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Fgf Signaling Controls the Number of Phalanges and Tip Formation in Developing Digits

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Summary

Tetrapods have two pairs of limbs, each typically with five digits, each of which has a defined number of phalanges derived from an archetypal formula [1]. Much progress has been made in understanding vertebrate limb initiation and the patterning processes that determine digit number in developing limb buds, but little is known about how phalange number is controlled. We and others previously showed that an additional phalange can be induced in a chick toe if sonic hedgehog protein is applied in between developing digit primordia [2, 3]. Here we show that formation of an additional phalange is associated with prolonged *Fgf8* expression in the overlying apical ridge and that an Fgf Receptor inhibitor blocks its formation. The additional phalange is produced by elongation and segmentation of the penultimate phalange, suggesting that the digit tip forms when Fgf signaling ceases by a special mechanism, possibly involving Wnt signaling. Consistent with this, Fgfs inhibit tip formation whereas attenuation of Fgf signaling induces tip formation prematurely. We propose that duration of Fgf signaling from the ridge, responsible for elongation of digit primordia, coupled with a characteristic periodicity of joint formation, generates the appropriate number of phalanges in each digit. We also propose that the process that generates the digit tips is independent of that which generates more proximal phalanges. This has implications for understanding human limb congenital malformations and evolution of digit diversity.

Results and Discussion

The skeleton of each individual digit has a characteristic morphology in terms of length, number, and shape of phalanges, but the molecular and cellular bases of how this is achieved, including the process of making a digit tip once pattern formation is complete, are not understood. Individual digits are set up as digit primordia, condensations of chondrogenic cells separated by interdigital spaces containing cells destined to die. These primordia elongate and then segment to form phalanges with intervening joints. Digit development is finally completed by formation of a terminal phalange that carries ectodermal derivatives such as nails or claws. When Shh beads are implanted in interdigital spaces between digit primordia at stages prior to segmentation (Figures 1A and 1B), several different effects can be produced in a dose- and stage-dependent manner. These include digit truncations, soft-tissue fusions, and fusions between the tips of adjacent digits [3] but, more strikingly, formation of elongated digits with an extra phalange [2, 3]. In a previous report [2], Bmp signaling was implicated in this latter effect, but no molecular mechanism was proposed. Here we demonstrate that Fgfs are involved and propose a mechanism for controlling phalange number.

The first clue that Fgf signaling could be involved was the observation that Shh beads placed far away from the apical ridge did not produce elongated digits with an extra phalange. The apical ectodermal ridge rims the entire limb bud at digit primordia stages and is known to mediate limb bud outgrowth via production of Fgf8 at these stages. When Shh beads were placed near the ectoderm between digit primordia, all toes could be induced to grow longer and form an extra joint (Figures 1D–1F, arrows). In contrast, in wing buds only digit 2 increased in length and formed an extra phalange, resulting in a triphalangeal thumb (Figure 1C, arrow); neither digit 3 nor digit 4 of the wing was ever significantly elongated, although the reason for this is not clear. Application of 4 mg/ml Shh in the first interdigital space in wings or the second interdigital space in legs at stages 27 and 28 was optimal in inducing elongation and formation of extra phalanges in nearby digit 2 and toe 2, respectively (Figures 1G and 1H). Analysis of *Fgf8* expression, 40–50 hr after Shh bead implantation, showed that *Fgf8* expression persisted in the ridge over digit 2 and toe 2 in treated limbs, whereas in the ridge over the same digits in contralateral limbs, expression had already disappeared ($n = 12/12$, legs; $n = 4/4$, wings, Figures 2A and 2B). Consistent with persistence of *Fgf8* expression, we also observed maintenance of a tall ridge over elongating digits and increased expression of *Msx1*, a gene expressed in undifferentiated mesenchymal cells under the influence of Fgf signals ($n = 4$).

To directly test involvement of Fgf signaling in Shh-induced digit elongation, we coimplanted Shh beads and beads soaked in Fgf receptor inhibitor SU5402 [4] in the first interdigital space in wings and the second interdigital space in legs at stage 28 and compared results with those obtained with coapplication of Shh beads and DMSO control beads. Elongation of digits was induced by Shh coimplanted with control beads in most cases (9/11, legs; 1/1, wings; Figures 2E and 2G), but when beads soaked in SU5402 inhibitor were coimplanted, most digits (9/12, legs; 1/1, wings) were not elongated (Figures 2F and 2H, respectively). These results suggest that sustained Fgf signaling from the ridge is responsible for continued elongation and segmentation of digit primordia. Indeed, when Fgf8-soaked beads

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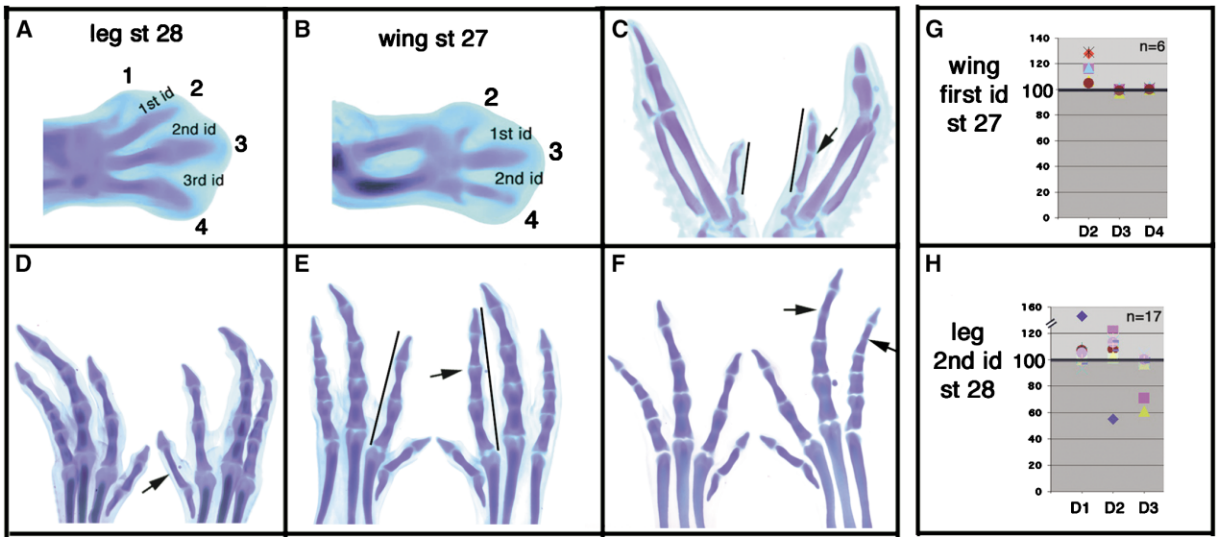

Figure 1. Application of Shh to Interdigital Spaces Induces Digit Elongations

(A and B) Alcian Green-stained legs and wings showing digit primordia at the time of operations (stages 27–28 [32]). "id" indicates interdigital spaces where Affigel Blue beads (soaked in the indicated dose of Shh) were implanted, according to [3]. Numbers indicate the identity of digits from anterior to posterior.

(C–F) Alcian Green-stained limbs at stage 36, five days after implantation of Shh beads. (C) Triphalangeal digit 2 (arrow) obtained in the wing after application of 14 mg/ml Shh to the second interdigital space at stage 28. Lines indicate measurements for (G). (D–F) Elongation of all toes can be obtained (arrows). (D) Shh (4 mg/ml) at stage 29 in the first interdigital space; elongated toe 1. (E) Shh (4 mg/ml) at stage 28 in the second interdigital space; elongated toe 2; note formation of a complete new joint. Lines indicate measurements for Figure 1H. (F) Shh (4 mg/ml) at stage 27 in the third interdigital space; elongated toes 3 and 4; note extra joints. (G and H). Scatter plots of the relative length (as a percentage) of phalanges (base of digit to tip, see Figures 1C and 1E) from operated versus control digits 5 days after implantation of Shh beads (4 mg/ml) to leg or wing buds at the position and stage indicated. Each symbol represents an individual experiment (three digits measured per limb; D1, digit 1; D2, digit 2; D3, digit 3; D4, digit 4). n = number of cases. Symbols above the line indicate elongated digits; symbols below the line indicate truncated digits.

were implanted at the tip of toe 2 or 3 at stage 27/28, digit elongation could be seen in nearby toes in 31% of the cases (n = 19).

Previous work reported that *Fgf8* expression in the apical ridge of chick leg buds switches off sequentially, first over primordia of more anterior toes and later over posterior toes [5]. We examined *Fgf8* expression in the apical ridge of chick wing buds and showed that this switches off first over digit 4, then over digit 2, and slightly later over digit 3 (Figure 2C). Thus, in both legs and wings, timing of *Fgf8* expression correlates with the number of phalanges in individual digits (*Fgf8* switching off later over digits with more phalanges), suggesting that, in normal development, duration of Fgf signaling is an important determinant of phalange number.

The endogenous signal(s) that determines duration of *Fgf8* expression in the apical ridge is unknown, but heterochronic recombination experiments [6] suggest a mesenchymal origin. In our experiments, application of Shh mimics this signal, suggesting that hedgehog signaling might be involved. Although *Shh* is not expressed at these stages near digits [2], *Ihh*, another member of the hedgehog family, is expressed during cartilage differentiation later in digit development and also in digit tips in the mouse [7]. Furthermore, mutations in *Ihh* underlie human brachydactyly type A [8], in which phalange number is reduced. Previous work [2] has suggested that the digit elongation/extra phalanges induced by Shh application could be mediated by Bmps; because Shh beads enhance Bmp expression in interdigital spaces, induction of an extra phalange can be blocked by coimplantation of Noggin (a Bmp antagonist) with Shh, and application of Noggin alone leads to a reduced number of phalanges. All these data would implicate Bmps as positive regulators of phalange number. However, it should be noted that Noggin is also known to interfere with chondrogenesis, and the cellular mechanism responsible for blocking the extra phalange has not been reported [9]. Moreover, Noggin misexpression in ectoderm leads to persistence of *Fgf8* expression in the ridge [10], and there is evidence of a direct negative effect of Bmps on *Fgf8* expression in the ridge at late stages [5]. This taken in conjunction with our present results would suggest that Bmps act as negative regulators of phalange number. We have confirmed that Shh beads affect interdigital *Bmp* expression, but they do so in a way similar to that in anterior mesenchyme of early limb buds [11]. After Shh application in interdigital spaces, expression of *Bmp2* (n = 7/7) and *Bmp7* (n = 7/13) was increased 16–28 hr later, in accordance with previous reports [2], but *Bmp4* expression was reduced (n = 28/32, Figure 2D). This result could explain the decrease in cell death and syndactyly observed after Shh application [3], and given the negative effect postulated for Bmps in ridge maintenance, the reduction in *Bmp4* expression could be linked to the prolonged expression of *Fgf8* in the ridge over digital primordia. It is also possible that Bmp signaling regulates *Fgf* expression in the ridge through a Bmp antagonist, and this could be controlled by Ihh. When all of these results are

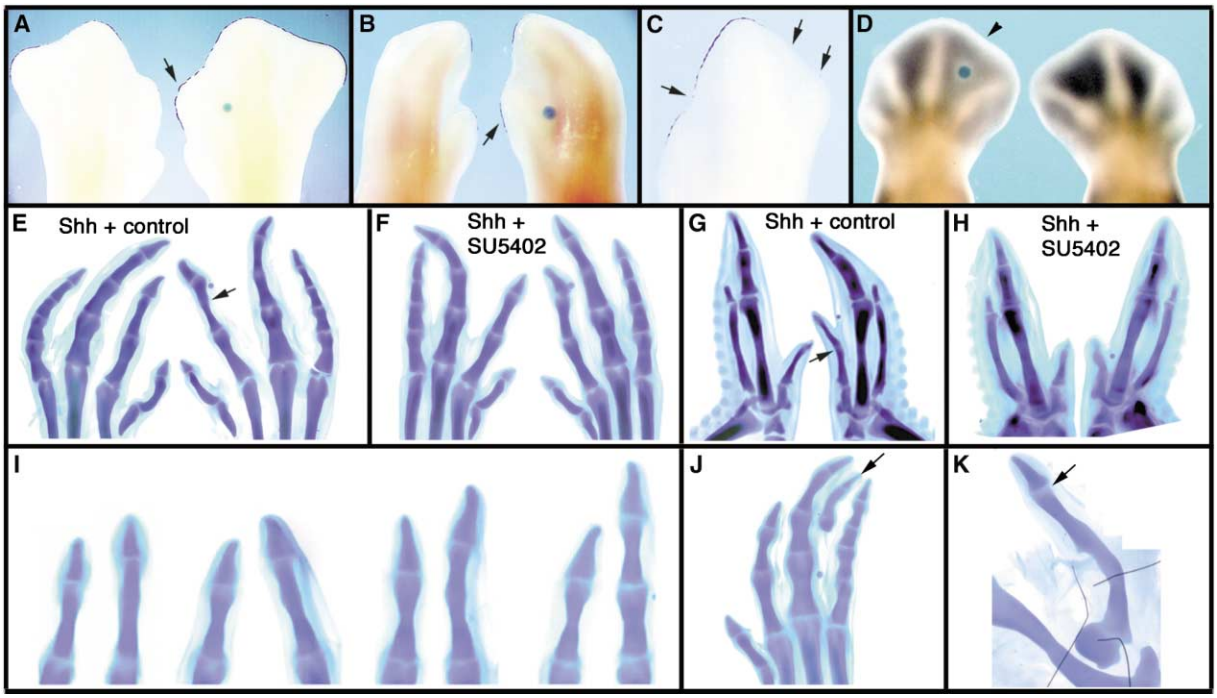

Figure 2. Sustained Fgf Signaling from the Apical Ridge Is Responsible for Digit Elongation and Segmentation of the Penultimate Phalange

(A and B) In situ hybridization showing persistence of *Fgf8* expression in the ridge ([A] leg, [B] wing, arrows) 42 hr after application of 4 mg/ml Shh beads to the first interdigital space in wings and the second interdigital space in legs, as compared with contralateral limbs, where expression has already disappeared.

(C) *Fgf8* expression in the apical ridge of wing buds at stage 32–33. Note its disappearance from the tip of digit 4 and the interdigital spaces (arrows). Expression persists over digits 2 and 3 up to stage 34–35.

(D) Twenty-two hours after Shh bead (4 mg/ml) implantation in the third interdigital space of leg at stage 27, *Bmp4* expression is reduced in the interdigital space (arrowhead).

(E–H) Digit elongations induced by Shh are blocked by Fgf receptor inhibitor. Control DMSO- (E and G) or SU5402 (AG1X2 beads, 1 mg/ml [F and H])-soaked beads were coimplanted with Shh beads (Affigel Blue beads, 4 mg/ml) in leg (E and F) or wing (G and H). Extra growth and joint formation occurred in (E) and (G) (arrows) but not in the presence of inhibitor (F and H).

(I) Different examples of digit elongations obtained after Shh bead application to leg buds. Pairs of tips and penultimate phalanges of Alcian Green-stained digits, control (left); operated (right). Note differing extent of elongation and steps in new joint formation. From left to right: elongation with no sign of joint; central swelling; incomplete segmentation; and complete new joint.

(J and K) Tip of digits develops independently of proximal phalanges. (J) Extra digit (arrow) induced by application of Shh bead. Note the presence of a normal last joint and tip. (K) Digit primordia of stage 27 toe 3 grafted in isolation to anterior stage 20 wing bud; an arrow marks the last joint; note the absence of other interphalangeal joints. This occured in 80% of cases (n = 15).

taken together, they suggest a signaling cascade (Figure 3A) similar to that in the posterior limb bud, in which Shh signaling (in digits likely to be Ihh) leads to expression of Bmps and either directly or indirectly to Bmp antagonists; Bmp antagonists then block Bmp signaling and hence maintain *Fgf8* expression in the ridge rimming digit primordia. Local and/or temporal regulation of *Bmps* and antagonist expression, specific effects of particular Bmps, or even different functions of Bmps in mesoderm versus ectoderm [12] could fine-tune Bmp signaling and could explain all the different effects observed, depending on signaling overcoming inhibition or preponderance of antagonist activity.

An unexpected feature of the elongated digits induced by Shh application is that it is always the penultimate phalange that segments to give the extra phalange. Elongation of more proximal phalanges was not observed, even when Shh beads were implanted at earlier stages (stages 25 or 26) or more proximally (n = 8, legs; n = 8, wings). In some cases, elongation of the penultimate phalange occurred without segmentation, and phalanges of different lengths and in varying stages of joint formation, such as thickenings or incomplete segmentation, were observed (Figure 2I). Thus, formation of a new joint may involve a threshold mechanism related to condensation length, and below a certain length, no segmentation occurs. An additional joint was frequently seen in toe 4 when the penultimate phalange was 15%–20% longer than the corresponding phalange in the same toe of the contralateral leg; in toe 3, when 25%–30% longer; in toe 2, 35%-40% longer; and in toe 1, 45%–50% longer. This correlates with the periodicity of segmentation in different toes in chick legs, with segmentation occurring at shorter intervals in posterior (toe 4) compared to anterior (toe 1) toes. The molecular mechanism underlying this characteristic periodicity for each digit is not known but is presumably determined by positional values previously established during limb bud patterning and probably involves both Wnt and Bmp signaling pathways [9]. Thus, phalange number depends

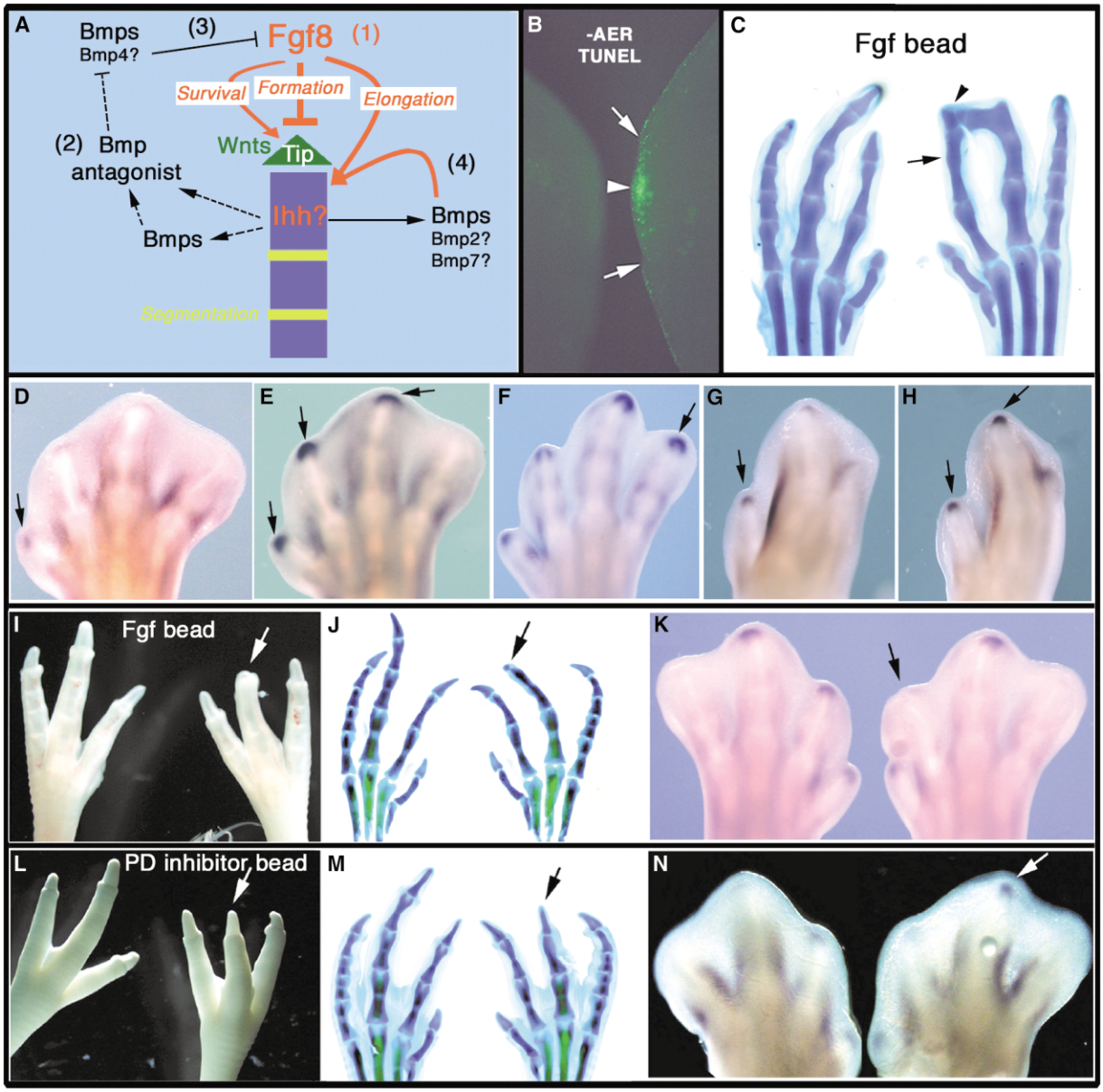

Figure 3. Fgf Signaling Controls Elongation of Digits and Tip Formation

(A) Model for digit formation and regulation by Fgf signaling (see also text). Fgf signaling (red, 1) is required for digit elongation and survival of presumptive tip cells but blocks tip formation. Digit primordia (blue), under the influence of Fgfs from the apical ridge, elongate and segment to form joints (yellow). When Fgf expression is extinguished in the ridge, the tip formation program, possibly involving Wnt signaling, is activated (green). Bmps and probably Bmp antagonists (2), induced directly or indirectly downstream of Hedgehog signaling (likely Ihh), could regulate Fgf expression in the ridge (3) [10] and participate in the formation of new phalanges (4) [2]. Dashed lines indicate postulated interactions.

(B) Six hours after apical ectodermal ridge removal from the tip of digit 3 in the leg, mesenchymal cell death is detected by TUNEL labeling in the distal part of the digit (arrowhead). Arrows mark the limits of the removed ridge. The control limb is on the left.

(C) An Fgf bead (Heparin bead soaked in 0.5 mg/ml Fgf8) inhibits tip formation (arrowhead) but induces distal digit fusion and elongation of the penultimate phalange (arrow).

(D–H) Expression of *Wnt14* at digit tips appears sequentially and correlates with the last joint/tip formation in the legs ([D] stage 30; [E] stage 32; [F] stage 34), and wings ([G] stage 33; [H] stage 34).

(I–K) Fgf application inhibits tip formation. (I) Fixed legs 5 days after bead implantation before staining; (J) Alcian Green cartilage staining of the same legs. (K) *Wnt14* expression in the tip (arrow) is reduced 40 hr after Fgf8 bead application.

(L–N) Inhibition of Fgf signaling by PD184352 (AG1X2 beads, 10 mM) induces premature tip formation. (L) Fixed whole mount; (M) Alcian Green staining of the same legs shows premature tip formation in digit 3, with only three phalanges (arrows). (N) Thirty-six hours after PD184352 bead implantation, *Wnt14* expression is prematurely activated (compare with the control digit on the left limb).

on duration of Fgf signaling and periodicity of joint formation. Modulation of duration of *Fgf* expression might be an important evolutionary factor leading to morphological diversity of limbs and explaining, for example, hyperphalangy of cetacean flippers [13].

Another striking finding was that the digit tip appeared to be normal in most cases, despite the penultimate phalange being longer or an extra phalange developing (Figure 2I). Extra digits induced in interdigital spaces by surgical manipulations [14] (Figure 2J) and digits resulting from grafts of isolated digit primordia (Figure 2K) also often have a normal tip, even though proximal elements are reduced. Many examples of digits with normal tips despite proximal alterations can be found in mouse mutants [15–19] and human conditions including brachydactyly types A and B [8, 20]. Also, ossification of the terminal phalange in the mouse precedes that of more proximal ones. Genes known to be expressed specifically at digit tips in mouse embryos include *Dlx5*, *Sa1I*, *Msal-3*, *dach* and *bambi* (another Bmp antagonist); *activin ßB* is expressed at the time of tip formation in chicks [21]. We found that *Wnt14*, a gene implicated in joint induction [9], is also expressed sequentially at digit tips in a pattern reciprocal to that of *Fgf8* (Figures 3D–3H).

These observations suggest a model (Figure 3A) in which Fgf signaling from the ridge promotes elongation of digit primordia and is required for survival of tip precursors while negatively regulating tip formation. When *Fgf8* expression switches off in the ridge, the tip program, which may involve *Wnt14* expression, would be implemented. To test this model, we removed the apical ridge overlying the tip of toe 3 at stage 27–28. After 6 hr, cell death was detected by TUNEL labeling in distal cells in the toe tip (n = 9/11; Figure 3B), confirming that ridge signals are required for survival of tip mesenchymal cells. Moreover, implantation of Fgf8-soaked beads at the tip of toe 2 or 3 at stage 27–28 blocked tip formation in 79% of cases (n = 19, Figures 3C, 3I, and 3J), although distal digit fusion and/or elongation of the penultimate phalange (21% and 31% of cases, respectively) also occurred (Figure 3C, arrowhead, arrow). Interestingly, in human conditions such as Apert syndrome, in which mutations in Fgf receptors lead to hyperactivation of the pathway [22], distal digit fusions occur, similar to phenotypes observed here. Furthermore, 48 hr after Fgf8 bead implantation, *Wnt14* tip expression was reduced in 86% of cases (n = 7, Figure 3K).

Another prediction is that attenuating Fgf signaling distally during digit primordia development should induce premature activation of *Wnt14* expression and tip formation. When beads soaked in MAPK pathway inhibitor PD184352 [23] were implanted in the tip of toe 3 or 4 at stage 27–28, *Wnt14* expression was induced or appeared to be proximally extended at 36–48 hours in 58% of cases (n = 24, Figure 3N). In 20% of cases, digit tips formed prematurely, giving shorter digits with a decreased number of phalanges or fused medial phalanges with no joints, but normal tips (n = 49, Figures 3L and 3M; in remaining cases digits were normal). Shorter digits with normal tips were also seen in 33% of cases after beads soaked in SU5402 were implanted in the third interdigital space of stage 27–28 legs (n = 15; in remaining cases, toes were severely truncated distally). All these results taken together suggest that Fgf signaling blocks tip formation during normal digit development. According to the model, high Fgf concentrations are required for elongation but inhibit tip formation, whereas lower Fgf concentrations allow survival of tip cells. Thus, truncation of digits (no terminal phalange) could be produced both by hyperactivation of Fgf signaling (inhibition of the tip) or activity that is too low (inhibition of elongation and induction of cell death), suggesting that in normal development Fgf signaling levels must be precisely regulated for correct digit morphogenesis to occur. Remarkably, it has recently been proposed that a gradient of EGF receptor activity directs patterning of the tarsus, a segmented distal structure in *Drosophila* legs, independently of the distalmost claw [24, 25].

The fact that terminal phalanges are formed by a mechanism that is completely different from that which generates proximal phalanges has not been widely appreciated, despite many observations suggesting that this is so. We have provided experimental evidence here reinforcing the idea that there is a special program for making a digit tip. The fact that members of the Wnt signaling pathway, such as Wnt5a [26], their receptors Frizzleds (e.g., Fz4 [27]), and secreted inhibitors (e.g., FrzB [28]) are expressed at digit tips suggests that Wnt signaling is part of this program, and our results suggest that Wnt14 may be involved. Moreover, transgenic mice expressing an inhibitor of Wnt signaling (Dikkopf) in the skin [29] lack nails, consistent with Wnt-Wnt antagonist expression in digit tips being related to nail induction. Interestingly, regeneration of limbs in higher vertebrates is confined to digit tips [30, 31]. Therefore, understanding the molecular basis of tip formation could lead to development of new strategies to enhance the regenerative ability of limbs. Finally, similar mechanisms could also operate in the development of other appendages where a distal Fgf source directs outgrowth, such as genital tubercule, facial primordia, and tail. It is interesting to note that the tail tip also seems to express a special set of genes, suggesting an independent program for its formation.

Experimental Procedures

Surgical Manipulations

Fertilized chicken White Leghorn eggs were incubated at 38°C, windowed, and staged according to Hamburger and Hamilton [32]. Surgical manipulations were carried out with fine tungsten needles and forceps [3]. For delivering factors locally, microcarrier beads were used (Affigel Blue for Shh, Heparin-coated acrylic beads for Fgfs, formate-derivatized AG1X2 for inhibitors). Recombinant mouse Shh (14 mg/ml or 1–4 mg/ml, high or low dose, respectively), human Fgf4 (0.75 mg/ml), or mouse Fgf8 (0.5 mg/ml), all from R&D systems, were diluted in PBS-1 mg/ml BSA. SU5402 (Calbiochem) or PD1843542 (a gift from P. Cohen) was diluted in DMSO and used at 1 mg/ml or 10 mM, respectively [33]. Beads were soaked for 1 hr at room temperature and, in the case of inhibitors, rinsed in growth medium (MEM-25 mM HEPES) for 5 min before implantation. Control beads were soaked in PBS or DMSO. Beads were placed in interdigital spaces or tips of digit primordia. Embryos were reincubated for the required times. For grafting digit primordia, autopods were dissected from stage 27–28 leg buds, and condensing digital rays including the ectoderm and apical ridge were isolated with needles. Platinum wire was used for pinning these to wounds made

in flank or anterior wing buds of host stage 20 embryos. Tungsten needles were used for removing the apical ectodermal ridge from tips of digits 3 in stage 27–28 legs.

Alcian Green Staining, In Situ Hybridization, and Whole-Mount TUNEL

Embryos were fixed in 5% TCA (Tri-Chloro-Acetic Acid) for cartilage staining or 4% PFA (Paraformaldehyde) for whole-mount in situ hybridization and TUNEL. Alcian green staining was performed 5 days after operations (stages 35–36) as described [3]. In situ hybridization was done according to standard protocols [34], but instead of proteinase K digestion, limbs were subjected to permeabilization with 50% DMSO-50% Methanol and 30 min of 10% Triton X-100 treatment. For TUNEL labeling, the "In Situ Cell Death Detection Kit" (fluorescein) from Roche was used in a single-step labeling protocol, and fluorescein staining was visualized.

Measurements

Measurements were taken from digital pictures in Photoshop. The length of digits from the first phalange to tip, or the length of the penultimate phalange, was recorded, and the ratio (as a percentage) between the operated and contralateral control digits in each embryo was represented in scatter plots.

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