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Review

Achieving bilateral symmetry during vertebrate limb development

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ABSTRACT

While the various internal organs of vertebrates display many obvious left–right asymmetries in their location and/or morphology, external features exhibit a high degree of bilateral symmetry. How this external bilateral symmetry is established during development is largely unknown. In this review, we explore several mechanisms, in place during development, that regulate the final size of the limb. These mechanisms rely on the presence of positive signaling feedback loops during limb bud growth. Through the activity of these signaling loops and their eventual breakdown when the limb bud has reached a certain size, bilateral symmetry can be achieved.

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1. Introduction

The mythical French Dahu is a mountainous goat-like creature that has legs shorter on one side of its body allowing it to stand evenly on steep slopes. Although the thought of such an animal can be entertaining, it is also clear how poorly the Dahu would fare evolutionarily if it ever had to turn around or walk on flat ground. Thankfully, the body plan of most metazoans displays an obvious external radial or bilateral symmetry. The reason for this is clear, animals progress through their environment, and as such, need to move and interact equivalently in different directions. For aquatic animals a symmetrical hydrodynamic form is of great advantage and for tetrapods, efficient and balanced locomotion depends upon having symmetrical limbs. Yet achieving and maintaining symmetry through development is not a trivial problem. A mechanism relying only on initial symmetric conditions is dangerous. A slight deviation from equal cell number in, for example, the limb buds would be dramatically amplified by subsequent cell divisions in the vast growth leading to the adult limbs. Moreover, the establishment of symmetry must be layered on top of internal asymmetries that potentially alter the signaling environment the embryonic tissues are exposed to on the two sides of the body. Thus, in tetrapods, while appendages are paired and show a great similarity in length and size from side to side, there is a cascade of left–right asymmetric signals in the early embryo ensuring that many internal organs are asymmetrically distributed, for example: heart, stomach, pancreas and spleen on the left, liver and gall bladder on the right. Taken together, these considerations strongly imply that there must be internal mechanisms tightly controlling size to assure that, for example, the paired limbs end up being extremely close to the same

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size in spite of a lack of communication between them throughout their patterning and growth. While in general this remains an under-studied problem in developmental biology, in this review, we will discuss the developmental pathways that allow for this external symmetry to occur focusing on the tetrapod limb bud where some mechanisms of size control have recently been uncovered.

2. Limb initiation: establishing the initial conditions

While we have argued above that starting with near-identically sized bilateral limb buds is not sufficient to produce symmetric limbs, it seems clearly advantageous to maintain symmetry, that the initial conditions on the two sides be as equivalent as possible. Indeed, to a first approximation, the early limb buds do appear laterally similar in size.

Limb buds form from the outgrowth of the lateral plate mesoderm (LPM) on either side of the trunk. In spite of the widely held belief that directly or indirectly the location of the limb fields must be established by expression of several Hox family members in the LPM (for review see [1] and [2]), there is at present little evidence in support of this proposition. All of the individual *Hox* genes have now been genetically inactivated, and all of the double, triple and quadruple mutants have been constructed to remove each paralogous group. Yet there remains only a single example where a Hox mutation has led to a shift in the location of the limb buds, the rather unimpressive one-half segment anterior shift of the forelimb buds resulting from the targeted deletion of Hoxb5 [3].

While the entire flank has been shown to be competent to form limb buds under experimental conditions, it is only within the narrower fore- and hindlimb fields that limb buds form under the influence of local LPM expression of Fgf10 [4–7]. However, the mechanism by which Fgf10 is induced in these localized regions remains problematic. It was previously proposed that Fgf8 emanating from the intermediate mesoderm might be responsible for this [8]. However, more recently it has been shown that conditional inactivation of Fgf8 in the intermediate mesoderm has no effect on limb formation [9]. The ability of Fgf8 to experimentally induce limb bud formation most likely relates to its later physiological role, emanating from the apical ectodermal ridge (AER) at the top of the limb bud, in maintaining Fgf10 expression in the distal limb bud mesenchyme [4,5,10].

While we do not know the mechanisms that specify the location of the limbs fields, they are marked by the mesenchymal expression of T-box transcription factors, Tbx5 in the forelimb and Tbx4 in the hindlimb. In spite of early clues that these factors might be involved in limb-type identity, they are now known to have equivalent activities, necessary for limb bud formation [11,12]. The role of *Tbx* genes is best understood in the context of the forelimb where Tbx5 is located upstream of the signaling factor Wnt2b and together positively regulate the expression of Fgf10 in the LPM and thereby the expression of Fgf8 in the AER [13,14].

In the current context, there are no apparent differences in expression levels of these key genes in the left limb buds versus the right limb buds, the similar levels of left and right Tbx and Fgf10 expression mirroring the similar size and morphology of the left and right limb buds as they first form. In spite of this apparent left and right equivalence, toxicological studies have provided clues that, during limb bud development, some left–right differences are present.

3. Teratogens and early limb bud development: a window on early limb asymmetries

Are left and right limb buds really created equal? The study of the teratogenic effect of two compounds, acetazolamide and cadmium has shed some light on limb bud formation and provided some hints of an early asymmetry during limb bud development. When administered to pregnant mice or rats both compounds can induce consistent unilateral limb reduction defects in embryos ranging from loss of digits (ectrodactily) to complete lack of limb (amelia) [15–20]. These limb deficiencies are mainly found on the right side of the embryo although in rats, cadmium can induce left-sided defects [17]. The reason behind the difference observed between rats and mice remains unclear and, although unlikely, may point to an inherent difference between the two rodent species. The timing of administration of the drug relative to the stage of limb bud development is more likely to be responsible for such effect. Indeed, a difference in 10 h in exposure of mouse embryos to cadmium can shift the limb reduction phenotype from bilateral to predominantly right-handed defects [19]. This led Messerle and Webster to look closely at the initial limb bud growth comparing the left and right side. They noticed that early on during mouse limb bud development, by embryonic day E9, the amount of mesodermal cells in the right forelimb appears larger than in the left forelimb. As limb development goes on, however, these differences are initially reversed [19,21,22] and then erased. These authors and others attributed the right bias of cadmium and acetazolamide limb defects to the vasculature that initially supply the forming limb buds until it is derived from the dorsal aorta. This early vascular network includes the umbilical vein which is about three times larger on the right than on the left and could account for an initial difference in blood flow, drug delivery and subsequent growth of the left and right limb buds [19,21]. In light of these rather dramatic differences, it is clear that there must be a strong mechanism in place to re-establish symmetry between the limb buds. The source of this mechanism lies in the signaling systems that pattern the early limb bud.

4. Signaling centers during limb bud growth and patterning

The last decade has seen a dramatic increase in our understanding of the developmental pathways governing limb growth and patterning. Distinct pathways direct the establishment and maintenance of the different axes of the limb: antero-posterior (thumb to pinky), proximo-distal (shoulder to hand) and dorso-ventral (back to palm of the hand). There is also significant interdependency of these pathways allowing for a coordinated growth of the bud along the three axes through time.

The patterning and growth of chick and mammalian limb buds are under the influence of two principal signaling centers termed the zone of polarizing activity, or ZPA, as well as the AER (for comprehensive reviews on patterning of the vertebrate limb see [2,23,24].

The AER consists in a thickening of the ectoderm made of pseudo-stratified cells overlying the distal aspect of the limb bud, at the dorsal–ventral border. The AER is required for growth of the limb bud along the proximo-distal axis and its removal at increasingly later stages leads to the loss of progressively more distal structures [25,26]. One of the main roles of the AER is to promote cell survival in the distal limb bud. Indeed, surgical removal of the AER causes cell death extending up to 200 μm from the distal end of the limb [27,28]. Fgf molecules are the central mediators of AER function. A bead soaked in Fgf4 can substitute for lack of AER and direct outgrowth of the limb bud [29]. While the concerted genetic deletion of several AER Fgfs, namely Fgf4, Fgf9 and Fgf17, has no effect on limb bud growth and patterning [30], removal of Fgf8 alone or an isoform of its receptor, FgfRII, causes a severe reduction in limb bud size [31–33]. Finally, double mutants for Fgf4 and Fgf8 also show increased cell death in the distal mesenchyme consistent with the AER removal experiments [34].

A second important signaling center in the limb bud is the ZPA. It was identified based on its ability, after transplantation to the anterior side of the limb bud, to induce mirror image duplication of the digits [35] and was found to be located at the distal posterior margin of the limb bud. The signaling factor Sonic hedgehog (Shh) is expressed in a domain consistent with the location of the ZPA based on the transplantation experiments and is solely responsible for its activity [36]. As Shh expression on the anterior side of the limb bud causes duplication of digits bearing a posterior identity, Shh is thought to act as both a mitogen and a morphogen promoting posterior fates. The role of Shh in determining anterior–posterior identity has been extensively studied and falls beyond the scope of this review (for more details see [24,37]). Succinctly, limb bud cells are patterned along the antero-posterior axis not only according to the concentration of Shh that they are exposed to (paracrine action) but also according to length of time these cells express Shh (autocrine action) [38]. This model postulates that the most posterior digits (part of digit 3 and the entirety of digits 4 and 5 in the mouse) are specified by exposure to the highest levels of Shh for the longest period of time. Cells making the other half of digit 3 and digit 2 never express Shh and are only sensitive to the actual concentration of Shh. Finally, the most anterior digit, digit 1, is the only digit that forms in a Shh mutant and is therefore not dependent on the action of Shh [38]. The transcriptional regulator Gli3 is the main target of the action of Shh and the complex interaction between Gli3 and Shh ultimately specifies digit identity [39,40].

Beside its role as morphogen specifying antero-posterior identities, Shh also promotes cell division [41,42]. This was suggested early on by transplantation experiments where increasing number of ZPA cells moved to the anterior side of the limb bud leading to the formation of increasing number of digits [43]. Paralleling these tissue recombination experiments, genetic removal of Shh at various time points after mouse embryonic day E9.5 leads to the loss of an increasing number of digits [41]. Although deletion of Shh leads to a marked increase in apoptosis, this phenotype can be attributable to the downregulation of AER Fgfs in the Shh mutant (see below). Shh also appears to have a direct role in inducing certain cell cycles genes in the mesenchyme [42] although it is not alone sufficient to induce proliferation [44]. In some contexts, Shh can also prevent cell death as shown by application of Shh protein in the anterior necrotic zone and the inter-digital mesenchyme, regions where active apoptosis is usually observed [45].

The AER expressing Fgfs and the ZPA expressing Shh are therefore two important signaling centers in the limb that not only control the patterning but also cell survival and division along the proximo-distal and antero-posterior axes, respectively.

5. Coordinating growth and patterning along the limb axes

The pathways described above do not act in isolation but interact with each other to coordinate patterning and growth along the proximo-distal and antero-posterior axes (see Fig. 1). Shh is required for the maintenance of several Fgfs in the AER, namely Fgf4, Fgf9 and Fgf17 [44,46,32]. Interestingly, Fgf8 is only indirectly affected by Shh removal, as the integrity of the AER is not maintained in the absence of Shh and Fgf8 is downregulated [47]. Fgf signaling in turn is required for Shh expression [46,44]. Following removal of the ZPA, Shh-expressing cells do not regenerate, suggesting that Fgfs are required for maintenance of Shh expression and but are not sufficient for its induction [48]. In accordance with these results, following AER removal, application of Fgf4 alone cannot induce Shh expression in the anterior mesenchyme [46]. Although Fgf8 alone is sufficient to allow normal limb development [30], several Fgf molecules, including Fgf4, are likely to cooperate to maintain Shh expression [33,34].

Shh appears to act indirectly to maintain Fgfs expression in the AER. Clues on the intermediate between the two signals came from the study of the spontaneous mouse mutant *limb deformity* (*ld*). In the context of the *ld* mutation, Shh expression is initiated but fails to be maintained and Fgf4 expression is absent from the AER [49]. Interestingly, this mutant fails to induce the bone morphogenic protein (BMP) inhibitor Gremlin in the distal mesenchyme in response to Shh signaling [50]. Furthermore, overexpression of Gremlin in chick limbs can upregulate Fgf4 expression [51] suggesting that Gremlin is a likely intermediate between Shh and Fgf4. These results were confirmed by targeted deletion of Gremlin coding sequence which as expected leads to a failure to maintain Shh and Fgf4 expression [52]. Further analysis revealed that the different alleles of the *ld* mutation disrupt a *cis*-regulatory region required for proper Gremlin expression [53].

Shh appears to regulate Gremlin expression via modulation of BMP2 expression. Viral overexpression of Shh or implantation of a bead soaked in Shh protein can induce expression of BMP 2 [44,54]. Furthermore, the expression of Gremlin, although itself a BMP inhibitor, is dependent on BMP signaling [54]. Once induced, Gremlin functions to antagonize BMP activity in proximity of the AER. Distal BMPs are negative regulator of AER function and limbs treated with Bmp2 show a reduction in outgrowth [29]. Furthermore, overexpression of Noggin, a BMP inhibitor, is able to expand Fgf4 expression domain anteriorly [55]. Through downregulation of BMPs, Gremlin can maintain Fgfs expression in the AER.

6. Termination of the Shh–Fgf positive feedback loop and growth control

We have seen that Fgf and Shh are both involved in regulating proliferation and that their expression is maintained through reciprocal positive feedback loops. Shh expression is maintained in the ZPA by Fgfs from the AER. In turn Shh, through BMP2, maintains the expression of Gremlin which inhibits distal Bmps allowing for Fgfs expression in the AER. If this loop were not broken, one could imagine that the limb would continue growing. Indeed, experimentally maintaining the activity of Shh and Fgf after the time of endogenous expression terminates produces continued outgrowth and a longer digit containing an extra phalanx [56].

To assure that mesenchymal proliferation ceases at the appropriate time of limb formation, the Shh–Fgf feedback loop is broken through internal mechanisms based on the growth of the limb bud itself (see Fig. 1). The key player in this process turns out to be Gremlin. As discussed previously, Gremlin is induced indirectly by Shh and, also indirectly, acts to promote Fgfs. However, over time, the domain of Gremlin expression is increasingly displaced away from both the ZPA and AER. These displacements ultimately mean that the components of the feedback loop are no longer close enough to maintain their feedback interactions. This occurs in slightly different ways in the chick and the mouse. In the chick, it is the gradual separation between the ZPA and Gremlin that appears to be crucial.

In the chick embryo, Gremlin, Fgf and Shh expression cease more or less simultaneously, making it difficult to judge where the feedback loop first breaks down. In order to understand its dynamics, each component of the loop was individually added back, just prior to breakdown of the loop. Fgf4 bead implantation or Gremlin viral overexpression could maintain Shh or Fgf4 expression, respectively, when expression of these genes is normally no longer detectable. However, a Shh bead implanted at the posterior end of the limb failed to induce Gremlin expression, indicating a break in the loop between these two molecules [57]. Importantly, a Shh-cre recombinase based fate mapping revealed that cells that have a history of Shh expression are refractory to Gremlin expres-

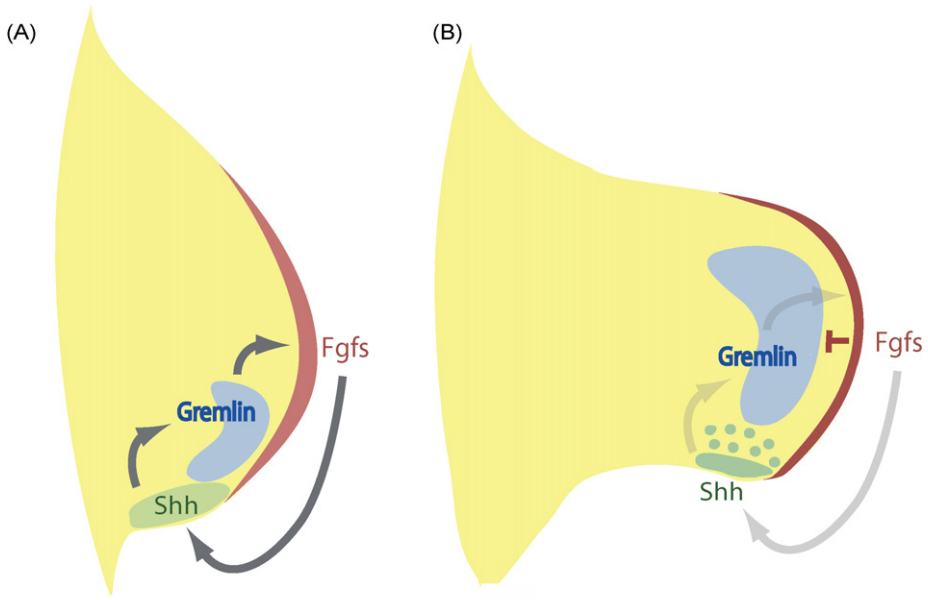

Fig. 1. Shh–Fgfs feedback loop and its breakdown. (A) During early limb bud development, Shh expression in the ZPA (green) positively regulates the expression of the BMP inhibitor Gremlin in the medial mesenchyme (blue). Gremlin, in turns, inhibits BMPs which allows for Fgfs expression in the AER (red). Fgf signaling is required for the maintenance of Shh expression in the ZPA. (B) The descendants of Shh-expressing cells (green dots) that no longer express Shh eventually populate the posterior half of the limb bud and are refractory to Gremlin expression. The distance between the ZPA and the mesenchyme competent to express Gremlin is therefore continuously increased until Shh can no longer maintain Gremlin expression. This part of the loop is the first to breakdown during chick limb bud growth. Gremlin expression is also downregulated by the high Fgf levels that are achieved later during limb development. Thus, the distance between the Gremlin expression domain and the AER also increases with time until Gremlin can no longer maintain Fgfs expression leading to downregulation of Shh and failure to maintain Gremlin expression. This mechanism allows for the initial break in the loop during mouse limb bud growth. These two modes of regulation of Gremlin expression likely coexist in both species.

sion [38,57] even when exposed to upstream pathway components such as BMP2 [54]. As the size of this population is continuously expanding and these cells eventually populate the posterior third of the limb bud, Gremlin expression is pushed further and further away from the Shh-expressing domain. When the Gremlin domain is too distant from the Shh domain, Shh cannot maintain Gremlin expression and the loop is broken. A prediction of this model would be that experimental reduction of the limb size, in particular of the distance between these two domains, would result in the maintenance of the loop for a longer time and compensation for the tissue lost. That is exactly what is observed when a wedge of limb bud located between Shh and Gremlin expression domains is removed. The limb bud shows continuous growth until reaching its final size and loop breakdown. This cut limb is indistinguishable in size from its un-operated contralateral limb [57]. This is an example of regulative development: the ability of embryos to fully recover following major extirpation of tissue to produce structures of the same form and size as would have developed without the surgery. It can also be seen how the same mechanism can act to ensure equal size of limb buds on left and right side of the embryo in situations where an initial small disparity in the number of cells on the two sides is present. Like the chick limb buds where tissue is surgically removed, the limb bud on the side of the embryo with fewer cells would simply grow for a slightly longer period of time until the Gremlin expression domain is displaced to the same extent.

In the mouse, the sequence of the loop breakdown differs from the chick. Careful examination of Shh, Fgf4 and Gremlin expression patterns in limb buds between E10.75 and E12 show that Fgf4 expression is first to terminate followed by Shh and Gremlin [58] indicating that maintenance of Fgfs fails in the older mouse limb bud. Interestingly, a second size control mechanism seems to be acting in concert with the Shh/Fgf feedback loop. While, as mentioned above, Gremlin expression is positively regulated by Shh, Gremlin is also negatively regulated by high Fgf levels from the AER. Once the feedback loop has been established and Shh, Gremlin and Fgfs expression is at its peak, high Fgf levels repress Gremlin expression in the region directly underneath the AER. As this region expands with further growth of the limb, Gremlin expression is continuously repressed in the distal limb bud, until its domain becomes too distant to maintain Fgf4 expression in the AER and the loop is broken. It is probable that the two mechanisms leading to interruption of the feedback loop actually act simultaneously in both chick and mouse. Together, they would insure that limb bud growth arrests when a fixed size has been reached along the antero-posterior axis (Shh–Fgf feedback loop) as well as along the proximo-distal axis (Fgf–Gremlin loop).

7. Buffering mechanism to achieve symmetry

The growth-regulating mechanisms discussed above depend on having a consistent level of signaling emanating from the ZPA and the AER on the two sides. This would predict that disruption or removal of either of these signaling centers will affect final limb size. As described previously, this is indeed what is observed in Shh [59,38,41] and Fgf mutants [31,34,30] as well as in several other mutants affecting these pathways. Agents causing massive cell death throughout of the limb, such as ionizing radiation [60] or cell cycle inhibitors [42], also lead to permanent shortening of the limb not only by removing progenitor pools behind the region of the limb involved in distal growth control, but also by potentially weakening of each signaling center and subsequent premature breakdown of the Shh–Fgf feedback loop.

If the level of signaling from the ZPA and the AER need to be equivalent in strength on the two sides of the embryo for the feedback loop to break down in a consistent manner, then there must be buffering mechanisms in place to assure Shh and Fgf activities are at the appropriate levels. Although not understood in detail, there

is evidence that such internal buffering mechanisms do exist. Over a limited range, the ZPA shows an ability to compensate for loss or addition of Shh-expressing cells [45]. Augmentation of Shh levels by Shh-coated bead implantation in the posterior end of the limb bud leads to upregulation of apoptosis in this region effectively reducing the size of the ZPA. Conversely, surgical or chemical diminution of the size of ZPA causes a transient decrease in the overall number of Shh-expressing cells. Twenty-our hours following treatment, however, the number of Shh-expressing cells is similar to un-operated contralateral limb buds and in half of the cases Shh expression domain is actually expanded. In this context, cell death levels in the posterior mesenchyme are reduced [45]. Although such an autoregulation of AER function through apoptosis has not been described, Fgf8 negatively regulates the expression of other AER Fgfs, namely Fgf4 [31,58]. This negative regulation of AER Fgfs could control for the total Fgf activity present in the AER.

8. Loss of buffering and unveiling of cryptic asymmetries

If such buffering mechanisms are really critical for maintaining symmetry, then the matched growth of the left and right limbs should be compromised in situations where the modulation of signal levels is disrupted. Interestingly, two mutations disrupting the integrity of the AER, *R-spondin 2* [61] and *footless* [62], show left-sided biased hindlimb reduction defects. More surprising is the phenotype of the *Sall4* mutant where unilateral hindlimb deficiencies are observed but with no preference for laterality [63]. How these mutations affect a limb on one side more than the other remains to be elucidated in detail, but presumably point to intrinsic differences between limb bud development along the left–right axis. In support of this idea, the right-sided limb reduction defects observed following exposure of embryos to acetazolamide are mainly left-sided in the mouse mutant *iv* which displays *situs inversus* or inversion *in situs* of the internal organs [16]. Similar observations were made in the insertional mouse mutant *legless* that disrupts the function of Sp8, a zinc-finger transcription factor required for proper AER formation [64,62]. In this mutant, the right forelimb is more affected than the left but this bias is reversed in the double homozygous mutant *legless*/*iv* displaying *situs inversus* [64]. These results direct us to two non-exclusive considerations. As postulated previously, it is possible that together with the asymmetric distribution of internal organs, other anatomical features established during development and differing along the left–right axis, can sensitize the limb to specific genetic alterations or the action of teratogens. Perhaps, limb development can also be influenced by factors that act during the establishment and/or maintenance of left–right identity. In order to develop symmetrically, left and right limb buds would need to be shielded from the influence of these left–right determinants. This is highlighted by the example of Pitx1, a hindlimb-specific transcription factor that is involved in imparting hindlimb morphology [65,66] as well as being required for hindlimb outgrowth [67]. Pitx1 is expressed equivalently in the left and right limb buds. However, loss of Pitx1 activity in a variety of animals including mouse, stickleback fish and manatees, results in remaining limb rudiments that are consistently larger on the left than on the right [67–69]. This is because the highly related gene *Pitx2* is expressed very early on in the left but not the right limb fields, as part of a broader left-specific expression domain critical for the asymmetric morphogenesis of the internal organs. Thus Pitx2 in the left hindlimb field compensates for the loss of Pitx1 in inducing outgrowth. In the current context, this means that Pitx1 must be expressed at a high enough level in both hindlimb primordiae so that it is above the threshold for saturation. This ensures that the additional Pitx activity imparted on the left by Pitx2 expression does not lead to preferential initial growth on that side. Other, currently unknown, activities likely operate, in a redundant fashion with Pitx activity in the forelimb primordia to assure that the early asymmetric activity of Pitx2 does not prevent the formation of bilaterally symmetric limb buds. This example is highly reminiscent of what has been observed during the formation of somites where retinoic acid can efficiently override the influence of sided determinants and coordinate symmetrical somitogenesis [70–72].

9. Conclusion

Work over the past few decades has unraveled a complex network of signaling centers and gene interaction that not only coordinates limb patterning but also regulates its growth along the various axes. As these exquisite cellular and genetic interactions are sensitive to distances within the limb field, they ultimately specify the final size of the limb bud. Further work is needed to help understand how specific teratogens and genetic mutation display a unilateral effect on limb development.

This review has focused on mechanisms that assure that the limb buds are of equivalent size on the left and right. Subsequent growth of the limb occurs by lengthening of the skeletal elements generated by condensation of the mesenchyme through the process of endochondral ossification. As significant alterations in the size of the limb buds through surgical or genetic manipulations result in defects in the final limb size, any later stage mechanisms of growth control cannot be sufficient to overcome gross differences imposed at early stages. Nonetheless, there need to be buffering mechanisms to assure that any slight differences in the sizes of the limb buds are not amplified with further growth.

Such example of regulative growth takes place during longitudinal growth of the long bones. During endochondral ossification, the length of the growth plate is established by the range of action of parathyroid hormone-related protein (PTHrP) produced at the end of the skeletal elements (reviewed in [73]). The level of PHTrP is maintained at an appropriate level through the activity of Indian hedgehog (Ihh) in a negative feedback loop, tying the length of the growth plate with the rate of differentiation, thereby assuring that the proliferation of the chondrocytes is maintained at a constant rate. Such mechanisms buffering skeletal growth assure that the limbs on the left and right sides of the embryo continue to grow in concert to achieve the bilaterally symmetric body plan needed to move about in the three-dimensional environment.

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