Developmental Cell
Perspective

On the Formation of Digits and Joints during Limb Development

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Critical steps in forming the vertebrate limb include the positioning of digits and the positioning of joints within each digit. Recent studies have proposed that the iterative series of digits is established by a Turing-like mechanism generating stripes of chondrogenic domains. However, re-examination of available data suggest that digits are actually patterned as evenly spaced spots, not stripes, which then elongate into rod-shaped digit rays by incorporating new cells at their tips. Moreover, extension of the digit rays and the patterning of the joints occur simultaneously at the distal tip, implying that an integrated model is required to fully understand these processes.

The distal aspect of the vertebrate limb is characterized by an iterative set of digits (fingers and toes in humans), forming in a repeated series, yet at the same time each differing from the next in morphology. It has long been clear that the developmental mechanism that establishes the number of digits is separate from that giving them distinct identities (Zwilling, 1964) (digit "identity" being a term encompassing the relative size, shape, and number of segments in a digit, differentiating it from its neighbors).

Digit Specification

A conceptual framework for considering the specification of digit identities was provided by the concept of "positional information," essentially a series of positional coordinates along an axis of a developing field that cells can use as guide posts to make different fate decisions (Wolpert, 1969). It was moreover proposed that distinct positional values could be established through the action of a diffusible signal, a so-called morphogen, forming a spatial gradient across the field of cells. In a critical, distinct second step, cells were suggested to interpret this positional information in a context-dependent manner. For example, the same positional values lead to the formation of fingers or toes depending on whether the mesenchyme being patterned originates in the forelimb or hindlimb bud (Saunders et al., 1957). The model of positional information conveyed by a morphogen fits extremely well with the experimental results of grafting cells from the zone of polarizing activity (ZPA) at the posterior digital margin of one limb bud into the anterior distal margin of a second (Tickle et al., 1975), a procedure that leads to a mirror-image duplicate of the digits (Saunders and Gasseling, 1968). In this view, the concentration of a morphogen produced by the ZPA is interpreted by the digit primordia within the limb field as positional information, to yield distinct digit identities. However, importantly, the morphogen model does not instruct the location or spacing of the digits, only their morphotype.

Digit Periodicity

In principle, a morphogen/positional information model could be used to generate a periodic pattern of digit and interdigit, if alternating concentrations of the morphogen induced and inhibited chondrogenesis, in addition to specifying different types of digits at increasing concentrations. However, this was not built into the model, as experimental evidence argued against it. For example, a periodic series of morphologically similar digits are formed when limb cells are dissociated, reaggregated, and grafted back onto an embryo (Zwilling, 1964), indicating that a periodic series of digits is formed in a context where there is no difference in positional information across the limb. However, when a ZPA is transplanted into a recombinant limb, anterior-posterior polarity is restored, demonstrating that these two independent properties of early limb formation (generating an array of digits and specification of distinct digit identities) are smoothly integrated once positional information is provided (Piedra et al., 2000). Moreover, when the limb field is expanded, for example, by a viral application of fibroblast growth factor 2 (FGF-2) (Riley et al., 1993), extra digits are formed, but the duplicated digits do not undergo a change in polarity. Rather, they appear to have the same identity as their immediate neighbors, again indicating that establishing the location of digits is not based on positional information, even if digit identity (differences between digits) is based upon it.

The specification of different types of digits and the specification of the correct number of digits are, however, linked. A ZPA graft (or ectopic application of the morphogen responsible for ZPA activity, Sonic hedgehog [Shh]) causes an increase in the number of digits as well as a polarization of digit identities. It was recognized early on that this depends on a second effect of a ZPA transplant, an increase in cell proliferation, which widens the digit field (Summerbell, 1981). More recent studies have shown that tissue growth and digit specification are indeed separate events integrated through the action of Shh (Towers et al., 2008; Zhu et al., 2008). Thus Shh activity drives the widening of the limb progenitor field, and digits and interdigits form in an alternating series with constant spacing across the distal limb to the extent that there is space. Shh then independently acts to provide positional information, leading the digit primordia to take on distinct identities.

If not through positional information, how then does the repeated series of digits and interdigits emerge? A number of researchers over the last 30 years (e.g., Newman and Frisch, 1979; Miura and Shiota, 2000a) have suggested that the underlying mechanism could be a self-organized Turing pattern. In a canonical Turing pattern, interactions between diffusible activator and inhibitor molecules lead to the spontaneous emergence of periodic concentration profiles (Figure 1A) (Turing, 1952). The patterns generated can take the form of, for example, a periodic array of spots or a series of stripes. However, Turing systems can also give rise to much more complex patterns. Indeed, part of the early appeal of a Turing mechanism as possible explanation underlying the generation of digits was that the same sets of equations that could generate stripes resembling the periodic digit-interdigit patterns could also neatly mimic the mosaic cartilage condensation patterns seen when limb mesenchyme is plated at high density in vitro (Newman and Frisch, 1979; Miura and Shiota, 2000b). While initially proposed on a purely theoretical basis, without any reference to actual biological molecules that might act in the proposed Turing systems, in subsequent studies a number of activator/inhibitor pairs were suggested as candidates, e.g., transforming growth factor β (TGF-β)/Noggin (Miura and Shiota, 2000a; Zhu et al., 2010) and bone morphogenetic protein (BMP)/BMP receptor (Badugu et al., 2012). In addition, other mechanisms that are not strictly Turing networks, but are "Turing-like," have been used to model digit-interdigit patterning (Hiscock and Megason, 2015), including more complex molecular circuits (Raspopovic et al., 2014) as well as mechanical models involving cell movements and deformations of the extracellular matrix (Murray and Oster, 1984).

Stripes or Spots?

As appealing as such models were, until very recently it was unclear how exactly a Turing mechanism would be implemented in the limb. An important advance, in this respect, came from the observation of changes in the digital pattern of compound mouse mutants deficient for Gli3 (a transcriptional repressor that mediates Shh signaling in the limb), and also missing (to varying extents) copies of the distal Hox genes Hoxa13 and Hoxd11–Hoxd13. In this allelic series, decreasing levels of Hox activity in the Gli3 null background yield what appear to be progressively more and more densely packed digits (Figure 1B) (Sheth et al., 2012). This observation is consistent with a Turing-like mechanism in digit-interdigit patterning, a view reinforced by a computational Turing model in which the level of distal Hox genes modulates the wavelength of the digit-interdigit series (i.e., the distance between the digits) and, hence, their packing density. In this model, the digits are specified as a set of gene expression stripes oriented along the proximal-distal axis of the limb. To fit the observed pattern of digital condensation, the stripes must spread apart distally as the autopod grows, with digit rays fanning out radially. This requires the digit-interdigit wavelength to increase distally, which can again be explained by allowing the dose of distal Hox genes to regulate digit spacing.

This theoretical framework can be put in the context of what is known about how the digit cartilages actually form in the developing limb. If one observes the formation of the distal skeletal elements over time, one sees that they are initiated, in a dynamic sequence, as small dots of pre-chondrogenic condensations underneath the distal tip of the growing limb bud (Zhu et al., 2008) (Figure 1C). The digit primordia do not emerge all at once. Rather, they begin formation asynchronously and then each extends distally as the limb bud grows. For example, by the time the initiation of a digit 5 primordium is evident, the initial spot-like digit 2 and 4 primordia have already been elongated into rods (Figure 1C) (Raspopovic et al., 2014; Zhu et al., 2008). The initial pre-chondrogenic dots correspond to the start of digit organizing centers at the distal end of each growing digit ray that maintain Sox9 expression and drive the formation of rod-shaped digit elements. These organizing centers were independently described by Juan Hurle and John Fallon as domains of extremely high p-SMAD activity under the apical ectodermal ridge (AER), at the tip of the growing digit rays, where new chondrogenic progenitor cells are added to the growing skeletal elements, calling this domain the digital crescent (DC) or phalanx forming region (PFR), respectively (Montero et al., 2008; Suzuki et al., 2008). The PFR/DC is the location where digit rays are extended. It is also the location where digit identities (i.e., the spacing of the joints) are actualized (Suzuki et al., 2008).

As the hand plate grows outward, the PFR/DC organizing centers remain at the distal margin, "trailing" the growing digit rays of cartilage behind them, creating a stripe-like pattern (Figure 1D). But, importantly, the digit rays are not formed de novo as stripes. An implication of this is that there is no need to think of an expanding wavelength (establishing greater spacing between the digits) during outward growth, through graded Hox expression (for which there is no evidence) and/or through changes in FGF activity as the autopod grows (an idea that is contradicted by old heterochronic transplants of the AER, which have no effect on limb pattern). Rather, the spreading apart of the digit rays can be seen as simply a passive consequence of the broadening of the distal margin while the number of organizing centers remains constant (Figure 1D). This view also explains, in a trivial fashion, the observation that in some mutants (such as Gli3$^{-/-}$, HoxA$^{-/-}$, and HoxD$^{+/-}$) the forming digit rays are closer together in the anterior than in the posterior (see Figure 5 in Sheth et al., 2013). Instead of requiring some new parameter to differentially affect wavelength along the anterior-posterior axis, one simply needs to invoke some degree of differential growth in the posterior, as the autopod extends distally.

Given these prior observations, it seems that the interpretations of the compound mutant phenotypes in Sheth et al. (2012) can be reconsidered. First, it is important to note that when one compares the spacing of the distal skeletal elements of the Hoxa13$^{+/-}$, Hoxd11–13$^{-/-}$, Gli3$^{-/-}$ mutant with those found in the wild-type, they are indeed much more closely spaced than the wild-type digits but, on the other hand, there does not appear to be an obvious change in wavelength relative to the base of the wild-type metacarpals, where the digit rays originate (Figure 1B, compare blue and red boxes). This visual impression is substantiated by a closer examination of the quantitative measurement of digit spacing in the various Hox/Gli3 mutants provided by Sheth et al. (2012) in their supplement. They measured the spacing between digit rays in each mutant background, at four different proximodistal levels. However, if

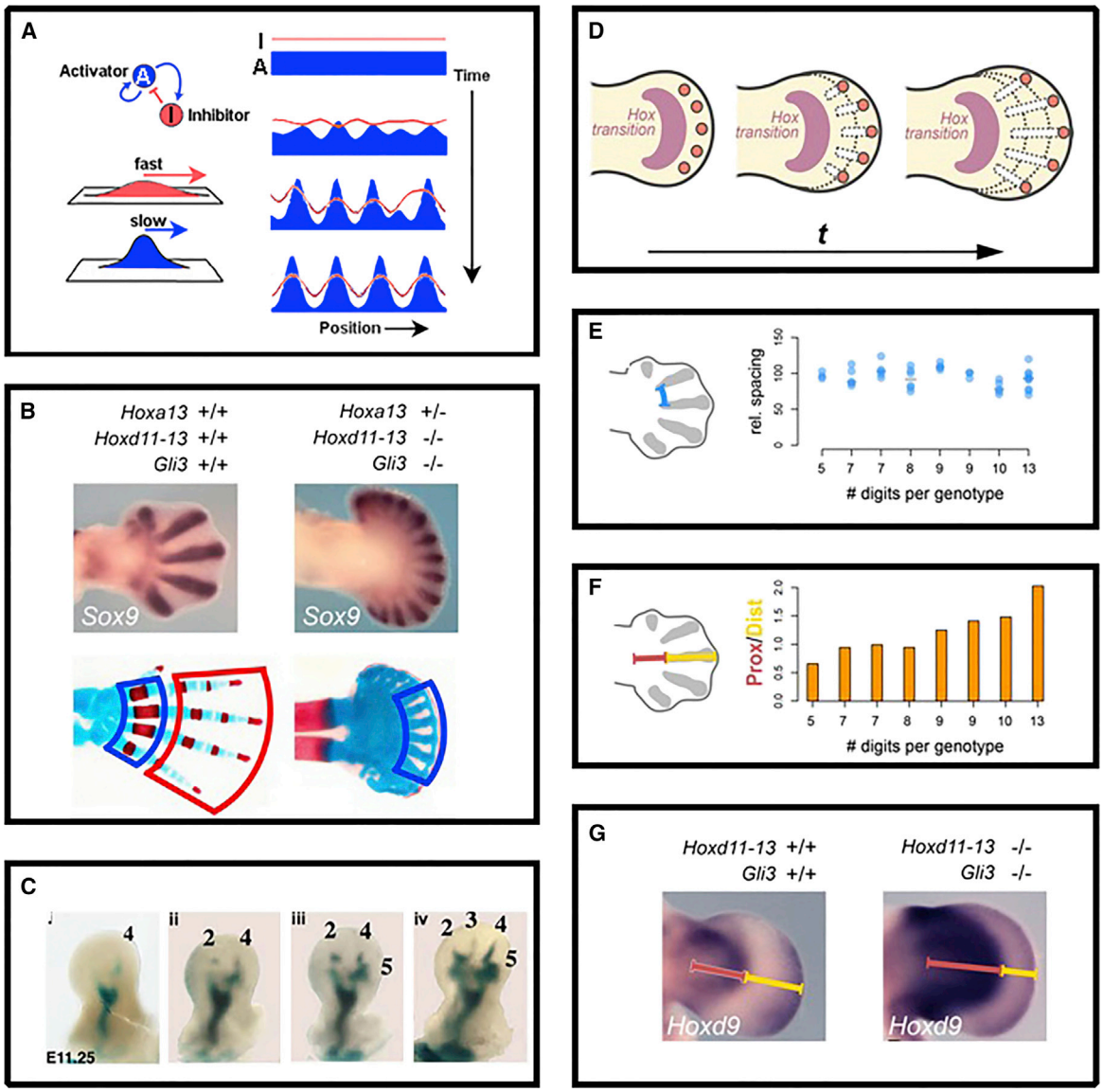

Figure 1. Digits Initiate as Regularly Spaced Spots

(A) Digit formation has been modeled on the basis of Turing-like mechanisms. The classical Turing formulation is of a two-component reaction-diffusion system involving an activator (A, blue) and an inhibitor (I, red). A autoactivates itself as well as activating I, while I inhibits A. In addition, I diffuses faster than A. In this setting, slight fluctuations in initial conditions trigger self-reinforcing interactions, ultimately leading to a stable state of alternating spatial domains of activator and inhibitor activity.

(B) Sox9 in situ hybridization and skeletal preparations of wild-type and compound mutant forelimb autopods (reprinted with permission from Sheth et al., 2012).

(C) Initiation of metacarpals/digit rays as dots of pre-cartilaginous condensations (reprinted with permission from Zhu et al., 2008).

(D) Initiation of digit organizing centers (red dots; PFR/DC, see main text), distal to the Hox transition zone and just underneath the AER, and subsequent fanning out of digit rays due to autopod growth patterns. Growing cartilaginous digit rays are depicted in white. Note that this diagram illustrates the relationship between the location where organizing centers form relative to proximal Hox gene expression and the process by which the digit rays are laid down as the organizing centers are displaced distally. It does not incorporate the differences in the timing of condensation of the different digit organizing centers, shown in Figure 2A.

(E) Spacing of developing skeletal elements, measured at their proximal base, for the Gli3/Hoxa13/Hoxd11–13 allelic series as reported in Sheth et al. (2012). (Note: the values are those reported in Supplementary Figure 4 of Sheth et al., 2012 for digit spacing at level 1 [most proximal level in their analysis]. For numbers of independent samples measured and statistical validation, please refer to that article.) The most anterior and posterior positions were excluded from the analysis, due to the difficulty in obtaining reliable measurements for these elements due to curvature of the hand plate at the margins.

(F) Length ratios of the proximal, Sox9-negative domain (red) to the distally developing skeletal elements (yellow) in embryos of the same allelic series.

(G) Distal shift in Hoxd9 expression boundary in compound mutant forelimb autopods (adapted with permission from Sheth et al., 2007).

one plots their measured values taken at the base of each digit ray where the dot-like condensations first form, it is clear that spacing is indeed invariant regardless of genetic background in this allelic series (Figure 1E).

If the wavelength is not changing, how can we explain the phenotype? What clearly *is* changing is that, in the mutants, the base of the metacarpal condensations is initiated at more distal locations within the hand plate (Figures 1B and 1F). By

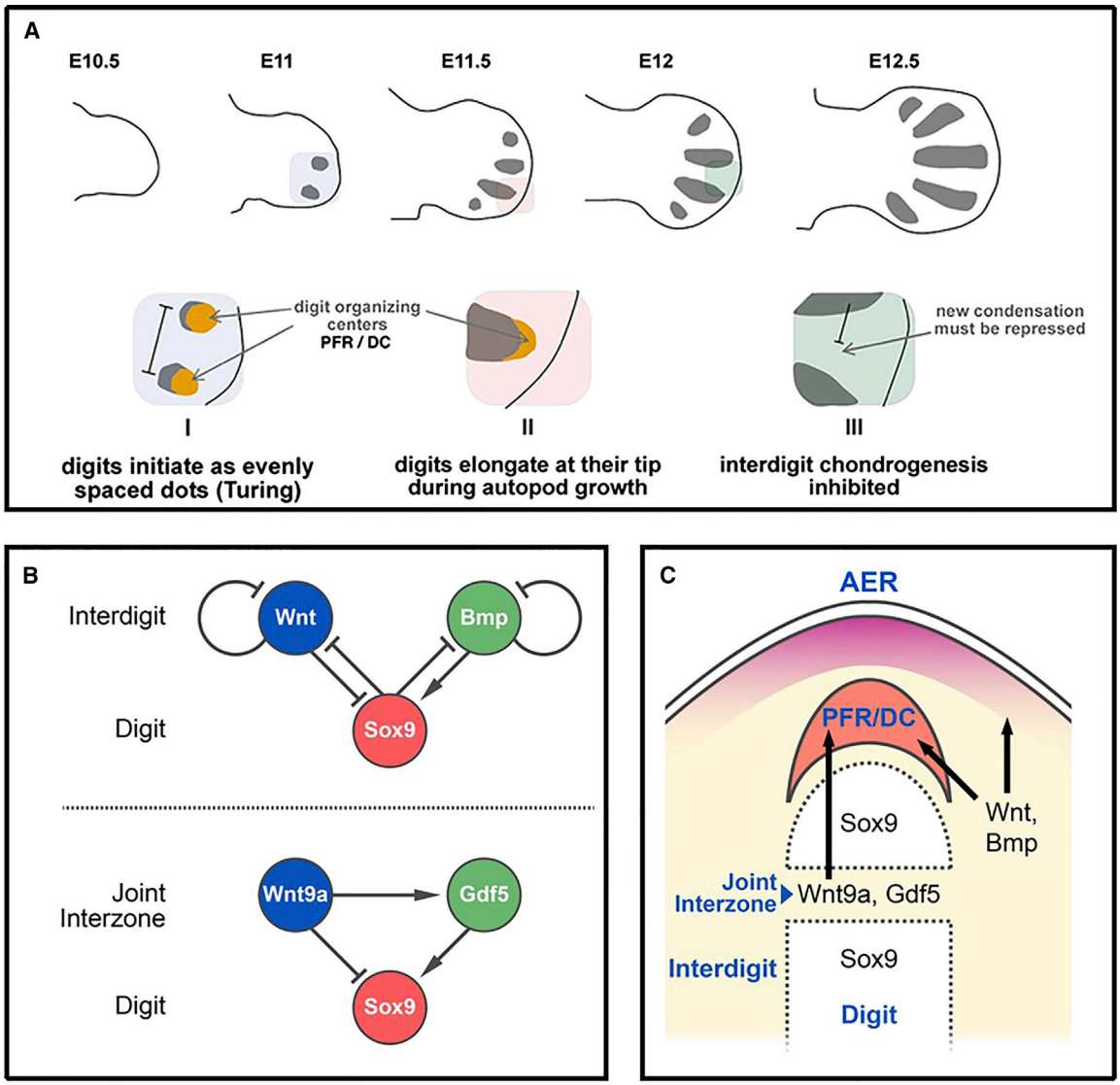

Figure 2. Molecular Similarities in Digit/Non-digit and Digit/Joint Fate Specifications
(A) Sequential initiation of what ultimately became a series of evenly spaced digit organizing centers establishes the pattern of the digit rays.
(B) Regulatory interactions between signaling molecules involved in the establishment of digit versus non-digit fate decisions (top, adapted with permission from Raspopovic et al., 2014), and digit versus joint interzone fate decisions (bottom).
(C) Integration of a complex array of signaling interactions occurs at the phalanx forming region (PFR) or digital crescent (DC), to concomitantly drive fate decisions of digit versus interdigit, as well as digit versus joint cell fate during autopod outgrowth.

initiating the formation of these skeletal elements, with similar wavelength, but at a more distal location where the developing hand plate is broader, more elements are formed.

The shift in location of the presumptive base of the metacarpals correlates with a distal shift of the boundary of proximal Hox genes, such as Hoxd9, within the limb bud (Sheth et al., 2007) (Figure 1G), a consequence of cross-regulatory relationships between the Hox genes themselves (Sheth et al., 2014; Beccari et al., 2016). It has previously been described that there are, in fact, two distinct phases of Hoxd expression in the limb: an early proximal phase 1 primarily influencing the formation of the zeugopod, and a later distal phase 2 that encompasses the autopod (Nelson et al., 1996; Tarchini and Duboule, 2006), with the mesopodium forming between the two domains (Woltering and Duboule, 2010). We would propose that the transition between phase 1 and phase 2 also allows for the initiation of digit organizing centers at what will become the base of the metacarpals, and that it is at this stage that the spacing of the future metacarpals is established, through a Turing-type mechanism. As the organizing centers move farther away from each other, due to expansion of the distal margin, there is a continual need to prevent any additional organizing centers from being formed at the distal margin between the existing condensations.

The emerging picture of digit patterning involves three processes (Figure 2A): (1) sequential initiation of what come to be evenly spaced pre-chondrogenic dots that will form digit organizing centers, likely established through a Turing-like mechanism; (2) maintenance and elongation of these dots into

digit rays as the autopod extends; and (3) inhibition of chondrogenesis in the distal interdigit region to prevent additional organizing centers from forming as the distal margin broadens. The importance of considering this alternative view is that it reframes the questions that need to be probed experimentally to further understand the process of digit specification. If a Turing-like mechanism sets up a series of discrete digit organizing centers, with an invariant wavelength, at the transition from phase 1 to phase 2 Hox expression, one needs to understand how that wavelength is fixed and why organizing centers only form distal to the phase 1 Hox domains.

Molecular Underpinnings

Candidate molecules involved in controlling the spacing of organizing centers were identified in a subsequent paper (Raspopovic et al., 2014). In this study, Bmp and Wnt signaling were shown to be active in a periodic pattern corresponding to digit and interdigit regions, respectively. According to the model proposed, Wnt activity inhibits chondrogenesis (and inhibits expression of the chondrogenic transcription factor Sox9), specifying the interdigit; while Bmp activity promotes chondrogenesis (and induces Sox9) outside the range of Wnt signaling, thereby specifying the digits. Sox9 represses both Wnt and Bmp production within the digit itself, forming the basis for a three-node Turing model (Figure 2B) (Raspopovic et al., 2014). While the simulations in this paper generated two-dimensional stripes of chondrogenic digit domains, a Turing mechanism based on the same molecular interactions can easily be tweaked to yield spots (representing the formation of digit organizing centers as argued above). Indeed, the Sox9-Bmp-Wnt-based Turing mechanism was recently modified to model the initiation of the series of round condensations that form at the base of the catshark pectoral fin (Onimaru et al., 2016). We note, however, that there are many other hypothetical Turing-like mechanisms that could generate these spot-like patterns, and more work is required to understand exactly how the digit patterns are initiated. Indeed while the epistatic relationships between Bmp, Wnt, and Sox9 activity are sufficient to generate an in silico pattern reminiscent of that found in the distal limb, it is likely a simplification of the endogenous regulatory mechanisms involved. Other signaling systems such as TGF-β and non-canonical Wnt/planar cell polarity (among others) are very likely to play roles as well.

As noted above, it is important to prevent the initiation of additional PFR/DC digit organizers as the autopod expands. One plausible candidate could be secreted Wnts, which are known to act as anti-chondrogenic factors in the limb (Hartmann and Tabin, 2001; ten Berge et al., 2008) and are a key interdigit-promoting component of the Turing-like model proposed for digit specification by Raspopovic et al. (2014).

While additional digit organizing centers, and hence additional digit rays, do not normally form once the organizing centers are established at the base of the metacarpals, the interdigital mesenchyme does maintain the ability to form ectopic digits when experimentally challenged. Digit rays can be bifurcated via application of ectopic Bmp delivered in a bead inserted at the tip of the growing digit (Ganan et al., 1996; Duprez et al., 1996). In the context of what we know about the formation of digit rays described above, this would reflect a splitting of an organizing center such that a single cartilaginous ray is laid down proximally before the splitting event, bifurcating into two extending branches once the organizing center is split. Fully independent ectopic digits can be induced between the endogenous digit rays by the removal of the overlying ectoderm, application of ectopic TGF-β, or delivery of retinoic acid antagonists (Hurle and Ganan, 1987; Ganan et al., 1996; Macias et al., 1993). Notably, in these studies, the ectopic digit is initiated as a single spot, just distal to the bead (e.g., carrying TGF-β) implanted into the distal margin of the interdigit and then extends to form a digit-like structure as the autopod grows distally (Lorda-Diez et al., 2011); i.e., these procedures induce the formation of an ectopic digit organizing center in the interdigit mesenchyme, which then acts like the endogenous organizing centers during subsequent growth of the hand plate. These data provide an additional test of the model presented here. If digits were to form as Turing-generated stripes, rather than dots, intercalating a new stripe between two existing digit rays should alter the distribution of activators and inhibitors, leading to a bending or distortion of the neighboring, endogenous digit rays. In contrast, if the spacing of the digit rays is established by dot-like organizing centers, and digits are then passively extended and fanned apart by subsequent growth of the hand plate, then inserting an additional digit organizing center at a later stage should have no effect on the growth of the endogenous digits. This is indeed what is observed.

Integrating Joint Formation into the Picture

There are intriguing parallels between the periodic patterning of the digits—interdigits and the periodic patterning of the phalanges (i.e., digit bones) and the synovial joints connecting them to one another. First, similar signaling molecules have been implicated in both processes. As discussed above, Bmp activity is produced by the non-chrondrogenic interdigit and is an activator of chondrogenesis in the establishment of the digit rays. Bmp signaling also acts to specify digit identity, a fundamental property of which is the spacing of the joints (Suzuki et al., 2008). That this Bmp activity originates in the interdigit has been shown genetically in the mouse (Huang et al., 2016), where it is modulated through Hoxd-Gli3 antagonism. Moreover, GDF5, a divergent member of the Bmp superfamily, is a very early marker of the non-chondrogenic future joint cells (Ray et al., 2015), and has been shown to induce chondrogenesis both in vitro and in vivo (Francis-West et al., 1999; Storm and Kingsley, 1999). Wnt activity is also present in the non-chondrogenic interdigit domains. Like GDF5, Wnt9a is an early marker of joint formation, and Wnt9a activity has been shown to downregulate chondrogenesis and induce the joint-forming program both in vivo and in vitro (Hartmann and Tabin, 2001). Thus, the network of signaling molecules described as being critical for digit-interdigit determination and the signals implicated in establishing the distinction between joint interzone and digit show striking parallels in their epistasis (Figure 2B), a similarity also pointed out by Cooper (2015).

In addition to the involvement of similar signaling pathways in joint initiation as in digit-interdigit formation, there is at least suggestive evidence that the periodic establishment of joints may also utilize a self-organizing patterning process, potentially even a Turing-like mechanism. For example, inducing an ectopic joint within a growing digit ray leads to the repression of the

formation of the neighboring endogenous joints, indicating the action of a joint inhibitor/pro-chondrogenic factor produced by the early joint interzone (Hartmann and Tabin, 2001). In the context of the PFR/DC, this can be seen as feedback from a newly formed joint, preventing cells leaving the PFR/DC from becoming joints themselves and instead directing them to join the subjacent growing cartilage segment. There is also evidence for joint-inducing, anti-chondrogenic factors being produced by the digit ray that affect the fate of cells differentiating at the PFR/DC. For example, when an impermeable barrier is placed through a newly formed joint, blocking its signals from feeding back to the PFR/DC, the next formed phalanx is longer (Kavanagh et al., 2013). This suggests there is also a joint-promoting activity emanating from each newly formed joint acting on the PFR/DC. Taken together, these indications of both activating and inhibitory signals are at least consistent with a self-organizing mechanism for the initiation of joint formation. However, an added complication is that each successive phalanx is shorter, following a constrained set of proportions (Kavanagh et al., 2013), rather than being all identical in spacing as are the digit rays.

Joint formation and digit interdigit specification are thus both tied to signaling events integrated at the PFR/DC. Moreover, it is important to note that the decision of cells to become part of the digit ray or interdigit, and the decision of those entering the digit ray to become phalanx or joint, are happening at the same time and place, both occurring at the distal margin of the growing hand plate. For example, GDF5, an early marker for joint formation, is initiated at the very tip of the growing digit ray (see, for example, Figure 4M in Ray et al., 2015). Moreover, regulation of joint specification by Hoxd-Gli3 antagonism and Bmp signaling occurs at the distal tip of the digit, i.e., the PFR/DC (Huang et al., 2016). This consideration brings us back full circle to the study by Sheth et al. (2012) which demonstrated that Hoxd-Gli3 interactions also influence the number of digit rays that form, or, as argued here, the proximodistal level at which the dots of condensation initiating the digit rays first form. Thus, it becomes inescapable that digit-interdigit and phalanx-joint specification are not separable processes. This suggests the need for an integrated model, incorporating the known signals described above (Figure 2C) and additional factors, in a common network determining cell fate and working together to specify where skeletal tissues form in the developing autopod.

Conclusions

The cartilage condensations that are the first sign of digit formation arise as dots at what will become the base of the metacarpals. This is well established. Once the digit rays are initiated, no further condensations are intercalated between them (no new PFR/DC organizing centers are established at the distal margin). The rays passively fan apart from each other as they grow, with a spread determined by the broadening of the hand plate itself. During this process, the digit rays are continually lengthened through terminal addition of cells at the PFR/DC organizing centers.

The idea the digit/joint/interdigit decisions are made at the tip of the growing elements is certainly not new, having been previously described and explored in Suzuki et al. (2008), Montero et al. (2008), and elsewhere. However, placing the proposed Turing-like mechanisms for digit formation into this context, compels one to think in terms of spots and not stripes in the specification of digits, and it affects the way we need to conceptualize the process: future mechanistic studies should refocus from the mesenchyme of a paddle-shaped hand plate to the action at the tip of the extending digit (Figure 2A). In a first step, the spots are sequentially laid down in an alternating pattern of pro-chondrogenic PFR/DC digit organizing centers and interdigits, the timing and spatial aspects of this being controlled through a Turing-like mechanism and, in a yet undetermined manner, through the transition in phases of Hox gene expression. This is followed by a maintenance phase during outgrowth of the autopod, whereby the PFR/DCs remain at the distal edge of the hand plate below the AER, trailing digit rays of cartilage behind them as they extend. During this phase, inhibitory interactions prevent the induction of any additional DC/PFRs at the distal margin, between those that have already formed, even as the original set of digit organizing centers grow apart to an extent that their spacing exceeds the original wavelength at which they were specified. The process of extending the digit rays from the PFR/DCs, while blocking induction of new PFR/DCs in between them, is integrated with the process of sequential and alternating specification of phalanges and joints, in response to the signaling networks at play at the distal edge of the limb bud.

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