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MEETING REVIEW

Size regulation blossoms in Kobe

Iswar K. Hariharan*

ABSTRACT

Coincident with the blossoming of the *sakura* was the 14th annual CDB Symposium hosted by the RIKEN Center for Developmental Biology in Kobe, Japan. This year's meeting, 'Size in Development: Growth, Shape and Allometry' focused on the molecular and cellular mechanisms underlying differences in size and shape and how they have evolved. On display was the power of using diverse approaches ranging from the study of organoids to whole organisms.

KEY WORDS: Hippo pathway, Growth control, Morphogen, Regeneration, Tissue shape

Introduction

The growth of organisms is driven by the synthesis of biomass by individual cells. The growth and proliferation of individual cells is, in turn, regulated by their immediate microenvironment; cells grow and/or divide when nutrients are available and in the presence of extracellular signals that promote survival, growth and cell cycle progression. The size and shape of an organ or organism, which may be composed of thousands or millions of cells, is a collective property of all of its cells. Unraveling the mechanisms by which cell growth and proliferation are regulated to generate organs and organisms of a predictable size and shape is one of the greatest challenges faced by developmental biologists. The organizers of the CDB Symposium 'Size in Development: Growth, Shape and Allometry' – Shigeo Hayashi, Hidehiko Inomata, Mitsuru Morimoto (all from RIKEN CDB, Kobe, Japan) and Stefano Piccolo (University of Padua, Italy) – brought together a diverse group of researchers to discuss this topic. As summarized here, the presentations showcased important progress in addressing this problem on many fronts. Owing to space constraints, I have covered only the talks given by the invited speakers and not the many excellent short talks and posters presented at the symposium.

Organoids: a simpler way of studying size and shape

Until recently, it was generally thought that the mechanisms that regulate organ size and shape could only be studied in the context of an organism or occasionally in organ explants. This notion has been shattered by the demonstration that many types of organoids can be generated from embryonic stem cells (ESCs). Remarkably, the structures generated by these organoids can be of a predictable size and shape. Mototsugu Eiraku (RIKEN CDB, Kobe, Japan) discussed experiments in which mouse ESCs can generate an optic cup with a layered neural retina (Eiraku et al., 2011) (Fig. 1). This seems to occur independently of external forces and attests to the self-organizing properties of retinal progenitor cells. James Wells (University of Cincinnati, USA) demonstrated how human ESCs could be directed to differentiate to generate specific portions of the intestinal tract that were even capable of performing a variety of functions related to digestion (Watson et al., 2014). The mechanisms that regulate organoid size and shape might be simpler to unravel than those that function in organs.

Alexander Aulehla (European Molecular Biology Laboratory, Heidelberg, Germany) showed that even somite formation, a complex developmental process characterized by oscillations in the activity of multiple signaling pathways, could be studied in cultures of cells dissociated from the presomitic mesoderm (PSM) (Lauschke et al., 2013). In monolayers of mouse PSM cells of different linear dimensions, the oscillation period was unchanged but the phase gradient (the shift in the oscillation phase over a given distance) was inversely proportional to the linear dimension of the monolayer. Thus, smaller monolayers possess steeper phase gradients. If somite boundaries are separated by a constant difference in phase, then the inverse relationship between the phase gradient and the length of the PSM can potentially explain why smaller embryos generate proportionately smaller somites.

Morphogen gradients *in vivo*

Although the importance of morphogen gradients in development is widely appreciated, we still have a poor understanding of how morphogen gradients robustly and accurately regulate tissue size. Hidehiko Inomata (RIKEN CDB, Kobe, Japan) discussed experiments that show how the gradient of BMP signaling in the early *Xenopus* embryo can adjust to compensate for changes in embryo size (Inomata et al., 2013). Spemann's organizer in the dorsal portion of the embryo secretes a group of BMP inhibitors. A gradient of one of these inhibitors, Chordin, antagonizes BMP ligands secreted from the ventral side of the embryo to set up graded values of BMP signaling along the dorsoventral axis. Remarkably, when the embryo is bisected, the dorsal portion can still develop into a normally patterned larva, suggesting that the gradient of BMP signaling scales with the altered dimensions of the embryo. This is because the level of expression of the Sizzled protein, which stabilizes Chordin, is dependent on the size of the embryo. Smaller embryos with less Sizzled degrade Chordin more rapidly and hence have a steeper gradient of Chordin and BMP signaling.

Also looking at signaling gradients *in vivo*, Yingzi Yang (Harvard School of Dental Medicine, Boston, USA) described a pathway by which graded expression of Wnt5a in the developing mouse limb bud promotes limb growth by regulating planar cell polarity in chondrocytes (Gao et al., 2011). In the chondrocytes of the limb bud, the gradient of Wnt5a regulates Vangl2 phosphorylation, which in turn regulates Vangl2 activity. By a series of feed-forward interactions, this eventually results in the asymmetric localization of Vangl2 to the proximal surface of the chondrocytes; this appears necessary to promote their growth and limb elongation.

Linking properties of cells to the size and shape of tissues and organs

How can differences in the properties of cells influence the structures that they collectively form? Richard Schneider (University of

Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA.

*Author for correspondence (ikh@berkeley.edu)

I.K.H., 0000-0001-6505-0744

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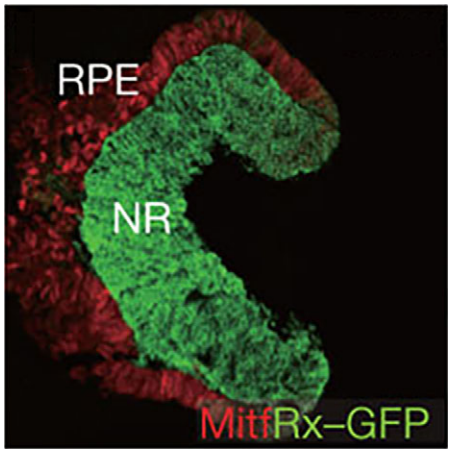

Fig. 1. An optic-cup-like structure generated in a 3D culture of aggregated mouse ESCs. The structure shows both a retinal pigment epithelium (RPE; Mitf, red) and a neural retina [NR; Rx (Rax)-GFP, green]. Reprinted with permission (Eiraku et al., 2011).

California, San Francisco, USA) addressed the cellular basis of differences in mandible size between ducks and quails. Ducks have larger mandibles than quails. The facial skeleton derives from neural crest cells, and hence the mechanisms responsible for the differences in mandible size can be addressed by generating chimeras by transplanting quail neural crest cells into duck embryos. Studies on these 'quck' chimeras have identified multiple factors involved: the progenitor pool is larger in duck embryos and continues to proliferate for a much longer period, whereas the quail tissue has a much higher rate of bone resorption, which is likely to contribute to the formation of a smaller mandible (Ealba et al., 2015; Fish et al., 2014).

In addition to proliferation rate and duration – which are among the major driving forces of mandible shape in birds – cell size, cell shape and the orientation and pattern of cell divisions are key regulators of tissue morphology in both plants and animals. Mechanisms that operate to generate the form of leaves in plants were highlighted by Mitsuyasu Hasebe (National Institute of Basic Biology, Okazaki, Japan), who discussed the evolution of the pitcher-shaped leaves found in the carnivorous pitcher plant from ancestors with planar leaves (Fukushima et al., 2015). Since plant cells have rigid cell walls, almost no cell migration occurs during development. Rather, localized changes in the orientation of cell division can result in growth in a different plane and hence in different leaf shapes.

What is the relationship between nuclear size and cell size? Mary Baylies (Sloan Kettering Institute, New York, USA) studies this interesting question in the larval muscles of *Drosophila*, which are multinucleate cells. In these muscles, cell size scales with the number of nuclei and the total cross-sectional area of nuclei, thus suggesting a close and possibly causal relationship between the size of nuclei and of cells.

Insights from tube growth

Shigeo Hayashi and Mitsuru Morimoto (both from the RIKEN CDB, Kobe, Japan) gave talks on size regulation in trachea. Shigeo Hayashi discussed the tracheal system in the *Drosophila* embryo, while Mitsuru Morimoto studies the mammalian trachea. The two systems differ in at least one important biological detail. In the *Drosophila* embryo, the tracheal branches are generated from a set of postmitotic placodes. By contrast, tracheal development in mice occurs concurrently with cell proliferation and involves a complex interplay between different types of cells.

The geometry of the tracheal system in the *Drosophila* embryo illustrates how interactions between cell membranes and the apical extracellular matrix (aECM) can regulate tube dimension (Dong et al., 2014). The overall length of the tracheal system has to match that of the embryo and individual branches also achieve a predictable size and diameter. How are these parameters regulated? An increase in the area of apical membrane drives tube elongation, which is resisted by the elasticity of the aECM. Proteins, including the enormous protein Dumpy, which is anchored to the apical membrane, and polymers, including a deacetylated form of chitin, are important contributors to the physical properties of the aECM. Prior to tube elongation, an increase in tube diameter is driven by increased production of aECM and resisted by circumferential actin cables in the epithelial cells. The aECM also serves to equalize forces along tubes so as to maintain a constant diameter within sections of the tracheal system. The development of the tracheal system in *Drosophila* represents, to date, one of the best-understood morphogenetic processes that occur in the absence of cell division.

In mice, tracheal development involves three different processes. Early on there is an elongation of the tube that is driven, in significant part, by an increase in cell number. Subsequently, cell proliferation decreases and the tube lumen expands. During this phase, the stratified epithelium changes to a pseudostratified epithelium. The combination of continued growth and reduced cell proliferation eventually results in the stretching and smoothing of the lumen. Importantly, the Morimoto group is now linking specific signaling pathways to each of the cellular processes that occur during the formation of the trachea.

The Hippo pathway in size, shape and beyond

The Hippo pathway has received increasing attention in recent years as a pathway capable of regulating cell growth in response to a wide variety of external signals. At the core of the pathway is a protein kinase (Warts in *Drosophila*, LATS in mammals) that phosphorylates a transcriptional co-activator (Yorkie in *Drosophila*, YAP/TAZ in mammals) resulting in its retention in the cytoplasm. In the nucleus, Yorkie associates with DNA-binding proteins such as Scalloped (Sd; TEAD in mammals) to promote the transcription of genes that promote growth or inhibit apoptosis.

Both Georg Halder (University of Leuven, Belgium) and Stefano Piccolo (University of Padua, Italy) (Zanconato et al., 2015) presented compelling arguments that growth promoted by alterations in Hippo signaling are important in regeneration following tissue damage and in several instances of tumorous overgrowth. Both investigators showed data that highlighted the proximity of Sd or TEAD sites to binding sites for the AP-1 transcription factor (a Fos/Jun heterodimer), which is activated by JNK signaling, suggesting that the two pathways might function together. This, together with relatively modest phenotypes obtained by eliminating the ability of Sd to both activate and repress gene expression, suggest that the Hippo pathway might be more important under conditions where accelerated growth is necessary rather than regulating the extent of growth under normal physiological conditions. Alternatively, growth during normal development might be regulated robustly by the combined activity of multiple pathways functioning with some degree of redundancy.

Several speakers highlighted functions of the Hippo pathway that are distinct from growth regulation. Hiroshi Sasaki (Osaka University, Japan) described the role of the Hippo pathway in specifying and stabilizing trophectoderm and inner cell mass fates in mouse blastocysts. During progression from the 8-cell stage to the

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32-cell stage, outer cells have an apical domain and are polarized, while inner cells are apolar and are attached to other cells by E-cadherin-mediated homophilic adhesion via their entire surface. The presence (in apolar cells) or absence (in polar cells) of Amot family proteins that interact with Hippo pathway components regulates YAP activity, such that nuclear YAP is only found in outer polar cells. This accumulation of YAP in the nucleus promotes the expression of genes appropriate for the trophectoderm fate. Thus, both cell position and polarity function together in regulating the extent of Hippo pathway activation (Hirate et al., 2013). It will be of great interest to determine whether other situations in which cells can divide asymmetrically to generate both polarized and apolar cells are also characterized by differences in Hippo pathway activity.

Makoto Furutani-Seiki (Yamaguchi University, Japan and University of Bath, UK) described the analysis of the *hirame* (*hir*) mutant in the medaka fish (*Oryzias latipes*), which is a loss-of-function mutation in the gene encoding YAP (Porazinski et al., 2015). The cells in *hir* mutant embryos have reduced actomyosin cortical tension, resulting in a flattening and collapse of their bodies suggestive of an inability to withstand gravitational forces. This phenotype is recapitulated in 3D spheroids of human cells, where YAP promotes the expression of a RhoGAP; when the level of YAP is reduced, there is excessive and ectopic actin polymerization. This results in abnormal deposition of extracellular matrix components and an inability to withstand mechanical deformation. These studies point to a potentially understudied function of the Hippo pathway in regulating tissue rigidity via the extracellular matrix.

Finally, Duojia Pan (Johns Hopkins University, Baltimore, USA) discussed recent findings that show that the Hippo pathway is activated by Toll signaling in cells of the *Drosophila* fat body (Liu et al., 2016). Surprisingly, Yorkie is normally mostly found in the nucleus in fat body cells (without causing major growth perturbations). When infected with gram-positive bacteria, Toll signaling activates the Hippo pathway by promoting the phosphorylation and degradation of a subunit of the phosphatase that inactivates the Hippo kinase. Hippo signaling results in a reduction of Yorkie-mediated transcription of IκB (*cactus*). This enables increased nuclear levels of the NFκB proteins Dorsal and Dif, thus promoting the synthesis of antimicrobial peptides. This nexus between the Toll and Hippo pathways also appears to function in imaginal discs. Here, Toll-like receptors (TLRs) and the Hippo pathway have both been separately shown to function in cell competition, a process by which cells can influence the survival of their neighbors that display altered growth properties. The link between these two pathways raises the possibility of a unification of at least some of the pathways that regulate cell competition.

Regeneration: why can some do it and not others?

For some animals, the processes of growth and tissue patterning are not restricted to their development; they can regenerate a variety of structures following damage. The capacity to regenerate differs widely between animal species. Moreover, even within a given species, animals frequently become less capable of regeneration as they mature and age. What underlies these differences? Nadia Rosenthal (The Jackson Laboratory, Bar Harbor, USA) discussed the role of the immune system in regulating the ability of a tissue to regenerate. Differences in early events in wound healing are evident following amputation in salamanders, which regenerate their limbs, as compared with mammals, which do not. Reducing the number of macrophages in a salamander compromises regeneration. However, a subsequent amputation of the stump results in regeneration if the number of macrophages is allowed to return to near-normal levels (Godwin et al., 2013). The secretion of Igf-1 by macrophages reduces inflammation and promotes regeneration.

Iswar Hariharan (University of California, Berkeley, USA) discussed the reduced ability of *Drosophila* imaginal discs to regenerate as the larva matures (Harris et al., 2016). A reduction in regeneration correlates with reduced damage-responsive expression of the Wnt genes *wingless* and *Wnt6*, which is mediated by an enhancer located between the two genes. As the larva matures, localized epigenetic silencing of this damage-responsive enhancer results in reduced Wnt expression following damage and thus attenuates regeneration. This type of mechanism would preserve the ability of those same Wnt genes to be activated by distinct developmentally regulated enhancers.

Genetic and environmental regulation of the evolution of size and shape

An age-old discussion in the evo-devo field is whether changes in animal form during evolution are driven primarily by changes in non-coding regulatory sequences or in protein sequences. Some argue that changes in protein sequences are likely to be more deleterious to the organism because they would impact all tissues, whereas changes in individual regulatory elements might allow changes in expression patterns, particularly of patterning genes that would result in changes in animal form or size. Others argue that evolution occurs by both types of changes and that the vast preponderance of non-coding DNA in complex genomes makes it more likely that mutations driving evolutionary changes in non-coding sequences are identified far more often than mutations that alter protein sequence.

In this symposium, speakers described examples in which genetic alteration of either type could account for some of the morphological differences between organisms. Rebecca Heald (University of California, Berkeley, USA) discussed the differences in size of subcellular structures between the large pseudotetraploid frog *Xenopus laevis*, which has larger cells, and the smaller diploid frog *Xenopus tropicalis*, which has smaller cells. The size of nuclei and mitotic spindles can be studied in extracts prepared from the eggs of the two species and, not surprisingly, the larger eggs of *X. laevis* have larger nuclei and larger spindles. Mixing of extracts between the two species results in nuclei and spindles of intermediate size. Using this system, her laboratory showed that altered properties of the nuclear import proteins importin α and Ntf2 of *X. laevis* increased the rate of nuclear import and thus nuclear size (Levy and Heald, 2010). The presence or absence of a phosphorylation site in katanin, a protein that regulates microtubule severing, can change the size of the mitotic spindle (Loughlin et al., 2011). These are clear instances of evolutionary changes in proteins that result in changes in the size of subcellular structures. Of course, changes that alter gene expression patterns on a tissue scale are unlikely to be detected in this type of system.

David Kingsley (Stanford University, USA) discussed the genetic basis of morphological differences between marine and freshwater populations of the three-spine stickleback fish. Using quantitative genetics and genomic association studies, his laboratory has shown that major morphological differences between two strains of sticklebacks can often be accounted for by differences in a few loci with major phenotypic effects. Moreover, the alterations in loci that have the largest effects usually involve changes in genes encoding major regulators of development. In the majority of these cases, there are alterations in *cis*-regulatory elements that can alter the level or spatiotemporal pattern of gene expression. There are instances in which the same phenotypic

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Fig. 2. The *sakura* in full blossom in Kyoto at the beginning of April, 2016. Photo by author.

change in different populations has resulted from independent and distinct alterations in the same regulatory element, as has occurred with an enhancer in the *Pitx1* gene resulting in pelvic reduction (Chan et al., 2010). The Kingsley laboratory has also looked for differences in conserved *cis*-regulatory sequences between chimpanzees and humans, and identified human-specific deletions upstream of *GADD45* and other tumor-suppressor genes that might have contributed to the increased size of the human brain (McLean et al., 2011). The functional consequences of such deletions are now being investigated in mice.

Elaine Ostrander (National Human Genome Research Institute, Bethesda, USA) described how the remarkable differences in the size and morphology among different dog breeds can be investigated using genetic methods (Schoenebeck and Ostrander, 2014). Nine loci can together account for 60-80% of the variation in standard body weight. These loci include both *IGF1* and the IGF1 receptor. An alteration in *FGF4* has been found in some breeds that have chondrodysplasia, a condition that results in short legs, and a change in *BMP3* is associated with craniobrachycephaly, as observed in breeds with rostrocaudal flattening of the cranium. The mutations in IGF1 and BMP3 are synonymous changes that are likely to affect the properties of the protein. The alteration in FGF4 appears to increase expression from a retrotransposed copy of the *FGF4* gene (Parker et al., 2009). Thus, using certain obvious physical differences as readouts, breeders have selected mutations that could either be in coding regions or in regulatory sequences. Taken together with the work presented by the other speakers, it appears that diversity in form can arise via both coding and non-coding genomic changes.

Ehab Abouheif (McGill University, Montreal, Canada) and Toru Miura (Hokkaido University, Sapporo, Japan) discussed the remarkable phenomenon of polyphenism in insects: the ability of the same genome to generate alternative morphological forms in response to environmental signals. Abouheif discussed wing polyphenism in ants (Rajakumar et al., 2012; Shbailat and Abouheif, 2013), while Miura presented data on termites (Toga et al., 2016). Wing polyphenism in ants is thought to have evolved once, ∼125 million years ago, such that reproductive castes have wings whereas sterile castes do not. The exploration of the mechanisms underlying wing polyphenism is still at an early stage but principles have started to emerge when comparing the wing specification network in the rudimentary discs of worker larvae of different species. These networks show consistent (stable) changes, as well as more labile changes. Both speakers discussed subdivisions in sterile castes that can generate individuals of vastly different sizes (e.g. soldiers versus workers). These differences can be elicited by exposing larvae to different levels of juvenile hormone at different stages of development. Moreover, the number of individuals of each type can influence the outcomes of development. For example, the addition of soldiers to an ant colony can promote the development of larvae to become worker adults rather than soldiers. Miura also discussed a stag beetle species that shows dramatic size variation but only in males (Gotoh et al., 2011). The gene expression changes that might mediate these differences are currently being explored. These talks illustrated that the phenomenon of polyphenism merits considerable attention and is likely to inform on how environmental factors can regulate animal form without necessitating genetic changes.

Concluding remarks

While the annual blossoming of the *sakura* (Fig. 2) is transient, the field of size regulation will only keep growing. The range of approaches presented in the meeting attest to how each type of experimental system can help us chip away at the big problem of size regulation from multiple sides. Although we still have much to learn, the progress made in recent years suggests that important breakthroughs in our understanding are on the horizon.

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Competing interests

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