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# A Literature Review on the Development of Upper Limbs in Humans

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## Cover Page Footnote

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# A Literature Review on the Development of Upper Limbs in Humans

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**ABSTRACT**

The development of tetrapod upper limbs shares an evolutionary origin and has been adapted and specialized for different functions for different species, such as flight in birds, swimming and balance in sea mammals, and coordination and grabbing objects in humans. The basis of tetrapod limb development has common developmental patterns, starting with the formation of the limb bud via Sonic hedgehog (Shh) signaling, where later developmental steps are modified for specialized functions. This review covers the basic developmental patterns of mammalian tetrapod development seen in humans, beginning with the formation of the limb bud, to the axis development of the limb bud, segmentation of the limb bud, then to cartilage formation in limb bud segments, digit ray formation, and lastly digit elongation and segmentation. Tetrapod limb development is a big focus in developmental biology, since there are many limb malformations caused by many different factors, such as over-expression of Sonic hedgehog (Shh) or under-expression. Understanding the formation of limbs help shed light on why limb malformations occur and future implications may be determining ways to prevent such malformations from occurring.

## INTRODUCTION

The early tetrapod is credited for the emergence of digits from the upper limb, which has been adapted and specialized to function differently for various species (Hu and He, 2008), such as how birds use their upper limbs for flight and how humans use their upper limbs for coordination and grabbing objects. The mechanism for upper limb development is similar in tetrapods, with slight variations and modifications due to species differentiation (Capdevila and Izpisua Belmonte, 2001). Many early studies on upper limb development were focused on studying chick limb development since it is easily manipulated in the embryo (Niswander et al., 1993), and since the general limb development mechanisms are conserved, the mechanisms discovered in the chick were able to be generalized to other species, such as on mice, salamanders, and humans (Tabin, 1991). Upper limb development is tightly regulated early on in embryonic development to ensure that the anterior-posterior axis, the dorsal-ventral axis, and the proximal-distal axis are correctly formed (Wolpert, 1999). Studying the development of upper limbs is crucial in understanding developmental biology in vertebrates, especially since upper limb development is highly conserved among vertebrates (Marigo et al., 1996).

## LIMB BUD FORMATION

Limb development occurs around 24-26 days after fertilization, followed by the migration and proliferation of the lateral plate mesodermal cells into the upper limb-forming field (Tickle, 2015). The lateral plate mesoderm cells form a lateral bud of undifferentiated mesodermal cells surrounded by ectoderm cells (Tickle, 2015). The initiation of the limb bud results from the expression of Sonic hedgehog (Shh) from the notochord at around 22 days after fertilization (Al-Qattan and Kozin, 2013).

There are four sets of HOX genes in humans, labeled HoxA, HoxB, HoxC, and HoxD, which are all located on a different chromosome (Guero, 2018). The lateral plate mesodermal cells are signaled by HOXC6 gene, which tells the lateral plate mesodermal cells to secrete T-box 5 protein and Wingless-related integration site (WNT), ultimately producing fibroblast growth factor (FGF) to increase mesodermal cell proliferation to form the preliminary limb bub into a larger bulge (Guero, 2018). The Sonic hedgehog (SHH) expression from the notochord is activated by the expression of the FGF, indicating a feed-forward mechanism to increase the formation of the limb bud (Hirashima et al., 2008). The mesodermal cells proliferate into the ectoderm, forcing the ectoderm to bulge and form into the apical ectodermal ridge (AER) (Al-Qattan and Kozin, 2013).

In the case where the AER was removed experimentally, it ceases the growth of the upper limb (Guero, 2018). The presence or lack of AER in congenital malformations have yet to been studied more in-depth. The further the AER cells stay in the progress zone (PZ), the more distal structures they develop (Koussoulakos, 2004). The AER and PZ move away from each other along the proximal-distal axis, from the embryonic axis to differentiate into a

proximal zone, a distal zone, and an intermediate zone (Guero, 2018). The formation of the AER helps determine proximal-distal limb axis (Sheeba et al., 2016). The zone of polarizing activity (ZPA) secretes Sonic hedgehog (Shh) which helps form the anterior-posterior axis (Sheeba et al., 2016). There is some conservation among vertebrates in regard to Shh signaling; for example, there are approximately 60% similarities among the signaling pathways between chicks and humans, where there was even complete conservation in the 4th and 5th zinc finger involved in the Shh pathway (Marigo et al., 1996). When the ZPA is removed during early limb bud formation, their digits show no pattern and the opposite of limb truncating occurs—the limb does not stop extending (Hirashima et al., 2008; Pagan et al., 1996). With no ZPA, the ulna and ulnar-side fingers do not develop, leading to an underdeveloped ulnar club hand (Anderson et al., 2012; Guero, 2018). With an overabundance of Shh, the ZPA becomes inactive, forming an ulnar club hand, where there is a severe ulnar hypoplasia with the 4th and 5th digit missing on the hand (Guero, 2018).The highest level of ZPA activity is furthest from the AER, and the distance between the ZPA activity and the AER is where the limb bud is able to elongate, along with the ulnar artery forming (Al-Qattan and Kozin, 2013; Hirashima et al., 2008). The concentration gradient of the ZPA was along the anterior-posterior axis of the limb, indicating that the morphogen was signaling and specifying which cells become posteriorized and which become anteriorized (Bastida and Ros, 2008). The dorsal-ventral axis is formed by the expression of wnt7a and Lmx1b in the dorsal limb ectoderm surrounding the lateral plate mesodermal cells (Sheeba et al., 2016). After all of the signaling regions have been established, the limb bud is able to develop autonomously (Tickle, 2015).

After the emergence of the limb bud, HoxC6 begins to signal the lateral plate mesoderm cells to secrete T-box 5 protein (TBX5) and Wingless-related integration site (WNT) (Bastida et al., 2009). The secretion of TBX5 and WNT increase the release of fibroblast growth factor 10 (FGF10), an essential growth factor required for rapid cell proliferation (Guero, 2018). The four segments are positioned along the proximal-distal axis formed earlier from the AER (Sheeba et al., 2016). Approximately 26-31 days after fertilization, the limb bud is divided into four segments, the stylopod, the zeugopod, the mesopod, and the autopod (Al-Qattan and Kozin, 2013). The stylopod is a proximal segment which includes the humerus. The zeugopod is an intermediate segment, including the radius and the ulna. The mesopod is the future wrist (Al-Qattan and Kozin, 2013). The autopod is a distal segment, which includes the skeletal components of the hand, such as the carpals, which are numbered 1 through 5, starting with 1 for the thumb and ending with 5 for the little finger (Cole et al., 2009). Hand2 directly binds to the ZPA regulatory sequence, also activates HoxD13, to further limb bud differentiation (Anderson et al., 2012; Galli et al., 2010). The AER develops into the PZ, where the first cells leave and differentiate into future stylopod (Al-Qattan and Kozin, 2013). In order to develop into the stylopod, the cells must first express MEIS-1 (Al-Qattan and Kozin, 2013). The next cells that leave later develop into the zeugopod cells, where they will express HoxA11 and HoxD9 (Al-Qattan and Kozin, 2013). The third group of cells that leave the PZ are the mesopod cells and express only HoxA13 (Al-Qattan and Kozin, 2013). The last group of cells that leave the PZ are the autopod cells and express HoxA13 and HoxD10-13 (Al-Qattan and Kozin, 2013).

## CARTILAGE FORMATION IN THE LIMB BUD SEGMENTS

After the four segments of the limb bud are formed, cartilage precursor cells migrate to the center of the limb bud, while connective tissue cells migrate to the periphery of the limb bud (Al-Qattan and Kozin, 2013; Cole et al., 2009), where the nerve trunks begin to enter the arm and chondrogenesis begins (Al-Qattan and Kozin, 2013). This occurs approximately 36 days after fertilization (Cole et al., 2009). The center cells form a blastema to differentiate into chondrocytes for endochondral skeletal bones or osteoblasts for membrane skeletal bones (Al-Qattan and Kozin, 2013). The chondrification occurs from the expression of FGFs, transforming growth factor-beta (TGF-ß), bone morphogenic proteins (BMPs), Indian hedgehog (IHH), and parathyroid hormones (Al-Qattan, 2011; Al-Qattan and Kozin, 2013; Daumer et al., 2004). Muscle fibers are beginning to develop at the surface, where the superficial ones appear first, followed by the deeper muscle fibers, as well as nerve ingrowth at the bottom of the limb bud (Al-Qattan and Kozin, 2013). The marginal vein develops from the capillary networks around day 31 of development, followed by the proliferation of the limb bud earlier (Al-Qattan and Kozin, 2013). The brachial artery forms the median arteries, which supplies the blood to the hand—along with the ulnar artery and the radial artery (Al-Qattan and Kozin, 2013; Zaleske, 1985).

After the first period of Sox9 expression and the arrangement of the skeletal elements, the second period, the condensation period begins, where the committed cells begin to undergo mesenchymal condensation via the increased activation of cell-cell adhesion genes and cell-extracellular matrix genes (N-Cam and Tenascin C, alpha 5 integrin respectively) (Marin-Llera et al., 2019). The third period is the pre-cartilage stage, where the previous genes are upregulated, as well as the expression of Sox9

to increase the development of the cartilage phenotype (Lorda-Diez et al., 2011; Marin-Llera et al., 2019).

The morphogen, Sonic hedgehog (Shh), varies in its concentration across the posterior-anterior axis (Guero, 2018). In the case where there is a deletion of the HoxB and HoxC gene, there was no visible abnormal phenotype; however, in the case where there was a deletion of both HoxA and HoxD, there was an early arrest of limb growth (Zakany and Duboule, 2007). If there are mutations in the GLI3 gene caused by Shh gene mutations, it can result in postaxial polydactyl or autosomal dominant genetic disorders, more commonly known as Greig cephalopolysyndactyly syndrome or Pallister-Hall syndrome (Guero, 2018). The polydactyly seen in both disorders are caused by the missing gradient of GLI3, causing lack of finger identity in the digit rays (Guero, 2018). Polydactyly can also arise without Shh gene mutations and are often caused by the domain that controls Shh expression, called the ZPA regulatory sequence (Guero, 2018; Montavon and Duboule, 2012). In cases where the ZPA regulatory sequence was partially duplicated, Haas syndrome occurs, which is severe polydactyly, resulting in the formation of eight fingers (Guero, 2018). It is important to note that while Shh is highly concentrated is on the ventral side, the dorsal side contains Shh antagonists, which demonstrates the variability in hand phenotypes, even with mutations in Shh (Guero, 2018; Quinn et al., 2012).

## INITIAL STAGES OF DIGIT FORMATION

Digits are formed as a single chondrogenic plate, where the autopod plate will form the digit rays, which are intercepted by interdigital tissue around 44-47 days after fertilization (Cole et al., 2009; Niswander et al., 1993; Vogel et al., 1996). The chondrogenic plate condenses, forming digit rays, followed by endochondral ossification (Murgai et al., 2018). Many cell types are required to form the adult limb, but the early precursors to the adult limb require cartilage, bone, dermis, ligaments, and tendon, all originating from the limb mesenchymal stem cells kept in an undifferentiated state (Marin-Llera et al., 2019). The limb mesenchymal stem cells are maintained in an undifferentiated state to serve as a pool of progenitor cells to later be differentiated into cartilage, bone, dermis, ligaments, and tendons (Marin-Llera et al., 2019). The pool of progenitor cells also differentiate into the cartilate required for the formation of the hand plate (Chimal-Monroy et al., 2011). The progenitor cell pools are maintained by the expression of FGFs and WNT, which will also inhibit cell differentiation and cell death, conserving the pool of undifferentiated mesenchymal cells (Mariani et al., 2008; Yu and Ornitz, 2008), as well as initiating the expression of Sox9 (Kumar and Lassar, 2014). Whenever the progenitor cell pool expresses Sox9, chondrogenesis initiates, expressing Sox5, Sox6, type II collagen, aggrecan, and sulfated proteoglycans (Lefebvre, 2019; Montero and Hurle, 2007).

Sox9 expression in the mesenchymal cells promotes cell aggregation as well as type II collagen, aggrecan, and sulfated proteoglycans, which are the cartilage synthesizing proteins (Marin-Llera et al., 2019; Montero and Hurle, 2007). The inhibition of Activin and Follistatin at the top of the developing digit inhibits BMP/SMAD signaling, which results in the truncation of the digit (Chimal-Monroy et al., 2011). In order for the digits to form from the hand plate, the interdigital mesenchyme undergoes apoptosis after receiving the signal from MSX2 (Al-Qattan and Kozin, 2013; Daluiski et al., 2001). When the AER fragments between the cartilage digit rays, apoptosis begins (Guero, 2018). Retinoic acid, fibroblast growth factors (FGFs) and morphogenic proteins (BMPs) are the main signaling pathways that induce interdigital apoptosis (Hernandez-Martinez and Covarrubias, 2011; Kaltcheva et al., 2016). Retinoic acid directly regulates interdigital cell death by interacting FGFs or BMPs, inducing interdigital BMPs upstream to regulate apoptosis (Kaltcheva et al., 2016; Murgai et al., 2018; Rodriguez-Leon et al., 1999). The phalanx forming region then develops between the AER and the distal areas of the digit rays, expressing Indian hedgehog (Guero, 2018). The posterior interdigital space controls the growth activity of the digital rays: the mesoderm is controlled by BMP 2,4,7, while the ectoderm is controlled by FGF and Wnt to control the size of the developing phalanges (Guero, 2018).

In cells that lose ß-catenin expression in type II collagen expressing cells, there was an observed joint fusion with no development of ligaments (Guo et al., 2004; Marin-Llera et al., 2019). If the interdigital mesenchyme is inhibited by BMP with TGF-ß and or Activin, Sox9 becomes inhibited, delaying the chondrogenesis and resulting in areas with lacking cartilage formation (Montero et al., 2008). When Sox9 is removed early on from the undifferentiated mesenchymal cells in the limb buds, it led to a loss of cartilage differentiation, which ultimately led to the inhibition of digit formation (Chimal-Monroy et al., 2011). At the tips of the developing digits, there was an increased concentration of SMAD 1,5,8, where the concentration was maintained until the last phalanx was developed (Montero et al., 2008). The distribution of SMAD 1,5,8 were expressing either bmp receptors or bmp genes on the developing digits (Montero et al., 2008).

## THE FORMATION OF DIGIT RAYS AND DIGIT ELONGATION

Interestingly enough, human embryos develop the finger rays in a different order as opposed to other vertebrates:

the 4th, 2nd, 5th, 3rd, and then lastly, the 1st digit (Guero, 2018). The thumb mainly uses GLI3R, located on the anterior region of the autopod for its identification (Guero, 2018). TBX5 affects the radial edge of the thumb, which also corresponds to the radial side of the forearm (Oberg, 2014). If TBX5 becomes mutated, it leads to the Holt-Oram syndrome, which is characterized as a missing forearm if the mutation was early in the induction phase of the limb or the formation of a forearm with missing fingers, also known as phocomelia (Guero, 2018). After the digit identifies have been determined, each primordium begins to distally elongate prior to the generation of phalanges (Hu and He, 2008). The distal phalanx begins as a cartilaginous skeleton, with the ossification initiating at the distal ends (Casanova et al., 2012). The nail begins to form at the distal side at each end of the terminal phalanx, which expresses Bambi, STP8, and MSX1, which retains the ability to potentially regrow a fingertip (Casanova et al., 2012; Guero, 2018). The AER regulates the outgrowth of the limb bud at this point, where removal of AER early in the limb bud development leads to massive cell death and failure of limb development completely (Hu and He, 2008). The amount of time Fgf8 is expressed corresponds to the elongation time of the digit primordia, where prolonged Fgf8 expression can lead to extra phalangeal development and where reduced Fgf8 expression can lead to shortened digit primordium and loss of phalanges (Hu and He, 2008; Stricker and Mundlos, 2011). The final number of phalanges that appear as well as the length of the phalanges depends on the intensity of the AER signaling in the digit crest, as well as the signaling of the BMP and Smad 1,5,8 (Stricker and Mundlos, 2011)

## DIGIT SEGMENTATION AND THE STAGES OF JOINT SYNTHESIS

After the elongation of the digits, the digits are segmented into metacarpals or metatarsals on the phalanges (Stricker and Mundlos, 2011). Similar to elongation, the segmentation of the digits occur in a specific order; segmentation occurs twice in the first digit while the other digits undergo segmentations three times (Stricker and Mundlos, 2011). The segmentation of the digits occurs from the proximal to distal end, which results in interphalangeal joints (Stricker and Mundlos, 2011). HoxD genes are active during the initial phases of joint tissue development (Khoa et al., 1999). In order for joints to form, chondrogenesis must be repressed and inhibited, allowing the future joint areas to express WNTN4 and WNT14 to activate cartilage-derived morphogenic protein 1 (CDMP1) (Al-Qattan and Kozin, 2013; Garciadiego-Cazares et al., 2004; Hartmann and Tabin, 2001). It must be noted that Wnt3a caused the mesenchymal cells to remain undifferentiated and chondrocytes with short-term exposure, but with long-term exposure to Wnt3a, the mesenchymal cells lost all of the chondrogenic potency and allowing them to be differentiated into other cell types, more specifically, soft connective tissue (ten Berge et al., 2008). In order for chondrogenesis to be repressed, Sox9 must be repressed while the joint synthesizing genes must be activated: Jun, Gfg5, Wnt4, and Wnt9a (Guo et al., 2004; Hartmann and Tabin, 2001; Kan and Tabin, 2013; Merino et al., 1999; Scoones and Hiscock, 2020; Sohaskey et al., 2008; Storm and Kingsley, 1999).

During the development of joint patterns, each set of joints is positioned within each digit ray, repeating as hinges form to give the joints movement and flexibility (Scoones and Hiscock, 2020). It is at the interzone, areas at the distal end of each digit ray where the joint progenitors accumulate, lengthening as more progenitor cells accumulate below the AER (Decker et al., 2014; Scoones and Hiscock, 2020). If the AER fails to maintain the progenitor cell pool, it causes severe limb malformations, such as the ectrodactyly syndromes, which are characterized by split-hands and split-feet (Stricker and Mundlos, 2011). The interzone has a high cell density where chondrocytes are able to mature and flatten, becoming fibroblastic tissue (Marin-Llera et al., 2019). During the formation of the interzone, the articular cartilage undergoes differentiation, where cavitation occurs, separating the skeletal elements into two parts to form the joint capsule (Marin-Llera et al., 2019). Interzone cells can differentiate into different phenotypes, depending on the different signaling pathways (Koyama et al., 2008). This demonstrates how the interzone cells can be differentiated into multiple different cell types, such as chondrogenic structures, non-chondrogenic structures, articular cartilage, and intra-joint ligaments (Koyama et al., 2008). Whenever Noggin, the BMP antagonist, malfunctions, it causes abnormal joint formation as well as abnormal joint fusion, indicating that BMP inhibition is important in the formation of joints (Stricker and Mundlos, 2011). A mutation in GDF5 that causes an increased activity of GDF5 causes joint fusion to occur (Stricker and Mundlos, 2011). After the condensation of the digital plate, the cells differentiating into cartilage is halted, while the chondrocytes in the growth plate start differentiating into reservoirs adjacent to the future joints (Stricker and Mundlos, 2011).

There are large phenotypic variations in upper limb malformations due to Shh concentration variations (Guero, 2018). Many types of upper limb anomalies correlate with specific syndromes, such as how 89% of patients with Townes-Brocks syndrome have thumb anomalies (Kohlhase, 1993), or how ulnar deficiencies correlate to ulnar-mammary syndrome (Webb et al., 2011). Mutations on HoxD13 lead to synpolydactyly, brachydactyly, and syndactyly (Garcia-Barcelo et al.,

2008; Guero, 2018). Polydactyly is a congenital malformation of the hands and feet, characterized by the fusion of multiple fingers or toes adjacent to one another (Zhou et al., 2014). Polydactyly occurs most commonly on the upper limbs (Malik, 2014). It can be caused by the defective patterning of the anterior-posterior axis during upper limb development (Biesecker, 2011). Brachydactyly is also a congenital malformation characterized by short digits that are unproportionate to the other digits (Temtamy and Aglan, 2008). Syndactyly is the fusion of two adjacent digits to each other (Zhou et al., 2014). Other phenotypes of HoxD13 mutations include Guttmacher's syndrome, which causes hypoplasia of the 1st and 5th digits, and Hand-foot-genital syndrome, which is characterized by hypoplasia of the distal phalanx (Guero, 2018; Imagawa et al., 2014). The analysis of the brachydactyly syndrome is characterized by the shortening of digits caused by hypoplasia or aplasia of individual phalanges or metacarpals or metatarsals (Stricker and Mundlos, 2011).

Joint patterning begins with multiple signals being expressed, mainly Wnt4, Wnt9a, as well as Wnt/ß-catenin (Guo et al., 2004; Scoones and Hiscock, 2020). When the interzone was removed, joint formation was inhibited, producing a lack of segmented skeletal elements, appearing as normal skeletal elements except with the lack of joints (Marin-Llera et al., 2019; Rux et al., 2019). Gdf5 is expressed at low levels in joint regions, due to Gdf5 inhibiting joint fate patterning—it will lead to local inhibition of joint formation when placed near a mature interzone (Merino et al., 1999; Scoones and Hiscock, 2020; Storm and Kingsley, 1999). In cases where GDF5 was expressed at high levels, there was no joint formation, rather cartilage growth (Hu and He, 2008). After analyzing the digit crescent, it was found that at the tip of the digit plate, there were low levels of cell proliferation that expressed Sox9, indicating that the area of proliferation overlapped the formation of the digit crescent (Montero et al., 2008). Whenever BMP was expressed at the tip of the digit, the size of the developing phalanx increased, indicating that the expression of BMP activated SMAD 1,5,8 to increase the DC domain (Montero et al., 2008). Shh can be downregulated with BMP signaling, as such when BMP was introduced to the ZPA, causing Shh to downregulate drastically, almost to being undetectable (Bastida et al., 2009). However, the level of interdigital cell death was not affected, indicating that cell death caused by BMPs are not associated with blocking Shh (Bastida et al., 2009). BMPs are unique, such that they can induce a cascade resulting in cell death or chondrocyte differentiation via Sox9 induction (Chimal-Monroy et al., 2011).

It was found that TGF-ß inhibits interdigital cell death in a dose-dependent manner, where higher doses of TGF-ß produces thicker regions of interdigital tissues that did not undergo interdigital apoptosis (Chimal-Monroy et al., 2011). BMP uses a negative feedback loop to inhibit their own signaling, by expressing Noggin in the developing digits as well as the pericardium, allowing Noggin to bind to BMPs, ultimately suppressing the BMP signal (Merino et al., 1998). IHH also discovered to be required in maintaining the interdigital mesenchyme during the proliferative stage of interdigital cells, preventing apoptosis from occurring (Murgai et al., 2018). Areas with high levels of BMP were observed on the digit tips in order to sustain digit growth and promote the cartilage differentiation of the mesenchymal cells (Murgai et al., 2018; Salas-Vidal et al., 2001).

## CONCLUSION

In conclusion, the development of upper limbs in vertebrates is a broadening field of developmental biology, with new discoveries every year. The limb bud is easy to observe since it occurs early on in development with a conserved general mechanism, allowing for rapid advances (Guero, 2018). The ZPA was discovered in chick embryos in 1968, by removing cells from the posterior region and transplanting them in the anterior region, forming the entire posterior rays in the anterior region (Guero, 2018). It was only in 1993, where Sonic hedgehog (Shh) was discovered to be involved in the formation of the ZPA (Riddle et al., 1993), and now, it has been discovered that Shh plays a large role in not only signaling digit formation but also signaling the development of the ulnar part of the forearm and the hand (Guero, 2018). Since the discovery of SHH, there have also been other morphogens discovered, but not all have been discovered yet. Currently, it is possible to identify where specific morphogens expressed (Guero, 2018). More areas of studies include the mechanisms that develop muscles, tissues, tendons, and ligaments in the hand (Johnson and Tabin, 1997). By identifying the location of where morphogens are expressed, it may be possible to identify mechanisms that form the tendons or the musculature in the hand. There is still much information regarding the roles of Sox9 in chondrogenesis as well as joint formation (Lefebvre, 2019). Upper limb development in tetrapods is a rapidly developing field of research due to the vast number of different phenotypic variations due to mutations, as well as the easy visibility of the different phenotypes (Vogel et al., 1996; Wolpert, 1999).

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