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Limb Development Takes a Measured Step Toward Systems Analysis

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Abstract

Growth and patterning of the embryonic vertebrate limb is regulated by feedback loops signaling between the epithelium and mesenchyme of the limb bud. Fibroblast growth factor (FGF) signaling from the epithelium regulates *Sonic hedgehog* (*Shh*) expression in the mesenchyme. In turn, SHH activity maintains the expression of *Gremlin1*, which encodes a bone morphogenetic protein (BMP) antagonist; the interaction between BMP and Gremlin1 regulates *Fgf* gene expression. A computational model using data from complex genetic analysis and quantitative measurements of gene induction kinetics demonstrates that limb development is robust and thus buffered against certain mutational alterations and epigenetic changes because of these feedback loops.

It was my understanding that there would be no math.

—Chevy Chase (1976)

Presidential debate skit (*Saturday Night Live*)

A reductionist approach (1-3) has brought us a certain distance toward one general goal of developmental biology, that is, understanding the molecular basis of embryogenesis. Thus, we have identified genes that are required for or play supporting roles during development, have studied their functions, and have examined how various signaling or genetic pathways affect cell behaviors that pattern the embryo. However, in this burgeoning new era of systems biology studies, such reductionist insights are beginning to be integrated and quantified to generate computational models of developmental processes, which are used to run in silico experiments and to generate data that then can be tested in vivo.

Such modeling can be used to study the “robustness” of embryonic development, which refers to the remarkable capacity of an embryo to develop normally despite being subject to a range of external and internal perturbations (4-6). Genetic mutations are examples of internal perturbations, whereas epigenetic effects, because they can be influenced by the environment, can be considered both external and internal perturbations. A straightforward cause of robustness is the widespread genetic redundancy that exists because of gene duplication (7) (the bane of many an embryologist who has needed to generate animals with multiple inactivated genes in order to uncover a phenotype). A different robustness

mechanism is provided by heat shock protein 90 (Hsp90), which, through its molecular chaperone activity, facilitates the correct folding of a large set of signal transduction proteins, even if these proteins harbor amino acid substitutions that otherwise impair proper folding (8, 9). Last, there is “distributed robustness” that, at first glance, may seem a more obscure concept. In distributed robustness, a function is distributed throughout different regions or modules of a system, each of which contributes to the function (but without straightforward duplications), so that a particular module may be compromised or, in the extreme case, inactivated without overall loss of function. This can be viewed as channeling of regulatory inputs into (several) alternate routes, so that when one route becomes blocked, the regulatory flow is maintained and the system is buffered (sometimes referred to as canalization). Robustness not only means that individual embryos might develop normally despite variation on account of mutation, but also that the population may harbor cryptic mutations that may be a source for evolutionary change (4, 5, 7, 8, 10). Developmental robustness has been modeled in *Drosophila* segment specification (11), *Caenorhabditis elegans* vulva formation (12), and *Xenopus* gastrulation (13). Now, we have an elegant example of distributed robustness from Bénazet and co-workers in mouse limb development (14).

Vertebrate limbs begin as buds of mesenchyme, jacketed in ectoderm, growing out of the embryonic flank. More than 50 years of experimental embryology, as well as loss- and gain-of-function genetics, have identified factors that are required for limb growth and patterning (15). These include Sonic hedgehog (SHH), produced by the zone of polarizing activity (ZPA) in the posterior bud mesenchyme, which is required for normal digit number and pattern (16), and four fibroblast growth factors (FGFs), which are secreted from a distal ectodermal structure known as the apical ectodermal ridge (AER) and which are required, to various degrees, for limb outgrowth (17) (Fig. 1). Normal limb formation depends on a feedback loop that coordinates these activities: FGFs released from the AER transcriptionally regulate *Shh* expression, and SHH, in turn, is required for inducing and maintaining expression of three *Fgfs* (*Fgfs* 4, 17, and 18) and for the normal expression of the fourth, *Fgf8* (17-22). In 1999, the Zeller laboratory observed that SHH indirectly regulates AER-specific *Fgf* expression by inhibiting an inhibitor: *Shh* maintains expression of *Gremlin1* (*Grem1*), which encodes a protein that directly antagonizes the activity of the bone morphogenetic proteins (BMPs), which, in turn, represses *Fgf* expression in the AER (23) (Fig. 1). *Grem1*-null mutants exhibited several limb defects, including a reduced complement of digits, thus confirming the role of *Grem1* in the epithelial-mesenchymal feedback loop for *Shh* and *Fgf* (24, 25).

A new study from the Zeller laboratory (14) began by asking which BMPs are antagonized by GREM1 during limb development, specifically, whether genetically reducing BMPs could rescue the limb defects caused by *Grem1* deficiency. Bénazet and co-workers found that, among the three primary *Bmps* expressed in the limb bud (*Bmps* 2, 4, and 7), *Bmp4* reduction had the strongest effect on rescuing digit number. Thanks to the availability of a hypomorphic allele (26), they generated a series of allelic mutants with progressive decreases in *Bmp4* dosage. In the context of the *Grem1*-null mutant, this stepwise reduction in *Bmp4* increasingly restored ZPA and AER signaling and rescued digit formation, which

demonstrated that the excess BMP activity causing the *Grem1*-null phenotype is largely because of BMP4.

Bénazet and co-workers next determined that dismantling this *Grem1*-*Bmp4* loop rendered limb development very sensitive to the amount of SHH. Complete *Shh* loss is disastrous for digit patterning, but if only one gene copy is deleted, then, as is the case for most genes, development is normal. Notably, the authors found that, without an intact *Grem1*-*Bmp4* feedback loop, the loss of one copy of *Shh* caused a reduction in the number of digits formed. Thus, the *Grem1*-*Bmp4* feedback loop acts as a “buffer,” rendering the system robust against perturbations in *Shh* amounts. Because this type of buffering depends on interconnecting feedback circuits and not on gene redundancy, it is an example of distributed robustness (6).

The authors then quantitatively measured the kinetics of *Grem1* induction using a mouse limb organ culture system that allowed direct in vitro application of BMP4, GREM1, or SHH protein (23). This analysis revealed a fast BMP4-GREM1 feedback loop [negative loop (Fig. 1)] that required 2 hours to increase *Grem1* expression, operating at a rate six times as fast as the slow, multistep epithelial-mesenchymal feedback loop between SHH and FGF [positive loop (Fig. 1)]. With concrete numbers in hand, a series of ordinary differential equations (ODEs) were used to model the regulatory input for the activity of each component of the regulatory circuits (BMP, GREM1, FGF, and SHH) (Fig. 1). Simulations using the ODEs generated dynamic gene expression curves that modeled normal limb development. When input values for specific genes were set to zero, the generated curves also mimicked expression patterns observed in the corresponding mouse singlegene knockouts, as well as compound mutants to a certain extent.

They then surveyed parameter space by altering input values and found that the computational model was insensitive to variability within a range of start conditions, which demonstrated the robustness of this model. But the ultimate test of any model is its predictive power. In this case, computations surprisingly predicted that *Bmp4* should be critical for the initiation of *Grem1* expression and should activate the regulatory circuits to establish morphogenetic signaling in the limb bud. Furthermore, *Grem1* should act as the critical “node” linking the two regulatory modules. Using inducible Cre recombinase technology (27), the authors found that early, but not late, removal of *Bmp4* dramatically disrupted limb development, which validated the prediction that an early *Bmp4* signal is essential to “kick-start” the system. However, *Bmp4* inactivation at a later stage resulted in an aberrantly elongated AER. That such different phenotypes occur with different timing of *Bmp4* inactivation is consistent with previous studies indicating that the function of BMP signaling in the limb is at least biphasic. Early BMP4 activity initiates *Grem1* expression, starting the limb development program, whereas later BMP activity terminates the program through regulation of AER morphology and function (28-30). During the “termination” phase, high FGF activity from the AER inhibits *Grem1* (31), which results in increased BMP4 activity, which then inhibits *Fgf* expression in the AER. With the introduction of additional parameters and ODEs to account for this (Fig. 1), the simulations could model how the signaling system passes through BMP-driven initiation, SHH-driven propagation,

and high FGF-driven termination phases, which describe the different impacts of BMP4, SHH, and FGF activity on *Grem1* expression over time.

A general lesson to be gleaned from this paper is that one should be cautious in interpreting data derived from conditional genetics in a rapidly changing system, which occurs in many embryological processes. For example, two previous papers had described Cre-mediated *Bmp4* inactivation in the limb, which primarily resulted in the elongated AER phenotype (32, 33), which would not have confirmed the computational model. The resolution to these conflicting data is in the details. In one study (33), the earlier *Bmp4* requirement was not revealed because Cre was used to inactivate two conditional alleles, whereas Bénazet *et al.* needed to inactivate only one. In the other study, Cre was used to inactivate a conditional *Bmp4* allele that encoded apparent wild-type BMP4 activity before inactivation, as do most conditional alleles (32). In contrast, Bénazet *et al.* used a *Bmp4* allele that encoded low, hypomorphic amounts of BMP4 before Cre inactivation. Presumably, this difference in genetics resulted in faster Cre-mediated clearance of the gene product in the Bénazet *et al.* study and thus revealed the earlier *Bmp4* function.

What does the future hold? The simulations using ODEs provide a good first approximation. To increase the veracity of the model, next steps may involve introducing cross terms in the ODEs (a few shown as dotted arrows in Fig. 1) to describe known, as well as yet-to-be-determined, regulatory interactions. For example, BMP signaling to the ectoderm is required for induction of both *Fgf8* and the morphological cell movements that result in the AER (28-30), although it is not clear what the relative contributions of ectodermal and mesenchymal BMP activity are to these functions. This positive role in the initiation phase of limb development (Fig. 1) was not modeled. Some *Bmps* are also up-regulated as SHH target genes (19, 22). Furthermore, BMPs act biphasically and inhibit *Grem1* expression above a certain threshold (34). These relationships generate new subloops and may introduce qualitative temporal changes in parameter activities that complicate the modeling. Additionally, to incorporate them, it may be necessary to experimentally measure specific parameter kinetics. Another level of regulation that has yet to be modeled is gene duplication. Bénazet *et al.* show that, although *Bmp4* may encode the primary BMP activity, other BMP genes, especially *Bmp7*, play supporting roles. Yet another feature of limb development is spatial constraints that affect signaling. For example, *Shh*-expressing cells and their descendants are refractory to *Grem1* induction by SHH, and the spatial expansion of this lineage is thought to be one mechanism that limits limb growth (35). Other spatial considerations include the decay of signaling activity over physical distance, which is usually governed by multiple variables. Such temporal and spatial constraints will require multivariable calculus and matrix algebra to be incorporated into current modeling. On the computational side, more sophisticated mathematical approaches are being developed to include such spatial and temporal variables, but the scanning parameter space is a capability that is still a critical limitation and requires a major expansion of computing capacity.

From the perspective of the experimentalist, more quantitative measurements of kinetics are needed; this is the key that makes the mathematical modeling possible. In a complex developmental system, the use of an allelic series also provides a very important semiquantitative genetic tool, which can describe an approximate dose-response curve for a

phenotypic outcome. In summary, it is clear that the hierarchical relations between elements in biological regulatory networks are complex and more akin to a web than to simple circuits, and understanding how they operate will require more than an intuitive approach. The combination of “quantitative” genetic approaches using allelic series, together with direct measurements of parameter kinetics and mathematical modeling as exemplified by the paper of Bénazet and co-workers, is an excellent step toward a predictive mathematical description of a complex system. There is still much to do ... and yes, there will be math!

References and Notes

1. Rosenberg A, Reductionism redux: Computing the embryo. *Biol. Philos* 12, 445–470 (1997).
2. Laubichler M, Wagner G, How molecular is molecular developmental biology? *Biol. Philos* 16, 53–68 (2001).
3. Gilbert SF, Sarkar S, Embracing complexity: Organicism for the 21st century. *Dev. Dyn* 219, 1–9 (2000). [PubMed: 10974666]
4. Kerszberg M, Noise, delays, robustness, canalization and all that. *Curr. Opin. Genet. Dev* 14, 440–445 (2004). [PubMed: 15261662]
5. Kitano H, Biological robustness. *Nat. Rev. Genet* 5, 826–837 (2004). [PubMed: 15520792]
6. Wagner A, Distributed robustness versus redundancy as causes of mutational robustness. *Bioessays* 27, 176–188 (2005). [PubMed: 15666345]
7. Kafri R, Springer M, Pilpel Y, Genetic redundancy: New tricks for old genes. *Cell* 136, 389–392 (2009). [PubMed: 19203571]
8. Rutherford S, Hirate Y, Swalla BJ, The Hsp90 capacitor, developmental remodeling, and evolution: The robustness of gene networks and the curious evolvability of metamorphosis. *Crit. Rev. Biochem. Mol. Biol* 42, 355–372 (2007). [PubMed: 17917872]
9. Rutherford SL, Lindquist S, Hsp90 as a capacitor for morphological evolution. *Nature* 396, 336–342 (1998). [PubMed: 9845070]
10. Gerhart J, Kirschner M, The theory of facilitated variation. *Proc. Natl. Acad. Sci. U.S.A* 104 (suppl. 1), 8582–8589 (2007). [PubMed: 17494755]
11. von Dassow G, Meir E, Munro EM, Odell GM, The segment polarity network is a robust developmental module. *Nature* 406, 188–192 (2000). [PubMed: 10910359]
12. Felix MA, Wagner A, Robustness and evolution: Concepts, insights and challenges from a developmental model system. *Heredity* 100, 132–140 (2008). [PubMed: 17167519]
13. De Robertis EM, Spemann’s organizer and self-regulation in amphibian embryos. *Nat. Rev. Mol. Cell Biol* 7, 296–302 (2006). [PubMed: 16482093]
14. Bénazet JD, Bischofberger M, Tiecke E, Gonçalves A, Martin JF, Zuniga A, Naef F, Zeller R, A self-regulatory system of interlinked signaling feedback loops controls mouse limb patterning. *Science* 323, 1050–1053 (2009). [PubMed: 19229034]
15. Mackem S, in *Cell Signaling and Growth Factors in Development: From Molecules to Organogenesis*, Unsicker K, Kriegstein K, Eds. (Wiley-VCH Verlag, Weinheim, Germany, 2006), pp. 523–618.
16. Riddle RD, Johnson RL, Laufer E, Tabin C, *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* 75, 1401–1416 (1993). [PubMed: 8269518]
17. Mariani FV, Ahn CP, Martin GR, Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature* 453, 401–405 (2008). [PubMed: 18449196]
18. Niswander L, Jeffrey S, Martin GR, Tickle C, A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371, 609–612 (1994). [PubMed: 7935794]
19. Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C, *Sonic hedgehog* and *Fgf-4* act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 79, 993–1003 (1994). [PubMed: 8001146]

20. Sun X, Lewandoski M, Meyers EN, Liu YH, Maxson RE Jr., G. R. Martin, Conditional inactivation of *Fgf4* reveals complexity of signalling during limb bud development. *Nat. Genet* 25, 83–86 (2000). [PubMed: 10802662]
21. Kraus P, Fraidenraich D, Loomis CA, Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech. Dev* 100, 45–58 (2001). [PubMed: 11118883]
22. Chiang C, Litingtung Y, Harris MP, Simandl BK, Li Y, Beachy PA, Fallon JF, Manifestation of the limb prepattern: Limb development in the absence of sonic hedgehog function. *Dev. Biol* 236, 421–435 (2001). [PubMed: 11476582]
23. Zuniga A, Haramis AP, McMahon AP, Zeller R, Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 401, 598–602 (1999). [PubMed: 10524628]
24. Michos O, Panman L, Vintersten K, Beier K, Zeller R, Zuniga A, *Gremlin*-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. *Development* 131, 3401–3410 (2004). [PubMed: 15201225]
25. Khokha MK, Hsu D, Brunet LJ, Dionne MS, Harland RM, Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. *Nat. Genet* 34, 303–307 (2003). [PubMed: 12808456]
26. Kulesa H, Hogan BL, Generation of a *loxP* flanked *bmp4^{loxP-lacZ}* allele marked by conditional *lacZ* expression. *Genesis* 32, 66–68 (2002). [PubMed: 11857779]
27. Lewandoski M, Conditional control of gene expression in the mouse. *Nat. Rev. Genet* 2, 743–755 (2001). [PubMed: 11584291]
28. Pajni-Underwood S, Wilson CP, Elder C, Mishina Y, Lewandoski M, BMP signals control limb bud interdigital programmed cell death by regulating FGF signaling. *Development* 134, 2359–2368 (2007). [PubMed: 17537800]
29. Ahn K, Mishina Y, Hanks MC, Behringer RR, Crenshaw EB 3rd, BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* 128, 4449–4461 (2001). [PubMed: 11714671]
30. Pizette S, Abate-Shen C, Niswander L, BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development* 128, 4463–4474 (2001). [PubMed: 11714672]
31. Verheyden JM, Sun X, An *Fgf/Gremlin* inhibitory feedback loop triggers termination of limb bud outgrowth. *Nature* 454, 638–641 (2008). [PubMed: 18594511]
32. Selever J, Liu W, Lu MF, Behringer RR, Martin JF, *Bmp4* in limb bud mesoderm regulates digit pattern by controlling AER development. *Dev. Biol* 276, 268–279 (2004). [PubMed: 15581864]
33. Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ, Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.* 2, e216 (2006). [PubMed: 17194222]
34. Nissim S, Hasso SM, Fallon JF, Tabin CJ, Regulation of *Gremlin* expression in the posterior limb bud. *Dev. Biol* 299, 12–21 (2006). [PubMed: 16989805]
35. Scherz PJ, Harfe BD, McMahon AP, Tabin CJ, The limb bud Shh-Fgf feedback loop is terminated by expansion of former ZPA cells. *Science* 305, 396–399 (2004). [PubMed: 15256670]
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Fig. 1.

Progressive modeling of early limb development from three different perspectives. From a classical embryologic perspective, limbs arise as buds from the embryonic flank mesenchyme surrounded by ectoderm (**top**). The middle row diagrams the signaling loops relevant for mathematical modeling; note that Bénazet *et al.* focused primarily on the propagation phase (see text for details). The ODEs they used are shown below and describe the interactions and dependencies of SHH, BMP, GREM1, and FGF activities. For example, GREM1 activity is positively controlled by both BMP4 and SHH and is modeled by Hill functions with the maximal velocities p_{G1} and p_{G2} , whereas the half-maximal induction concentrations correspond to K_1 and K_2 , respectively. The half-life of GREM1 is defined as $1/a_G$. MATLAB simulations using these equations generated expression curves that mimicked the gene expression profiles in normal limb development. Likewise, if specific mutations were modeled by using certain input values—for example, setting $p_{G1} = p_{G2} = 0$ for a *Grem1*-null genotype—the resulting expression curves mimicked mutant expression profiles. To model FGF-mediated repression of *Grem1* during the termination phase, the GREM1 equation was modified to include dependence of activity on a hypothetical repressor that is itself regulated by FGF activity.