transiently, and may act more as a trigger than a morphogen. Later, Shh may be required mainly to ensure an adequate cell mass (via survival, proliferation) and enable formation of normal digit number.

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CtBP2 is expressed in developing joints of murine limb

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We used a transgenic mutant with ROSA beta-geo inserted 5' to the ORF of mCtBP2 (Baker et al. 1997 Dev. Biol. 185:201–214) to study joint development in murine fore and hind limbs. mCtBP2 encodes a transcriptional corepressor protein known to interact with the transcription factor delta EF1, among others. Homozygous mCtBP null mice on a B6/CH3 background die before limb bud formation. Homozygous delta EF1 null mutant mice exhibit joint fusions (Takagi et al. 1998 Development 125:21–31). We find cartilaginous anlagen of limb skeletal elements in heterozygous embryos from E12.5 express mCtBP2 as indicated by X-gal staining. However, in limbs from E13.5 to E16.5 X-gal staining diminishes in mature chondrocytes, remains in the perichondrial area, and strongly increases around the articular cartilage at developing joints. Using an antibody to Collagen I, immunocytochemistry of sectioned limbs from E15.5 show mCtBP2 expression is strongest just peripheral to the perichondrium, suggesting that mCtBP2 may participate in delineating a boundary between adjacent perichondria of the joint. Moftah, et al. 2002 (Dev Bio 249:270–282) showed that chick limb bud micromass cell cultures treated with FGF2 and FGF8 exhibit peripheral and perinodal inhibition of chondrogenesis resulting in well-spaced nodules whereas chick embryos treated with morpholino to FGFR2 in ovo exhibit joint fusions. In micromass cultures of the transgenic limb bud cells, mCtBP2 expression is strongly enhanced by treatment with FGF2 but unaffected by FGF8 or FGF10. J.S. is grateful for the help and support of Gary E. Lyons, U.W. Medical School, Madison WI and of Goucher College.

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BMP signaling is required for limb bud mesenchyme survival

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BMP signaling has been implicated in patterning the developing vertebrate limb. To dissect the role of BMP signaling in the limb bud mesenchyme, we have inactivated the BMP receptor 1A (Bmpr1a) using the Ap2Cre mouse line. In the mutant autopod, phalanges are absent, metacarpals/metatarsals are fused and carpals/tarsals are malformed. To study the molecular mechanism involved in this phenotype, we examined Fgf4 and Fgf8 expression, which encode the principle activity in the apical ectodermal ridge (AER) required for proximal–distal outgrowth. However, we found almost no change in their expression except for an expanded expression domain in the anterior hindlimb AER at E11.5, thereby excluding a lack of AER-FGF as an explanation of this phenotype. We also detected no significant difference in the expression of genes from different Hox clusters, suggesting that early patterning may not underlie the Ap2Cre; BmpR1a defect. Sox9 expression, which is an early indicator of chondrogenic fate, showed little change in E10.5 embryos. However, soon after E10.5, Sox9 expression begins to change and is transformed in 11.5 embryos, recapitulating the skeletal pattern. During this period of changing Sox9 expression (E10.5–E11.5), we detected an increase in cell death in mutant limb buds. Thus we speculate that until E10.5 cartilage precursors in mutant buds are present but excess apoptosis impedes production of a sufficient progenitor population to form condensations of an adequate size for progression to normal differentiation. Thus, a principle role of BMPs in the early limb bud may not be patterning per se but cell survival.

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bHLH transcription factor regulation of vertebrate limb pattern

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Precise execution of developmental programs requires that signaling center activity is appropriately regulated. We are addressing how the bHLH transcription factors Twist1 and Hand2 control signaling activity during vertebrate limb development.

Defining the role of Twist1 in limb patterning has been difficult. While Twist1 null embryos have early molecular defects, early lethality precludes morphological analyses. We generated a Twist1 allelic series using Twist1 hypomorph and null alleles. These embryos allow correlation of early molecular and late skeletal phenotypes. As Twist1 activity is reduced, there are progressively more complex patterning defects, including preaxial polydactyly, anterior element loss and girdle defects. Many defects are rescued by reducing Hand2 dosage, underlining the importance of relative Twist1 and Hand2 levels.

Twist1 has multiple molecular roles that vary temporally and spatially, that are subject to quantitative and qualitative thresholds, and that involve multiple targets including transcription factors and signaling molecules. Interestingly, the molecular defects are not the most obvious that might be predicted from the morphological phenotypes, likely due to

nonautonomous effects that follow from disrupted signaling center regulation. Furthermore many of the skeletal and molecular phenotypes are encompassed by the sum of Twist1 target mutant phenotypes.

Our data have begun to reveal how balanced networks of antagonistic transcription factors control multiple aspects of limb patterning.

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Knockdown of alpha-1-microglobulin bikunin precursor (AMBP) causes ocular, and craniofacial defects

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We, as part of a of a large-scale morpholino-based screen of commonly translated and transported proteins (transcripts with signal peptides), identified the zebrafish homologue of human alpha-1-microglobulin bikunin precursor (AMBP). Knockdown of AMBP with 2 nonoverlapping translational blocking morpholinos results in a quantifiable large pupil and microphthalmic eye. Transverse sections of 3-day post-fertilization larvae demonstrate a protruding lens and reduced globe size when compared to uninjected controls. Hematoxylin and eosin staining of eye sections show a considerable disruption of retinal layering and a nearly absent photoreceptor layer. The larvae also exhibit jaw and branchial arch anomalies with smaller pectoral fins. Lastly, we see a disruption in expression of an enhancer trap line in the 4th ventricle choroid plexus. We are currently pursuing the hypothesis that the bikunin subunit of the protein functions as a mitogen.

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Adhesion ligand nanopatterning influences differentiation of preosteoblast cells: A combined experimental and computational approach

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We combine experimental and computational approaches to study the role that RGD presentation plays in regulation of osteogenic differentiation. An artificial extracellular matrix, alginate hydrogel, is used to present nanopatterns of RGD. Specifically, RGD is patterned into multivalent islands that are distributed throughout the matrix to promote integrin clustering and drive osteogenic differentiation. The nanopatterns are characterized using a multi-scale Monte Carlo modeling approach to predict both ligand presentation within islands and island distribution throughout the matrix. To test specific nanopatterns, MC3T3 preosteoblast cells are cultured in the hydrogel and assayed for osteocalcin secretion. Our computational work predicts that island distributions must be dense enough to include groups of islands and 5–25 RGD/island are necessary to observe an impact on osteogenic differentiation. These patterns can then be tested experimentally. For example, when MC3T3 cells are cultured both in nanopatterned matrix (using patterns suggested by the models) and in non-patterned matrix with the same bulk RGD density, cells in the patterned gels show a two-fold increase in osteocalcin secretion on day 10 and a 25% increase on day 21. Both the valency of the islands and the island distribution appear to impact osteogenic differentiation. These studies suggest that osteogenesis can be manipulated by the nanoscale organization of synthetic ECM.

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N terminal variation in zebrafish calcium channel beta subunit genes

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In many organisms, the embryonic heart circulates blood before the organ itself is fully formed. From the outset, calcium coordinates excitation–contraction coupling and may indirectly affect growth and morphology of the heart as well. L-type calcium channels comprise the primary mode of calcium entry into cardiac myocytes. Cardiac L-type calcium channels are oligomeric complexes composed of a pore-forming alpha1C subunit, and auxiliary beta and alpha2-delta subunits. The beta subunits fine-tune L-type calcium channel function by modulating their gating properties and enabling the cell-surface expression of the alpha subunit. We report the cloning of four new zebrafish calcium channel beta (CACNB) subunit genes. All four genes show alternative splicing of N terminal exons. In humans, alternative N terminal CACNB4 isoforms are functionally significant, in that the encoded subunits differentially affect calcium channel gating in Xenopus oocytes. Zebrafish CACNB N terminal exons are short, separated by large genomic distances, and likely subject to individual regulatory control. All four genes are expressed in the embryonic heart, but isoforms show different temporal patterns of expression in embryonic development and adults. Morpholino data indicate that three CACNB genes are essential for cardiac function, but suggest an earlier, pre-gastrulation requirement for one gene. We hypothesize that heterogeneity in CACNB protein composition during development provides a mechanism to modulate L-type calcium current as the heart grows and to facilitate changes in contractile properties of the developing heart.

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