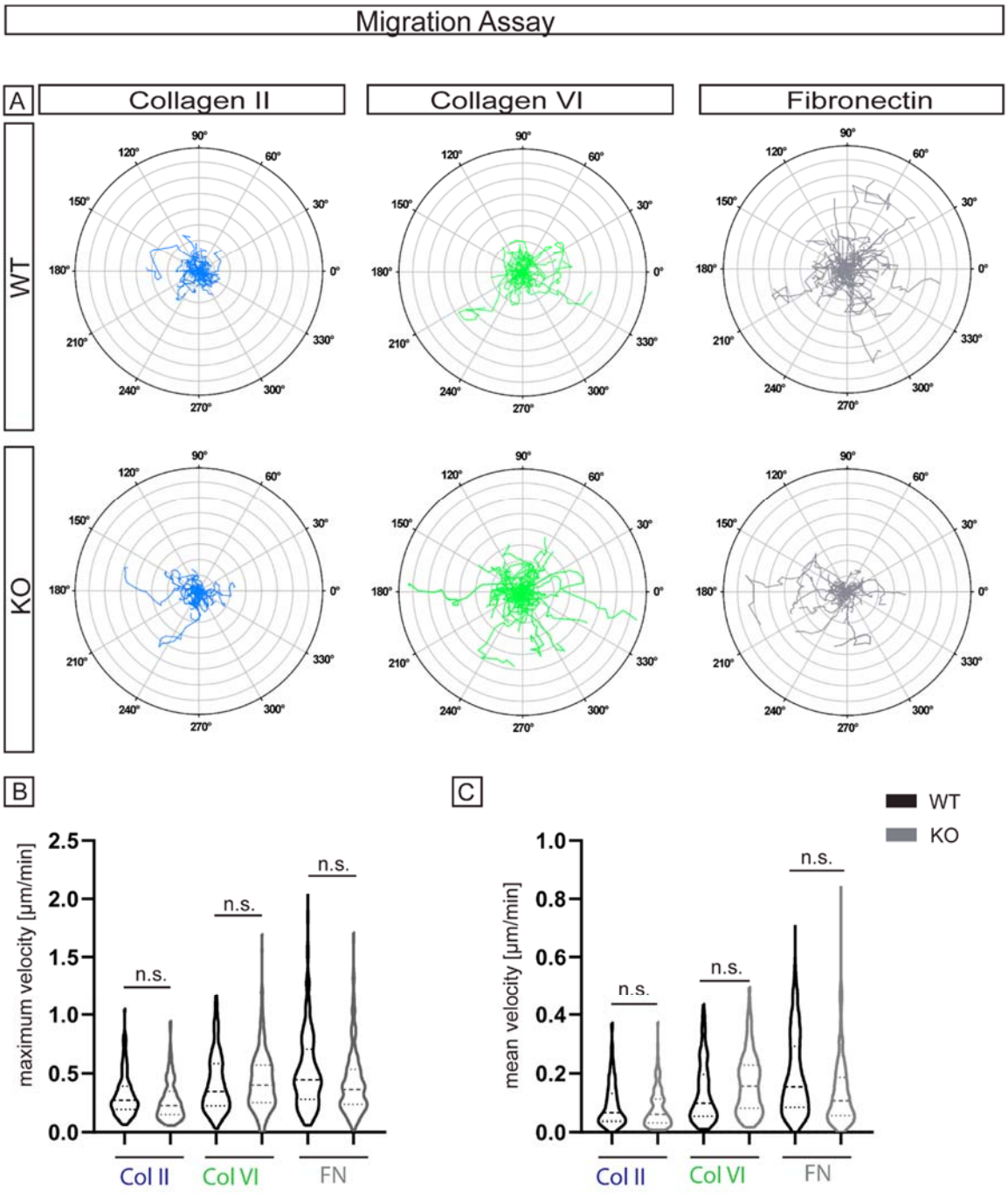
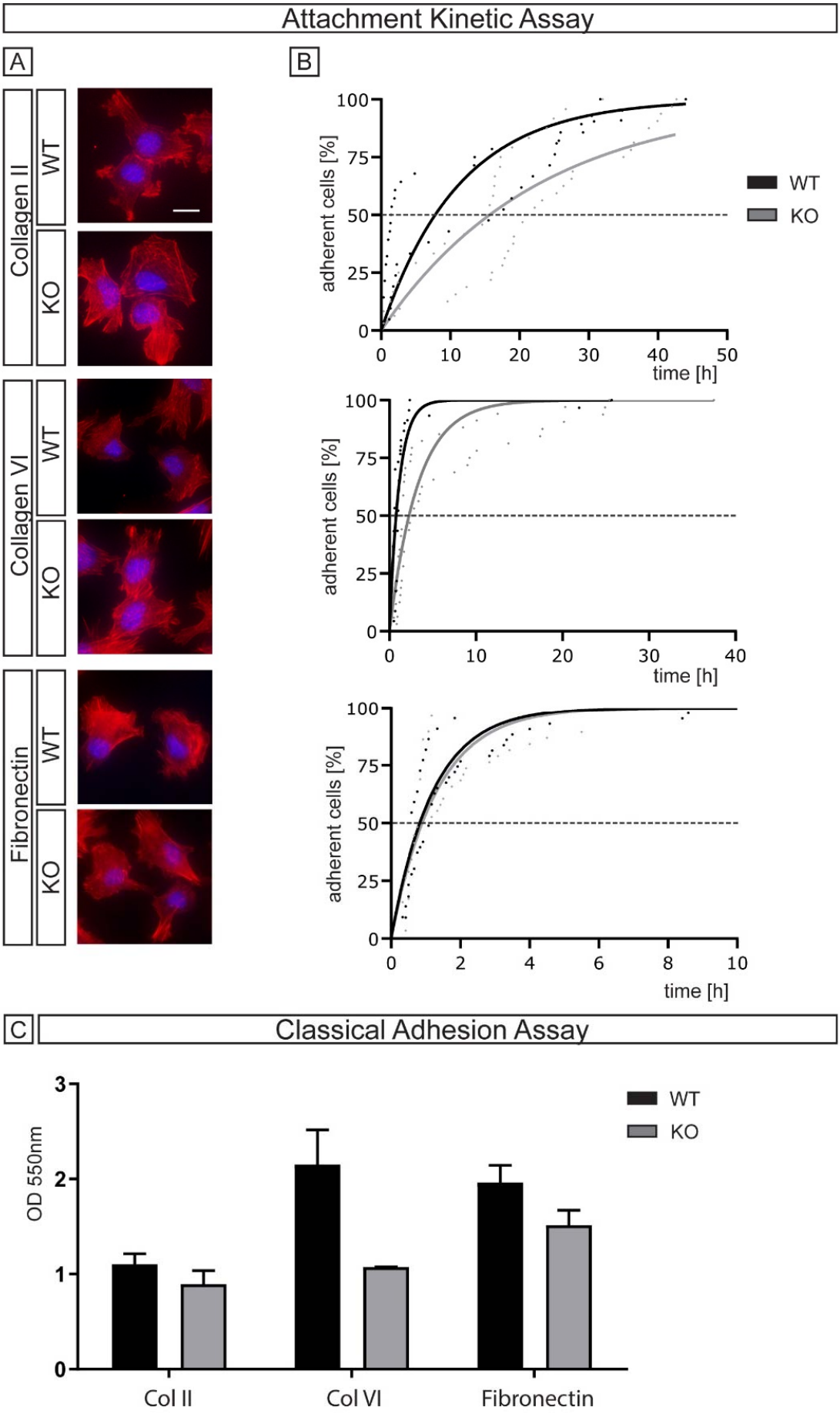


Figure 17: Cell migration assay on fibronectin (FN, grey), collagen II (Col II, blue) and collagen VI (Col VI, green). (A) Polar plot of the migration of WT and KO E18.5/19.5 chondrocytes. Cells were tracked by the MTrackJ plugin of ImageJ. For each plot, 45-50 cells were analysed over the last 24 h of a 96-h time lapse video. Two independent experiments were performed with independent chondrocytes. (B) Violin plots of the maximum velocity in µm/min during the last 24 h. Black plots represent the WT, grey plots the KO. (C) Violin plots of the mean velocity in µm/min during the last 24 h. (B, C) Both violin plots show 2 independent experiments (N:2). For each experiment 3 videos per condition (*e.g.* KO on FN) were analysed (n:3). n.s.–statistically not significant.

4.3 INFLUENCE OF INTEGRIN α10 ON THE CELL ATTACHMENT KINETIC AND ADHESION

To analyse a possible difference in the cell adhesion process, WT and integrin α10 KO primary chondrocytes were cultivated on different coatings, namely Col II, Col VI and FN. Because the actin cytoskeleton also plays a role during cell adhesion, its organization was analysed on the different substrates by immunohistochemistry. The staining of the actin cytoskeleton by phalloidin did not reveal a substantial difference between WT and KO chondrocytes cultivated on different substrates (Fig.18A). For the attachment kinetic assay, cells were monitored over 48 h by time-lapse video and the time of the appearance of the first cell protrusion, as a sign of attachment, was recorded. The experiment was done twice with independent and freshly isolated chondrocytes. Integrin α10 KO cells, cultivated on Col II and Col VI, showed a much slower attachment compared to the WT, while on FN no difference was seen (Fig.18B). To notice, cells attached much faster to FN than to Col II and Col VI (50% after 1 h on FN). On Col II, the chondrocytes showed the slowest attachment and the biggest difference between WT and KO chondrocytes as 50% adhesion was observed after 8 h for the WT and after 16 h for the KO (Fig.18B). The attachment rate on Col VI reached 50% within 45 minutes for WT and 2.5 h for KO chondrocytes. These data indicate that α10 integrin deficiency impaired initial adhesion of mutant chondrocytes on Col II- and Col VI-coated, but not on FN-coated surfaces.

Classical adhesion assay was also performed by optical density (OD_{550nm}) measurements of chondrocytes stained with crystal violet. Cells were seeded and left to adhere to ECM coated surfaces for 1 h, then the wells were washed with PBS. The adhered chondrocytes were lysed by Triton-X. At 550 nm, the absorbance between the WT and KO showed the biggest differences for cells seeded on Col VI (WT: 2.15+/-0.37 vs KO: 1.07+/-0.003). On Col II surface the OD_{550nm} values were 1.1+/-0.11 for WT and 0.89+/-0.14 for KO; whereas on FN the OD_{550nm} values were 1.96+/-0.18 for WT and 1.51+/-0.16 for KO. While the values ranged around 2 for WT cells cultivated on FN and Col VI, it was only the half on Col II, indicating a possible slower or worse attachment to this collagen in general (Fig.18C).


Attachment Kinetic Assay
A
B
Collagen II
WT
KO
Collagen VI
WT
KO
Fibronectin
WT
KO
adherent cells [%]
time [h]
WT
KO
C
Classical Adhesion Assay
OD 550nm
Col II
Col VI
Fibronectin
WT
KO

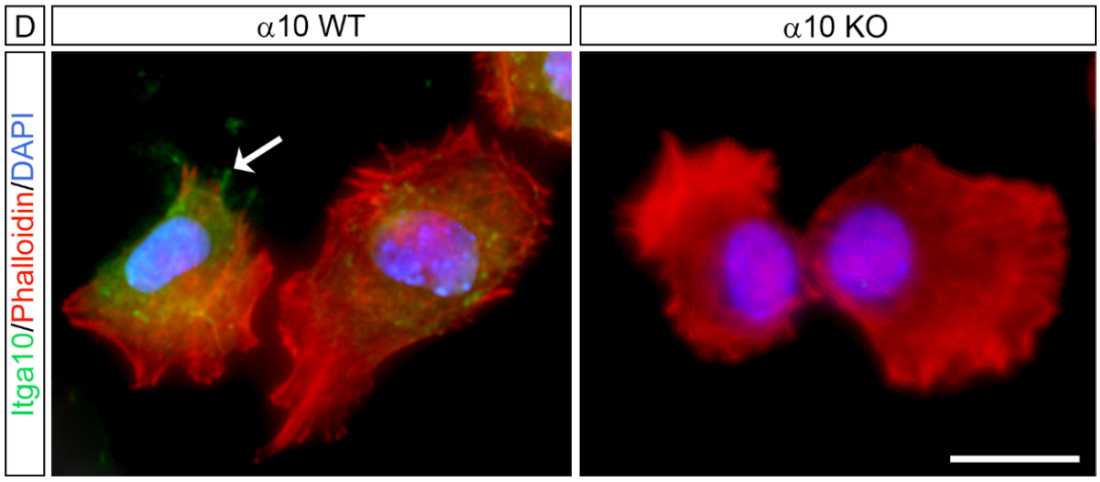


Figure 18: Cell adhesion on fibronectin (FN), collagen II (Col II) and collagen VI (Col VI). (A) Representative immunofluorescence pictures of E18.5/E19.5 WT and KO chondrocytes cultivated on FN, Col II and Col VI. In red the actin cytoskeleton stained by Alexa Fluor 546 Phalloidin, in blue the nucleus visualized by DAPI. (B) Cell kinetic attachment analysed by time-lapse videos over 48 h. The black line represents the WT, the grey line the KO. N:2. (C) Classical cell adhesion assay analysed by optical density measurements at $OD_{550\ nm}$ after staining with crystal violet. Graph shows one representative experiment out of two independent assays performed in triplicates. (D) Example of WT and KO primary chondrocytes stained for integrin α10 and phalloidin on Col II coating. Note the concentration of integrin α10 at the end of cells protrusions (white arrow) where focal adhesion is taking place. The scale bars indicate 20 µm.

5 DISCUSSION

The proper cytoarchitecture of biological tissues relays on their specialized ECM composition and its interaction with the surrounding cells. The main mediators for such interactions are represented by the integrin receptors. In the cartilaginous growth plate integrins have been shown to play a pivotal role in the development and maintenance of the correct function of this dynamic tissue. The most abundant integrins in cartilage are α1β1, α2β1 and α10β1, which bind to collagens; α5β1, αvβ3 and αvβ5 bind among others to fibronectin and α6β1 connects to laminin. (Loeser, 2000; Raducanu et al., 2009) Conditional loss of the β1 subunit in chondrocytes leads to perinatal lethality and severe chondrodysplasia characterized by a disrupted cytoarchitecture of the growth plate including rounding up of the normally elongated chondrocytes within the proliferative zone, which could not align into vertical columns. β1-deficient chondrocytes showed a loss of attachment to collagen II and laminin; reduced adhesion to and less spreading on FN; disrupted cortical actin cytoskeleton, increased apoptosis and less proliferation due to a delayed G1/S progression. In addition, integrin β1 appeared to influence chondrocyte maturation and thus hypertrophy. (Aszódi et al., 2003; Raducanu et al., 2009) In contrast to the β1 subunit, integrin α10 and its specific role in cartilage function has been only partially elucidated. Knockout experiment in mice revealed that loss of integrin α10 partly mimics the phenotype of the β1 mutant mice. Chondrocytes of the proliferative zone of the growth plate showed a tendency for rounding and abnormal column formation; and exhibited reduced proliferation with a slightly impaired cell cycle progression. Concerning the cartilage ECM, integrin α10 deficient mice displayed a reduced collagen network, which was also observed upon the loss of the β1 subunit. (Bengtsson et al., 2005) Underlying the importance of the α10 subunit, Kyöstilä *et al*. identified a truncating mutation in the integrin α10 gene (*Itga10*) of Nordic hunting dogs leading to mild chondrodysplasia (Kyöstilä et al., 2013). Furthermore, it was shown that FGF-2 treatment of bone marrow-derived MSCs upregulates the surface expression of integrin α10, which correlates with improved chondrogenic differentiation potential in pellet culture and better adhesion of these cells to osteochondral defects (Delco et al., 2020; Uvebrant et al., 2019; Varas et al., 2007).

These previous studies suggest that integrin α10, first discovered as an important collagen II-binding integrin in cartilage, may play a unique role in cartilage homeostasis and development.

To better understand the specific role of integrin α10 during development, it is indispensable to take a closer look to the functions of this subunit during the process of endochondral ossification. In this regard, knockout mice provide a perfect platform to analyse the impact of specific gene loss on growth plate functions. A crucially important factor for correct morphogenesis of the growth plate beside chondrocyte differentiation, proliferation and apoptosis is the proper cytoarchitecture, which itself is highly dependent on integrin-mediated cell-matrix interactions.

Loss of integrin α10 in cartilage impairs chondrocyte geometry and column elongation

In this work the cytoarchitecture of the growth plate was analysed in WT and α10-integrin deficient mice by phalloidin staining of the actin cytoskeleton followed by confocal microscopy and morphometric measurements. The significant influence of integrin α10 on the geometry of proliferative chondrocytes was indicated by the lower shape index compared to WT cells, indicating a mild failure of chondrocyte elongation during growth plate morphogenesis. The flattened shape of chondrocytes in the proliferating zone of the normal growth plate, maintained even during mitosis, is a peculiar characteristic of endochondral bone formation (Aszódi, 2017). Nearly all others cell types in the body undergo mitotic rounding and cytoskeletal reorganisation, which is believed to play an important role for the right assembly of mitotic spindle and subsequent cell division (Cadart et al., 2014; Kunda & Baum, 2009). The correct positioning of the spindle is the basis for the right segregation of the chromosomes and thus for forming of the cleavage plane, which in turn ensures proper tissue growth and regeneration (Li & Jiang, 2018; Stevermann & Liakopoulos, 2012). If the correct cell shape plays such an important role, one could hypothesize that the rounded shape observed for the integrin α10 KO chondrocytes may result in impaired spindle formation and cell division, and thus proliferation, leading to a different cell content per column in KO growth plates. This was observed at E18.5 and 2 months of age, where the KO growth plates showed significantly fewer cells/column than the WT. Chondrocytes divide according to the Hertwig's rules with spindles forming along the mediolateral (ML) axis and the dividing plain orientated perpendicular to it (Hertwig, 1893). Therefore, it would be interesting to analyse a putative impaired spindle

formation, for example by anti-tubulin staining. Furthermore, disturbed proliferation was already found by Bengtsson *et al*. 2005, who showed delayed G1/S progression in integrin α10 KO chondrocytes by BrdU (5-bromo-2′- deoxyuridine) incorporation and cyclin-D immunostaining primarily in new born and two week old mice (Bengtsson et al., 2005). An impaired BrdU incorporation implies impaired S-phase, while cyclin D plays an important role during the G1 phase progression (Baldin et al., 1993; Crane & Bhattacharya, 2013). However, it would be interesting to analyse the cell proliferation in terms of spindle formation in the integrin α10 deficient mice. Bengtsson *et al.* reported a comparable but much weaker proliferation phenotype compared to the β1 integrin loss (Bengtsson et al., 2005). In β1-deficient mice, chondrocyte proliferation was severely impaired, being nearly completely absent at the age of 6 weeks (Aszódi et al., 2003). Furthermore, β1 null chondrocytes, which were completely rounded up in the proliferative zone, displayed a binucleated phenotype suggesting that β1 integrins-mediated attachment to the ECM is pivotal to initiate and/or complete the cytokinesis. These observations were explained, among others, by reduced cyclin D1 expression and the impaired organisation of the cleavage furrow due to loss of integrin β1. (Aszódi et al., 2003) The impaired cyclin D1 expression may result from impaired Ihh signalling due to a disrupted prehypertrophic zone, which was reported for the β1-deficient mice (Aszódi et al., 2003; Long et al., 2001; St-Jacques et al., 1999). As already mentioned in the introduction, Ihh is primarily expressed within the prehypertrophic zone and activates PTHrP near the developing articular surface influencing chondrocyte proliferation and maturation (St-Jacques et al., 1999). The diffusion of Ihh could be affected by alterations within the ECM, in particular a reduced fibrillar network, as found in integrin β1 and α10 deficient mice (Aszódi et al., 2003; Bengtsson et al., 2005). Not only Ihh but also other factors like FGF influence chondrocyte proliferation. Upregulation of the FGF3 receptor leads to dwarfism due to less proliferation (Ornitz & Marie, 2002). Aszódi *et al*. reported elevated levels of the *Fgfr3* expression in β1 deficient chondrocytes, accompanied by augmented levels of Stat proteins (Stat1/Stat5a), followed by up-regulation of inhibitory cell cycle controllers like p16 and p21 (Aszódi et al., 2003). In contrast, Bengtsson *et al.* found no changes in the *Fgfr3* expression but indeed moderate changes in Stat1/Stat5a translocation and p16 up-regulation in α10 deficient mice (Bengtsson et al., 2005). These explanations for reduced chondrocyte proliferation could be at least partly true for the integrin α10 deficient columns, which contained significantly fewer chondrocytes at the ages E18.5

and 2 months. It is important to note that in this study, we found only a slight tendency for chondrocyte rounding in α10 integrin-deficient growth plate, and no evidence of binucleation. Those findings suggest that, while all β1 integrin heterodimers are essential for anchoring the elongated chondrocytes to the ECM, the α10β1 integrin heterodimer has only a moderate effect on cell shape determination in the proliferative zone. Nevertheless, α10 is the only α subunit that can influence chondrocyte geometry in the growth plate.

Integrin α10 KO growth plates have more cell clusters at the age of E15.5 as a sign of developmental retardation

The analysed orientation of the cells compared to the proximodistal (PD) axis of the tibia showed no significant difference at any age stage. Cells are normally orientated with their long axis perpendicular to the PD axis of the bone. The average angle of orientation observed was around the expected 90°. During skeletal development, the arrangement of the chondrocytes into columns in mice begins around the embryonic age E14.5 and is well established at birth (Aszódi, 2016; Prein et al., 2016). Interestingly, well-arranged chondrocytes were already observed in columns of E15.5 mice, with a mean orientation of 88.26°+/-12.95° for WT and 86.24°+/-12.36° for the KO. In the cell clusters observed at age E15.5, the orientation of the chondrocytes was not analysed as they are not yet arranged perpendicular to the long axis of the bone. Our results indicate that integrin α10 did not have a major influence on cell orientation. However, there were significantly more cell clusters in the E15.5 KO growth plates compared to WT (Fig.11G), which may be a sign of mild developmental retardation due to loss of integrin α10.

Significantly affected column orientation due to integrin α10 loss at E18.5

The columns within the growth plate should normally be orientated along the long axis, thus a measured angle can vary around 0°. The observed column orientation in E18.5 mice averaged 14°+/-9° for WT and 22°+/-16° for KO. Therefore, the KO columns were not aligned as close to 0° as the WT columns and showed a huge variation within their orientation. Comparing the ranges of orientations obtained, we found values between 0° - 39° for WT and 0° - 116° for KO in newborn mice. Thus, supported by the highest number of analysed mice, integrin α10 apparently influenced column formation at this age. Column formation takes place in the proliferative zone of the growth plate, according to the rules first described by Dodds in 1930. The model describes that semi-

circular postmitotic cells arranged side to side in a lacuna. Afterwards, these daughter cells undergo shape change, flattening and intercalation. Following this intercalation and gliding movement mediated by cell-matrix interactions, both postmitotic cells lie on top of each other rather than side by side, resulting in a new or extended column. (Dodds, 1930) Another more recently discovered theory is that the semi-circular cells spread back into the column due to temporary mediation via cell-cadherin and β-catenin dependent cell-cell adhesion (Romereim et al., 2014) (Fig.4). As the heterodimeric integrins mediate both cell-matrix and cell-cell interactions, it is not surprising that α10 deletion may affect column formation by interfering with either matrix-mediated intercalation or cell-mediated spreading motions. This observation is consistent with that observed in the case of loss of integrin β1, where the growth plate of the mice was completely disrupted and no columns were formed due to an affected attachment of the KO chondrocytes to the ECM (Aszódi et al., 2003). At least for the E18.5 and 2-month-old mice, this process of column formation appeared to be slightly impaired by loss of integrin α10, as indicated by the altered column orientation and cell content of the columns (Fig.12B, C; Fig.15B). The number of mice analysed was between 2-4 animals per group, which is sufficient to see a difference in between the WT and the KO for highly affected parameters as SI. For only slightly differences, more mice should be analysed to overcome normal and random variances between individuals, for example due to the gender. It is well known that females show certain differences within the skeleton compared to males (e.g. thickness, length, bone mineral content and microstructure of the long bones) (Almeida et al., 2017; Khosla et al., 2006). Even if this difference may not already alter bone and cartilage architecture during embryogenesis and in neonatal mice, puberty begins around 1 month of age with an slightly early onset in females (Cross et al., 2021) and leads to diverse structural and functional changes within the growth plate (Eshet et al., 2004; Hunziker & Schenk, 1989). It is tempting to speculate that slight differences due to gender could interfere our results, as gender was not considered. Thus, the analysis of more mice, possibly with respect to sex, would be of especially interest for the column orientation and the cell content, which were significant only at some age stages (column orientation E18.5, cells/columns E18.5 and 2 months) and could have been influenced by random selection of female or male mice primarily for 1 and 2 months old mice (Fig.12B, C; Fig.15B). Another parameter of interest for further studies could be the detection of the primary cilia, which shows a random distribution on chondrocytes in

the resting zone, while on cells in the proliferating and hypertrophic zone they are located parallel to the bone long axis (Ascenzi et al., 2007). Primary cilia are hypothesized to function as mechano- and chemo-sensors and influence cell polarity (Ascenzi et al., 2007; De Andrea et al., 2010; Farnum & Wilsman, 2011). It would be interesting to know whether the loss of integrin α10 affects the positioning of primary cilia and thus also oriented column formation.

Loss of integrin α10 affects chondrocyte adhesion to Col II and Col VI

Integrins play a pivotal role in mediating cell-matrix interactions (Hynes, 1992). In human adult articular cartilage, the cellular component only contributes to approximately 2% of the total tissue mass, while the ECM and water account for the majority of the total volume (Poole et al., 2001; Stockwell, 1978). Cell-matrix interactions therefore appear to play a very important role in cartilage maintenance and development. In particular, integrins of the β1 family are known to contribute to proper cartilage development (Loeser, 2014). Compared to the other α subunits, integrin α10 is highly expressed at the onset of chondrogenesis (E11.5) and later stages of chondrocyte differentiation and maturation, suggesting that this subunit also plays an important role during cartilage development (Camper et al., 2001). Integrin α10β1 was first discovered and described as a type II collagen binding integrin on chondrocytes (Camper et al., 1998), further studies demonstrated its binding capacity to collagens I, IV, VI and IX (Lundgren-Åkerlund & Aszódi, 2014; Tulla et al., 2001). It is likely that the loss of integrin α10 may result in a lower capacity of chondrocytes to bind the above-mentioned collagens, which could also be an explanation for the slightly rounded cell shape. Contrary to this hypothesis, previous experiments failed to detect differences in the attachment of integrin α10 KO chondrocytes to Col II, Col I and FN compared to WT cells (Bengtsson et al., 2005). However, our study showed that mutant chondrocytes adhere less to Col VI. The adhesion assay in the present work still did not reveal a significant difference between KO and WT chondrocyte in adhesion to Col II, but there was an observable difference in the adhesion to Col VI and slightly to FN (Fig.18C). Bengtsson *et al.* proposed a compensation by other collagen-binding integrins for the undetectable adhesion difference of WT and KO chondrocytes to Col II (Bengtsson et al., 2005). Collagen-binding integrins consist of a β1 subunit associated with an α1, α2, α10 or α11 subunit (Heino, 2000; Tulla et al., 2001). While integrin α1β1 and α2β1 have been studied and characterised for decades, the others, namely α10β1 and α11β1, are not as well investigated (Davidenko et al., 2018; Hamaia

& Farndale, 2014). Integrin α1β1 is most commonly found in the mesenchyme, vascular and visceral smooth muscle cells and the immune system (Gardner, 2014), and α2β1 is located primarily on epithelial, endothelial cells and platelets (Ghatak et al., 2016). The more recently found integrins α11β1 and α10β1 were first discovered in foetal muscle and cartilage, respectively (Camper et al., 1998; Velling et al., 1999). Although all those integrins have binding preferences for different type of collagens, their I-domains are quite conserved. As for the putative compensation of the α10 loss, the most likely candidate is α1β1. Integrin α1 has similar amino acid responsible for Col IV and VI binding within the I-domain (Arg-218) and was found to be expressed on mutant and wildtype chondrocytes. (Bengtsson et al., 2005; Tulla et al., 2001) In contrast, integrin α2 and α11 do not appear to be highly expressed on chondrocytes (Bengtsson et al., 2005; Camper et al., 2001). The putative compensation by integrin α1 may explain the milder growth plate disorganization and normal chondrocyte adhesion to Col II in integrin α10 deficient mice compared to integrin β1 knockout mice. Therefore, it is interesting to compare the expression levels of the different collagen-binding integrins on growth plate chondrocytes in α10 knockout and the wild-type mice. This was done for α1, α2, α10, α11 and β1 subunits in WT and KO chondrocytes by Bengtsson *et al*. The results showed that integrin α10 was not expressed and integrin β1 was less expressed on primary KO chondrocytes. Integrins α2 and α11 were not expressed on both mutant and WT chondrocytes, while α1 was equally expressed on both. (Bengtsson et al., 2005) Interestingly, α1β1 does not seem to be expressed in high levels within the growth plate cartilage (Camper et al., 2001). Regarding adhesion to Col VI, the present results could provide a possible explanation for the mild shape change of KO chondrocytes due to an impaired adhesion to this collagen. This would support the findings of Tulla *et al*., who showed that actually the α10 I domain has a higher affinity for the non-fibrillar Col IV and VI than for Col II. The slightly lower adhesion of KO chondrocytes to FN was not expected, as α10β1 integrin does not bind FN. Whether the expression of the classical FN-binding integrins such as α5β1, α3β1, αvβ1, αvβ5 and αvβ3 (Loeser, 2014; Pankov & Yamada, 2002; Zollinger & Smith, 2017) is changed in α10 KO chondrocytes is remained to be elucidated.

In contrast to the classical adhesion assay, the attachment kinetic assay, which was performed by time-lapse analysis, revealed a difference in the attachment of the cells to both Col II and VI. This experimental design may be more appropriate because it takes into account the entire kinetics of cell attachment, not just an one-hour stage as

the adhesion assay. We observed that the KO chondrocytes adhered much slower to Col II (50% adherent cells after 8 h for the WT and after 16 h for the KO) and Col VI (50% adherent cells after 30 min for the WT and after 2.5 h for the KO) (Fig.18B). Thus, the putative compensation between integrin subunits discussed above could not fully restore integrin α10 function as shown by this experiment. Since it was discovered as Col II binding integrin, it is not unreasonable that the loss of integrin α10β1 could result in an impaired binding kinetics to this collagen, as observed in the attachment assay. In addition, the attachment assay did not show a difference between KO and WT chondrocytes cultivated on FN as was expected. The fast attachment to FN (50% after 1h) may be due to the broader spectrum of integrin heterodimers specific for this substrate or a possible higher expression of major FN-binding integrins like integrin α5β1. (Enomoto-Iwamoto et al., 1997; Kurtis et al., 2003; Maylin et al., 2018). The attachment assay was done twice with independent and freshly isolated chondrocytes. Each single cell was analysed individually every 5 minutes, while in the adhesion assay grouped cells were analysed at one time point and only after 1 h. This may be the reason for the more accurate results in the attachment assay, indicating that binding to Col II in general was slower than to Col VI and FN, and that loss of integrin α10 resulted in an impaired attachment of chondrocytes to Col II and Col VI. The much slower adhesion to Col II could also explain the not observable difference in the adhesion to this collagen between the genotypes in the adhesion assay. The 1-hour time slot to adhere was probably too short for WT as well as for KO chondrocytes. This is also supported by the OD_{550}, which was only half as much as for chondrocytes cultivated in Col II compared to cells analysed on Col VI and FN (Fig.18C).

After a qualitative analysis of the shape of chondrocytes cultured on Col II, Col VI or FN, we conclude that there was no significant difference in the shape and thus spreading (Fig.18A). Different spreading was expected due to an altered attachment. This was found for integrin β1 deficient chondrocytes cultivated on FN or vitronectin, where spreading on FN was much more affected (Aszódi et al., 2003). In order to make a more objective statement on the spreading of α10 KO chondrocytes cultured on different substrates compared to WT, it should be analysed and quantified, for example by time lapse video microscopy. This would also be of interest in terms of binding, as altered spreading could affect the results of the adhesion assay.

Integrin α10 loss does not affect migration of primary chondrocytes on Col II, Col VI and FN

As a highly dynamic process, cell migration plays a central role in many biological processes, like embryogenesis, wound healing, regeneration and general responses to biochemical and biomechanical stimuli (Kirfel et al., 2004). In cartilage, chondrocyte migration is essential during growth plate development and tissue growth, and is hypothesised to play a role in remodelling in response to articular cartilage pathologies in adulthood (Aszódi et al., 2003; Frenkel et al., 1996; Morales, 2007). Furthermore, cell migration is of paramount importance for proper column formation in the growth plate. Experiments with integrin β1 deficient mice by Aszódi *et al*., demonstrated that postmitotic chondrocytes lacking integrin β1 are unable to flatten and rearrange (Fig.4) into columns (Aszódi et al., 2003). Cell migration generally relies on different aspect, like the formation of filopodia or lamellipodia by protrusions of the actin cytoskeleton, accompanied by the organized assembly and disassembly of focal adhesion complexes (Kirfel et al., 2004; Stossel, 1993). Focal adhesion itself requires, beside other proteins, integrins as transmembrane proteins that connect the actin cytoskeleton of the cell to its ECM surroundings (Kirfel et al., 2004). Altered cell adhesion to the ECM components may therefore also influence cell migration. In contrast to this hypothesis and to the results of the attachment assay, interestingly the cell migration assay performed in this work could not reveal a significant difference between the cell migration velocity for WT and integrin α10 KO chondrocytes cultured on FN, Col II and Col VI. Tracking and plotting of the random cell migration revealed a possible, but still discussable, difference, especially for cells cultured on Col VI and FN. It appeared that integrin α10 KO chondrocytes moved slightly more on Col VI and less on FN compared to the WT (Fig.17A). Only 3 to 5 cells were responsible for the observed differences, for more reliable result many more cells should be tracked. On the other hand, many different theories have been proposed previously about cell migration. Migration consist of different steps, namely (1) cell polarisation and pseudopod formation by actin polymerisation at the anterior side; (2) attachment to the ECM by integrins; (3) protease-mediated detachment from the ECM; (4) contraction by actomyosin remodelling; (5) retraction of the opposite cell side and movement of the cell body (Friedl & Wolf, 2009; Wolf et al., 2013). It is therefore still questionable whether the loss of integrin α10 would result in more or less, faster or slower migration. Weaker or less adhesion to the ECM by a specific integrin loss could facilitate the

detachment and thus lead to a higher migration velocity. Conversely, absent or weak adhesion could also result in less migration, which can lead to skipping the second step of the process, resulting in insufficient focal adhesion complexes and impaired migration. Previously, is has been shown that blocking of the integrin β1 subunit by β1-antibodies in melanoma cells resulted in nearly absent migration (Friedl et al., 1998), therefore it would be tempting to speculate that loss of integrin α10 could result in less migration. Nevertheless migration differs between substrates, cell types and integrin subtypes (Chen et al., 2012). Strong binding of integrins at the anterior cell side seems to build up a greater traction force through the cell´s actin cytoskeleton, which helps to overcome interactions between the cell-body and the ECM (Schmidt et al., 1995). Integrin binding associated with cell migration is believed to be a highly adhesive and high-affinity process that, in combination with integrin independent low affinity bindings and non-adhesive processes, ensures migration (Friedl et al., 1998). Furthermore, integrins interact with several different focal adhesion proteins that interact with catalytic enzymes. In cell migration, focal adhesion kinase (FAK) plays an important role, influencing migration through the assembly/disassembly of focal contacts and modulation of the actin cytoskeleton, and thus formation of lamellipodia, filopodia and stress fibres via Rho-family GTPases. (Mitra et al., 2005; Sieg et al., 2000) Again, the similar migration rate between the WT and integrin α10 KO chondrocytes suggests a compensatory mechanism from other collagen-binding integrin α subunits. Cell migration is also dependent on the composition of the ECM or the used substrate (Chen et al., 2012). This was supported by the different random migration behaviour and velocity observed for chondrocytes on Col II, Col VI and FN in this work. Both WT and KO chondrocytes appeared to move the least on Col II and also moved the slowest on this substrate (maximum velocity: WT: 0.31 µm/min vs KO: 0.27 µm/min). In contrast, the chondrocytes moved fastest on FN (maximum velocity: WT: 0.52 µm/min vs KO: 0.43 µm/min) (Fig.17B, C), which is known to have many different integrin binding partners (for review see Plow et al., 2000). Interestingly, FN was previously shown to enhance migration in a number of different cell types, such as hMSCs (Kalkreuth et al., 2014; Kulawig et al., 2013), fibroblasts (Missirlis et al., 2017) and chondrocytes (Krüger et al., 2019). This fact fits well with the observations of faster migration on FN in the migration assay of the present work.

6 CONCLUSION

Integrin α10β1 is the major collagen binding integrin in cartilage. While the β1 subunit appears to be essential for proper cartilage and bone development through the modulation of growth plate architecture, ECM composition, chondrocyte maturation and proliferation, the α10 subunit seems to be at least partly dispensable. This has been demonstrated by the analysis of the integrin α10 KO mice, which partly mimic the β1 growth plate phenotype, but to a milder extent. In this study, close examination of the growth plate from integrin α10 deficient mice by immunohistochemistry and confocal microscopy allowed for the first time to quantify the consequence of α10 integrin loss on growth plate cytoarchitecture. Pathological changes included (1) mild alteration of chondrocyte geometry in the proliferative zone, (2) a slight effect on column orientation and a (3) moderately affected proliferation represented by a less cell content per column in the KO growth plates at the age E18.5 and 2 months. The hypothesis of an attachment deficit due to the integrin α10 loss and hence shape, was further supported by the time-lapse attachment assay, where we clearly demonstrated impaired attachment of KO chondrocytes to Col II and VI. The reduced binding to Col VI was also confirmed by the adhesion assay. These results lead to the conclusion that the integrin α10 subunit is not fully substitutable by other collagen binding integrins and is indeed required for proper interaction of chondrocytes with the ECM molecules Col II and VI. Furthermore, it supports the thesis, that integrin α10 is not only a major Col II receptor but also interacts quite strongly with Col VI. In contrast, *in vitro* time-lapse videos could not show obvious differences of integrin α10 KO chondrocyte migration rates, which on the other hand we speculated to perceive because of known *in vivo* intercalation movements for column formation within the growth plate. As it is well known that the adhesion of the chondrocytes to specific matrix components can affect ECM biomechanical properties, which in turn are pivotal for proper morphogenesis of the growth plate, it would be of great interest as follow up study, to investigate whether the growth plate ECM of the KO mice displays altered biomechanics compared to their WT littermates. This, together with our data on the effect of integrin α10 on chondrocyte shape, column formation and adhesion, will complete the picture of the unique role of this integrin subunit in growth plate morphogenesis and thus bone and cartilage development.

7 LIST OF ABREVIATIONS

AC	Articular cartilage
bp	base pair
BSA	Bovine serum albumin
COMP	Cartilage oligomeric matrix protein
Col	Collagen
CS	Chondroitin sulfate
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EO	Endochondral ossification
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FGF	Fibroblast Growth Factor
FN	Fibronectin
GAG	Glycosaminoglycans
GFOGER	Gly-Phe-Hyp-Gly-Glu-Arg
GLOGER	Gly-Leu- Hyp-Gly-Glu-Arg
Het	heterozygote
Ihh	Indian-hedgehog
Itg	Integrin
KO	Knockout
KS	Keratan sulfate
LA	Long axis
LRR	Leucine-rich-repeat
MES	2-(N-Morpholino)ethansulfonacid
hMSC	human Mesenchymal stem cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PCM	Pericellular matrix
PG	Proteoglycan
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PFA	Paraformaldehyde
PTHrP	Parathyroid hormone - related peptide
QE	Quick extract
RGD	Arginine, Glycine, Aspartate
RT	Room temperature
Runx	Runt-related transcription factor
SA	Short axis
SI	Shape index
Sox	Sex-determining region Y box
Tris	Tris(hydroxymethyl)aminomethane
VW	von-Willebrand
WT	Wildtype

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DECLARATION

I, the undersigned, hereby declare that this dissertation entitled “The role of integrin α10 in the growth plate cytoarchitecture” is my own work, and that all the sources I have used or quoted have been indicated or acknowledged properly by means of included references.

Augsburg, 16.11.2022 — Christina Marie Völcker

Place, Date — Signature

AFFIDAVIT

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