Deconstructing the genesis of animal form

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Summary

Santa Fe – with its museums and galleries full of art and crafts inspired by natural forms – was the perfect setting for a Keystone conference on vertebrate organogenesis in February 2004. Organized by Gail Martin and Cliff Tabin, the conference sessions were loosely subdivided into anatomical systems – 'skin, hair, teeth', 'pancreas, liver, gut', 'skeleton', and so on. However, from the outset, common themes emerged that transcended particular organ systems and generated a sense of unity and excitement among the participants.

The first common theme to arise at this conference was clearly articulated by the keynote speaker Mark Krasnow (Stanford University School of Medicine, Stanford, CA, USA). He argued that the only way to identify all the genes controlling the development of a complex organ is to break the process down into simpler events that can be described at the cellular level. In other words, organogenesis must ultimately be reduced to a sequence of changes in parameters such as cell shape, polarity, movement, adhesion and proliferation. This approach enables the investigator to focus on specific events and cell populations in order to achieve a more-complete genetic analysis (e.g. by using gene arrays or proteomics). Other speakers also evoked the concept of deconstructing embryonic development into simple morphometric modules. They did so in the context of understanding how dramatic changes can occur during evolution in the size and shape of organs as diverse as the skeleton and brain.

A second theme to emerge during the meeting was the importance of using organ culture systems to study discrete steps in morphogenesis. The power of in vitro approaches has long been recognized. However, newer technologies for lineage tracing, time-lapse fluorescence microscopy and gene inactivation are making it easier to pinpoint the function of specific factors during morphogenesis. Emerging technologies in mouse genetics, including conditional gene manipulation and small interfering RNA (siRNA) gene knockdown in early embryos, are also having a strong impact on the field of organogenesis. In particular, they are enabling investigators to transcend the early lethality of null mutations and to take on heroic projects to determine the redundant functions of multigene families in specific tissues during organ development.

Finally, several speakers touched on the theme of tissue-specific stem cells in organogenesis, the plasticity of progenitor cells and the regulation of cell fate.

Deconstructing morphogenesis

One of Krasnow's take-home messages to students was the importance of carefully describing the cellular anatomy of organogenesis. He illustrated this concept with recent work from his own laboratory on the formation of the fine terminal branches of the *Drosophila* larval tracheal system. These branches, which ramify deeply into internal tissues, are generated from single cells containing a lumen 1 μm or less in diameter. This lumen is thought to be formed by the coalescence of multiple apical membrane vesicles that line up in the center of the cell. Using mosaic analysis to circumvent any requirements for tracheal genes early in development, members of his laboratory identified a set of larval mutants with defects in lumen formation. Some mutants completely lack lumens, including those with mutations in genes that encode highly conserved components of the vesicle trafficking machinery. One of the most dramatic tracheal mutants, called 'tendrils' because of the supernumerary, convoluted lumena seen in mutant terminal cells (Fig. 1), has mutations in a cytoskeletal protein. Krasnow suggested that this constitutes part of a novel scaffold system, somehow constructed in the center of the terminal cell, on which the vesicles line up before they coalesce. He raised the interesting idea that information on how prelumenal vesicles are aligned could shed light on how endothelial tubes are formed during blood vessel development in vertebrates.

The importance of vesicle fusion in organogenesis was also highlighted by Christopher Walsh (HHMI and Beth Israel Deaconess Medical Center, Boston, MA, USA), but in a completely different context – that of human brain development. He showed that brain size is reduced in mice mutant for the gene *Napa*, which encodes a component of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-mediated vesicle fusion complex. In *Napa* mutants, defects in the transit of membrane vesicles from the trans-Golgi apparatus to the apical cell surface in neuronal progenitor cells of the embryonic brain ultimately disrupt the highly ordered production and migration of neurons that are needed to form the laminae of the cerebral cortex (Chae et al., 2004). These and other studies of mouse mutations that affect cortex size and complexity have lead to the idea that changes in very basic cell biological processes – vesicle trafficking or spindle orientation, for example – can ultimately influence a biological process as profoundly important as the evolution of the human brain (Zhang, 2003).

The concept that simple changes in cell behavior underlie the development of complex organ systems was also the message of Didier Stainier (University of California, San Francisco, CA, USA), who works with the zebrafish embryo. He described how both the development of the heart and the looping of the gut ultimately depend on mesodermal cell migration and polarization. In the case of heart formation, for example, mutant analysis shows that fibronectin is required for the correct epithelial organization of the migrating myocardial precursors (Trinh and Stainier, 2004). Asymmetric gut looping requires that the right lateral plate mesoderm (LPM) migrates ventrally underneath the endoderm, pushing it to the left, while the left LPM simultaneously moves horizontally towards the midline. The gene *heart and soul* encodes an atypical protein

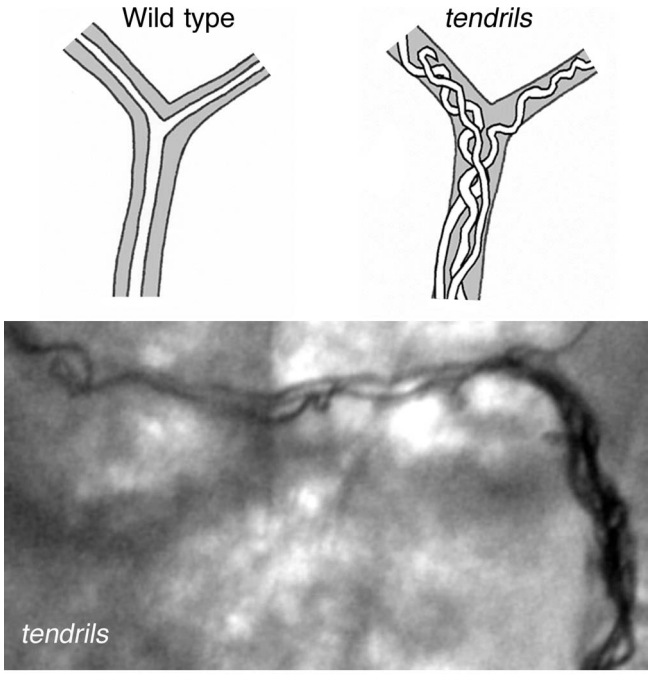

Fig. 1. Effect of the *Drosophila tendrils* mutation on lumen formation in the terminal branches of the tracheal system. Terminal branches are formed by long cytoplasmic projections of tracheal terminal cells that develop a single membrane bound lumen within each projection (top left panel). In *tendrils* mutant terminal cells (top right and bottom panels), multiple convoluted lumina are seen within individual projections. Image courtesy of Boaz Levi, Amin Ghabrial and Mark Krasnow (Stanford University School of Medicine, Stanford, CA, USA).

kinase C (PKC) required for establishment of cell polarity. Mutations interfere not only with cell polarization and lumen formation in the gut tube itself, but also with the epithelialization and migration of the LPM, leading to secondary effects on the looping of the gut (Horne-Badovinac et al., 2003).

Cell behavior during gut development was also the topic of discussion by Ken Zaret (Fox Chase Cancer Center, Philadelphia, PA, USA), although in relation to much earlier stages in mouse embryogenesis. By labeling definitive endoderm cells around the developing anterior foregut (known as the anterior intestinal portal or AIP) with the lipophilic fluorescent dialkycarbocyanine dyes DiI or DiO at the two-somite stage and then culturing the embryos in vitro, members of his laboratory were able to trace the origin of specific populations of foregut cells that contribute to the liver bud. Unexpectedly, he found that these liver bud cells come from two locations. Some derive from the lateral walls of the AIP that come together as the endoderm tube 'zippers up' ventrally. Others arise from a small population of endoderm cells in the anterior midline that gives rise to a line of cells along the ventral foregut tube, from the thyroid to the liver. A similar population of cells has recently been described in the chick embryo at an earlier stage (Kirby et al., 2003). It remains to be seen whether these midline foregut cells have organizer properties or fates different from the more lateral cells.

Morphogenetic modules and vertebrate evolution

The central dogma of evolutionary biology is that changes in the shape of anatomical structures over time are brought about by natural selection working on genetic variants. But precisely how such changes are brought about is not always well understood. Although emphasis is usually placed on changes in amino acid sequences in proteins, David Kingsley (Stanford University, Stanford, CA, USA) argued for the importance of variation in DNA regulatory elements, especially in genes encoding embryonic growth factors and other morphogenetic signaling proteins. He cited as evidence work from his laboratory on the mouse bone morphogenetic protein 5 (*Bmp5*) gene, which is required for the normal development of many cartilages and bones. Members of his laboratory have identified discrete DNA regulatory regions that drive *Bmp5* gene expression in specific anatomical sites such as the perichondrium of the ribs, sternum, ears or thyroid cartilage during mouse embryogenesis (DiLeone et al., 2000). In the case of the ribs, two different DNA elements were identified, lying hundreds of kilobases apart on the chromosome. One element promotes *Bmp5* expression on one lateral surface of the rib, while the other DNA element drives expression on the geometrically opposite side of the same rib! The significance of this spatial segregation became obvious when they investigated how ribs – which are cylindrical structures – dynamically change their shape during embryonic and postnatal development: to increase curvature, bone is deposited on one side of the cylinder and broken down by osteoclasts on the other. It therefore makes sense for the embryo to be able to control the activity of genes such as *Bmp5* in each domain independently. Evolutionary changes in the shape of the ribs (or any other skeletal element) could thus be brought about by selection for changes in the activity of one regulatory element versus another. Using the three-spined stickleback as a model genetic system, David Kingsley, Chuck Kimmel (University of Oregon, Eugene, OR, USA) and others are now testing such ideas in natural fish populations. They are identifying genes that control the striking morphological differences in skeletal elements such as spines, jaws and bony plates that occur in sticklebacks isolated in freshwater and marine locations around the world (for more information see Stanford Genome Evoution Center http://cegs.stanford.edu).

The application of embryology to evolution was also the subject of a fascinating talk by Cliff Tabin (Harvard Medical School, Boston, MA, USA). His laboratory is exploring the origin of the differences in beak shape and size among Darwin's finches in the Galapagos: the warbler finch, for example, has a thin long beak suited to catching insects, while the large ground finch has a thick short beak more appropriate for cracking nuts. Studies in chick have shown that the beak develops from facial buds, each consisting of an ectodermal jacket filled with neural crest and head mesoderm. The outgrowth of the buds depends on epithelial-mesenchymal interactions that are driven by signaling factors such as BMPs, sonic hedgehog (SHH) and fibroblast growth factors (FGFs) expressed in the different cell populations (e.g. Schneider and Helms, 2003). It is not unreasonable to suppose that the beak morphology of different finch populations depends on variations in the temporal and spatial patterns of expression of these signaling genes. To test this hypothesis, gene expression

is being analyzed in the developing beaks of finch embryos collected in the wild.

In vitro organ culture to explore organogenesis

Several investigators described the use of in vitro culture systems to study specific steps in organogenesis. Organs examined in this way included kidney and urogenital sinus [Costantini (Columbia University, New York, NY, USA), Carroll (Harvard University, Boston, MA, USA), Herzlinger (Cornell University, Ithaca, NY, USA), Mendelsohn (Columbia University, New York, NY, USA)], submandibular gland [Hoffman (NIH, Bethesda, MD, USA)], and inner ear [Wu (NIH, Bethesda, MD, USA)]. Joan Brugge (Harvard Medical School, Boston, MA, USA) described three-dimensional basement membrane cultures in which a mammary cell line undergoes a morphogenetic program leading to the formation of a hollow acinar-like structure. Formation of a lumen in these structures involves selective death of cells within the center of a solid mass (Fig. 2). Brugge's in vitro studies suggest that this cell death proceeds by two parallel pathways: apoptosis and a distinct, but poorly understood, mechanism known as 'autophagy', which is elicted by an extracellular ligand known as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (Mills et al., 2004). These findings are important because evasion of programmed cell death by either pathway appears to be a crucial step in the progression of breast cancer. The studies also raise the question of whether the processes observed in this in vitro model recapitulate events associated with clearing of the end buds of growing mammary glands. Initially, the end bud consists of a dense cluster of cells that become polarized and organized into an epithelial layer surrounding a lumen. Anatomical studies of normal mammary gland development by Cheryl Tickle (University of Dundee, Dundee, UK) had shown that end bud cells that do not undergo polarization are fated to die (Hogg et al., 1983). It will also be important to see if TRAIL-induced autophagy plays a role in other examples of lumen formation involving cell death (Coucouvanis and Martin, 1995).

Early kidney development lends itself well to analysis by in vitro culture, as the rudiment continues to develop normally for several days. Frank Costantini described the use of a cell autonomous *Hoxb7-GFP* transgene to follow the branching morphogenesis of the ureter bud in these kidney rudiments (Srinivas et al., 1999). During normal development, the ureter bud grows out from the caudal end of the Wolffian (mesonephric) duct into the surrounding nephric mesenchyme. It first gives rise to a T-shaped epithelial tube with an ampulla-like swelling at the tip of each primary branch. The tip cells then produce new branches and the process is repeated until a tree-like system of trunks and tips is generated. Costantini asked the simple question of whether the epithelial cells of the ureter bud are already committed to tip and trunk lineages. To address this, he made chimeras between *Hoxb7-GFP* transgenic and wild-type embryos. He then selected for organ culture those kidney rudiments in which, by chance, a relatively small number of *GFP*-expressing cells was clustered at the tip of the ureter bud. Following the fate of these cells by time-lapse videomicroscopy, he showed that the tip cells initially mix only with each other and do not mingle with the trunk cells, which stay together as they extend. However, as the ampulla undergoes branching, labeled tip cells are segregated into both new trunks and new tips. This disputes the irreversible commitment of ureter bud cells to tip or trunk fates. Prevailing models of kidney development, which are based on both genetic and experimental data, strongly suggest that the initial outgrowth of the ureter bud is driven by a local chemotaxic gradient of the transforming growth factor β (TGFβ)-related signaling factor glia-derived neurotrophic factor (GDNF) in the adjacent intermediate mesoderm (IM). This acts through the RET tyrosine kinase receptor in the duct and bud. Costantini challenged this model by using genetic techniques in the intact embryo to drive GDNF expression throughout the Wolffian duct and ureter bud while removing the gene from the mesenchyme. Contrary to expectations, the duct gave rise to numerous buds extending into the mesenchyme. Even more surprising, some of the ectopic buds even developed into normal-looking and well-patterned kidneys! It therefore appears that as long as GDNF is available for epithelial cell survival and proliferation, there is a redundant system for driving the branching morphogenesis of the epithelium.

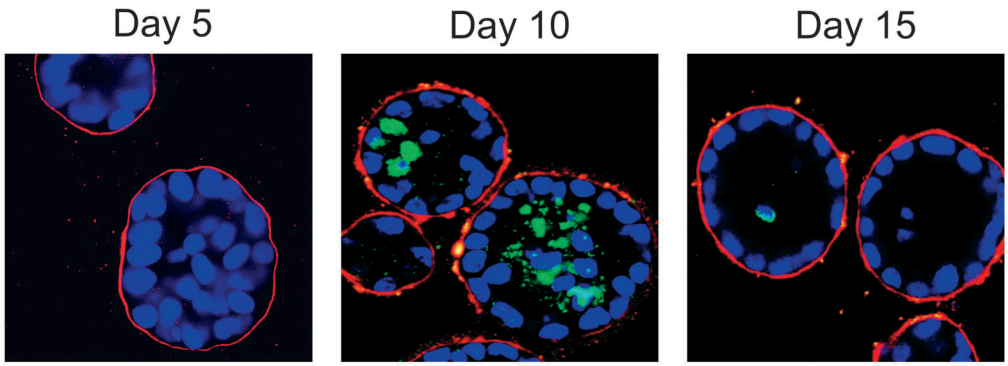

Fig. 2. The progressive stages in the morphogenesis of hollow acini-like structures that are generated by culturing MCF-10A immortalized mammary epithelial cells in basement membrane gels (Matrigel). Confocal images show solid masses at day 5 (left), structures with centrally localized apoptotic cells at day 10 (middle) and hollow structures at day 15 (right). Apoptotic cells were identified using an antibody to caspase 3 (green); laminin 5 was detected with an antibody to the g2 laminin chain (red); and nuclei were stained with DAPI (blue). Images courtesy of Kenna R. Mills and Jay Debnath (Brugge's Laboratory, Harvard Medical School, Boston, MA, USA).

Axonal guidance molecules in branching morphogenesis

New ideas about ureter bud morphogenesis were also presented by Uta Grieshammer, from Gail Martin's laboratory (University of California, San Francisco, CA, USA). She provided evidence for a role in kidney development of SLIT2 and ROBO2. These evolutionarily conserved proteins are best known as axonal guidance molecules, although there is evidence that they function in the branching morphogenesis of the *Drosophila* tracheal system (Englund et al., 2002). *Slit2* encodes a secreted protein that either repels or attracts neurons, whereas *Robo2* encodes one of its receptors. Grieshammer showed that in mouse embryos lacking *Slit2* or *Robo2*, numerous ectopic ureter buds emerge from the Wolffian duct anterior to the normal bud (Fig. 3), and some of them give rise to ectopic kidneys. *Slit2* is normally expressed in the Wolffian duct and in the adjacent intermediate mesoderm, while *Robo2* is expressed only in the mesoderm. In *Slit2*- and *Robo2*-null mutants, *Gdnf* expression, which initially extends along the IM, does not become restricted posteriorly to the site where the single ureter bud normally emerges. However, the mechanism underlying this defect is not clear. Based on various experiments, including lineage labeling, Grieshammer argued against the simplest hypothesis, that SLIT2 drives the posterior repulsion of *Gdnf*-expressing IM cells so that they aggregate around the normal site of ureter bud formation, and instead suggested that SLIT2/ROBO2 controls the extent of the *Gdnf* expression domain via effects on transcriptional regulators of *Gdnf* (Grieshammer et al, 2004).

A role for the axonal guidance molecule, netrin 1 (NTN1) and its receptor, neogenin, in mammary bud morphogenesis was the topic of a talk by Lindsey Hinck (University of California, Santa Cruz, CA, USA). She had observed that NTN1 protein is localized through the mammary end buds while expression of the receptor, neogenin, is confined to the overlying cap cells. *Ntn1*-null mutants die soon after birth, but Hinck exploited the fact that mammary rudiments can be transplanted into isogenic hosts to follow the early development of mutant buds as they rapidly extend throughout the fat pad. Hinck argued that, under these conditions, subtle effects caused by the absence of netrin proteins might be revealed. This was indeed the case, suggesting that netrin normally promotes adhesion between the end bud cells and cap cells (Srinivasan et al., 2003). Hinck has preliminary evidence that netrin proteins may collaborate in this process with other axonal guidance factors such as SLIT2. Evidence that axonal guidance molecules regulate branching morphogenesis in other organ systems came from Brigid Hogan (Duke University Medical Center, Durham, NC, USA). Members of her laboratory have found that genes encoding several netrins and netrin receptors are expressed in the epithelium and/or mesenchyme of the embryonic mouse lung. By combining three-dimensional culture of isolated endodermal lung buds in extracellular matrix with time-lapse videomicroscopy, they showed that NTN1 or NTN4 protein inhibits secondary budding elicited by exogenous FGF7. Taken together, their data point to netrins playing a role in controlling the precise shape and size of lung buds during development. However, *Ntn1*- and *Ntn4*-null mutants show no obvious morphological abnormalities in lung branching, suggesting that in this organ, as in the mammary gland, several axonal guidance molecules function redundantly to control branching morphogenesis (Liu et al, 2004). As discussed below, the problem of genetic redundancy was encountered by several speakers at the meeting and was, in some cases, overcome with the help of conditional gene inactivation in the mouse.

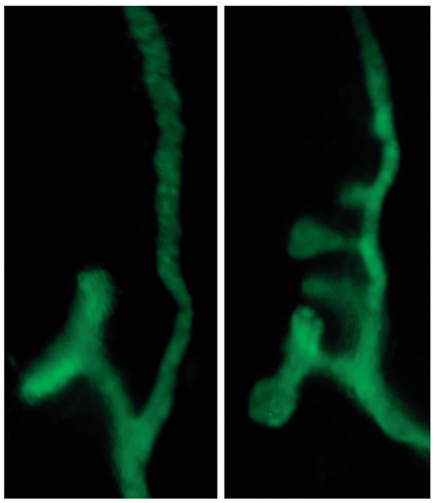

Fig. 3. Loss of SLIT2 function causes the formation of supernumerary ureteric buds. Expression of a *Hoxb7-GFP* transgene marks nephric duct-derived epithelia in mouse embryos at 11.5 days of gestation (anterior towards the top). (Left) The normal ureteric bud has emerged from the nephric duct. (Right) Supernumerary ureteric buds have formed anterior to the normal one in a *Slit2*-null mutant.

Genetic redundancy and new technologies in mouse genetics

It is now apparent that the development of most vertebrate organs is regulated by a relatively small set of evolutionarily conserved signaling factors – the FGF, BMP, WNT and hedgehog (HH) proteins, for example. However, each class may include more than 20 family members, which are encoded by different genes and/or by multiple splice variants of the same gene. Moreover, several family members may be expressed in the same or overlapping populations of cells at different stages of organogenesis. These redundancies frequently complicate the genetic analysis of vertebrate organogenesis. Sometimes it is possible to overcome the problem by brute force, generating a few embryos with multiple mutant alleles. This approach was used by Ryoichiro Kageyama (Kyoto University, Kyoto, Japan) in his work, to be described later, on the role of basic helix-loop-helix (bHLH) genes in neural stem cell maintenance.

In other cases of genetic redundancy, it is necessary to resort to making floxed alleles of several gene family members and then inactivating them simultaneously using a tissue or cell type-specific Cre recombinase. Gail Martin described a very successful use of this approach. She used a transgene that drives Cre in the apical ectodermal ridge (AER) of the mouse limb bud to facilitate inactivation of three out of the four FGF genes co-expressed in this tissue. Surprisingly, when *Fgf4*, *Fgf9* and *Fgf17* are inactivated, limb patterning is normal, being driven by the remaining family

member, *Fgf8*. Martin then asked whether there is a special requirement for *Fgf8* or whether its function can be replaced by *Fgf4* if the temporal and spatial expression pattern of this gene is changed to be more like that of *Fgf8*. This was achieved using a conditional gain-of-function transgene to express *Fgf4* in the limb bud in the absence of *Fgf8*. Under these conditions, *Fgf4* is able to substitute very well for *Fgf8*. Martin further outlined how the powerful genetic toolkit that she has generated is being used to test new ideas and models for the mechanisms patterning the skeletal elements of the limb (Mariani and Martin, 2003). The talk by Rolf Zeller (University of Basel Medical School, Basel, Switzerland) also brought home to the audience the fact that, in spite of all the research on limb development over the past decade, the story is still undergoing elaborations and revisions. Zeller presented very detailed and convincing new genetic evidence that the limb deformity phenotype is not due to inactivation of the formin gene, as previously thought, but to inactivation of the adjacent gremlin gene (*Cktsf1b1* – Mouse Genome Informatics), which is expressed in the developing limb bud and encodes an antagonist of BMP signaling (Khokha et al., 2003; Zuniga et al., 2004).

A new approach to mouse genetic analysis was well illustrated by Benoit Bruneau (Hospital for Sick Children, Toronto, Canada) in the context of exploring the function of one of the components of the SWI/SNF chromatin-remodelling complex. The particular subunit, SMARCD3 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily d, member 3), is expressed specifically in the early heart tube. To knock down the gene encoding this subunit, Bruneau collaborated with Janet Rossant's laboratory in Mount Sinai Hospital (Toronto, Canada). They used a recently described method in which DNA that encodes a neomycin-resistance cassette and the human H1 RNA polymerase III promoter to drive expression of a short hairpin RNA is electroporated into mouse embryonic stem (ES) cells (Kunath et al., 2003). Cells providing good expression of the siRNA are then aggregated with tetraploid embryos, so that the resulting fetus is derived entirely from the ES cells. Using another new technique, optical projection tomography (Sharpe, 2003), in conjunction with regular histology, Bruneau found that reduction in SMARCD3 levels leads to abnormal cardiac chamber morphogenesis, including specific defects in the outflow tract and ventricular trabeculations of the embryonic heart. Moreover, by comparing embryos derived from different cell lines, he was able to generate an 'epiallelic series' correlating the extent of gene knockdown with the severity and localization of the morphological defects. Finally, he used in vitro assays to show that SMARCD3 functions to potentiate the activation of *Pitx2* and other genes important in heart development.

Stem cells and switches in cell fate

As might be expected, stem cells featured in several of the talks at the meeting, with the emphasis being on tissue-specific stem cells contributing to organogenesis and tissue turnover and repair, rather than on pluripotential embryonic stem cells. Both Bruce Morgan (Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA) and Fiona Watt (Cancer Research UK, London, UK) considered stem cells in the epidermis of the adult mouse. One question is whether the normal turnover of epithelial cells in the skin relies, in the long term, on only a single source of multipotent stem cells, located in the bulge of the hair follicle. Morgan described preliminary studies in which he and his collaborator, Brian Harfe (UFGenetics Institute, Gainsville, FL, USA) have used a cell-autonomous lineage label to follow the fate of hair follicle placode cells that once expressed SHH. They found that all of the cells of the hair follicle, including the bulge, expressed the label, consistent with them being derived originally from the placode. However, even after a year, the epidermis between the follicles was not extensively labeled. This suggests that under normal conditions when there is no injury, the interfollicular epidermis is renewed from stem cells in this compartment and does not require migration of progenitor cells from the bulge. Fiona Watt presented evidence that activation of β-catenin in interfollicular epidermal cells could lead to the generation of new hair follicles and sebaceous gland cells. Whether the ectopic hair follicles have bulge stem cells is not yet known. However, Watt argued that the level and duration of β-catenin signaling in the interfollicular epidermal cells could lead to a change in the lineage of their descendants from interfollicular epidermis to hair follicle or sebaceous gland. Brigid Hogan also presented evidence that transient overexpression of a constitutively active Lef1/β-catenin fusion protein in progenitor cells of the embryonic mouse lung switches their fate to intestinal lineages (Okubo and Hogan, 2004). Thus, levels of WNT signaling may affect progenitor cell fate in more than one organ system.

The regulation of neural stem cell fate was the topic of a talk by Ryoichiro Kageyama. During normal brain development, a pool of stem cells has to be carefully maintained in the ventricular layer. Progenitors leave this pool at different times to migrate to layers where they give rise to different kinds of neurons. Kageyama showed that in animals double mutant for the genes encoding the bHLH transcriptional repressors, HES1 and HES5, progenitor cells in the ventricular layer differentiate into neurons prematurely, thus reducing the pool available to make later-born neurons. In triple mutants also lacking *Hes3*, the reduction in the pool of stem cells available to generate later neurons was even further reduced, and virtually all neural stem cells become early neurons without generating late neurons and glia. Precisely how the expression of HES genes regulates the individual behavior of stem cells and their topographic organization in the ventricular zone is under investigation.

Conclusion

In the space available here it has not been possible to cover all of the excellent talks, nor to include the data discussed so enthusiastically in the poster sessions. Taken together, the presentations contributed to a most stimulating and inspiring meeting. The feelings of the participants as they left Santa Fe are perhaps best summed up in the words of a Native American people of the Southwest.

May it be beautiful before me.
May it be beautiful behind me.
May it be beautiful above me.
May it be beautiful all around me.
In beauty it is finished.

from a Diné Night Chant

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