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MEETING REVIEWS (SHOP TALK)

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Discussing limb development and regeneration in Barcelona: The future is at hand

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From July 2 to 5, the city of Barcelona hosted the EMBO Workshop on Limb Development and Regeneration (the 15th international meeting on this topic). Wrapped by the literal warmth of the Spanish city by the sea, scientists from all over the world presented and discussed the new developments of a classic field that has rapidly and enormously benefited from the use of state-of-the-art tools. Here, we present a summary of the latest advances on gene regulation, patterning, morphogenesis, growth, and evolution of limb development and regeneration.

The *Gene Regulation* arena was dominated by the use of new approaches. The lab of keynote speaker **Len Pennacchio** (Lawrence Berkeley Laboratory, Berkeley, CA) has developed an enhancer ranking method based on the intensity of H3K27ac or P300 peaks, which correlates well with the activity of the element. Moreover, they have improved transgenic assays by using targeted insertion via CRISPR/Cas9, minimizing nonspecific and silencing effects. The increased efficiency now allows for high-resolution interrogation of whole regulatory regions. Continuing with new approaches, **James Sharpe** (EMBL Barcelona, Spain) gave a live demo of the new tool his team has built, LIMB-NET. This browser-based application allows the user to upload, edit, and test different models of the gene regulatory networks of interest, simulate the outcome on a 2D model of mouse limb outgrowth, and interactively explore the predicted gene expression patterns over time and space. Besides new tools, a common theme in the session was the robustness of regulation. **Aimee Zuniga** (University of Basel, Switzerland) described a small TAD rich in enhancers for *Grem1*, the removal of which yields a loss-of-function phenotype. Surprisingly, removal of several of this enhancers lead to weak or no effect on expression or in skeletal phenotype, revealing remarkable robustness, probably due to plasticity of chromatin interactions upon deletion of some of these regions. **Denis Duboule** (University of Geneva/EPFL, Switzerland) went out on a limb to show regulation of 5′ Hox genes in genitalia. Surprisingly, when they studied one region responsible for 70% of 5′ Hox gene expression, they discovered that none of the four presumptive enhancer sequences was individually necessary for that regulation. It remains to be seen if and how combinatorial deletion impacts gene expression. There were also new proposals on how some transcription factors work. **Steve Vokes** (University of Texas at Austin, TX) showed evidence suggesting GLI proteins mediate repression via recruitment of histone deacetylases that deactivate enhancer activity without affecting histone methylation. **Marie Kmita** (Clinical Research Institute of Montreal, Canada) described new roles for HoxA13 and HoxD13 in facilitating chromatin accessibility, enabling other transcription factors to bind their motifs. This could contribute to mechanisms

underlying tissue-specific variation in Hox targets. **Marian Ros** (Institute of Biomedicine and Biotechnology of Cantabria, Spain) showed her lab's studies on the binding sites of SP6 and SP8 factors in the establishment of the apical ectodermal ridge and dorsoventral polarity. Interestingly, SP8 shows a dual mode of action: indirect binding through interaction with DLX, that tends to occur in enhancers, or direct binding to GC-rich motifs with equal distribution between promoters and enhancers.

In the *Patterning* session, the oft-forgotten soft tissues got their fair share of attention. **Malcolm Logan** (King's College London, UK) showed the requirement of *Tbx5* within the muscle connective tissue to provide positional information to the forming muscle masses. It remains an interesting puzzle how this broadly expressed gene conveys its positional effects. **Maëva Luxey** (Tschopp Lab, University of Basel) presented light-sheet microscopy data to reveal muscle and nerve development with unprecedented resolution. In the case of mirror-image retinoic acid-induced digit duplications, she showed evidence of perfect symmetry in the case of muscles but imperfect for the nerves. Still in digit territory, **Susan Mackem** (National Cancer Institute, Bethesda, MD) proposed that during early AP patterning, sonic hedgehog (SHH) direct action would be very short (autocrine) and limited to forming digits 4 and 5, whereas indirect relay signals specify anterior digits 1-3. Surprisingly, later-stage direct SHH signaling acts long range but mainly controls survival and expansion. The nature of the relay is still uncertain, but **Jianjian Zhu**, also from her lab, proposed that Hox genes such as Hox13 members could be involved. Switching to the PD axis, **Irene Delgado** (Torres Lab, Spanish National Center for Cardiovascular Research, Spain) presented loss-of-function studies of *Meis1* and *Meis2* genes. The data support a model in which Meis absence in the distal region would trigger *Hoxa13* activation and autopod specification, while a gradient of Meis expression controls stylopod and zeugopod fates. It remains to be determined whether a gradient of distal FGF is enough to explain the opposing gradient of Meis. **Amitabha Bandyopadhyay** (Indian Institute of Technology Kanpur, India) showed that shifting the retinoic acid-FGF balance by fine-tuned pharmacological manipulation shifts the position of the stylopod-zeugopod joint in chicken. This correlates with the expression of the gene *Barx1*, expressed very early at sites of future joint formation. Gain- and loss-of-function studies showed partial interconversion between cartilage and joint tissue, suggesting that other components are also involved.

In the *Patterning and Regeneration* session, a big theme was the dichotomy between intrinsic regenerative abilities and the role of the wound environment. **Ken Muneoka** (Texas A&M University, TX) made the point that in mice the inability to regenerate might just be a problem of the environment. Indeed, digit stumps normally do not regenerate after amputation at the second phalanx level (P2), but Muneoka showed that increased bone formation and even joint cavitation took place when beads soaked in bone morphogenetic proteins (BMPs) were implanted into the amputation wound. Another important point was the fact that non-regenerative healing is dynamic and transitions from antiregenerative to permissive then back to anti-regenerative. **Caroline Dealy** (UConn Health, Farmington, CT) added to these results showing that the outcome of P2 amputation is very much dependent on the level of amputation in BL6 mice. Indeed, partial regrowth after P2 amputation correlated with the presence of a progenitor population in the periphery of the phalanx stump. She also showed that stimulation of EGFR signaling promoted P2 regenerative responses. On the other hand, keynote speaker **Elly Tanaka** (Institute of Molecular Pathology, Austria) used some grafting experiments to show that the regenerative failure in Xenopus limbs is due in part to their intrinsic inability to respond to a regenerative environment. She also showed that, in axolotl, injuries induce a retinoic acid responsive state, and that retinoic acid produced by the nerves is required for expression of *Meis*, a proximal limb marker. Interestingly, the inability of lower arm cells to induce *Meis* seems to be a combination of their environment and their intrinsic properties. **Koji Tamura** (Tohoku University, Japan) is also studying positional memory and position-dependent effects on regeneration; most remarkably the fact that proximal amputations of the zebrafish caudal fin regenerate faster than distal amputations. They discovered that the distribution of growth rate and growth period follows the lobulated shape of the fin, rather than being a flat front, suggesting that the final shape is already encoded in the proliferative capabilities of the regenerating fin cells.

Two common themes in the *Morphogenesis and Modeling* session were the application of new live imaging systems for direct visualization of cell movements during limb development and understanding the interface between tissue forces and developmental outcomes. **Jerome Gros** (Institut Pasteur, France) presented an experimental set-up for explanting developing mouse autopods and imaging the direct chondrogenesis that occurs in the digit tip. His group showed data regarding the connection between morphogenetic events and specification of chondrocytes during phalanges formation. A new 3D computer simulation of limb morphogenesis, taking into account tissue growth, cell polarization and cell intercalation, was introduced by **Miquel Marin-Riera** (lab of James Sharpe, EMBL Barcelona, Spain). **Sevan Hopyan** (The Hospital for Sick Children, Canada)

also presented modeling work, here as a product of experimental measurements made via direct imaging, a genetically encoded tension reporter, and magnetic tweezers. **Yoshihiro Morishita** (RIKEN Center for Biosystems Dynamics Research, Japan) used comparative embryology between chick and Xenopus to create tissue deformation maps for the developing limb, concluding that rescaled deformation dynamics for both species have high similarity each other. **Sarah Rubin** (lab of Elazer Zelzer, Weizmann Institute, Israel) used light sheet microscopy to explore chondrocyte cell morphology and behavior. She discovered that cell and nuclear morphology change in correspondence to their differentiation state in the growth plate. Additionally, she found that nuclear, and not cell, morphology was defective in *gdf5* mutants. Finally, she found that normally, columns of proliferative chondrocytes are arranged in straight rows rather than spirals, but this typical column organization was disorganized in *gdf5* mutants, possibly connected to a defect in amount of extracellular matrix rather than cell shape. A little-understood aspect of limb development is the formation of the supporting proximal structures, the pelvic girdle and shoulder. **Christian Bonatto Paese** (lab of Mark Lewandoski, National Cancer Institute) presented work defining the time window of pelvic girdle development using a Sox9 marker, and he demonstrated that loss of FGFs expressed by the apical ectodermal ridge causes loss of two of the three pelvic girdle skeletal elements.

The *Growth* session featured diverse questions and model systems. **Megan Davey** (University of Edinburgh, UK) presented findings interrogating mechanisms of digit reduction in ratite birds. Using experiments, which included the generation of chicken-emu chimeric limbs, her group concluded that rate of embryonic development, "embryonic tempo," is a species-specific trait, which is not influenced by the embryonic environment. **Alberto Rosello-Diez** (Australian Regenerative Medicine Institute, Monash University, Australia) presented his lab's studies on catch-up growth of the embryonic limbs in mice. Whole-mesenchyme and cartilage-localized genetic disruption of cell survival reveal that catch-up growth is subject to both intrinsic and extrinsic control and that these compensatory mechanisms for ensuring scaling can be over-ridden by localized expression of specific factors such as Connective Tissue Growth Factor (CTGF). **Hanh Nguyen** (lab of Nadine Peyrieras, project led by Elena Kardash, CNRS, France) turned to zebrafish to leverage live-imaging to investigate the growth of the pectoral fin. Using customized imaging and semi-automated tracking, she has been able to identify and count up to 700 cells in each fin, with more than half being tractable. This method allowed her to conclude that pectoral fin anisotropic growth is largely achieved through cell compaction along the dorsoventral axis. **Neil Vargesson** (University of Aberdeen, Scotland, UK) discussed the role and importance of blood vessels in normal limb development and presented further data demonstrating how blood vessels could mediate thalidomide-induced damage. He also discussed recently identified molecular targets from his group and others, how they may interact with blood vessels, and how this data together are beginning to finally explain thalidomide-induced limb damage. His group have also screened for structural variants of thalidomide and identified some with clinically relevant actions with no side effects—these will be discussed at the next meeting. Canonical limb development factors were investigated in developing axolotl limbs, which lack a morphological AER, by **Sruthi Purushothaman** (lab of Ashley Seifert, University of Kentucky). While *fgf10* is expressed in limb mesenchyme similar to chick and mouse, she found that transcripts ordinarily expressed by the AER in those species (such as *fgf8*, *9*, and *17*), are instead made only in mesenchymal cells in axolotl limb buds demonstrating that salamanders lack a molecular AER. They also showed that *shh* expression is proximalized in axolotl. Furthermore, using Fgf inhibitors, they discovered that Fgf-signaling does not control *shh* signaling or proximo-distal patterning and instead controls cell proliferation and limb size.

In the *Evolution* session, several presenters revisited the loss-of-flight question in birds. Also using comparative studies between chicken and emu, **John Young** (lab of Cliff Tabin, Harvard Medical School) found that the epithelial-to-mesenchymal transition necessary to form a limb bud as well as the recruitment of muscle progenitors appear to be similar in both species. However, emus exhibit dramatically less cellular proliferation in the limb bud, and differences in Fgf signaling were discovered between forelimb buds of the two species. Emu embryos with transplanted pieces of chick forelimb mesenchyme showed rescue of cellular proliferation and expression of key AER signaling factors, such as *fgf8*, arguing that there is an intrinsic difference between chick and emu mesenchyme that accounts for differences in forelimb bud growth and, ultimately, overall wing development. Turning to trait acquisition, **Karen Sears** (University of California, Los Angeles, CA) addressed the question developmental novelties using two flight membranes in bats. She showed that the plagiopatagium, or the central-most membrane, develops as a de novo outgrowth from the body and then fuses to the limb. The shape of the wing membranes correlates with the type of diet and therefore flight behaviors that each species exhibits, with fruit-eating bats having shorter wings and insect-eating bats having longer, narrower wings. The question of how individual features are specifically changed over evolutionary

FIGURE 1 EMBL limb development and regeneration workshop in Barcelona, July 2-5, 2019

time was also addressed by **Kim Cooper** (University of California, San Diego, CA). Focusing on skeletal proportions in limb long bones, she shared RNAseq results implicating several genes in the disproportionate growth of the jerboa hindlimb compared to mouse and provided evidence that *mab21l2*, identified in these studies, is a negative regulator of skeletal growth possibly via modulation of the BMP signaling pathway. Another theme was evolutionary transitions between limb forms; **Joost Woltering**, **Renata Freitas**, and **Brent Hawkins** each presented work on the fin-to-limb transition. Two independent zebrafish mutants were described by Brent Hawkins (lab of Matt Harris, Harvard Medical School), each of which form aberrant skeletal structures in the pectoral fin that were characterized to be supernumerary radials complete with ectopic joint. This work uncovered a latent potential in fish to form transition-type features that may have been used along the evolutionary pathway toward limb development.

The meeting concluded with a keynote address by **Deneen Wellik** (University of Wisconsin, Madison, WI). First, she reviewed the paradigm-shifting work her lab published a few years ago about continued regional expression of Hox genes in stroma of the mammalian skeleton from embryonic stages through adulthood. In this work, they identified these cells as mesenchymal stem/stromal cells (MSCs) residing in the bone marrow and implicated them in fracture repair. New results using a Hoxa11 lineage-trace model demonstrates that this pool of MSCs both self-renews throughout the lifetime of the animal and gives rise to chondrocytes, osteoblasts, and adipocytes in vivo. New work from the lab demonstrates continued function for Hox genes in both the adult skeleton and muscle.

In summary, the 2019, Limb Development and Regeneration conference in Barcelona was a great success, highlighting the broad range questions and model organisms for which modern technologies are now illuminating specific molecular mechanisms (Figure 1).

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