Joint Development

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Abstract

Synovial joints between different skeletal elements are essential for mobility. The joint is encased in a capsule, lined by a synovial membrane inside, and enforced by ligaments. Within the synovial joints, articular cartilage lining bone surfaces provide a smooth, wear-resistant structure that reduces friction and absorbs impact forces. Joint formation is complex with structures of different shapes and sizes that are fit for purpose. In adult life, these structures need to be maintained, as loss or damage to articular cartilage is the hallmark of arthritic diseases. The joint when damaged does not repair well and the reason is not clear. However, understanding the developmental process will provide critical insights into how early limb patterning is linked to later skeletal morphogenesis. This chapter focuses on our current understanding at the cellular and molecular levels, from creation to maturation of a synovial joint. Morphologically, we know there is the formation of interzone regions at the presumptive sites of the future joint. Molecularly, we have some insights into signals that direct the initiation and progression of interzone regions toward a joint. And through innovative technologies in mouse genetics and genomics, we are beginning to understand the developmental processes, with the identification of progenitor cell pools, and to trace the origin of cells and track the fate of descendent cells from initiation to formation of the complete joint. The information gained from development will enable potential therapeutic strategies, from activation of endogenous repair mechanisms to the use of appropriate progenitors for cell therapy.

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7.1 Skeletal Joints

A joint is where two or more bones meet, allowing motion of the skeletal elements. Diarthrosis (synovial) joints are highly mobile, amphiarthrosis (intervertebral disk) joints are less mobile, and synarthrosis (suture and gomphosis) joints are relatively immobile. Most common in the human body are synovial joints and their formation, maintenance, and degeneration are best studied (Li et al. 2013). This chapter will focus on synovial joints that are complex structures as a unit, consisted of articular cartilage at the surface of opposing bones, ligaments, synovium, and the joint capsule (Ward et al. 1999; Archer et al. 1999). The synovial fluid provides lubrication for the joint during movement, whereas the synovial membrane, a saclike structure, encloses the joint cavity and synovial fluid. These joints differ in shape with distinct architecture for the required movements and loading, exemplified by the different joints of the limbs and digits.

Synovial joints when damaged through trauma do not repair well, and the reasons are not well understood. The repair processes that do occur make fibrous cartilages that cannot fulfill compressive and articulation functions. Repair of many tissues rely on reactivation of developmental cues and availability of progenitor cells, in which a damaged articular cartilage appears to be unable to activate and/or due to limited/absence of progenitor cells. With aging, as with other tissues, the articular cartilage will degenerate leading to conditions such as osteoarthritis (OA). While aging is a natural process, there are strong evidences for genetic contributions that could influence maintenance and repair potentials or abnormal developmental processes that lead to enhanced abnormal responses to daily wear and tear. Thus, a clear understanding of the developmental processes in joint formation would facilitate the identification of related processes or progenitor cells that need to be reactivated for better repair of articular cartilage and the associated joint tissues.

7.2 Structure of Synovial Joints

Synovial joints are composed of articular cartilage, synovial membrane, ligaments, and a fibrous capsule but diverse in shape, construction, and biomechanical function. For example, the shoulder and hip have universal "ball and socket" joints allowing multidirectional movements, the elbow has hinge joint for flexion and extension in one plane, while the knee has a modified hinge joint allowing flexion, extension, abduction, and adduction movements. Some joints have additional structures such as the meniscus and intra-joint ligaments in the knee, phalangeal joints have externally positioned collateral ligaments, and the hip displaced a centrally located ligamentum teres of the femur head. The anatomical functions of the different joints and structures are well understood; however, how these structures come about in development with the appropriate shape is not clear at all. Recent developmental studies in mice suggest that structures of joints are derived from a pool of cells with progenitor properties within the developing interzones, sites of the future joints.

The cellular and structural organization of the articular cartilage is similar between various types of joints with minor difference due to loading requirement.

Articular cartilage at the surface of joints has been extensively studied in knee joints because of pathological relationship with osteoarthritis. With articular cartilage of the knee as an example, the superficial zone contains elongated and flattened cells oriented parallel to the articular surface that produces lubricin and hyaluronic acid (HA) to lubricate joint movement (Jay et al. 2001). Chondrocytes in the middle zone are round in shape, usually organized in vertical rows of cells, and produce and maintain extracellular matrix components such as type II collagen and aggrecan for biomechanical function. Chondrocytes in the deep zone tend to be larger in size with hypertrophic appearance at the tidemark, the boundary articular cartilage, and the underlying subchondral bone (Broom and Poole 1982).

7.3 Developmental Processes of Joint Formation: An Overview

In the developing limb, skeletal elements are formed from a proximal to distal sequence, through temporally and spatially regulated processes that include mesenchymal condensation to give rod-shaped cartilage elements (anlagen), followed by elongation, branching, and/or segmentation (Sanz-Ezquerro and Tickle 2003a; Goldring 2012). For example, in the developing forelimb, the humerus element is formed first and then through segmentation and branching the radius and ulna, followed by the carpal and metacarpal elements, with the phalangeal elements forming last when a single skeletal condensation is segmented into 2 (thumb) or 3 (digits II to V) smaller segments through the formation of synovial joints (Hall and Miyake 2000). The general processes in joint development are depicted diagrammatically in Fig. 7.1a.

At the site of the future joint, the chondrogenic mesenchyme remained undifferentiated or undergoes a "dedifferentiation" process to form interzone regions, represented by a localized high-density region of cells. These cells appear flattened and begin to lose chondrogenic characteristics. With progression, the interzone further refines to a three-layered structure, with two outer layers of higher cell density flanking a central region of lower cell density where cells are thought to undergo apoptosis in some joints, forming a joint cavity. Cells within the high-density outer layers contribute to the formation of the future articular cartilage (Bland and Ashhurst 1996; Mitrovic 1978). The cartilage element proximal to the joint undergoes hypertrophy, initiating the process of endochondral ossification and the establishment of a growth plate at the epiphyseal regions. Importantly, growth plate chondrocytes appear not to be contributed by cells of the articular cartilage in development (Koyama et al. 2008). This is in support of the notion that the articular cartilage is structurally and functionally different to the growth plate cartilage.

While the morphological changes are well characterized and the role of interzone cells is established, the molecular regulation and changes in the differentiation and fate of interzone cells are not so clear (Pacifici et al. 2005; Decker et al. 2014). A study in mice using genetic activation of a reporter gene in interzone cells provided important clues to the progenitor status of interzone cells and their contribution to the articular cartilage and other structures of the joint (Koyama et al. 2008).

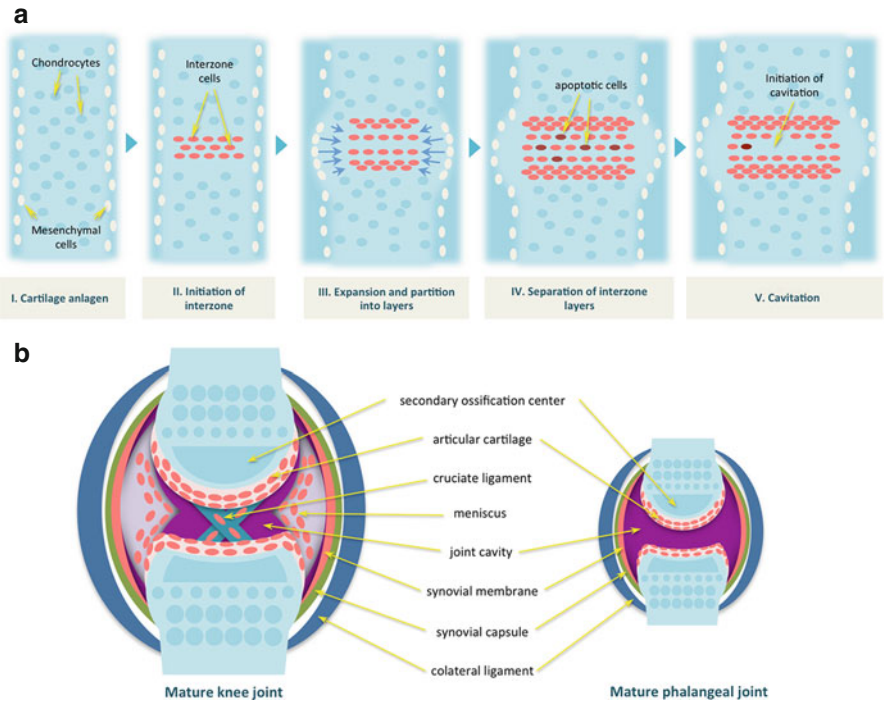

Fig. 7.1 Development of synovial joints. (**a**) Schematic representation of the developmental processes in synovial joint formation with the following steps: (*I*) condensation of mesenchymal cells in the formation of the cartilage anlagen; (*II*) cells at the future joint site undergo dedifferentiation that become flattened and arrange into layers to form an interzone; (*III*) the interzone expands by recruiting the cells from the surrounding mesenchyme and partitions into two outer regions with densely packed cells and the central/intermediate region with more loosely packed cells; (*IV*) initiating separation at the center of interzone with some cells that undergo apoptosis. (*V*) Cavitation with physical separation with cavity filled with synovial fluid, and the partitioned cells contribute to the formation of the articular cartilage, cruciate ligaments, synovial membrane, and meniscus. (**b**) Illustrations showing the anatomical structures of mature knee and phalangeal joints. The proposed contribution of interzone cells to the different structures is indicated by the position of the orange-colored interzone descendent cells

Such studies will provide further insights to more thorough understandings of the key events in joint formation.

7.4 Skeletal Patterning Through Formation of the Interzone Regions

7.4.1 Morphological Changes

Skeletal patterning in limb development is regulated in part by the formation of interzone regions within cartilage elements that will become future joints. The role of the interzone cells in joint formation is clear as their removal by microdissection

in the developing chick embryo in ovo resulted in the loss of the joint in the specified region (Holder 1977).

Cartilage elements in the developing limbs are formed through chondrogenic processes involving condensation of the mesenchyme and differentiation of mesenchymal cells toward the chondrocyte lineage under the regulation of key transcription factors such as *Sox9* in the initial stage and *Sox5/6* at a later stage (Akiyama et al. 2002; Archer et al. 2006), with specific changes in the extracellular matrix proteins and cell adhesion molecules, such as type I collagen and N-cadherin (DeLise et al. 2000) CD44 (Toole 1991), hyaluronic acid (HA), tenascin, and fibronectin (Dessau et al. 1980; Pitsillides et al. 1995).

The mechanism by which the interzone regions are initiated is not clear. It is likely to be complex involving intrinsic and extrinsic factors that may vary depending on the skeletal element concerned. While there may be some common factors in initiating interzone formation, studies have shown that the specificity of joints formed is dependent on additional autonomous and nonautonomous factors in directing joint morphogenesis. In the formation of the stylopods (humerus and femur) and zeugopods (radius and ulna, tibia, and fibula), the formation of the three cartilage elements, in the respective forelimbs and hind limbs, appears to be as discrete entities forming a Y-shape structure, and with elongation of the anlagen, a region of prechondrogenic cells is retained at the intersect that will become the interzone region corresponding to the future elbow and knee joints (Hamrick 2001; Hinchliffe and Johnson 1980). This is common from studies in mouse and chick limb development.

Formation of the autopod (metacarpal and phalangeal) joints appears to differ slightly, with an initial mesenchyme condensation forming a continuous cartilage element for the metacarpal in the digital rays that are segmented sequentially with elongation of the digital rays and formation of the phalangeal joints. This is the consensus in mouse development that the interzones in autopod develop through dedifferentiation of chondrogenic cells at sites of the future joints. However, in the developing chick autopods, the sequential condensation of digital cartilage anlagen suggests a mechanism similar to elbow and knee joints with the appearance of an intervening region of prechondrogenic cells that will become the interzone regions concomitant with the sequential condensation of the distal cartilage elements, suggesting species variations in the initiation of digit joints. It was suggested that this difference could be due to the presence of a phalange-forming region at the tip of the developing digit that is responsive to BMP signaling in the avian system (Suzuki et al. 2008). However, a similar region can be identified in the mouse (Stricker and Mundlos 2011). Thus, while this region may be responsible for the recruitment of mesenchymal cells into the condensing mesenchyme as the cartilage anlagen grows and elongates, the initiation of phalangeal interzones is different between avian and mammal.

Irrespective to how the interzone cells may arise, they are presented as more flattened cells than the surrounding rounded chondrocytes in the cartilage anlagen. These flattened cells are organized into layers aligned perpendicular to the proximal-distal axis of the developing limb and begin to lose their chondrogenic characteristics with a downregulated expression of *Sox9* and a change in the extracellular

matrix from a type II collagen- to a type I collagen-rich environment (Craig et al. 1987). Once initiated, the interzone becomes an important signaling region and a source of progenitor cells for the subsequent formation of the joint structures. With development, the number of cells within the interzone increases; it does not appear to be due to active proliferation of the interzone cells, but rather further recruitment of mesenchymal cells from the surrounding tissues (Niedermaier et al. 2005).

7.4.2 Molecular Regulation of Interzone Initiation

The specification of skeletal elements along the proximal-distal axis in the developing limb is likely to have some predetermined cues influencing the initiation and position of the future joint. For example, as joints are formed around skeletal elements of the stylopod and zeugopod, the disruption of paralogous *Hox* genes (*Hoxa11* and *Hoxd11*) in mouse that lead to the loss of radius and ulna bones (Davis et al. 1995) will influence the formation of the elbow joint, and the *Hoxd13* mutant mice with skeletal abnormalities restricted to the autopod will affect phalangeal joint formation (Dolle et al. 1993). It has been shown that the proximal-distal progression in digit development can be influenced by the prolonged FGF signaling from the apical ridge that results in the formation of an extra phalangeal joint/bone and that an FGF receptor inhibitor can block its formation (Sanz-Ezquerro and Tickle 2003b). Similarly, an additional phalange can be induced in a chick toe if sonic hedgehog (SHH) proteins are placed in between developing digital rays (Sanz-Ezquerro and Tickle 2000; Dahn and Fallon 2000).

Interestingly, cell-matrix interaction can also be a contributing factor, as demonstrated by inhibition of α5β1 integrin signaling using specific antibodies or arginine-glycine-aspartic acid (RGD)-blocking peptides, with the formation of an ectopic interzone between proliferating chondrocytes and hypertrophic chondrocytes in forelimb cartilage elements in mouse embryos at E14.5 (Garciadiego-Cazares et al. 2004). The ectopic interzone expresses interzone markers (*Wnt9a/Wnt14*, *Gdf5*, *chordin*, *autotaxin*, *Col1a1*, and *CD44*), while chondrocyte markers (*Ihh* and *Col2a1*) are downregulated, consistent with the initial stages of joint formation. Clearly, the positional specification and signals that initiate interzone formation will be complex that require more detailed molecular genetic studies in chick and mouse development.

At the molecular level, joint formation correlates with downregulation of type II collagen (*Col2a1*) and aggrecan (*Acan/Agc1*) at the specified joint region, with concomitant expression of genes such as *Gdf5*, *Gdf6*, *Bmp7* and *Bmp2* (Francis-West et al. 1999b; Merino et al. 1999; Storm and Kingsley 1996, 1999), *Noggin* (Brunet et al. 1998), *Wnt9a*/(*Wnt14*) (Hartmann and Tabin 2001), *Wnt4* and *Wnt16* (Guo et al. 2004), *Gli3* (Spater et al. 2006a), *CD44* (Pitsillides 2003), and *Erg* (Iwamoto et al. 2000). Overexpression of *Bmp2*, *Bmp4*, *Bmp7*, and *Gdf5* genes in the limb bud or inactivation of BMP antagonist Noggin causes overgrowth of the cartilage and inhibition of joint formation (Brunet et al. 1998; Storm and Kingsley 1999; Duprez et al. 1996), whereas deletion of *Gdf5* or *Gdf6* results in fusion of joints (Settle et al. 2003).

Gdf5 and *Wnt9a* are two early markers of the interzone. Therefore, it has been proposed that they have a role in early interzone formation, and there is a balance between chondrogenic (GDF5) and anti-chondrogenic (WNT9a) signals that regulate the initiation and progression of interzone formation. GDF5 is a member of the TGF-β superfamily. Mutations in this gene are associated with acromesomelic dysplasia, Hunter-Thompson type, brachydactyly type C, and chondrodysplasia Grebe type (Baldridge et al. 2010; Mundlos 2009), consistent with its role in chondrogenesis and joint formation. Furthermore, inactivation of *Noggin*, a secreted BMP antagonist, results in the absence of joints (Brunet et al. 1998), and mutations in *Noggin* lead to multiple synostoses (Gong et al. 1999). However, loss of TGF-β responsiveness from inactivation of the TGF-β type II receptor gene (*Tgfbr2*) in limbs of mice resulted in the absence of interphalangeal joints (Spagnoli et al. 2007). Thus, TGF-β signaling is needed to promote joint formation as signaling via TGFBR2 regulates *Noggin*, *Wnt9a*, and *Gdf5* expression (Spagnoli et al. 2007). Given that overexpression of *Gdf5* fails to induce joint formation but results in overproduction of cartilage and loss of joints (Francis-West et al. 1999a), TGF-β signaling is likely to play a regulatory role that is necessary but not sufficient to induce joint formation.

Several Wnt genes, including *Wnt4*, *Wnt9a*, and *Wnt16*, are identified with overlapping and complementary expression in early interzone cells together with increased β-catenin level and activity; hence, Wnt signaling is likely to play an important role (Guo et al. 2004). Wnt4 was implicated in both canonical and noncanonical Wnt signaling, while Wnt9a and Wnt16 were implicated in the canonical β-catenin pathway (Guo et al. 2004). Many studies have shown that the canonical β-catenin pathway has a role, as removal of β-catenin in mesenchymal progenitor cells promoted chondrocyte differentiation and genetic removal in chondrocytes led to bone fusion (Guo et al. 2004; Spater et al. 2006a; Kahn et al. 2009).

Ectopic expression of *Wnt9a* or activation of the canonical β-catenin pathway induced ectopic joint-like structure, with expression of *Gdf5* (Guo et al. 2004; Hartmann and Tabin 2001; Tamamura et al. 2005). Thus, Wnt9a was considered to be critical in determining where the joint will be formed, and the Wnt/β-catenin signaling was shown to be necessary and sufficient to induce early steps of synovial joint formation (Guo et al. 2004). However, later studies find that Wnt/β-catenin signaling may not be required for induction but needed for the subsequent maintenance and cell fate, important for long-term joint integrity (Koyama et al. 2008; Spater et al. 2006a).

Gdf5 is expressed by condensing mesenchyme and immature chondrocytes (Kan et al. 2013); its expression in early interzone cells may represent an early sign of the dedifferentiation. It is clear that what signals are required for joint initiation is still poorly understood. Given that c-Jun can act at the enhancer level to regulate Wnt signaling at the initiation and joint progression (Kan and Tabin 2013), and *Sox11* expression, a transcription factor that becomes restricted to interzone cells in joint development, can stimulate expression of *Gdf5* (Kan et al. 2013), there are upstream regulators that form feedback loops in promoting/regulating the initiation process, likely to be a balance between TGF-β and Wnt signaling.

7.4.3 Interzone as a Reservoir of Progenitor Cells for Joint Formation

Given that the interzone emerges from sites that are previously occupied by chondrocytes (phalangeal joints) or chondrocyte precursors (knee and elbow joint), the consensus is that interzone cells are descendants of the dedifferentiated chondrocytes/chondrocyte precursors, with a history of expressing *Sox9* or *Col2a1*. In addition, *matrilin 1* (*Matn1*) is normally expressed in all chondrocytes except articular chondrocytes, and *Gdf5* is expressed at the onset of interzone formation; mice with *Cre* recombinase expressed under the transcriptional regulation of *Sox9* (Soeda et al. 2010), *Col2a1* (Sakai et al. 2001), *Matn1* (Hyde et al. 2007), and *Gdf5* (Rountree et al. 2004) have been used to perform cell lineage tracing-tracking for the interzone cells in joint development.

The fate of *Gdf5*-expressing cells that has been studied in a genetic cross between Gdf5-Cre and Rosa26-LacZ (R26R) mice (Rountree et al. 2004; Koyama et al. 2008) showed that descendants of *Gdf5*-expressing cells gave rise to many joint tissues, including articular cartilage, synovial membrane, and intra-joint ligaments (Koyama et al. 2008). Although the Gdf5-Cre used in this study was not an inducible *Cre*, and *Gdf5* expression remains till the formation of the articular cartilage, the data does provide the genetic evidence in support of the proposed lineage. This finding is in support of another study using the *Matn1-Cre*/R26R mice that showed cells of the developing articular cartilage are not tagged with *LacZ* expression, while chondrocytes in the rest of the cartilage element are positive for LacZ (Hyde et al. 2007). This is consistent with these cells not having a history of *Matn1* expression, suggesting early articular chondrocytes did not arise from chondrocytes of the cartilage anlagen.

However, cell tracing studies using the Sox9-CreERt2/R26R mice with CreERt2 induced with tamoxifen at E11.5 and embryos examined at E17.5 showed Sox9-descendant cells in articular and growth plate chondrocytes, as well as ligaments (Soeda et al. 2010), suggesting cells from these tissues originated from a common progenitor pool with a history of *Sox9* expression. This would be in line with the concept of interzone cells that arise from dedifferentiation of chondrocytes/chondrocyte precursors, expressing *Sox9* in the condensing limb mesenchyme. Similar finding in a study using endogenous doublecortin (*Dcx*) to drive expression of reporter genes (LacZ or GFP) supported this concept, showing expression of *Dcx* in much of the limb mesenchyme that later are restricted to interzone and articular cartilage (Zhang et al. 2010).

In a related study using the *Col2a1-Cre*, a cross with the *R26R* mouse revealed resident chondrocytes of the cartilage anlagen that have switch off expression of *Col2a1* contribute to the interzone at E13.5. However, following interzone formation, non-*Col2a1*-expressing cells migrate into the developing knee joint interzone that formed the lateral and outer medial meniscus, suggesting cells of the developing meniscus in the knee joint have a complex cell origin (Hyde et al. 2008).

Incorporating these cell tracing-tracking findings, it is clear that interzone cells have progenitor properties. Once established, these cells can contribute to the formation of the articular cartilage as well as non-cartilaginous structures such as synovium

and, in the knee, cruciate ligaments and meniscus (Fig. 7.1b). While these studies provided the genetic support for the proposed origin of the cells within the developing joint, the mouse tools used were not ideally designed for cell tracing, and more detailed and thorough analyses of the progenitor cell pools within the interzone await the availability of inducible joint tissue-/cell-specific Cre (such as the tamoxifen-inducible Cre) for in vivo "pulse-chase" style of cell lineage tracing-tracking studies.

7.4.4 Interzone as a Signaling Center

The interzone, once established, must be maintained and directed to progress along the correct lineage of cellular differentiation and organization to form the various tissues of the joint. While both TGF-β and Wnt signaling are known to be involved in joint initiation, the balancing act continues in the developmental process for the formation of cartilage and fibrous tissues of the joint. TGF-β signaling is clearly important as many of the genetic defects affecting joint formation, in particular brachydactyly (short digits) disorders. Recent advances in deciphering the molecular basis of these brachydactylies show that genes in the BMP/TGF-β signaling pathway are deregulated, suggesting this signaling pathway is pivotal for digit and joint development (Mundlos 2009).

As the interzone begins to organize into the zonal layers in preparation for the formation of the articular cartilage layers along a chondrogenic lineage, and cells within the middle/intermediate layer begin to organize along the lineage for fibrous tissues, it can be envisaged that nonskeletal signals should be maintained in this middle/intermediate layer that should be reduced with corresponding enhanced chondrogenic signals for the outer articular chondrocyte layers. Indeed, this was demonstrated in a recent gene expression analysis of the interzone layers through laser capture microdissection from a developing interzone of a knee joint from a mouse embryo at E15.5 (Jenner et al. 2014). This study showed a high expression level for genes related to chondrogenesis, endochondral ossification, and chondrocyte hypertrophic and cartilage matrix genes. Both BMP and Wnt signaling appear to be active for chondrocyte differentiation and maturation, respectively (Jenner et al. 2014). Within the intermediate layer, chondrogenic genes are not the main feature, although *Sox9* and *Sox6* are expressed and some matrix genes such as *Col2a1*, *Comp*, and *Agc1* suggest this layer may still possess some chondrogenic potential. This would be consistent with joint fusions in some disorders where the balance is tipped toward chondrogenesis. Interestingly, this study also shows a high level of expression for inflammatory genes in the intermediate layer. Their presence while interesting is not clearly understood, but perhaps as a response to remodeling processes in preparation for cavitation as suggested by the authors (Jenner et al. 2014).

The interzone as a signaling center is an interesting concept, not only within the developing joint, as well as its potential to influence development of adjacent tissues. It was postulated that the interzone might be an essential regulator of skeletal development, controlling chondrogenesis of the adjacent cartilage element (Hartmann and Tabin 2000). It was also proposed that signal emanating from the interzone could determine the position of the more distal joint. This proposal arises

from the study of *Wnt9a* mis-expression study, in which the endogenous joint formation was inhibited by the presence of an ectopic joint (Hartmann and Tabin 2001). How this functions is not clear, but a model for spacing of joint was proposed, where the initial step of joint formation involves induction of Wnt9a, and with the formation of the interzone, distinct gene expression patterns will occur that includes *Gdf5*, *chordin*, and other secondary signals secreted from the interzone, acting on neighboring cartilage elements to prevent the induction of a new interzone in the vicinity until their level is reduced to a permissive level (Hartmann and Tabin 2001). This model would be consistent with the observation of an additional joint form through elongation of the cartilage element with prolonged FGF signaling at the apical ectodermal ridge (Sanz-Ezquerro and Tickle 2003b). Thus, with elongation, the level of inhibitory factors from the proximal interzone region is reduced to a level permissive of another joint to form, and the length of the distal cartilage anlagen can be a determining factor.

Studies of IHH signaling within the developing joint also supported the interzone as a signaling center. In the developing interzone, HIP1, a hedgehog target and negative regulator of hedgehog diffusion, is expressed and localized to the margin between the proximal and distal cartilage anlagen (Gao et al. 2009). This was hypothesized to regulate a precise level of hedgehog signaling within the boundaries of the interzone, and one of the targets is PTHrP that signals to the distal tip where the receptor (Ppr) is located. Through a negative feedback loop similar to the growth plate, PTHrP regulates the level of *Ihh* expression in a group of cells at a distal region, a signal needed to regulate the recruitment of mesenchymal cells into the condensing mesenchyme for growth of the cartilage anlagen (Gao et al. 2009; Stricker and Mundlos 2011).

Missense mutations in the *IHH* gene are responsible for brachydactyly type A1 (BDA1) with affected individuals having shortened or missing middle phalangeal bones (Gao et al. 2009; Ma et al. 2011; Guo et al. 2010). In a mouse model for BDA1 with an E95K mutation in *Ihh*, it was shown that the interaction with the receptor patched 1 (PTCH1) and HIP1 was affected (Gao et al. 2009). A consequence is reduced signaling capacity and increased signaling range. Thus, in the BDA1 mouse, while the signaling capacity is reduced, the range is extended, and IHH signals much further into the developing interzone with enhanced PTHrP level, and PTHrP from this center signals to a distal group of cells expressing the receptor, and the level of IHH at this region is downregulated due to the negative feedback loop; the result is reduced recruitment of mesenchymal cells into the cartilage anlagen affecting joint formation further supporting the interzone that acts as a signaling center (Gao et al. 2009).

7.5 Joint Cavitation Formation

7.5.1 Progression to Cavitation

As the joint develops, interzone cells will become increasingly flattened, which attenuates with the continued expansion of the cartilage anlagens. With progression, the presumptive joint capsule establishing in concomitant with interzone becomes

vascularized, infiltrating to the periphery of the synovium. Tissue separation then begins within the center of the interzone that is avascular, and it would seem reasonable to propose that cells within this region would need to change in preparation for this event.

Morphologically, the middle/intermediate layer of the developing interzone can be considered as a "transition zone" and, in the knee, can contribute to the spindle-shaped cells lining the surface of the future joint and the formation of the joint menisci. It seems that the process is intrinsic to cells and tissues within the interzone, as the early process of interzone formation and progression is unaffected by removal of the surrounding cartilaginous tissues, but removal of the interzone region results in loss of a joint (Holder 1977). Thus, interzone cells receive intrinsic signals that influence progression, although these signals could be influenced by local cues such as mechanical loading that will be discussed later (Osborne et al. 2002).

In chicks, the homeobox-containing transcription factor, *Cux1*, was shown to be involved at the onset of joint formation, downregulating the expression of *Col2a1* and *Agc1*, consistent with interzone progression accompanied by reduced staining for cartilage matrix and changes in the extracellular matrix (ECM) (Lizarraga et al. 2002). Wnt9a should also have a role in directing the progression of joint formation as it can induce or maintained the expression of key joint-forming genes, including *autotaxin* (an enzyme for the synthesis of lysophosphatidic acid), *chordin* (a BMP antagonist), and *CD44* (receptor for HA) (Hartmann and Tabin 2001). Thus, *Wnt9a* may exert a later effect in joint formation by promoting the induction of *CD44* expression in the developing interzone and changes in the ECM. This would be expected, as the joint cavitation process must involve key changes in the local ECM environment and architecture for a separation to take place and contribution for the articular cartilage to take place.

Gdf5-expressing cells within the developing interzone also continue to have a role in regulating the formation of cartilaginous and fibrocartilaginous tissues. In addition, *Bmp4* and *Gdf6* are also expressed during joint development (Storm and Kingsley 1996; Zou et al. 1997), suggesting BMP signaling continues to modulate the joint developmental process. Thus, abnormal joint progression can be observed in mice when the receptor (*Tgfbr2*), BMP antagonist (*Noggin*), *Wnt4*, *Wnt9a*, or *ß-catenin* is inactivated in mice (Brunet et al. 1998; Spater et al. 2006a, b; Spagnoli et al. 2007; Koyama et al. 2008), caused by deregulated chondrocyte differentiation at the joint sites.

Indian hedgehog (Ihh) is better known for its role in regulating chondrocyte proliferation and differentiation in the cartilage growth plate (Kronenberg 2003). However, it also has a critical role in regulating synovial joint formation. Inactivation of *Ihh* in mice results in loss of phalangeal joints, but cells in the tissue surrounding the presumptive joint regions express *Gdf5* but the progress of joint formation is impaired (St-Jacques et al. 1999). Its relevance in digit joint formation is further supported by the identification of mutations in *IHH* causing brachydactyly type A1 (BDA1) (Gao et al. 2001), which was later shown to the range of IHH signaling with enhanced signaling into the developing interzone region, affecting the progression of joint formation, perhaps altering the balance between chondrogenic and

non-chondrogenic signals in cellular differentiation and ECM production, leading to the failure of joint formation in some cases (Gao et al. 2009). This notion is supported from a study of the *short digit* (*Dsh*) allele in mice, with an 11.7 Mb inversion in chromosome 5 encompassing the *Shh* locus (Niedermaier et al. 2005). The inversion alters the regulatory control of *Shh* expression, and the digit phenotype in heterozygous (*Dsh*/+) mice results from a dysregulated expression of *Shh* at E13.5 and E14.5 in the phalangeal anlagen with increased hedgehog signaling into the developing interzone altering chondrocyte differentiation and matrix production, resulting in a phenotype similar to BDA1 (Niedermaier et al. 2005).

7.5.2 Cavitation Process

For a synovial joint to function, smooth long-lasting articular cartilage surfaces at opposing ends of long bones need to be generated, separated by a cavity filled with synovial fluid for lubrication. This is an area of extensive research given it is of fundamental importance to the function of the joint. Morphologically, cavitation occurs within the interzone intermediate layer by separation of the elongated and spindle-shaped cells, accompanied by local change in ECM. Once cavitation occurs, the outer layer of spindle cells will localize to the future surface of the articular cartilage (Ito and Kida 2000; Kavanagh et al. 2002; Prehm 1984), and the cavity is filled with lubricants such as hyaluronic acid (HA) (Dowthwaite et al. 2003). HA is a long polymer of glycosaminoglycan units of molecular weight up to 10^7 daltons. Although non-sulfated, it is highly negatively charged due to the carboxyl groups present within the sugar moieties. It fills the cavity produced by attracting and holds water to create a hydrated environment for frictionless joint movements.

A number of cellular and biochemical events have been proposed to be associated with the cavitation process. These included cell death, remodeling of the extracellular matrix, and mechanical influences (Andersen 1961; Mitrovic 1971, 1972; Nalin et al. 1995). Cell death through apoptosis is a common occurrence in development, helping to shape and pattern tissues (Suzanne and Steller 2013). Indeed, some studies have reported cell death in the interzone just prior to cavitation. However, the extent was minimal. Furthermore, cell death was not observed in studies of the developing rat and rabbit knee (Ito and Kida 2000; Kavanagh et al. 2002). However, cell death does occur in interzones of phalangeal joints (Fernandez-Teran et al. 2006), and we have demonstrated this in the developing mouse digits (Fig. 7.2). Thus, the role of cell death is still not clear as it appears to not be essential for all developing synovial joints. This difference between joints may be due to size and shape requirements. Further, massive cell death may not be required, and a temporal cell death is sufficient to initiate the process, triggering downstream events to promote cavitation. Studies of genetic diseases with malformation in phalangeal joint development support the role of cell death, as in its absence, joint fusion and syndactyly occur (Niedermaier et al. 2005; Mundlos 2009).

Change in the ECM environment is observed with cavitation (Andersen 1961; Craig et al. 1987) suggesting the need for remodeling and the involvement of matrix

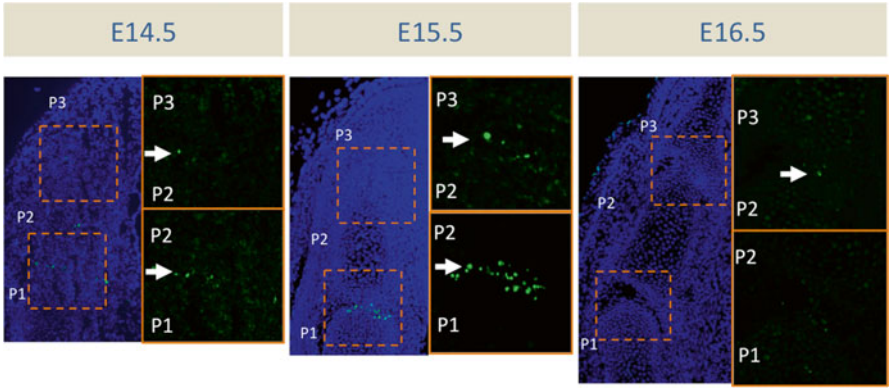

Fig. 7.2 Apoptosis and cavitation in mouse phalangeal joint development. Cellular apoptosis in phalangeal joint development. Apoptotic (TUNEL positive; GFP) cells are detected in the P1/P2 interzone (*white arrows*) of digit III at E14.5 that become more pronounced at E15.5 at the initial stage of cavitation, but with no apoptotic cells detected at E16.5 when a clear joint cavity is established. The distal P2/P3 interzone represents a later development that followed a similar trend. *P1* phalangeal bone 1, *P2* phalangeal bone 2, *P3* phalangeal bone 3. The *dotted boxes* represent magnified regions shown in the respective panels to the right of each of the developmental time points

metalloproteinases (MMPs). Interestingly, immunohistochemical studies show little evidence of MMPs in the interzone during cavitation (Edwards et al. 1994, 1996). MMP activity was detected post cavitation in articular cartilage (Gepstein et al. 2002) but likely to be involved in the establishment of the articular cartilage matrix. One possible way to enforce ECM changes without initial matrix degradation is to produce new matrix and physically push the "older matrix" apart. HA is produced at the plasma membrane of cells and is extruded directly into the extracellular space (Itano et al. 1999; Prehm 1984). Thus, in the developing interzone, it is possible that the presence of HA in the cavity would provide a swelling pressure to physically move the outer interzone layers apart. Furthermore, CD44 is a cell surface HA-binding protein and should have an important role in the cellular interactions with HA (Aruffo et al. 1990). HA-CD44 interaction can induce both cell adhesion and cell separation, depending on the HA concentration in the presence of receptor saturation (Toole 1991). During interzone cavitation, the increased HA synthesis binding to CD44 at cells of the intermediate interzone and cells at the surface of the future articular cartilage can further facilitate tissue separation. The relevance of HA-CD44 interaction is supported from exogenous application of HA oligosaccharides to displace the interaction of endogenous HA with cells that prevented joint cavitation in ovo (Dowthwaite et al. 1998).

Embryo movement and muscle contraction are known to play a role in joint development. Indeed, drug-induced muscle paralysis in chicken or mouse embryos inhibits joint formation (Fell and Robison 1934; Osborne et al. 2002). Muscle contraction is thought to influence the physical separation of the interzone, and HA has also been implicated in immobilization that inhibited cavitation in developing limb

joints (Osborne et al. 2002). Mechanical stimulus can increase the level and activity of the enzymes such as uridine diphosphoglucose dehydrogenase (UDPGD) activity and HA hyaluronan synthase (HAS), thereby increasing the level of HA (Mikic et al. 2000; Osborne et al. 2002). Furthermore, movement in joint development can stimulate the establishment of superficial cells of the articular cartilage with the production of HA and lubricin (Dowthwaite et al. 2003).

Genetic manipulations of muscle development and excitation-contraction coupling deficiency in mice provided mechanistic insights that show muscle contraction is needed to reinforce Wnt/β-catenin signaling, and its impairment in "muscleless" mouse embryos results in fused joints (Kahn et al. 2009). However, the specific mechanism by which muscle contraction regulates the levels of Wnt/β-catenin signaling in joint development is not known. Interestingly, while joint defects are observed in several limb joints, not all are affected with differences between joints in the fore- and hind limbs. For example, the elbow is affected but not the knee (Kahn et al. 2009). Intriguingly, in a gene profiling study for factors that direct development of the elbow and knee in mouse embryos, the authors found genes in the developing elbow joints are enriched in specification for muscle development genes (Pazin et al. 2012). Whether this has a direct link to the abnormal elbow joint development in the muscleless mouse embryos remains to be elucidated (Kahn et al. 2009).

7.6 The Joint Proper

Following cavitation, genetic cell lineage tracing experiments showed that cells in the outer interzone layers contribute to multiple joint tissues over time, including the future articular cartilage, synovial lining, and intra-joint ligaments that persisted into postnatal life (Decker et al. 2014). Cells in these tissues can be traced back to *Gdf5*-expressing dedifferentiation chondrocytes at the onset of interzone formation. Furthermore, expansion of the interzones included recruitment of mesenchymal cells from surrounding tissues of the joint sites into the *Gdf5*-expressing lineage (Niedermaier et al. 2005; Decker et al. 2014). Thus, cells outside the original cartilage anlagen contribute to interzone expansion and progression. These cells could be a population of *Tgfbr2*-expressing cells as demonstrated from a study using the *Tgfr2-LacZ* mice that are initially restricted to the surrounding tissues but, with time, contribute to the synovial lining, meniscal surface, outer ligaments, and groove of Ranvier (Spagnoli et al. 2007).

In mice, a thin layer of cells at the surface of the articular cartilage of the tibia at birth can be molecularly defined by the expression of genes such as collagen XXII (*Col22a1*) (Koch et al. 2006), lubricin (*Prg4*) (Rhee et al. 2005), and tenascin C (Mikic et al. 2000). These cells are derived from *Gdf5*-expressing cells from the interzone (Koyama et al. 2008; Rountree et al. 2004). By P10, the articular cartilage is thicker that can be roughly divided into three zones: a superficial zone of flattened cells at the surface, a middle zone of more rounded cells with typical chondrocyte appearance, and a deep zone of large cells more consistent with hypertrophic

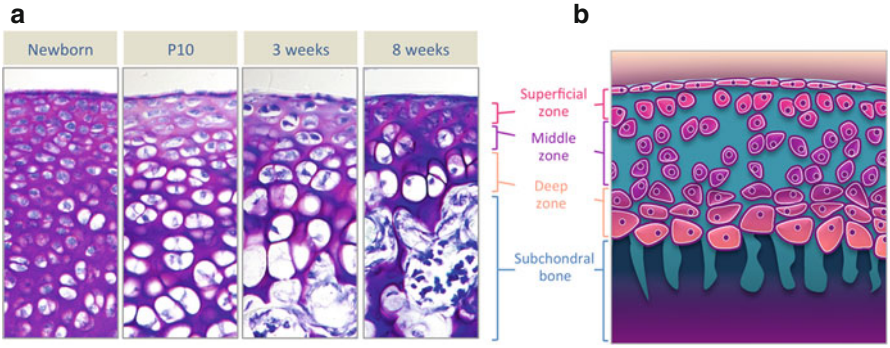

Fig. 7.3 Postnatal development and structure of articular cartilage. (**a**) Histological sections (toluidine blue staining) of the proximal tibia showing the progressive changes in the organization and differentiation of the chondrocytes in the articular cartilage from birth to 8-week-old mice. The progressive postnatal organization into the superficial, middle, and deep zone is illustrated. Cells in the middle and deep zone are progressively larger, consistent with hypertrophic chondrocytes. (**b**) Illustration shows the demarcated zonal organization of the articular cartilage in a mature joint

chondrocytes (Fig. 7.3). From 3 weeks, the articular cartilage exhibits a more mature organization with the onset of secondary ossification of the epiphyseal end of the proximal tibia that becomes a more permanent structure by 8 weeks of age (Fig. 7.3). Interestingly, a recent study tracing *Prg4*-expressing cells at the most superficial region of the articular cartilage of 1-month-old mice showed that they may serve as progenitor cells contributing to the cells of the deeper zones that persisted for at least up to 1 year (Kozhemyakina et al. 2015). Given that the origin of these *Gdf5*-expressing cells can be traced back to the interzone, it may be argued that interzone cells continue to play an important role in the growth and maintenance of the mature articular cartilage.

The joint is not complete without the capsule. It is a dense fibrous membrane attached to the whole circumference of each bone flanking the articular cartilage, forming a sleeve around the joint, providing a seal that keeps the lubricating synovial fluid within the joint space (Ralphs and Benjamin 1994). This capsule contributes to stability of the joint by limiting motion that is further supported by accessory ligaments inside and outside the capsule. It is also thought that the capsule itself may have a role in articulation function as part of the capsule is compressed during movement, which in time becomes more fibrocartilaginous as it adapts to compression. The capsule, despite its importance for joint function, is not well studied, in particular the developmental aspect.

7.7 Overall Perspectives

This chapter provided a brief account of our current understanding of synovial joint formation. With advances in mouse genetics and transcriptomic analyses, we have gained significant insights into the cellular origin of the joint tissues in development,

the molecular signals involved, and the fate of joint cells in adult life. It is a complex system of many players, with both intrinsic and extrinsic influences to the interzone. However, there remain many uncertainties and challenges that require detailed studies to better define the origin(s) of interzone cells. As the pool of Gdf5-expressing cells in the developing interzone is unlikely to be homogenous, there is a need to address potential subpopulations. Mice with better-defined cell-specific inducible *Cre* would be needed, and combining this with single-cell transcriptomics of interzone cells could provide vital clues to the contribution and presence of predetermined subpopulations. Further, more specific gene sets could be identified for the generation of *Cre* driver mice. For example, the Prg4-GFPCreERt2 mice presented as a useful tool to trace cells of the superficial zone in articular cartilage growth and maintenance, not only for the understanding of normal joint biology but also in disease states such as osteoarthritis. Similar *Cre* mice could be produced for the analysis of earlier developmental processes along the Gdf5 lineage toward articular chondrocytes and other joint tissues and to address their potential to become resident adult stem/progenitor cells in the joint (Dowthwaite et al. 2004; Candela et al. 2014). A big question in the field is to understand the reason behind the notoriously weak repair potential of adult cartilage. If there are stem/progenitor cells present in the joint, and they are similar to developmental progenitors "locked" away in a special niche, perhaps understanding the developmental cues may help to unlock these cells to enhance repair. In addition, we should not forget the power of mouse genetics with defined strains of mice with varying degrees of repair/regeneration potentials. For example, the recent genetic studies of the good (MRL, LG/J) and poor (C57Bl, SM/J) cartilage healer mice are aimed at discovering regenerative loci/genes that could promote repair (Rai and Sandell 2014), as the good healer mice can activate an effective repair of large defects in the articular cartilage (Fitzgerald et al. 2008). These mice are also good candidates to study reactivation of development progresses or the activation/mobilization of resident stem/progenitor cells in the repair process.

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