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Joints in the appendicular skeleton: Developmental mechanisms and evolutionary influences

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Abstract

The joints are a diverse group of skeletal structures, and their genesis, morphogenesis, and acquisition of specialized tissues have intrigued biologists for decades. Here we review past and recent studies on important aspects of joint development, including the roles of the interzone and morphogenesis of articular cartilage. Studies have documented the requirement of interzone cells in limb joint initiation and formation of most, if not all, joint tissues. We highlight these studies and also report more detailed interzone dissection experiments in chick embryos. Articular cartilage has always received special attention owing to its complex architecture and phenotype and its importance in long-term joint function. We pay particular attention to mechanisms by which neonatal articular cartilage grows and thickens over time and eventually acquires its multi-zone structure and becomes mechanically fit in adults. These and other studies are placed in the context of evolutionary biology, specifically regarding the dramatic changes in limb joint organization during transition from aquatic to land life. We describe previous studies, and include new data, on the knee joints of aquatic axolotls that unlike those in higher vertebrates, are not cavitated, are filled with rigid fibrous tissues and resemble amphiarthroses. We show that when axolotls metamorph to life on land, their intra-knee fibrous tissue becomes sparse and seemingly more flexible and the articular cartilage becomes distinct and acquires a tidemark. In sum, there have been considerable advances toward a better understanding of limb joint development, biological responsiveness, and evolutionary influences, though much remains unclear. Future progress in these fields should also lead to creation of new developmental biology-based tools to repair and regenerate joint tissues in acute and chronic conditions.

1. Introduction

The joints are a diverse and multi-faceted group of skeletal structures. They differ not only in anatomical location, architecture and size, but also in the type and degree of movement they allow and the nature and structure of their components. One current and useful classification of joint diversity is largely based on the degree of joint movement (Gray, 1988). Synarthroses allow minimum if any movement and consist of dense connective tissue separating the opposing skeletal elements, one example being the joints between cranial

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bones. Amphiarthroses permit some but delimited movement and display a fibrocartilaginous structure between the adjacent skeletal elements. Examples are the intervertebral joints and the pubic symphysis. Lastly, diarthroses permit free, reciprocal and nearly friction-less movement, and prominent members of this subgroup are the synovial joints in the appendicular skeleton. This classification emphasizes the strict relationship between the structural organization of the joints and their functional properties, each combination producing an organ exquisitely fitted to diverse anatomical locations and fulfilling specific biological and mechanical requirements. As it will be described later in this chapter, joint diversity also reflects evolutionary influences, processes and traits.

The synovial joints in the limbs have long attracted strong research attention not only for their importance in daily activities, overall skeletal function and quality of life but also for their susceptibility to acquired and congenital diseases, including osteoarthritis (OA), synphalangism, and developmental dysplasia of the hip (Archer, Caterson, Benjamin, & Ralphs, 1999; Goldring & Goldring, 2007; Hunziker, 2002; Seemann et al., 2005). These joints are composed of multiple tissues and structures. They all share a fibrous capsule that is continuous with tissues attached to the flanking skeletal elements including periosteum, insulating the joint from the internal body environment. The capsule's inner portion is covered by a synovial membrane—a distinct tissue composed of tightly-assembled and flat shaped synovial fibroblasts—which is rich in stem cells (Kurth et al., 2011). The joint cavity is filled with synovial fluid that contains phospholipids, hyaluronan, and glycoproteins such as *Prp4*/lubricin, each component contributing in its own manner to joint lubrication and friction-less motion (Jones & Flannery, 2007; Kosinska et al., 2012; Seror, Zhu, Goldberg, Day, & Klein, 2015; Temple-Wong et al., 2016). The epiphyseal ends of the opposing skeletal elements are covered by articular cartilage, a complex multi-zone tissue that is rich in collagen II, aggrecan, and other extracellular matrix molecules and provides resilience during movement (Bhosale & Richardson, 2008; Hunziker, Kapfinger, & Geiss, 2007). The limb joints also contain components required for certain joint-specific functions, including the anterior cruciate ligament and the patella in the knee and the teres in the hip, that are essential for regulation of motion directionality and joint stabilization and contribute also to proprioception (Ellison & Berg, 1985; Schutte, Dabezies, Zimny, & Happel, 1987). In sum, limb joint functioning requires the orchestrated contributions and efforts of multiple tissues and structures that potentially, last throughout life. As indicated above however, these physiologic traits and mechanisms often succumb to disease or injury since the innate repair capacity of limb joints—and articular cartilage in particular—is notoriously poor. This situation remains a major healthcare problem and challenge and has vexed scientists and clinicians for years. Enormous efforts have been—and are being—devoted to finding therapeutic means by which joint tissues could be repaired or regenerated by biological and bioengineering approaches, but this laudable task has yet to be fulfilled (Johnstone et al., 2013; Makris, Gomoll, Malizos, Hu, & Athanasiou, 2015). Thus, there has been much interest in recent years in deciphering the developmental biology of limb joints, with the hope that detailed information and understanding in this area may elicit the conception and creation of new repair means mimicking or reproducing developmental mechanisms of joint tissue formation (Caldwell & Wang, 2015; Longobardi et al., 2015). Our own group has contributed to studies on synovial joint development, growth, and morphogenesis (Decker et

al., 2017; Koyama et al., 2008, 2010), and this chapter focuses mainly on early joint determination events, tissue morphogenetic mechanisms, and evolutionary influences. Other important aspects of limb joint formation have been reviewed elsewhere (Decker, 2017; Longobardi et al., 2015; Pacifici, Decker, & Koyama, 2018; Pitsillides & Ashhurst, 2008; Salva & Merrill, 2017).

2. Onset of limb synovial joint formation: The interzone

For over a century, the developing limb in mammalian and avian embryos has served as a popular system to study skeletogenesis, owing to its experimental accessibility and relative simplicity compared to the trunk and head. Embryologists realized long ago that the limb skeletal primordia are initially laid down largely as uninterrupted mesenchymal cell condensations with no obvious traits of where the joints would form (Haines, 1947; Hinchliffe & Johnson, 1980). The primordia include the Y-shaped preskeletal condensation that depicts the prospective stylopod element (humerus/femur) continuous with the two zeugopod elements (radius and ulna or tibia and fibula), and the digit rays that correspond to carpal/tarsal and phalangeal elements. It was found that the first morphological indication of joint formation became histologically evident with the emergence at each prospective joint site of an “interzone” (Haines, 1947), also called “zwischenmasse” or “articular disk” by earlier investigators (Whillis, 1940). The interzone consists of mesenchymal cells that initially are closely bound to each other and are apparently interconnected, in a manner distinct from that of surrounding mesenchymal cells and chondrocytic cells within flanking long bone anlagen. One study indicated that the initially close cell-cell contacts among interzone cells reflect the presence of tight junctions and expression of connexin 32 and 43 (Archer, Dowthwaite, & Francis-West, 2003). Soon after, the interzone acquires a typical tri-layer configuration with two compacted cell layers, each bound to the epiphyseal ends of flanking long bone anlagen and one central and more dispersed cell layer. This arrangement is quite obvious in avian limbs where the overall interzone is thick and highly cellular, but is more subtle in mammalian limbs where the interzone is rather thin and sparse (Mitrovic, 1977, 1978). These species-specific differences in thickness, structure, and cellularity have remained largely unexplained in terms of possible developmental significance, but we provide some new insights below.

Though important, the studies above left several important questions unanswered, in particular whether the interzone merely represents an otherwise passive signpost or landmark specifying the anatomical location of the future joint or whether its cells and/or their progenies would have active roles in joint formation. A first experimental attempt to address this key question was carried out in a study in chick embryos (Holder, 1977). Microsurgical procedures were used to remove the prospective interzone from the developing right elbow joint site in stage 24–26 chick embryos *in ovo* (about 4.5 days old). Effects were monitored over time by anatomical examination and compared to the phenotype of unoperated contralateral left limb in the same embryos. The elbow joint failed to form in operated limbs and the epiphyseal cartilaginous ends of humerus, radius, and ulna did not separate and became fused, with penetrance of the phenotype of about 100%. These novel observations showed for the first time that interzone cells are required for formation of synovial joints and their roles cannot be compensated for by surrounding cells nor cells

within the flanking long bone cartilaginous anlagen. Despite its novelty and great importance, this study has not been extended and further verified. In addition, (i) the joints were analyzed anatomically but not histologically or by other means and (ii) it was not clear how selective the removal of the interzone had been effected and whether adjacent tissues were accidentally removed as well. Thus, we carried out similar microsurgical manipulations on developing stage 25–26 chick embryo elbow joints (Fig. 1), but varied our interventions as follows. In a first group of embryos, we mechanically disturbed the interzone with a dissecting needle, but left it in place. In a second group, we removed it, but carefully limited the resected tissue slice to about 100 μm in thickness, very close to the calculated thickness of native interzone at that stage. In a third set, we resected a larger segment of about 300–350 μm in thickness, thus removing not only the interzone but also an epiphyseal portion of flanking cartilaginous elements. After tissue resection, skeletal elements were stabilized by insertion of tungsten pins. Embryos were re-incubated and examined over time. In day 10 control unoperated elbows on the left side of the embryos, the joints displayed typical mature characteristics that included a well-developed articular cartilage, a large capsule, and synovial lining (Fig. 1A). Of note is the fact that a fairly conspicuous fibrous layer covered articular cartilage (Fig. 1B, yellow brackets), a trait typical of avian limb joints largely absent in mammalian joints. Mechanical disturbance of the interzone deranged joint formation (Fig. 1C and D). This was exemplified by delayed separation of the opposing skeletal elements and a poor definition of the epiphyseal ends of each element (Fig. 1D), indicating that the interzone is extremely sensitive to manipulation and cannot recover its function easily. Whole resection of the interzone completely prevented joint formation, and there was cartilaginous continuity between the humerus on one side and ulna and radius on the opposite side (Fig. 1E and F). The intervening ectopic cartilaginous tissue was composed of small-sized chondrocytes, resembling those present at the flanking epiphyses at that stage (Fig. 1F). A similar cartilaginous continuity was observed in a related study after removal of a 150- μm thick tissue slice from the prospective elbow joint in stage 27 chick embryos, though details about surgical approach and tissue slice identity were scanty (Ozpolat et al., 2012). In comparison, elbow sites from which a large and thick tissue segment had been removed not only lacked joints but also the flanking long bone cartilaginous elements remained largely separated from each other and were composed of hypertrophic chondrocytes undergoing endochondral ossification (Fig. 1G and H). Removal of such large prospective joint tissue segment thus appeared to have caused a conversion of the epiphyseal ends into a neodiaphysis. Overall, the data agree with Holder's observations that the interzone per se exerts a required and non-redundant function in limb joint development and its removal alters the developmental program of flanking skeletal cells. The data are also in line with intriguing observations and concepts reported several decades ago. For example, Hampe and Wolff transplanted thick slices of stage 20–22 hind limb buds containing the prospective knee region onto the chorioallantoic membrane of host chick embryos and found that a seemingly normal joint formed over time (Hampe, 1956; Wolff, 1958). Perceptively, they concluded that the cells within the transplanted tissue had acted autonomously and possessed all the determination cues and morphogenetic information needed to create a joint, a concept subsequently reiterated by others (Cosden-Decker, Bickett, Lattermann, & MacLeod, 2012; Wolpert, 1969).

3. Interzone cell function and fate

Given that interzone cells are needed for joint formation, what do they actually do? Some answers to this lingering central question have been provided by genetic cell lineage trace-track studies carried out in the last decade. Kingsley and coworkers were the first to show that the transforming growth factor- β superfamily member *Gdf5* is selectively expressed by interzone cells at the very onset of limb joint development in mouse and chick embryos (Storm & Kingsley, 1996, 1999). The group subsequently created a transgenic BAC-based *Gdf5-Cre* mouse line for conditional gene ablation studies, but used it also to genetically label the *Gdf5*-expressing cells with a reporter and monitor them (Rountree et al., 2004). Their data showed that reporter-positive cells persisted at the joint sites at a later examined time point, were present in joint tissues including articular cartilage, and did not appear to contribute much to formation of long bone shafts or other surrounding tissues. In collaborative follow-up efforts with that group, we used *Gdf5-Cre;R26R-LacZ* double transgenic mice and systematically monitored the topographical distribution and fate of β -galactosidase-expressing cells and their progenies (collectively termed *Gdf5*⁺ cells) over embryogenesis and postnatal life up to about 2 months of age (Koyama et al., 2007, 2008). We found that the *Gdf5*⁺ cells generated articular cartilage, intra-joint ligaments, meniscus, synovial lining, and the inner half of the capsule in limb joints including hip, knee, elbow, carpal/tarsal elements, and digits. The cells were restricted to joint tissues prenatally and postnatally and made little if any contribution to formation of surrounding tissues and structures, though occasional *Gdf5*⁺ cells could be observed in underlying cartilaginous shafts. Similar trends were observed in prenatal and postnatal wrists where the *Gdf5*⁺ cells were confined to the articulating surfaces, though wrist skeletal development is quite distinct from that of long bones (Hogg, 1980). The data agreed well with concurrent genetic cell lineage tracing studies in developing mouse embryo knees carried out by others indicating that *Col2*⁺ articular chondrocytes arise as a population distinct from *Matrillin-1*⁺ chondrocytes constituting the bulk of the long bone elements (Hyde, Dover, Aszodi, Wallis, & Boot-Handford, 2007). Considered together with the interzone extirpation data above (Fig. 1) (Holder, 1977), the genetic cell lineage studies showed that interzone cells are not only required for joint formation but also survive and thrive over time to produce most if not all joint tissues, supporting the notion that they represent a specialized cohort of progenitor cells with innate joint formation capacity.

Because the interzones emerge within preskeletal mesenchymal condensations that are initially uninterrupted but differentiate to chondrocytes soon after, it has long been debated whether interzone cells represent mesenchymal cells present (and determined) at each prospective joint site or are generated via de-differentiation of chondrocytes formed at those sites. Spatiotemporal analyses in developing day 4.0–4.5 chick limbs using immunohistochemistry indicated that the cartilage markers collagen II and keratan sulfate were present over the entire prospective metatarsalphalangeal digital rays prior to interzone formation (Craig, Bentley, & Archer, 1987). We reported similar *Col2a1* gene expression patterns at those stages as revealed by *in situ* hybridization (Koyama, Leatherman, Shimazu, Nah, & Pacifici, 1995). Mouse studies elicited comparable observations. Hyde et al. made use of *Col2a1-Cre;R26R-LacZ* mouse embryos and compared the spatiotemporal

distribution of β -galactosidase-positive cells (that includes progeny cells and collectively termed *Col2+* cells here) with the distribution of cells actively expressing *Col2a1* in developing knee joints (Hyde, Boot-Handford, & Wallis, 2008). At embryonic day 12.5 (E12.5) when the interzone had not become evident yet, there was continuous and overlapping distribution of *Col2+* cells with *Col2a1* transcripts throughout the Y-shaped preskeletal condensations. The interzone became appreciable by E13.5; at this stage, most interzone cells did not express *Col2a1*, but were still positive for β -galactosidase activity. These data agree with observations by Soeda and coworkers using knock-in *Sox9-LacZ* reporter mouse embryos (Soeda et al., 2010) in which the reporter is driven by the chondrogenic master regulator gene *Sox9* (Bi, Deng, Zhang, Behringer, & de Crombrughe, 1999). They found that reporter positive, *Sox9*-expressing cells were present in incipient interzones in E13.5 mouse embryo knee joints. Considered collectively then, the above studies indicate that the cells founding and constituting the initial interzone at the very onset of joint formation have a chondrogenic history and express chondrogenic markers, giving support to the notion that they largely derive from local de-differentiated chondrocytes.

As the cell lineage tracing-tracking studies above indicate, interzone cells and their progeny give rise to diverse joint tissues over time, including inner capsule, meniscus, intra-joint ligaments, and articular cartilage (Koyama et al., 2008; Rountree et al., 2004). However, it had remained unclear whether the cells are multipotent and produce different tissues within the developing joint or whether they are pre-specified into subgroups by local morphogenetic cues and endogenous determination mechanisms to produce different tissues at appropriate locations and time. Progress and insights into this complex but fundamental question have been reported recently. In a study from our group (Decker et al., 2017), we carried out additional analyses of joint progenitors with our new BAC-based *Gdf5-CreERT²* transgenic mice mated with ROSA reporter mice, aiming to trace and track the cells starting at different stages of joint development. Pregnant *Gdf5-CreER;R26-*zsGreen** mice received a single injection of tamoxifen at E13.5, E15.5, or E17.5, and the resulting *Gdf5-CreER+* cells and possible progenies were monitored in their pups over postnatal time. Focusing on digit joints that are structurally simpler, we found that cells labeled at E13.5 were mostly present in the developing capsule and synovial lining by postnatal day 0 (P0) and those patterns did not change much by P28 or thereafter. Cells labeled at E15.5 or E17.5 was present in both articular layers and capsule by P0, and these patterns also did not change significantly with postnatal age. In addition, the overall number of *Gdf5-CreER+* cells increased over time but not in a major way, indicating limited proliferative activity. To verify the latter and potentially important observation, we carried out experiments with (unbiased) *ROSA-CreER* mice mated with *R26-Confetti* reporter mice in which green (GFP), yellow (YFP), red (RFP), or cyan (CFP) fluorescent protein can be alternatively activated upon *Cre* action in different cells (Snippert et al., 2010). *ROSA-CreER/Confetti* mice were injected with tamoxifen once at E13.5, P0, P7, or P14, and we estimated proliferation by quantifying cells expressing the same reporter (representing daughter cells) over increasing postnatal time. For example, analysis at 2 months showed that *ROSA-CreER+* cells initially labeled at E13.5 produced the largest progeny, with cell clusters containing as many as 18 daughter cells. Cell cluster size declined markedly thereafter, and averaged about 3–4 cells after P0 induction and only about 1–2 cells with increasing age. Taken together, the studies above

indicate that *Gdf5*⁺ joint progenitors become quickly determined and committed within the developing joint, do not migrate much from their initial birth location, produce relatively small progenies, and give rise to distinct joint tissues over time.

Data and conclusions agreed well with a recent and very important study by Zelzer and coworkers (Shwartz, Viukov, Krief, & Zelzer, 2016). The authors created knock-in *Gdf5*-*CreER*^{T2} mice and crossed them with reporter mice to further analyze joint cell progenitor origin and fate. Reporter induction by a single tamoxifen injection at E10.5 failed to elicit *Gdf5*⁺ (*tdTomato*-positive) cells over the entire initial interzone by E11.5; only most epiphyseal cells were labeled in knee, elbow, and carpalphalangeal joints by E18.5. When tamoxifen was given three consecutive times at E11.5, E13.5, and E15.5, *Gdf5*⁺ cells were present in most joint tissues by E18.5. Comparison of reporter expression after a single tamoxifen injection versus real-time endogenous *Gdf5* expression pointed to a highly dynamic behavior of joint-forming cells. Groups of cells turned *Gdf5* expression off while maintaining *tdTomato* expression and other groups turned it on while all contributing to formation of different joint tissues. Cell proliferation measured by BrdU incorporation indicated that the mitotic activity of *Gdf5*⁺ cells decreased significantly between E13.5 and E18.5. The data led the authors to conclude that while *Gdf5*⁺ cells constituting the initial interzone are direct descendants of de-differentiated *Sox9*⁺ chondrocytes, additional *Sox9*⁺ progenitors are recruited from the joint's immediate surroundings, and generate additional *Gdf5*⁺ cohorts contributing to distinct tissue joint development. The data fit well with observations in previous studies suggesting that local progenitors immediately surrounding the incipient joint site are recruited into the *Gdf5*⁺ lineage over time and take part in joint formation (Hyde et al., 2008; Koyama et al., 2007; Niedermaier et al., 2005). This mechanism may contribute not only to specification of given tissues within the developing joint but may also sustain the considerable growth and lateral expansion of the developing joints to accommodate and cover the fast expanding epiphyses of long bones during late prenatal as well as postnatal time.

We mentioned above that developing chick embryo limbs display thick interzones with a clear triple-layer tissue organization, while the interzone in mammalian embryonic limbs is rather thin, but the possible implications of this structural difference have remained unclear. Fig. 2 shows the histology and collagen gene expression patterns in chick and mouse embryo knees at day 17 of embryogenesis. Clearly, the proximal epiphysis in chick embryo tibia (Fig. 2A and B) displays a developing articular cartilage expressing *collagen II* (Fig. 2D, ac) covered by a thick fibrous layer strongly expressing *collagen I* and facing the emerging joint cavity (Fig. 2C, fl), a dual tissue structure that persists through adulthood in this species (Pacifci, 1995). In comparison, mouse embryo tibia (Fig. 2E and F) contains similar incipient *collagen II*-expressing articular chondrocytes (Fig. 2H, ac) that exhibit some residual *collagen I* expression (Fig. 2G, ac). The latter is rapidly lost after birth, and the maturing articular cartilage expresses *collagen II* only already by P7 (Fig. 2I–L). Possibly then, the initial differences in organization, cellularity, and structure of interzones in chick and mouse may have developmental consequences, with the conspicuous interzone in chick leading to formation of a thick fibrous layer and thick cartilage and with a subtler mammalian interzone leading to absence of fibrous layer and thinner cartilage. We include

data below suggesting that differences in interzone characteristics and cell fates may have evolutionary significance as well.

4. Articular cartilage postnatal growth and morphogenesis

The key function of articular cartilage is to provide resilience during cycles of compression and relaxation during daily activities and to contribute to friction-less joint motion via elaboration of anti-adhesive and lubricant macromolecules (Hunziker et al., 2007). These properties are directly assignable to the unique composition and structure of the tissue. In limb joints such as the knee in adult mammals including humans, the tissue displays a characteristic zonal organization that includes: (i) a thin superficial zone abutting the synovial space that is composed of flat, elongated, and tightly-bound cells oriented along the major direction of movement, contains scant, and isotropic matrix, and contributes to joint lubrication by producing molecules such as lubricin (Prg4); (ii) a thick intermediate/deep zone containing large round chondrocytes in a columnar organization perpendicular to the surface and surrounded by abundant and anisotropic matrix in which collagen II fibrils and aggrecan-hyaluronan complexes provide tensile strength and elasticity, respectively; and (iii) a mineralized zone below the tidemark with very large chondrocytes that is bound to subchondral bone (Hunziker et al., 2007). These structural and organizational features allow articular cartilage to exert its biomechanical function and to endure and maintain tissue homeostasis through life. Unfortunately, structural derangements of articular cartilage due to acute injury or chronic conditions can lead to irreversible changes and joint pathologies including OA (Mow, Ratcliffe, & Poole, 1992). There are also pediatric joint pathologies such as osteochondritis dissecans in which large segments of articular cartilage become detached from the subchondral bone, causing pain, and joint damage and often requiring surgery (Shea, Jacobs, Carey, Anderson, & Oxford, 2013). As indicated above, current remedies for both acute and chronic joint maladies are unable to restore native structure and function (Johnstone et al., 2013; Makris et al., 2015). A key problem in this field is that we know relatively little about how articular cartilage acquires its functional zonal organization by adult age. In addition, it is not understood how articular cartilage remains phenotypically stable and persists through life under normal circumstances, while the remaining and preponderant cartilaginous skeleton is transient and is all replaced by endochondral bone by end of puberty with closure of the growth plates.

As exemplified by mouse studies, incipient articular cartilage in late embryogenesis and neonatal stages is rather thin and composed of small, randomly distributed, and variously shaped chondrocytes and scant isotropic extracellular matrix (see Fig. 2F) (Li et al., 2016; Rhee et al., 2005). The tissue undergoes remarkable growth in thickness over the first 2–3 weeks of age. While small flat cells continue to populate its incipient surface zone, the bulk of underlying chondrocytes grow in average size and become progressively separated by ever increasing cartilage matrix. By 6–8 weeks of age, the tissue had acquired its characteristic and fully functional mature multi-zonal organization with a clear superficial zone, an intermediate/deep zone with chondrocyte columns and abundant anisotropic matrix, and a mineralized zone at the bottom (Decker et al., 2017). Similar postnatal maturation and structuring of articular cartilage characterize other and larger mammalian species (Hunziker

et al., 2007). Thus, how does articular cartilage evolve from a thin and matrix-poor tissue at neonatal stages to a highly structured, thick, and zonal tissue in adults?

One explanation was originally suggested by the identification of cells with a distinct character in postnatal articular cartilage (Dowthwaite et al., 2004). The authors isolated cells from the superficial, intermediate, and deep zones from juvenile bovine articular cartilage by sequential enzymatic treatment and then characterized the cells by *in vitro* and *in vivo* assays. Superficial zone cells displayed high affinity binding to substrate-bound fibronectin and effective colony unit-forming ability *in vitro*, and expressed the progenitor cell marker gene *Notch1* *in vitro* and *in vivo*. The cells displayed developmental plasticity in that they engrafted into, and became part of, several tissues once transplanted into chick embryos, including bone, tendon, and perimysium. In comparison, cells in the intermediate and deep zones exhibited the expected chondrocytic phenotypes. These and other data led the authors to propose that cells in the superficial zone have a progenitor/stem character. Over postnatal time, the cells would be responsible for articular cartilage growth and establishment of its zonal structure by a mechanism of appositional growth. The cells would proliferate and produce vertical columns of overlapping daughter chondrocytes spanning the entire tissue thickness from superficial to deep zone, a notion proposed also in previous studies (Hayes, MacPherson, Morrison, Dowthwaite, & Archer, 2001). Cells with a progenitor/stem character expressing markers such as CD90 and STRO-1 have been identified in human articular cartilage as well (Williams et al., 2010).

Appositional growth has also been invoked in a recent cell lineage tracing study to similarly account for postnatal articular cartilage growth and acquisition of its zonal organization in adults (Kozhemyakina et al., 2015). The study was based on previous data showing that *Prg4* is expressed in developing limb joints by late mouse embryogenesis (Rhee et al., 2005). The authors created knock-in *Prg4-CreER^{T2}* mice (*Prg4* heterozygous null) and mated them with *LacZ* reporter mice. After a single tamoxifen injection of pregnant mice at E17.5 of gestation, reporter-positive *Prg4+* cells were found to be located exclusively in a single cell layer at the very surface of incipient tibial articular tissue in P0 pups. With increasing postnatal time, the *Prg4+* cells became more numerous and were then found to be present in vertical chondrocyte columns spanning the whole tissue thickness in adult mice. When mice were injected with tamoxifen at postnatal times, the reporter-positive chondrocyte columns appeared to be partial and did not span the whole tissue thickness in adults. BrdU incorporation was used to estimate mitotic activity, and the data indicated that over 70% of superficial cells were proliferative even in 1-month-old mice. These and other data led to the conclusion that the *Prg4+* cells located at the articular surface of neonatal tibia serve as stem cells and elicit postnatal growth of articular cartilage by proliferation and apposition, generating columns of daughter cells spanning the entire thickness of articular cartilage in adults. Data and conclusions were further examined in a follow-up study by Chagin and coworkers (Li et al., 2016). Using the same knock-in *Prg4-CreER^{T2}* mice but mated to *Rosa-Confetti* reporter mice, the authors found that *Prg4+* cells present at the tibia articular tissue surface expressed stem cell traits and renewed themselves or generated chondrocytes by symmetric and asymmetric cell divisions at neonatal stages. With increasing postnatal time, the progeny of those cells formed daughter cell clusters and could be found within the growing and thickening articular cartilage. This was accompanied by increases in average

cell volume, but at variance with the previous study (Kozhemyakina et al., 2015), no columns of daughter cells (expressing the same reporter) were found spanning the entire articular cartilage in adults. The authors concluded that articular superficial cells are self-renewing progenitors able not only to maintain themselves but also to produce progenies reconstituting articular cartilage over postnatal life.

Because of the broad relevance of this research area, we carried out analogous studies, but with the following considerations in mind. The exclusive presence of *Prg4*⁺ cells in a single and most superficial articular cell layer observed at P0 (Kozhemyakina et al., 2015; Li et al., 2016) does not actually match endogenous *Prg4* that is expressed throughout the 6–8 layers of incipient articular cartilage at that stage (Koyama et al., 2008; Rhee et al., 2005), raising some concern about the reliability of knock-in heterozygous null *Prg4-CreER*^{T2} mice. Appositional growth of postnatal articular cartilage would require high rates of cell proliferation, but proliferation in neonatal and juvenile cartilage is relatively modest (Decker et al., 2017; Li et al., 2016). It would also require considerable matrix turnover when in fact the articular matrix—and collagen in particular—is quite stable (Eyre, 2002; Poole et al., 2001). To carry out follow-up studies, we made use of our new BAC-based *Prg4-CreER*^{T2} transgenic mice (in which the endogenous *Prg4* alleles are intact) mated with single and *Rosa-Confetti* reporter mice. We found that when *Prg4-CreER;R26-tdTomato* mice were injected with tamoxifen once at E17.5, reporter-positive cells were present throughout the 6–8 cell layers in tibia articular cartilage in P0 pups, matching endogenous *Prg4* expression at that stage. Identical patterns were observed in *Prg4-CreER;R26-Confetti* mice injected at E17.5 and harvested at P0, though recombination efficiency and reporter expression were overall lower as expected (Snippert et al., 2010). *Prg4-CreER;R26-Confetti* mice injected at E17.5 were then harvested at successive postnatal time points up to 2 months of age. With increasing time, the *Prg4*⁺ cells produced small and locally-restricted groups of daughter cells (same reporter color) averaging 3–6 cells/cluster, suggesting that their mitotic activity was low as was their mobility. There was evidence of horizontal and vertical cluster expansion over time, but single color cells or clusters did not span the full thickness of adult articular cartilage nor did they form columns of same-color daughter cells. Indeed, stereological imaging and 3D reconstruction throughout articular cartilage in late juvenile and adult mice indicated that articular chondrocytes were not perfectly aligned and did not fully overlap each other, thus forming stacks rather than columns. The data were confirmed and verified using unbiased *ROSA-CreER;Confetti* mice injected once at E17.5 and *Gdf5Cre;R26-Confetti* mice, both harvested at successive time points as above.

Additional stereological and reconstruction analyses showed that the average cell volume of articular chondrocytes increased markedly over postnatal time and did so in a zone-specific manner. Computation of data actually indicated that such increases were a major driver of overall articular cartilage thickening and growth over postnatal life. Interestingly, this mechanism is reminiscent of chondrocyte hypertrophy in the growth plate that is by far the major driver of skeletal elongation and growth while cell proliferation plays a minor role (Breur, VanEnkevort, Farnum, & Wilsman, 1991). Importantly also, the maximum volume displayed by deep zone chondrocytes did not exceed about 60% of that of growth plate hypertrophic chondrocytes, suggesting that chondrocytes can set and maintain distinct volumes in different settings and that articular chondrocytes normally avoid full

hypertrophy. Second harmonic generation with two-photon microscopy indicated that the collagen fibrils in the matrix were isotropic and scattered at neonatal stages and became anisotropic and aligned along the chondrocyte stacks in adult articular cartilage (Decker et al., 2017). These and other data led us to conclude that rather than by apposition, articular cartilage in large joints such as the knee grows and thickens mainly by formation of non-daughter cell stacks and increases in average cell volume, with important contribution by matrix deposition and accumulation but modest contribution by proliferation. Because the chondrocyte stacks are made of non-daughter cells, it is possible that they are produced by a process of realignment and reorientation of neighboring cells and clusters, possibly aided by mechanisms such as convergent extension (Tada & Heisenberg, 2012). Our data and conclusions differ in some respects from those reported in the studies above (Kozhemyakina et al., 2015; Li et al., 2016), and further studies are thus needed to sort out and explain these differences and move ahead in this research field. It will also be important to determine whether articular cartilage grows and matures by similar mechanisms in different joints in the limbs and other skeletal sites.

5. Evolutionary considerations

Joints have undergone significant and remarkable changes through the evolutionary transition of vertebrates from a cartilaginous to a bony skeleton, and also from aquatic to terrestrial habitats. Analyses of such evolutionary changes offer unique insights into mechanisms of joint determination, diversification, and organization as a function of changes in environments, body locomotion and physical demands. The evolution of joints in the appendicular skeleton followed the transition from water to land to air over hundreds of millions of years as it adapted to the unique and ever more sophisticated capabilities in higher species culminating in primates and humans. Others have analyzed and previously reviewed important themes regarding the conserved signaling pathways involved in joint development and the transcriptional control of joint diversification among vertebrate and non-vertebrate species (Salva & Merrill, 2017; Shubin, Tabin, & Carroll, 1997). Here we highlight salient aspects of joint organization, structure, architecture, and function in the appendicular skeleton during the progression from fins to limbs as vertebrates adjusted to terrestrial life and how these evolutionary considerations provide insight into the developmental mechanisms of synovial joints.

Fishes are among the oldest documented vertebrates. These animals have stood the test of time and have adapted to drastic climate and environmental changes over hundreds of millions of years. The colonization of marine habitats by vertebrate life began around 450 million years ago (MYA) and dominated the following Devonian era, commonly referred to as the “era of the fishes,” lasting from about 415 to 360 MYA (Becker, Gradstein, & Hammer, 2012). Cartilaginous fishes were the first to evolve from primitive vertebrate animals that lacked appendages. The skeleton of those fishes was not ossified, contained mineralized cartilaginous structures that were flexible and therefore lacked the necessity for cavitated joints. Sharks are the living descendants of these primitive ancestors and first evolved around 425 MYA (Becker et al., 2012). Bony fishes first evolved around 420 MYA with ray-finned fish first followed by lobe-finned fish that evolved around 415 MYA (Fig. 3) (Becker et al., 2012; Botella, Blom, Dorka, Ahlberg, & Janvier, 2007; Clack, 2013). As their

names suggest, these two classes of fishes differed in the structure of their fins. Ray-finned fish exhibited fins that extended from the body wall as a series of spines and were connected together by thin tissue to form the overall fin (Fig. 3). Living examples of these fish include commonly hunted species such as tuna and salmon, and also lab-maintained species such as spotted gar and zebrafish. By contrast, lobe-finned fish displayed much thicker, fleshy fins attached to the body wall by a single skeletal element (evolved to the humerus in higher species) that articulated with several smaller, and more distal, bones. The connection of the fin to the trunk by a single bone in lobe-finned fish is a key trait that has been maintained through evolution of lobe-finned fishes and is a defining feature of all tetrapods including humans (Clack, 2013; Romer, 1958; Shubin, Daeschler, & Coates, 2004; William & Raven, 1941). Over additional time, the articulated bony elements of the lobe fins transitioned to the stereotypic limb pattern maintained by all amphibians, birds, and mammals: the proximal stylopod (humerus/femur), the medial zeugopod (radius/ulna and tibia/fibula), and the distal autopod (with several bones composing the wrist/ankle and digits) (Fig. 3) (Hinchliffe, 1994; Hinchliffe & Johnson, 1980).

The transition of lobe-finned fish from water to land represents one of the critical evolutionary advances in vertebrate history that required not only major changes in the skeleton and joints but also adaptation and diversification of body functions including hearing and breathing. The now extinct genus of lobe-finned fish *Panderichthys* ultimately gave rise to terrestrial tetrapods that colonized land in the post-Devonian era (Boisvert, Mark-Kurik, & Ahlberg, 2008; Vorobyeva & Schultze, 1991); however, a clear evolutionary path from fin-to-limb remained elusive until the landmark discovery of a novel fossilized animal that was first reported in 2006. Two studies published that year described *Tiktaalik roseae*, a fish that exhibited unequivocal transitional features of both fish and terrestrial tetrapods (Daeschler, Shubin, & Jenkins Jr., 2006; Shubin, Daeschler, & Jenkins Jr., 2006). A series of fossils were uncovered in the Devonian sediment of Arctic Canada on Ellesmere Island placing the evolution of this animal in the correct timeline during aquatic to terrestrial transition. Based on the fossilized structures, it is hypothesized that *Tiktaalik* displayed gills, lungs, and a neck that swiveled, and its pectoral fins revealed a clear progression toward amphibian limbs: a single proximal bone attached to the trunk (ancient stylopod), two smaller and more distal bones (ancient zeugopod), and several even smaller bones that shaped the remaining fin/limb-like elements (Fig. 3). It was dubbed “the fish that crawled out of water.”

The overall geometry and spatial orientation of the joint, curvatures of the opposing skeletal surfaces, and other anatomical features make it possible to generate hypotheses concerning the mobility and function of the skeletal elements in fossilized animals. The glenohumeral joint of *Tiktaalik* likely permitted a significant degree of motion freedom given the deep concave-convex contours of the opposing articulating surfaces. Such architecture suggests that this joint could have been fully cavitated and, perhaps, synovial in nature. The distal epiphysis of the humerus was part of an elbow-like joint that articulated with—and provided independent mobility to—two medial short bones (representing primitive radius and ulna). The two articulating surfaces on the humerus facing the medial bones were well separated from each other, a feature not seen in more primitive fishes (Fig. 3) (Andrews & Westoll, 1968; Shubin et al., 2004). However, they were not fully oriented along the longitudinal axis

suggesting that those medial elements were oriented laterally and experienced some motion limitation. There were several small bones distal to the primitive ulna and radius—including ulnare and radials—that displayed intervening joints with rather shallow concavities and convexities in between, suggesting some but probably limited mobility and flexibility (Shubin et al., 2006). Subsequent analyses of the pelvic girdle in *Tiktaalik* by the same groups further bridged the gap between fish and terrestrial tetrapods (Shubin, Daeschler, & Jenkins Jr., 2014). In addition, multiple discoveries of tetrapod trackways embedded in sediment from the Devonian era scattered throughout Europe, China, and Australia corroborate evidence of the fin-to-limb transition of the *Tiktaalik* fossils. Specifically, fossilized trackways in Poland allowed Paleontologists to predict that ancient tetrapods existing in that region likely ambulated with amphibian-like movements consistent with predicted mobility of *Tiktaalik* appendicular joints (Niedzwiedzki, Szrek, Narkiewicz, Narkiewicz, & Ahlberg, 2010). Discrepancies in the evolutionary timeline of the fin-to-limb transition do exist: *Tiktaalik* dating to the late-Devonian era, the Polish trackways to the mid-Devonian era, and other trackways ranging from early- to late-Devonian (Clack, 1997; Gouramanis, Webb, & Warren, 2003; Lu et al., 2012; Stossel, 1995; Warren, Jupp, & Bolton, 1985; Warren & Wakefield, 1972; Williams, Sergeev, Stossel, & Ford, 1997). These require further analysis, however, the combined studies provide consistent evidence that requirements for ambulation on land necessitated the evolution of joints to provide novel functions that are not seen in entirely aquatic fishes.

The above discoveries and insights are indeed striking, but the lack of preservation of soft tissues in fossils make it quite difficult to evaluate more specific joint features, including cavitation, mobility range, lubrication, and biomechanical tissue traits. One way to address these limitations has long been the analyses of anatomical features in extant animals carrying ancestral characteristics. The axolotl salamander (*Ambystoma mexicanum*) is one such species and remains a popular model of skeletal development, growth, and regeneration, and has been recognized more recently as a model for joint development/evolution and repair (Cosden et al., 2011; Cosden-Decker et al., 2012; Thampi, Liu, Zeng, & MacLeod, 2018). Axolotls display neoteny (Shaffer & Voss, 1996) and retain larval salamander characteristics (i.e., maintenance of gills) and an aquatic habitat. In rare cases axolotls undergo metamorphosis to terrestrial life in response to environmental stress (Thampi et al., 2018) or by administration of thyroxine hormone (Page, Monaghan, Walker, & Voss, 2009). This real-time transition offers insight on the biologic changes that occur in the transition from an aquatic to a terrestrial environment. Previously, one of us and colleagues carried out anatomical and histological analyses of the limb joints in aquatic axolotls of different ages. In 1-year-old skeletally mature axolotls, the hip, shoulder, and elbow joints were found to be cavitated, thus resembling full-fledged synovial joints. In sharp contrast, the knee, carpal/tarsal, and interphalangeal joints were not cavitated and were filled with a dense fibrous and highly cellular tissue interconnecting and bridging the epiphyseal cartilaginous ends of the opposing skeletal elements, thus resembling amphiarthroses. The fibrous tissue was still present at 2 years of age (the last time point examined) and as revealed by immunohistochemistry, was rich in collagen I and GDF5, but poor in aggrecan (Cosden-Decker et al., 2012). Fig. 4A and B shows additional histological images of knees from 2- to 10-year-old aquatic axolotls. At 2 years of age, the epiphyseal

and metaphyseal regions of femur, tibia, and fibula were cartilaginous and displayed a continuous uninterrupted histological organization, without an obvious distinction or boundary between articular cartilage area and underlying growth plate (Fig. 4A). By 10 years, the metaphysis was ossified, but the epiphyseal cartilage still displayed a seamless organization along with the remaining and shallower growth plate (Fig. 4B and C).

Major joint differences were observed in axolotls that had undergone spontaneous metamorphosis and adjustment to a terrestrial habitat. At 2 years of age, the articular cartilage area in their knees was compacted, narrower, and had slightly lower chondrocyte density, thus becoming more distinguishable from the underlying growth plate (Fig. 4D, yellow brackets). This trend had advanced and became quite obvious in 10-year-old animals. The articular cartilage area was not only further compacted and structured but also now separated by a clear tidemark from the underlying residual growth plate cartilage (Fig. 4E and F, arrowheads). Additionally, the intra-knee fibrous tissue exhibited reduced cellularity and histological density at 2 years in terrestrial axolotls (Fig. 5C) and displayed a significantly expanded overall thickness at 10 years (Fig. 5D, green bracket) compared to the compacted, crowded, and highly structured fibrous tissue in aquatic counterparts (Fig. 5A and B, green bracket). These data correlate quite well with a recent morphometric and gene expression study of 1-year-old aquatic and terrestrial axolotls where authors report reduced cell density in the articular cartilage and in the fibrous intra-joint tissue in the metamorphosed animals (Thampi et al., 2018). Interestingly, the authors also reported that fibrous tissue retained appreciable expression of *Gdf5* in these adult animals, expression that is rapidly lost by early postnatal stages in the limb joints of mammals (Koyama et al., 2008). Taken together, the combined data provide suggestive and intriguing evidence that as axolotls transition to the new physical and locomotive demands of life on land, knee joint tissue organization, and probably biomechanical function, is altered accordingly. Most impressive is the response of epiphyseal end cartilaginous tissue that is rather generic and undefined in aquatic animals, but becomes distinct and separated by a tidemark in terrestrial animals. The accompanying reduction in cell number and histological density in intra-joint fibrous tissue in the latter animals point to higher elasticity, joint flexibility, and range of motion and may even depict morphogenetic steps in the direction of cavitation. The maintenance of strong *Gdf5* expression in both aquatic and terrestrial axolotls highlights the likelihood that these cells are descendants of embryonic interzone cells, as they are in mammals. However, these axolotl joint cells may persist and multiply throughout postnatal life underscoring their distinct capacity for tissue regeneration (Cosden et al., 2011).

As we have highlighted above, it has long been held that full-fledged synovial joints first emerged in the appendicular skeleton of lobe-finned fish and were eventually passed on to amphibians and terrestrial tetrapods (Bemis, 1986). However, due to their superiority in providing mobility and possible evolutionary advantages, they could have evolved earlier. Indeed, synovial joint-like structures were proposed to occur in the jaw (temporomandibular joint) of ray-finned fishes such as longnose gar and sturgeon (Haines, 1942). While this early study fell short of describing the presence of defining synovial features including capsule, articular cartilage, and lubrication, a very recent study has provided strong evidence that the jaw and pectoral fin joints in ray-finned zebrafish, stickleback, and gar are synovial in nature and express *Prx4/lubricin* homologs (Askary et al., 2016). Genetic deletion of

zebrafish *Prg4b* gene resulted in joint degeneration similar to that seen in *Prg4*-deficient mice and humans (Koyama et al., 2014; Marcelino et al., 1999; Rhee et al., 2005). Evolution of synovial joints thus appears to have preceded the ray-finned to lobe-finned fish divergence. In this context, it is relevant and thought-provoking to consider the fin joints in dolphins and whales, mammals that evolved from a terrestrial tetrapod around 40 MYA. While their scapular-humeral joints are synovial and freely movable (Klima, Oelschlager, & Wunsch, 1980; Rommel, 1990), the more distal joints are not and contain fibrous disks and resemble amphiarthroses (Cozzi, Huggenberger, & Oelschlager, 2016). In aquatic mammals then, distal limb joints appear to have reverted to more ancestral and less mobile forms, possibly caused by reduced weight bearing and range of motion requirements in the aquatic environment. On the other hand, when extant aquatic axolotls undergo metamorphosis into terrestrial animals, their joints quickly adapt and acquire characteristics of tetrapod joints, including a well-defined articular cartilage. In sum, the genetic instructions to create a synovial joint first evolved in early fishes and have been passed along through evolution while endowed with considerable adaptability to divergent physical and environmental demands.

6. Conclusions and implications

The above synopsis of past and most recent literature on key aspects of limb joint determination, growth, and morphogenesis shows that much has been learned in these important areas of research. It is clear now that mesenchymal interzone cells are endowed with the ability to generate joint tissues, and their microsurgical removal prevents joint formation and can alter the developmental behavior and fate of chondrocytes in adjacent long bone shafts. Genetic cell lineage tracing studies have provided evidence that interzone cells are heterogeneous in origin, with the centrally located ones derived directly from chondrocytes and the more lateral ones recruited from joint site-flanking mesenchyme. The data raise the tantalizing possibility that this diversity of origin reflects diversity of fate and function, with the laterally recruited cells largely engaged in formation of capsule and synovial lining and expansion of the joint, and with the centrally located cells mainly engaged in intra-joint ligament and articular cartilage formation. More work will of course be needed to verify and extend these predictions. If correct however, they would signify that interzone cells are not equivalent and quickly become committed to generating distinct local tissues, despite homogenous expression of genes including *Gdf5* and *Wnt9a* (Koyama et al., 2008). In this regard, it will be crucial to clarify how interzone cells in different joints are able to produce and/or elicit formation of joint-specific structures, such as the meniscus only in the knee. These studies could also contribute to understanding the developmental basis and mechanisms of joint diversity along the limbs, possibly clarifying whether the cells also influence overall joint morphogenesis, shape, orientation, and configuration.

Among the various joint tissues, articular cartilage has attracted the most attention over the years, a reflection of its indispensable biomechanical function and unfortunate susceptibility to malfunction with aging and poor repair capacity after injury (Trippel, Ghivizzani, & Nixon, 2004). Major efforts continue in order to address this medical need and to find biological and bioengineered tools to solve it. As addressed above, the current limited success in that field likely reflects our current poor understanding of basic developmental

aspects of articular cartilage and most importantly, how articular chondrocytes become permanent and avoid the transient fate of chondrocytes that form the bulk of the growing skeleton. In addition, we lack a mechanistic understanding of how articular cartilage acquires its intricate and functional stratified architecture by maturity. Studies we describe above have provided a measure of progress regarding the basis of articular cartilage zonal structure, but we remain utterly ignorant on how articular chondrocytes acquire a permanent state. The latter is a major issue since hypertrophic degeneration of articular cartilage and its switch to a transient fate are often observed during osteoarthritis (OA), leading to the demise of tissue and cells (Buckwalter & Mankin, 1998). Indeed, unwanted chondrocyte hypertrophy often undermines bioengineered attempts to repair articular cartilage with mesenchymal stem cells isolated from marrow, fat, or other tissues (Benz, Breit, Lukoschek, Mau, & Richter, 2002; Schnabel et al., 2002).

It is important to cite two recent studies contributing important new information on basic aspects of articular cartilage developmental biology. In the first study (Gamer et al., 2018), the authors examined the roles of BMP2 in postnatal maturation and maintenance of murine knee joints and found that *Bmp2* is expressed in both embryonic and adult articular cartilage, unlike *Gdf5* expression that predominates only during embryonic stages. Joint-specific ablation of *Bmp2* caused derangement of articular cartilage structure in adults and progression to early onset OA, in addition to disrupting meniscus organization and long-term functioning. A second study focused on the important question of how articular cartilage regulates its overall thickness (Kobayashi & Kozlova, 2018), an intriguing issue given that cartilage thickness is joint specific and can even vary significantly on the proximal and distal sides of a given joint (Koyama et al., 2008). Based on their previous studies (Papaioannou, Inloes, Nakamura, Paltrinieri, & Kobayashi, 2013), the authors further explored the roles of Lin28a, an RNA-binding protein regulating growth and metabolism, and found that *Lin28a* over-expression, starting in mouse embryos, up-regulated Erk kinase signaling and caused an increase in knee articular cartilage thickness in adults. The same was seen after over-expression of constitutive active *Kras* that also up-regulated Erk signaling. These studies represent important progress toward a much needed in depth understanding of how articular cartilage acquires its distinct multi-zone organization and how articular chondrocytes are endowed with a permanent phenotype. Progress in these areas will not only be of fundamental basic value but is likely to also have major repercussions in joint translational medicine.

The study of joint evolution is instructive in several respects. As delineated above, it appears clear that the first joint to cavitate and acquire a synovial character was the temporomandibular joint (Askary et al., 2016), likely providing a major asset for species survival, nutrition, and propagation. The emergence of similar joints at most proximal sites in the fins is likely to have derived from similar evolutionary demands and advantages, and it is truly remarkable that extant axolotls still display synovial joints in shoulder, hip, and elbow only, but not in the knee. We can surmise that the limited movement and range of motion permitted by the thick fibrous tissue in their knee are fully compatible with survival and thriving in water, but the sudden changes we describe here following metamorphosis to land speak volumes about joint adaptability and responsiveness to new environments and physical forces. In this context, axolotls could represent an ideal model system to decipher

the mechanisms by which the interzone cells in shoulder, hip, and elbow sustain and permit cavitation while those in the knee and more distal joints do not. Cavitation requires production of joint lubricants and the insulation of the synovial fluid from the body environment by a capsule. Thus, those comparative analyses could shed light on mechanisms regulating the genesis of these traits as well, providing once again important basic information but also key clues on how to rebuild faulty joint tissues in disease.

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Fig. 1.

The interzone is essential for limb joint formation. Stage 25–26 chick embryos *in ovo* were subjected to microsurgical intervention to alter or remove the elbow joint interzone on the right side. The left elbow was left untouched and served as internal control. Embryos were re-incubated until day 10 (E10) at which point the elbows were dissected out and processed for histological analysis and Safranin O-fast green staining. (A) Images of E10 control elbow showing the prominent Safranin O-positive humerus (*h*), radius (*r*), and ulna (*u*) epiphyses flanking the developing synovial tissues and cavity. Note also the well-developed capsule (arrowhead). (B) High magnification image of boxed area in (A) showing the compact fibrous tissue (yellow brackets) covering the cartilaginous ends of the skeletal

elements and an intervening and slightly less dense tissue (pink bracket). (C and D) Images of E10 elbow in which the interzone had been mechanically damaged but not removed. Area boxed in (C) is shown at higher magnification in (D). Note the considerable delay in joint development depicted by substandard separation of the opposing elements and by defective definition of the epiphyses compared to controls. Capsule appears to be unaffected (arrowhead). (E and F) Images showing absence of elbow joint and fusion of the opposing cartilaginous epiphyses after resection of the interzone. Area boxed in (E) is shown at higher magnification in (F). Note the small and relatively uniform size of chondrocytes in both intervening tissue and flanking epiphyses. (G and H) Images showing that removal of interzone and neighboring tissue led to absence of a joint and physical separation of neighboring skeletal elements. Note also the hypertrophic phenotype of chondrocytes in the opposing and truncated elements suggesting that the epiphyses were undergoing ectopic endochondral ossification typical of the diaphysis at this stage. Scale bar in (A) for C, E, and G, 250 μm ; scale bar in (B) for D, F, and H, 100 μm .

Fig. 2.

Tissue structure and gene expression in developing tibia articular cartilage in chick and mouse. (A and B) Images show Safranin O/fast green-stained proximal tibia epiphysis in E17 chick embryo. Area boxed in (A) is shown at higher magnification in (B). Note the presence of a prominent, thick, and fast green-positive fibrous layer (*fl*) facing the synovial cavity and overlaying the Safranin O-positive articular cartilage (*ac*). (C and D) *In situ* hybridization images of serial sections showing strong expression of *collagen I* (C, *Col I*) and *collagen II* (D, *Col II*) in fibrous layer and cartilage, respectively. (E and H) Images show Safranin O/fast green-stained proximal tibia epiphysis in E17.5 mouse embryo. Area boxed in (E) is shown at higher magnification in (F). Note that incipient articular cartilage (*ac*, yellow bracket in F) is rather narrow at this stage and is identified based on *Gdf5*⁺ cell lineage tracing (Decker et al., 2017). Its cells strongly express *collagen II* (H, *Col II*) and exhibit some residual *collagen I* expression (G, *Col I*), possibly stemming from their former mesenchymal character. (I–L) Images show Safranin O/fast green-stained proximal tibia epiphysis in neonatal P7 mouse. Area boxed in (I) is shown at higher magnification in (J).

Note that the developing articular cartilage has grown in thickness (*ac*, yellow bracket in J), still expresses *collagen II* strongly (L, *Col II*) but no longer expresses *collagen I* (K, *Col I*). Scale bar in (A) for E and I, 350 μm ; scale bar in (B) for all remaining panels, 50 μm .

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Fig. 3. Timeline of vertebrate evolution through the fin-to-limb transition. Solid and dashed lines represent evolutionary timelines and currently extant species. Pictured at the bottom are enlarged cartoons of the pectoral fins/limbs of the animals indicated in the timeline and depicting the evolutionary transition from ray fin to lobe fin to *Tiktaalik* fin to amphibian limb. Key aspects of skeletal transitions at each stage are highlighted within these cartoons.

Fig. 4.

Epiphyseal cartilage changes with adaptation to terrestrial life in spontaneously metamorphosed axolotl salamanders. Hematoxylin and eosin-stained sections of aquatic and terrestrial axolotl knee joints are depicted: femur (fe), tibia (t), and fibula (fi). (A) In 2-year-old aquatic animals, the presumptive epiphyseal cartilage (yellow bracket) is thick and continuous with the underlying growth plate (green bracket). (B and C) In 10-year-old aquatic animals, the epiphyseal cartilage is reduced in thickness (yellow brackets) but maintains morphological continuity with growth plate cartilage (green bracket). (D) In a 2-year-old metamorphosed terrestrial sibling, epiphyseal cartilage (green bracket) is much reduced in thickness compared to aquatic animal shown in (A). (E and F) By 10 years of age, a clear tidemark is visible and creates a clear histological separation from the underlying ossified growth plate (F, arrowheads). Scale bar for all panels, 100 μ m.

Fig. 5.

Intra-joint tissue modifications during aquatic to terrestrial transition in axolotl salamanders. Images from specimens shown in Fig. 4. (A and B) The intra-joint tissue in aquatic axolotls is densely packed with fibrous cells (A) and thickness is reduced with age (B, green bracket). (C and D) By contrast, the intra-joint tissue in terrestrial axolotls displays reduced cell density, increased matrix deposition (C), and maintains thickness with age (D, green bracket). All tissue sections are stained with hematoxylin and eosin. Scale bar for panels A and C, 50 μ m; scale bar for panels B and D, 150 μ m.