Initiation of Proximal-Distal Patterning in the Vertebrate Limb by Signals and Growth

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Two broad classes of models have been proposed to explain the patterning of the proximal-distal axis of the vertebrate limb (from the shoulder to the digit tips). Differentiating between them, we demonstrate that early limb mesenchyme in the chick is initially maintained in a state capable of generating all limb segments through exposure to a combination of proximal and distal signals. As the limb bud grows, the proximal limb is established through continued exposure to flank-derived signal(s), whereas the developmental program determining the medial and distal segments is initiated in domains that grow beyond proximal influence. In addition, the system we have developed, combining in vitro and in vivo culture, opens the door to a new level of analysis of patterning mechanisms in the limb.

The mechanisms that pattern the vertebrate limb mesenchyme so that the correct size, shape, and number of elements condense at precise locations have been argued in the literature for decades. Broadly, models of proximal-distal (PD) patterning can be divided into two general classes. One, exemplified by the progress zone model (1), posits that progressive distalization of limb pattern is based on an autonomous clocklike mechanism inherent to the undifferentiated mesenchymal cells. The second postulates that instructive cues from surrounding tissues are responsible for specifying the PD segments (2, 3). It has proven surprisingly difficult to differentiate between the autonomous and nonautonomous models experimentally. Here we try to address this issue by focusing on the establishment of the most proximal segment, the stylopod, as distinct from the more distal limb.

In the early vertebrate limb bud, mesenchymal cells encounter members of the fibroblast growth factor (FGF) family produced by the distal ectoderm and retinoic acid (RA) produced in the flank (2, 4). To clarify the roles these signals play in PD patterning, we have taken advantage of recently described conditions that allow limb bud cells to be maintained and manipulated in an undifferentiated state in vitro. When primary limb bud cells from Hamburger and Hamilton (5) stage 18 (HH18) chick embryos are cultured at high density, they quickly differentiate into chondrocytes (6). However, in the presence of Wnt3a and FGF8 proteins, both of which are normally secreted from the ectoderm, the cells remain proliferative and undifferentiated (7). As these

cells are cultured, they continue to express markers, such as Axin2, Dusp6, and Msx1, which are characteristic of undifferentiated limb mesenchyme (7). The expression of PD markers in these cultured cells has not been examined. Whereas none of the known segmental markers are themselves required for PD specification [see discussion in Tabin and Wolpert (3)], at later stages during development in vivo, Meis1, Hoxa11, and Hoxa13 domains are congruent with the eventual stylopod, zeugopod, and autopod limb segments, respectively. We used quantitative reverse transcription polymerase chain reaction (RT-PCR) to detect

these segmentally expressed limb markers in cells cultured in vitro (8). It has been proposed that cells falling out of range of distal signals in the limb bud become fixed in their PD pattern as they begin to differentiate—the so-called "differentiation front" (3). Consistent with this, dissociated primary distal HH18 mesenchymal cells expressing Meis1, but not Hoxa11 or Hoxa13, when first placed in culture with serum alone, maintained this profile at the onset of differentiation as Sox9 was up-regulated, before the formation of cartilage nodules. In contrast, we found that over time cells cultured with Wnt3a and FGF8 lost expression of the proximal marker, Meis1, and up-regulated expression of Hoxa11, a marker of the middle limb segment, followed by a distal marker, Hoxa13, an expression profile similar to distal cells of an intact limb bud (Fig. 1).

In vivo, early limb bud cells are also exposed to RA from the flank in addition to FGF and Wnt activity. RA was previously shown to induce Meis1 expression, and it has been proposed to act as a proximal patterning signal (2, 3). Although this endogenous role of RA has recently been challenged, at least in the developing mouse limb bud (9, 10), the chick results indicate that RA, at minimum, may mimic or share redundancy with additional factor(s). As such, exogenous RA may act as a proxy for endogenous factors with analogous proximalizing activity (2, 11). Therefore, we next added all-trans RA at physiological concentrations (12) to the cultures with Wnt3a and FGF8. When primary limb cells were cultured with all three factors and, hence, exposed to a
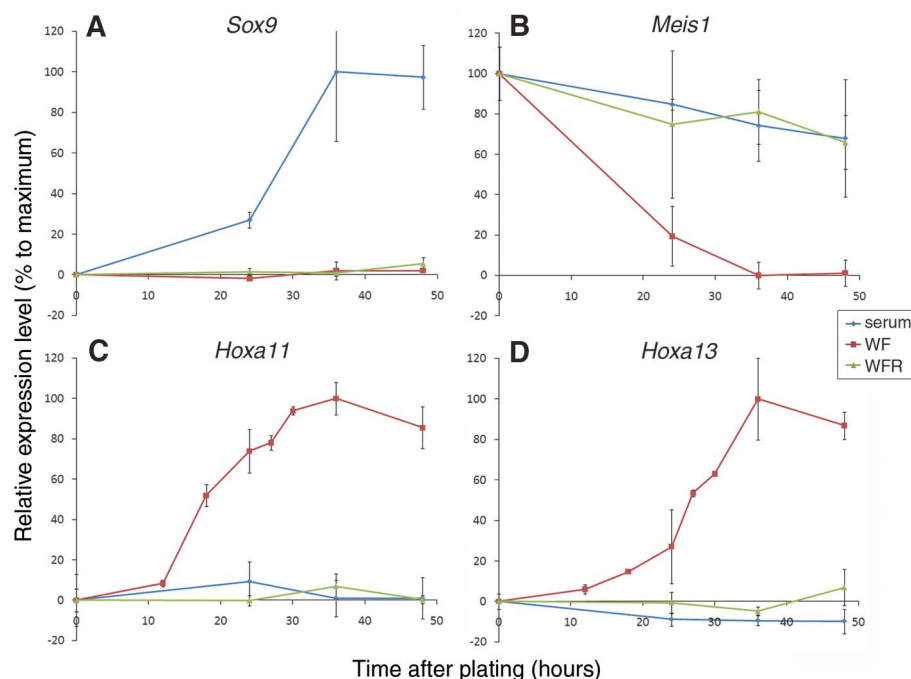
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Fig. 1. Wnt3a, FGF8, and RA act together to maintain markers of early limb mesenchyme in culture. Dissociated fresh HH18 distal limb bud cells were cultured with serum only (serum), serum + Wnt3a and FGF8 (WF), or serum + Wnt3a, FGF8, and RA (WFR) for increasing amounts of time. (**A**) Sox9, (**B**) Meis1, (**C**) Hoxa11, and (**D**) Hoxa13 expression levels were measured by quantitative PCR and normalized to β-actin expression.

signaling milieu comparable to what is seen by the early limb bud mesenchyme, *Meis1* expression was maintained, and *Hoxa11* and *Hoxa13* were not up-regulated (Fig. 1). Although this expression profile is similar to that of primary mesenchymal cells cultured in serum alone, the latter rapidly differentiate. In contrast, cells cultured with Wnt3a, FGF8, and RA remain undifferentiated while the expression of genes characteristic of the early limb mesenchyme is maintained. At higher doses, FGF8 appears to overcome the effect of RA to a limited extent, which results in a partial decrease in *Meis1* expression and a concomitant increase in *Hoxa11* expression (fig. S1).

To directly assess the developmental potential of cultured primary cells after exposure to various combinations of signals, we made use of a classic technique referred to as constructing a "recombinant limb." Dissociated mesenchymal cells are reaggregated, placed within a jacket of limb bud ectoderm, and grafted onto a host embryo (*13*). After several days of development in the host egg, recombinant limbs are patterned by endogenous signals and form recognizable skeletal structures (*14*, *15*).

Recombinant limbs, made from limb mesenchyme cultured under various conditions, were first assessed 3 days after grafting to determine how expression of the segmental markers resolved in this in vivo setting. As in normal limb development, these markers are expressed in a segment-specific manner in recombinants generated from freshly dissociated HH18 limb mesenchyme (Fig. 2, A to C). In contrast, recombinants made from mesenchymal cells cultured for 36 hours in Wnt3a and FGF8 lacked proximal *Meis1* expression but did express *Hoxa11* in a middle domain and *Hoxa13* distally (Fig. 2, D to F). Thus, limb mesenchyme cultured without RA shuts off *Meis1* expression in vitro and does not reactivate its expression when reexposed to flank signals in vivo. However, recombinants made from cells exposed to Wnt3a, FGF8, and RA in culture continued to express *Meis1* proximally, *Hoxa11* centrally, and *Hoxa13* distally (Fig. 2, G to I), comparable to fresh recombinants and normal limb buds.

Similar to previous studies, recombinant limbs made from fresh HH18 leg bud mesenchyme that were allowed to develop for 14 days after grafting formed segmented skeletons approximating the PD organization of normal limbs including apparent femur, fused tibia and fibula, and digits (Fig. 3A) When made from cells cultured for as little as 12 hours in medium with serum alone, which (as noted above) rapidly initiate chondrogenic differentiation in vitro, they lost the ability to form more than a small cartilaginous nodule in ovo (fig. S2A). When similar recombinants were made from limb mesenchyme that was cultured with Wnt3a and FGF8 for 18, 24, or 36 hours, there was a progressive loss of proximal structures (Figs. 4, D and F, and 3C, respectively; fig. S2; and table S1), such that those cultured for 36 hours were reduced to a cartilage nodule em-

bedded in the flank and a single digit. This loss of ability of cells cultured in Wnt3a and FGF8 to form proximal skeletal elements was not due to detectable decrease in proliferation, increase in apoptosis, or inhibition of chondrogenesis within the recombinants (fig. S3). In contrast, primary limb cells cultured for 36 hours with Wnt3a, FGF8, and RA and then assayed in recombinant limbs gave rise to multiple well-formed segments, similar to those produced in recombinants made with fresh HH18 mesenchyme, although typically smaller in size and often exhibiting a bend or break at the thinnest point in the middle of the second skeletal segment (Fig. 3B, and table S1). Our best interpretation of these skeletons is one of three segments—stylopod, zeugopod, and autopod, which is also consistent with the three distinct domains of segmental gene expression at earlier stages, discussed above.

This interpretation critically depends on our ability to correctly identify the skeletal structures resulting from recombinant limbs. Most problematic is the identification as a digit of the small rod-like ossified element that forms in recombinants made from mesenchyme cultured for 36 hours with Wnt3a and FGF8, but in the absence of RA (Fig. 3C). We therefore used a second criterion for establishing the identity of these structures using cultured leg bud mesenchyme repackaged in wing bud ectoderm. The identity

of ectodermal appendages, feathers and scales, is induced by the underlying mesenchyme late in embryonic development (*16*). The proximal part of the chick leg is covered with feathers, whereas scales cover the feet (including metatarsals and digits). Similarly, recombinant limbs generated from freshly dissociated HH18 leg mesenchyme formed feather-covered proximal elements and scale-covered feet with claws, another digit-specific structure. The elements we identified as digits in the recombinants made after culture with Wnt3a and FGF8 were invariably covered by scales and ended in claws (Fig. 3D). Similarly, the multi-segmented recombinants produced by cells cultured in Wnt3a, FGF8, and RA displayed scales only over the distal elements we identified as digits, also terminating in claws (Fig. 3, E to H). Although this approach identifies the distal-most element as digit, both of the proximal segments of recombinants made with cells grown in all three factors are exclusively covered in feathers. Section in situ detection of *Meis1* surrounding the proximal-most cartilage of recombinants harvested after 4 days in ovo clearly delineates this element as stylopod (fig. S4).

Thus, mesenchymal cells cultured in the combination of all three signaling molecules to which early limb cells are normally exposed maintain the capacity to form both proximal and distal structures despite the passage of time and con-
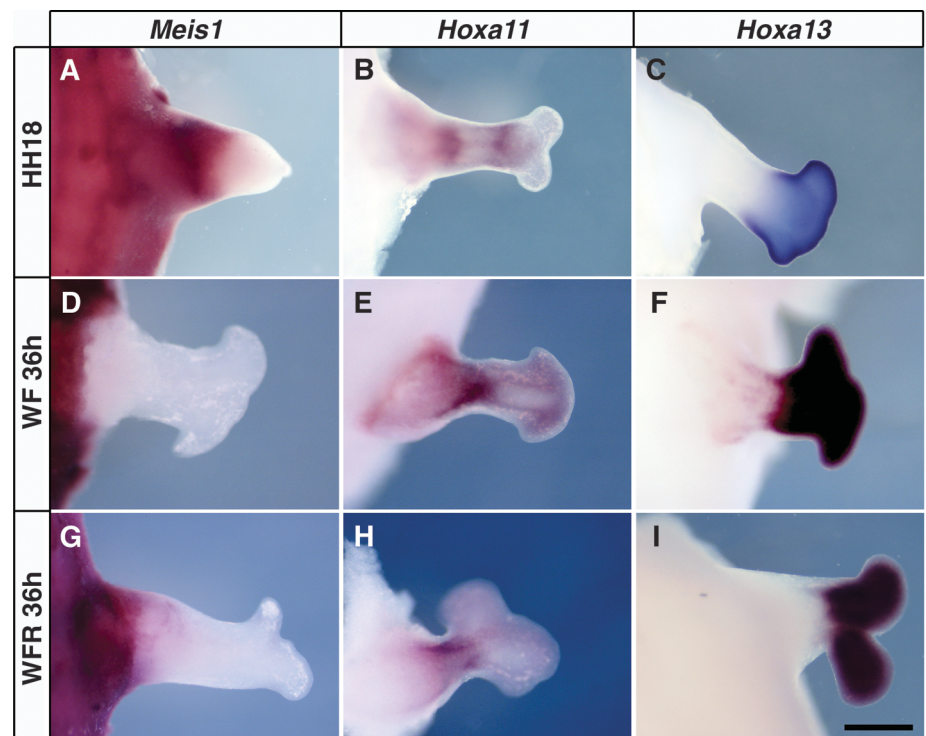

Fig. 2. Expression of *Meis1*, *Hoxa11*, and *Hoxa13* delineate segmental domains in recombinant limbs. Whole-mount in situ hybridization with *Meis1*, *Hoxa11*, and *Hoxa13* probes 72 hours after grafting. (**A** to **C**) Recombinants using freshly dissociated HH18 hindlimb cells. (**D** to **F**) Recombinants using HH18 hindlimb cells cultured for 36 hours in Wnt3a and FGF8. (**G** to **I**) Recombinants using HH18 hindlimb cells cultured for 36 hours in the presence of Wnt3a, FGF8, and RA. Scale bars: 500 μm in (A) to (C) and 800 μm in (D) to (I).

tinued proliferation. Indeed cells cultured in either Wnt3a and FGF8 or Wnt3a, FGF8, and RA divide with a cell cycle time of ~11 hours (11.43 ± 1.4 hours and 10.82 ± 1.25 hours, respectively) (movie S1), comparable to what has been reported for early limb mesenchyme in vivo (17, 18). This strongly argues against a mechanism linking PD specification to a cell cycle–based internal clock (1, 19, 20).

Freshly harvested HH18 limb bud cells give rise to multiple segments of the PD axis in a recombinant limb. Previous studies using a dif-

ferent experimental approach have also indicated plasticity of proximal HH20 limb bud cells in response to their environment (21). However, undifferentiated cells from the distal HH24 limb bud are committed to forming only autopod structures [Fig. 4A and Dudley et al. (22)], in spite of exposure to endogenous proximal signals after grafting to a host embryo. To determine whether this fate restriction is irreversible under culture conditions that maintain the ability of HH18 limb cells to form all three segments, we cultured dissociated distal HH24 limb mesenchyme in the presence of Wnt3a, FGF8, and RA for 36 hours. We found that when placed in a recombinant limb, these cells were at most capable of forming a single digit with terminal claw (Fig. 4B and table S1).

Although the combination of Wnt3a, FGF8, and RA cannot reverse digit specification once it starts, our data indicate that these factors are sufficient to maintain early limb mesenchyme in a state capable of giving rise to the full PD pattern. We propose that the trigger for initiating the process of specification of the zeugopod and autopod is the cessation (due to displacement) of RA exposure. If this model is correct, then cells initially cultured with Wnt3a, FGF8, and RA, and hence held in an early limb mesenchyme–like state, should start to lose the ability to form proximal structures in vitro as soon as RA is removed from the media. Indeed, we found that primary HH18 leg bud mesenchyme cultured for 18 hours in all three factors and then for 18 hours in only Wnt3a and FGF8 developed two segments, comparable to primary cells assayed immediately after culture for 18 hours in Wnt3a and FGF8 (Fig. 4, C and D; fig. S2B; and table S1). Similarly, HH18 limb cells cultured for 12 hours in all three factors followed by 24 hours in Wnt3a and FGF8 alone formed a digit with a shorter proximal element similar to those assayed after culture in Wnt3a and FGF8 for 24 hours (Fig. 4, E and F; fig. S2C; and table S1).

These data strongly suggest that exposure to the combined activities of Wnt3a, FGF8, and RA in the early limb bud or in culture maintains the

Fig. 3. Wnt3a, FGF8, and RA together maintain the potential of cells to form the complete PD axis. (**A**) Freshly dissociated HH18 hindlimb cells formed three distinct limb segments 14 days after grafting. Although not shown, proximal and middle segments were covered with feathers, and digits with terminal claws were covered by scales. (**C** and **D**) Cells cultured for 36 hours in the presence of Wnt3a and FGF8 (WF) lost the ability to form all but a single scale-covered digit extending from a cartilage nodule embedded in the flank (n = 25 out of 28 with one segment). (**B** and **E** to **H**) Cells cultured for 36 hours in Wnt3a, FGF8, and RA (WFR) formed an elongated feather-covered proximal segment, a short feather-covered middle segment, and a scale-covered digit with a terminal claw (n = 13 out of 24 with three segments). F, femur; T/F, tibia and fibula; M, metatarsal; D, digits. Scale bars: 5 mm in (A) and (E), 2.5 mm in (B) and (C), 1.6 mm in (D), and 1 mm in (F) to (H).
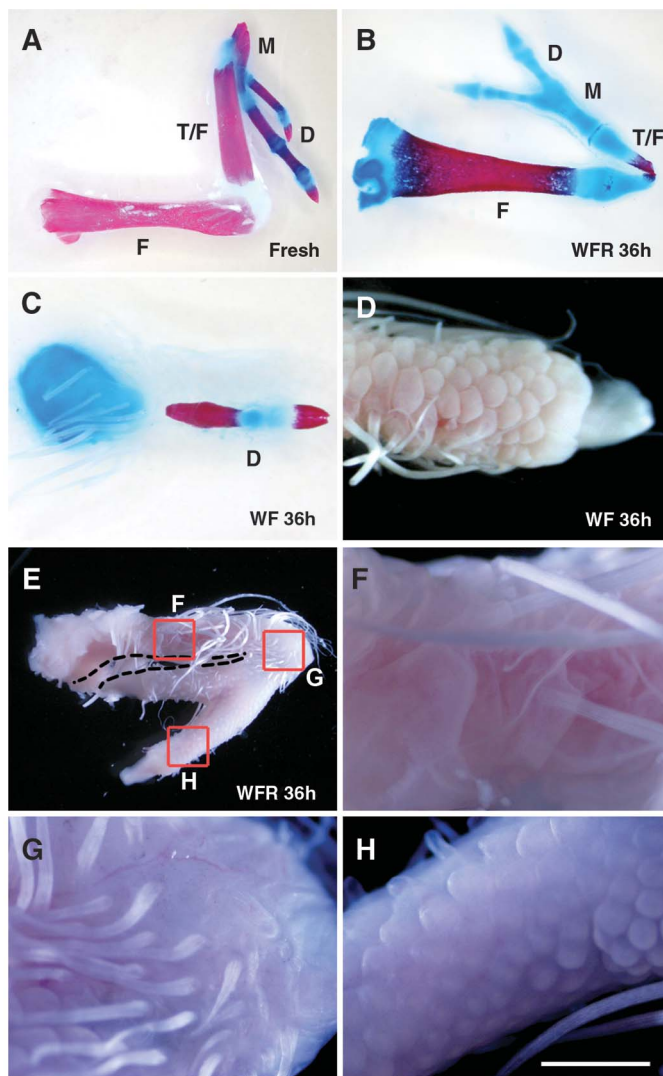

Fig. 4. PD potential is restricted by time spent out of the influence of RA. (**A**) Recombinants made from freshly dissociated distal HH24 hindlimb cells. (**B**) Recombinants of distal HH24 cells cultured for 36 hours in Wnt3a, FGF8, and RA (WFR) (n = 8). (**C** and **D**) Recombinants of HH18 cells cultured for 18 hours in the presence of all three factors followed by 18 hours with Wnt3a and FGF8 (WF) resembled recombinants grafted immediately after culture in Wnt3a and FGF8 for 18 hours (C) n = 28; (D) n = 12. (**E** and **F**) HH18 cells cultured for 12 hours in all three factors followed by 24 hours in Wnt3a and FGF8 formed recombinants that resembled those cultured for 24 hours in the two factors alone (E) n = 12; (F) n = 9. Scale bars: 1.5 mm in (A) and (B) and 1 mm in (C) to (F).
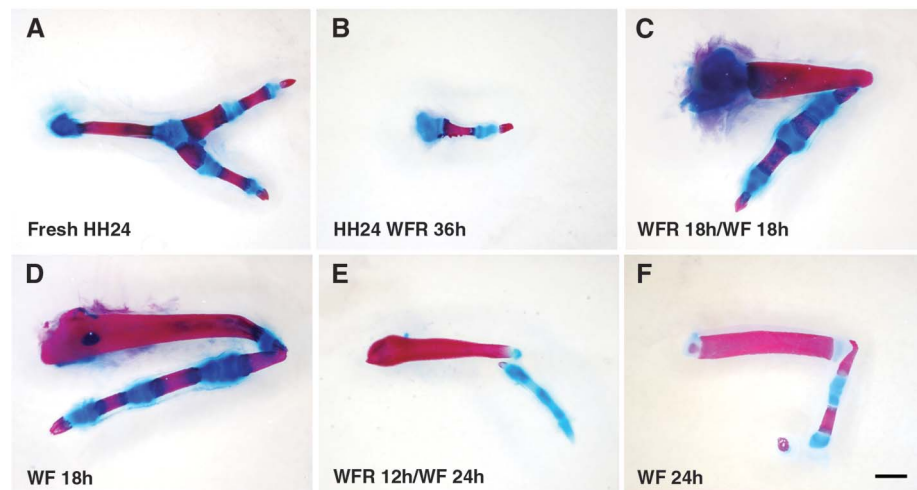
potential to form both proximal and distal structures. As the limb bud grows, the proximal cells fall out of range of distal signals that act, in part, to keep the cells undifferentiated (7). Cells closer to the flank therefore differentiate and form proximal structures under the influence of proximal signals. Meanwhile, the potential of distal mesenchymal cells becomes restricted over time to zeugopod and autopod fates by virtue of their growing beyond the range of proximally produced RA. Similar conclusions were reached independently by Roselló-Díez et al. (11), as discussed in the accompanying paper.

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Diffusible Signals, Not Autonomous Mechanisms, Determine the Main Proximodistal Limb Subdivision

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Vertebrate limbs develop three main proximodistal (PD) segments (upper arm, forearm, and hand) in a proximal-to-distal sequence. Despite extensive research into limb development, whether PD specification occurs through nonautonomous or autonomous mechanisms is not resolved. Heterotopic transplantation of intact and recombinant chicken limb buds identifies signals in the embryo trunk that proximalize distal limb cells to generate a complete PD axis. In these transplants, retinoic acid induces proximalization, which is counteracted by fibroblast growth factors from the distal limb bud; these related actions suggest that the first limb-bud PD regionalization results from the balance between proximal and distal signals. The plasticity of limb progenitor cell identity in response to diffusible signals provides a unifying view of PD patterning during vertebrate limb development and regeneration.

The vertebrate limb bud arises from the lateral plate as a bulge of mesenchymal cells encased within an ectodermal hull. Late limb buds of all tetrapods contain three proximodistal (PD) segments, each expressing specific homeobox genes. The stylopod (upper limb) expresses Meis1/2, the zeugopod (lower limb) expresses Hoxa11, and the autopod (hand/foot) Hoxa13 (1, 2), although none of these markers is sufficient to specify limb-segment identity. The transition between stylopod (proximal) and nonstylopod (distal) structures represents the main PD subdivision of tetrapod limbs (3, 4). In the distal limb bud, the pool of undifferentiated cells responsible for limb generation is maintained by fibroblast growth factor (FGF) and Wnt signals produced by a distal epithelial structure called the apical ectodermal ridge (AER) (5). However, the importance of these and other signals in PD patterning remains controversial. Whereas the progress zone model proposes autonomous progressive distalization of undifferentiated cells under permissive AER influence (6, 7), classical transplantation experiments provide evidence for nonautonomous signals (8, 9). More recently, a two-signal model was proposed, with retinoic acid (RA) as proximalizer and FGFs as distalizers (10–13); however, endogenous proximal signals have not been identified, and the role of endogenous RA has been questioned by genetic analyses in the mouse (14).

To investigate limb-proximalizing signals in the chicken embryo, we transplanted distal leg tips [200 μm thick, Hamilton-Hamburger (15) stage 19 to 20 (HH19-20)] to two potentially proximalizing regions: the somites and proximal

wing bud of HH20 embryos (16) (fig. S1, A and B). These transplants were compared with transplants to tissues not expected to contain limb-proximalizing signals: HH24 distal wing bud (prospective zeugopod) and anterior HH20 hindbrain (fig. S1, A and B).

Graft development in these experiments was not influenced by the grafting site (Fig. 1, A and B and fig. S1D). These results thus support previous reports indicating autonomy of distal limb grafts (6, 7, 17). An alternative explanation, however, is that proximalizing signals were suppressed by distalizing FGFs from the graft's AER (10). We tested this by treating the grafts with the FGFR1 inhibitor SU5402. Whereas untreated grafts transplanted to the somites did not express the proximal marker Meis1 and maintained Hoxa11 22 hours post grafting (hpg) (Fig. 1, C and D), SU5402-treated grafts expressed Meis1 along the entire PD axis and lost Hoxa11 expression (Fig. 1, E and F). Presumably, SU5402 action is enhanced by its effect on AER degeneration, which further diminishes FGF signaling. Notably, neither control nor SU5402-treated grafts to prospective HH24 zeugopod or anterior HH20 hindbrain activated Meis1 or down-regulated Hoxa11 expression 22 hpg (Fig. 1, I and J, and fig. S2, B to I), which indicated that the changes observed require a specific signal from the somites and not just release from FGF signaling. The somite region thus specifically contains signals that proximalize the limb bud expression profile, but these signals are counteracted by strong FGF activity from the AER.

The somite region expresses the RA-synthesizing enzyme RALDH2 and the RA target RARβ (18) and contains biologically active RA levels (13), whereas anterior hindbrain and distal limb bud do not (fig. S1, A and B). To test whether endogenous RA was required for the proximalizing activity of the somites, we treated grafts with beads soaked in SU5402 plus RA antagonist (RAA). In this case, Meis1 expression was not

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