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MEETING REVIEW

The endoderm from a diverse perspective

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ABSTRACT

The historic town of Taos, New Mexico, with its rich multicultural history of art and craft, was the site of the second Keystone Symposium on 'Endoderm Development and Disease', which was held in February 2018. The theme of the meeting was 'Cross-Organ Comparison and Interplay', emphasizing an integrative and multisystem approach to the broad topics of organ physiology, homeostasis, repair, regeneration and disease. As we review here, participants shared their recent discoveries and discussed how new technologies developed in one organ system might be applied to answer crucial questions in another. Other integrative themes were how agents such as parasites, microbes, immune cells, physical forces and innervation can affect tissue organization and progenitor cell dynamics, and how defects in the development of an organ can impact its adult function. Participants came away with a broader vision of their field and a renewed sense of collective energy empowered by novel tools and fresh ideas.

KEY WORDS: Endoderm, Organoids, Human development, Keystone

Introduction

Many internal organ systems of the body, including the lungs, liver, gastrointestinal tract and pancreas, as well as the often forgotten thyroid, prostate, bladder and tongue, are all derived from the germ layer known as the definitive endoderm (DE, Fig. 1). The quest to understand the origin of the DE and its subsequent transformation into organ systems with such a wide variety of three-dimensional architectures, has led to many fundamental discoveries in developmental biology. The search has also established an 'embryonic roadmap' that guides strategies to generate replacement endodermal tissues for regenerative medicine and devise stem cell culture systems for disease modeling and drug screening.

The road to DE development begins in the mouse embryo during the process of gastrulation. This initiates around embryonic day (E) 6.5 when a specialized doorway – the primitive streak – opens in the posterior of the cup-shaped epiblast. Multipotent epithelial epiblast cells ingress through the streak and emerge as either mesoderm or DE. By E8.5, the DE forms a sheet on the ventral surface of the embryo. As development continues, this sheet rolls up into a tube and positional cues establish first the general and then the more-localized domains along the antero-posterior and dorso-ventral axes where specific organ primordia will form. Reciprocal interactions between the overlying mesoderm and DE then initiate organ outgrowth, patterning and morphogenesis, processes that are fully integrated with vascularization and innervation. The final three-dimensional architecture of each organ is unique, highly reproducible and optimal for organ function.

Besides understanding how tissue architecture impacts progenitor cell behavior and how specific signaling pathways function in organogenesis, several recurrent themes emerged during the conference, which was expertly organized by Xin Sun (University of California, San Diego, USA), Kat Hadjantonakis (Memorial Sloan Kettering Cancer Center, New York, USA) and Didier Stainier (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany). These included the importance of taking the study of lineage commitment, organogenesis and regeneration to the single-cell level, and the importance of using real-time and 3D imaging as well as a variety of models, including organoids, for gaining a more complete understanding of endoderm development.

Endoderm-omics

Over the last few years, there has been an explosion of studies employing single-cell RNA sequencing technologies (scRNA-seq) to reveal the RNA repertoire of a single cell (reviewed by Griffiths et al., 2018). Such analyses, together with other 'omics' type techniques, can unmask previously unappreciated cellular heterogeneities and provide novel insight into signaling pathways and lineage relationships. Indeed, Liz Robertson (University of Oxford, UK) complemented elegant genetic studies with several 'omics' approaches to dissect the molecular mechanisms involved in mouse primitive streak formation and DE specification – two process that are known to be controlled by the Nodal signaling pathway. In particular, she provided a high-resolution view of how the Nodal effector proteins Smad2/3, Eomesodermin and Lhx1 govern DE formation.

At present, there appears to be no limit (except from funding) to the number of cells that can be sequenced from normal tissue, tumors, embryos or embryonic tissues. This was particularly apparent in a talk by Sonja Nowotschin (Memorial Sloan Kettering Cancer Center, New York, USA). Using the Chromium platform introduced by Stéphane Boutet from 10× Genomics (Pleasanton, CA, USA), she was able to heroically sequence over 100,000 single cells from the developing mouse embryo. Her goal was to comprehensively classify all endoderm types, from the emergence of the extra-embryonic primitive endoderm in the pre-implantation blastocyst to the beginning of regionalization in the gut tube at the 13-somite stage. To achieve this, Nowotschin took advantage of an *Afp::GFP* reporter mouse line in which GFP labels the visceral endoderm (VE), an extra-embryonic lineage that covers the gastrulating embryo and gives rise to the yolk sac (Kwon et al., 2006). During gastrulation, epiblast-derived DE cells (GFP^-) emerge from the primitive streak and intercalate into and intermix with cells in the overlying VE (GFP^+). Using a computational method that clusters cells based on the relatedness of gene expression, she identified 15 clusters among ∼42,000 cells that

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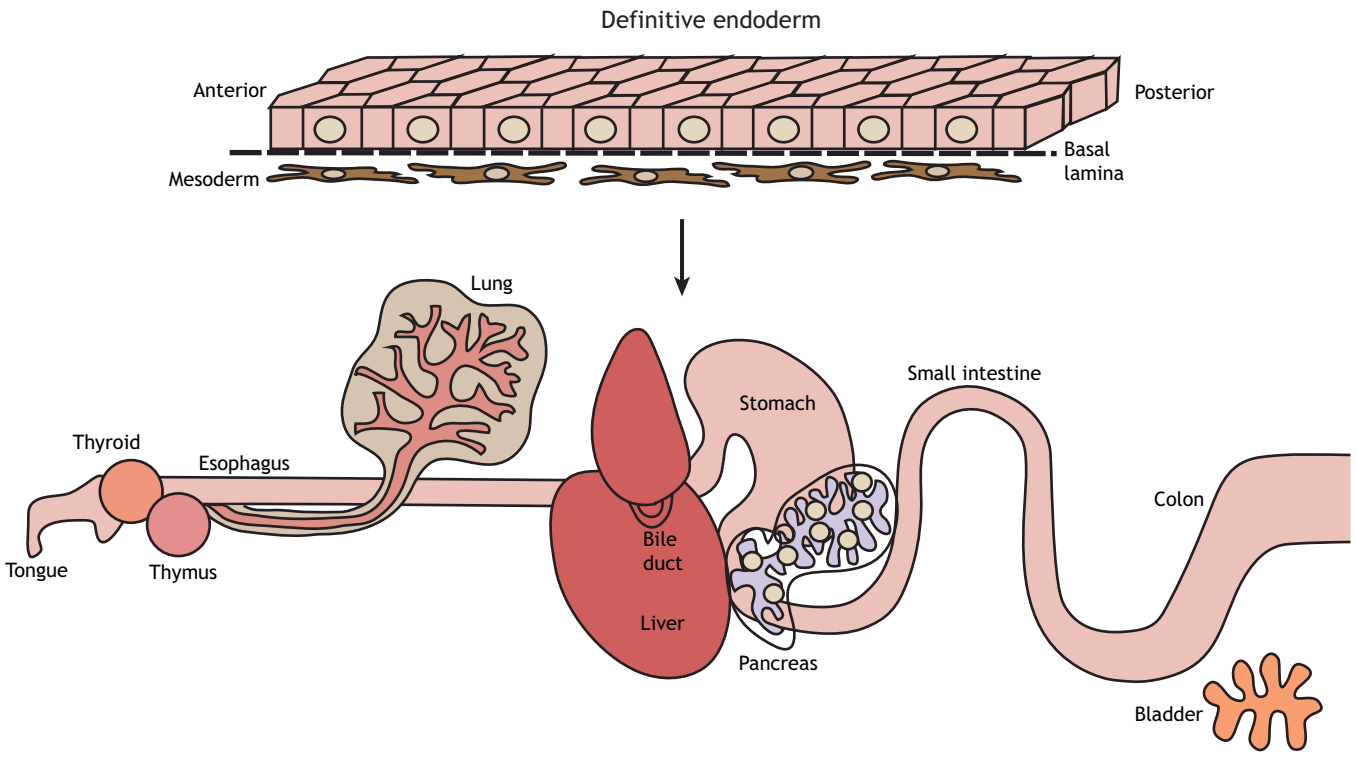

Fig. 1. Schematic representation of some mammalian endodermal organs. The definitive endoderm (DE) is initially a simple sheet of epithelial cells attached to a basal lamina and associated with mesoderm. Through a sequence of patterning and cell fate specification/lineage allocation events, the positions of the rudiments of the major organs are established along the anterior-posterior axis. Reciprocal interactions between the endoderm and associated mesoderm (not shown) then lead to organ-specific processes of proliferation, morphogenesis, cell differentiation and maturation, in association with vascularization and innervation. Each mature organ has a unique three-dimensional tissue architecture and contains one or more population(s) of stem cells. Some are localized in distinct niches that function to coordinate stem cell proliferation and differentiation in response to physiological need. Other facultative stem cell populations are only revealed during repair after injury. However, no stem cells have yet been identified in the thyroid.

were harvested and sequenced from the 13-somite stage, 10 of which were of endodermal origin and represent cell identities distributed along the anteroposterior axis of the gut tube. As previously reported, some descendants of the VE are incorporated into the gut tube (Kwon et al., 2008). Such GFP^+ VE cells predictably emerged in Nowotschin's scRNA-seq data; they were transcriptionally distinct from DE descendants and more frequently located posteriorly. It will be interesting to determine whether these GFP^+ VE cells persist postnatally and remain transcriptionally different or become more identical when incorporated into a developing organ. Overall, these data make exciting inroads into establishing a complete 3D transcriptional map of the gut tube over space and time.

Pamela Hoodless (Terry Fox Laboratory, Vancouver, Canada) applied scRNA-seq technologies to map the ontogeny and cell identities within the developing mouse liver. She started with Nowotschin's 13-somite stage data and complemented it with ∼2600 cells from microdissected E9.5 liver buds. These cells were computationally clustered into 13 groups, including, among others, mesoderm, endoderm, endothelium and erythroid/macrophage progenitors. Hoodless highlighted the fact that the program correctly segregated several well-known growth factor-receptor pathways between ligand source and target cell types. For example, oncostatin M (*Osm*), which is known to be required for liver development, is expressed in erythroid cells, whereas the gene encoding its receptor, *Lifr,* is expressed in liver progenitors. Hirofumi Kiyokawa (RIKEN Center for Developmental Biology, Kobe, Japan) also performed organ-specific scRNA-seq on ∼3500 cells harvested from the developing mouse trachea between E12.5 and E18.5. His analysis yielded 15 distinct clusters and suggests the existence of novel cell populations, including bi-potential cells. Lineage-tracing experiments designed to test the existence of these progenitors are ongoing.

Francesca Spagnoli (Max Delbrück Center for Molecular Medicine, Berlin, Germany) used RNA-seq to explore how the liver and pancreas, two organs important for regulating metabolism, initiate distinct transcriptional and morphogenetic programs even though they arise in close proximity. She and her colleagues previously established, using bulk RNA sequencing, the transcriptional profiles of microdissected regions of the developing mouse gut at different stages, including before the buds emerge from the ventral foregut (E8.5) and slightly later (E10.5), when discrete liver and ventral and dorsal pancreatic buds are visible (Rodríguez-Seguel et al., 2013). Mining these RNA-seq datasets for transcriptional differences, Spagnoli and colleagues uncovered an unexpected role for the Robo-Slit signaling axis – better known for its roles in axonal guidance and angiogenesis – in the divergence between liver and pancreatic lineages. *Robo2* transcripts are abundant in the foregut at E8.5, but by E10.5 specifically increase in the ventral pancreas and remain low or absent in the dorsal pancreas and liver. *Slit3* is expressed in the mesenchyme surrounding the ventral pancreatic bud. In *Robo1;Robo2* double mutant embryos, the ventral pancreas activates the pancreatic lineage marker *Pdx1*, but by E9.5 some cells lose *Pdx1* expression and begin to express canonical liver markers such as *Alb* and *Afp*. In addition, there is a striking reduction in organ size, suggesting that Robo2 controls the size of the pancreatic

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progenitor pool and preserves pancreatic identity. These findings raise the possibility that the Robo-Slit signaling pathway is unexpectedly involved in the early development of other endodermal organs. Indeed, Sun discussed how Robo expression labels rare pulmonary neuroendocrine cells in the mouse lung airway epithelium that serve as 'airway sensors' (Branchfield et al., 2016).

Organoids a go-go

Several examples of decoding the 'embryonic roadmap' using organoid culture models were showcased during the meeting. Organoids can be derived from different sources: pluripotent stem cells (PSCs), established cell lines, stem/progenitor cells from adult or fetal organs, or fragments of an organ itself. Under defined culture conditions, the isolated cells are capable of self-renewal, differentiation and spontaneous self-organization into 3D structures that mimic the architecture of the native organ (Little, 2017). Among endodermal organs, organoids of intestine, lung and liver have received considerable attention, as they have provided valuable models of human disease as well as insights into the cellular mechanisms driving tissue physiology, homeostasis and repair. Highly efficient protocols are now available to direct the differentiation of human PSCs into DE (Yiangou et al., 2018). However, it is now clear that protocols designed to direct DE into organs such as stomach, intestine and colon can lead to the specification of splanchnic mesoderm that is spatially patterned alongside the endoderm. This finding can be exploited to model human diseases, as elegantly discussed by Jim Wells (Cincinnati Children's Hospital Medical Center, OH, USA) in his Keynote lecture. His group recently developed protocols in which organoids mimicking the human colon can be obtained by giving a BMP pulse that normally promotes posteriorization of the embryo (Múnera et al., 2017). When the human colonic organoids (HCOs) are transplanted under the mouse kidney capsule for several weeks, they yield outgrowths that display a typical colon structure with mesoderm-derived smooth muscle. However, HCOs still lack several other cell types that can contribute to disease, such as immune cells and enteric neurons. To overcome this problem, the Wells group exploited the 'multiplexing' of organoids, namely by combining organoids with specific cell types derived in parallel from the same population of PSCs. This was illustrated by the introduction of enteric neurons, which are deficient in Hirschsprung's disease. Enteric neurons are derived from vagal neural crest cells (NCCs) that migrate into the gut, and they are responsible for GI motility, secretion, blood flow and epithelial permeability. To introduce neurons into their organoids, the Wells group aggregated PSC-derived NCCs with, in this instance, intestinal organoids by simple centrifugation (Workman et al., 2017). The NCCs migrated into the mesenchyme, sandwiched themselves between smooth muscle fibers, self-organized, differentiated into an array of neuronal and glial cell types, and showed neuronal activity. It is anticipated that this multiplex approach, perhaps next incorporating hemogenic vascular endothelial cells, will allow the *in vitro* production of organoids with immune cells whose organization more faithfully mimics *in vivo* tissue structure and function.

Several other talks showcased the diverse uses of organoids derived from or differentiated into other endodermal organs. The R-spondin receptor Lgr5 is expressed in a number of adult stem cell compartments, such as intestinal crypts and the hair follicle (Leung et al., 2018). In the adult liver, however, Lgr5 is not expressed but is activated upon injury (Huch et al., 2013). When recovered from the damaged liver, such $Lgr5^+$ cells can seed organoids. Previous work has shown that precursors to $Lgr5^+$ organoid-forming cells reside in the biliary ducts of the liver (Dorrell et al., 2014), and Markus Grompe (Organ Stem Cell Center, Portland, OR, USA) described his group's recent attempts to further define the origins of these cells in the uninjured organ. He reported that the cell-surface marker ST14 subdivides clonogenic versus non-clonogenic cholangiocytes (Li et al., 2017): only sorted $ST14^{hi}$ cells are able to generate organoids that can be serially passaged. Interestingly, the $ST14^{hi}$ and $ST14^{lo}$ populations both express equal levels of cardinal cholangiocyte markers, whereas other genes are highly differentially expressed (over tenfold). One of these, *Pkhd1l1*, was used as a surrogate in attempt to localize $ST14^{hi}$ clonogenic cholangiocytes in the adult liver. Indeed, *Pkhd1l1* mRNA was restricted to a subpopulation of duct cells. Only formal confirmation by lineage tracing, perhaps after generating new Cre driver lines, will confirm that these $ST14^{hi}$/*Pkhd1l1*-expressing cells are an important population of facultative stem/progenitor cells *in vivo*.

Organoids are also proving useful for studying the progression of endodermal tumors. This was illustrated by work on a little studied endodermal organ, the bladder, by Michael Shen (Columbia University, New York, USA). He described his group's recent success in establishing biobanks of organoids from biopsies of human bladder cancers. These organoids retain the heterogeneity of the tumors from which they are derived and additionally display clonal evolution with serial passaging (Lee et al., 2018).

Germ layer conversations

All endoderm-derived organs require intimate interactions with cell types that originate from the other two primary germ layers: the ectoderm and mesoderm. For example, aortic endothelial cells of mesodermal origin provide inductive signals that specify pancreatic endocrine cell fate in the developing mouse embryo (reviewed by Cleaver and Dor, 2012). Later, the optimal function of pancreatic islets, which sense fluctuating glucose levels in real time and quickly secrete insulin, is ensured by crosstalk with the dense islet microvasculature. Sarah Knox (University of California, San Francisco, USA) described an interesting dialogue between ectoderm and endoderm in the mouse salivary gland. The transcription factor *Sox2* is expressed in progenitors of both duct cells and saliva-secreting acinar cells, but its genetic loss specifically impacts formation of the acinar lineage, resulting in a smaller gland. Knox provided several intriguing lines of evidence, including elegant *in vitro* recombination experiments, that *Sox2* expression within these acinar progenitors is maintained by parasympathetic nerves via acetylcholine-muscarinic-calcium signaling. The adult salivary gland is highly innervated, which explains, Knox quipped, why we are able to 'drool over cake', and also contains $Sox2^+$ lineage-restricted acinar cells. Severing the chorda tympani, a branch of the facial nerve, in adult mice results in denervation of the salivary gland and loss of $Sox2^+$ cells. After about a month, the glands are reinnervated and, remarkably, $Sox2^+$ cells reappear. Last, Knox highlighted that both salivary glands and parasympathetic nerves degenerate in head and neck cancer patients undergoing gamma irradiation treatment, and she presented data using human salivary glands showing that *SOX2* levels increase upon treatment with the acetylcholine mimetic, carbachol. She posited that therapies directly targeting $SOX2^+$ cells in cancer patients might restore salivary gland function.

Dynamic changes in epithelial cell behavior during organ building and repair

A recurring theme of the conference was the importance of real-time and 3D imaging of cell behavior for understanding basic mechanisms driving organogenesis and regeneration. Two stimulating talks centered on imaging progenitor epithelial cells in

Development (2018) 145, dev163550. doi:10.1242/dev.163550

the developing mouse pancreas as they generate nascent islets of Langerhans, which contain precursors of various hormone-producing endocrine cells. Islet formation begins around mid-gestation and involves the localized emigration of post-mitotic endocrine-committed cells from a web-like plexus of tubules composed of $Sox9^{+}$ progenitor cells. Distributed along the tubules are clusters of mitotic $Ngn3^{lo}$ progenitors that are already strongly biased (lineage-primed) towards forming endocrine cells. Chris Wright (Vanderbilt University School of Medicine, Nashville, TN, USA) showed time-lapse movies of delaminating $Ngn3^{+}$ cells, cleverly labeled with mCherry nuclear and GFP plasma-membrane fluorescent tags (Bechard et al., 2016). The egressing cells exhibit protrusive filipodia on the basal surface but surprisingly keep a long-lived narrow apical contact at their luminal surface, which may prevent them from breaking through the basal lamina around the emergent proto-islet structures. Wright argued that confining the process of endocrine-cell emigration to localized bulges allows a transient niche or 'way station' to form, in which there is very tight feedback control between the egressing cells and the remaining progenitor pool as a way of regulating the supply and status of cells colonizing the nascent islets. Similarly, Nadav Sharon (Harvard University, Cambridge, MA, USA) showed that the cells that form islets do not go through the presumed process of dissociation and aggregation. Using microscopic analysis, he reported that extensive buds grow out of the tubules to become the full islet. Furthermore, the organization of these buds, which he termed 'peninsulas', correlates with the temporal order in which the various cell types differentiate and reflects their final organization in the mature islet. There was lively discussion as to whether the localized departure of the endocrine-committed cells constituted an example of 'epithelial-mesenchymal transition' (EMT). It was argued that as epithelial cells transition from one location to another without adopting a robust 'mesenchymal' cell state, a more appropriate term was 'epithelial motility transition' (EMoT).

Further discussion about changes in epithelial motility and differentiation followed talks that showcased the *Drosophila* intestine (midgut) as an endodermal organ system. The midgut epithelium is maintained by intestinal stem cells (ISCs) that both self-renew and generate absorptive enterocytes and secretory enteroendocrine (EE) cells. Kyra Campbell (University of Sheffield, UK), working in collaboration with Andreu Casali (Biomedical Research Institute of Lleida, Spain), used the *Drosophila* midgut as a model for studying colorectal cancer, tumor progression and metastasis (Martorell et al., 2014). Activation of the WNT pathway, KRas and EMT in these cells leads to tumors, very few of which (1%) metastasize to distant sites. Campbell presented lineage-tracing and imaging studies suggesting that epithelial cells do not leave the tumor individually but rather through a process of collective migration that is driven by partial EMT, whereby cells gain some mesenchymal characteristics without losing all their epithelial traits (see Campbell and Casanova, 2016). Lucy O'Brien (Stanford University School of Medicine, CA, USA) also skillfully exploited the *Drosophila* midgut stem cell system, in her case to gain insights into how feedback mechanisms regulate tissue homeostasis and organ size. Her previous work had shown that when enterocytes die and undergo apoptosis they generate a signal triggering neighboring stem cells to divide (Liang et al., 2017). By opening up and flattening the gut tube, and imaging cells within it, she was able to determine, with the help of computer algorithms, the distribution of stem cells under different conditions. This revealed that as the tube grows or undergoes remodeling after damage, the stem cells actively disperse. If this dispersion is inhibited the repair is less effective. This system of active dispersal may ensure that no region of the gut is left without the means to recover from damage and that no enterocyte dies in vain. Norbert Perrimon (Harvard Medical School, Boston, MA, USA) also presented very recent studies in which the *Drosophila* midgut undergoes remodeling after damage (He et al., 2018). His lab found that the *Piezo* gene, encoding an ion channel that senses mechanical tension in cell membranes, is expressed in a subset of stem cells that are specifically EE progenitors. They showed that Piezo plays a role in promoting the differentiation of these precursors into mature EE cells, revealing a previously unappreciated heterogeneity in the ISC population. As in many other tissues, this finding is being explored further using single cell transcriptomics.

Alveolar stem cells: a tale of two CreERT2s

Lung alveoli are the gas exchange units of the adult lung. They are composed of two endoderm-derived epithelial cell types – extremely large and thin type I (AT1) cells and cuboidal type II (AT2) cells that produce surfactant – surrounded by closely apposed capillaries and several different subtypes of fibroblasts. Alveolar cell turnover is very slow at steady-state, but significant proliferation is triggered upon injury or genetic ablation of AT2 cells. Lineage-tracing studies have shown that, during repair, AT2 cells self-renew and give rise to AT1 descendants. An important issue is whether this reparative potential is confined to a small subpopulation of 'elite' AT2 stem cells or whether many or most AT2s can be activated in response to injury. Both Edward Morrissey (University of Pennsylvania, Philadelphia, USA) and Ahmad Nabhan (Stanford University School of Medicine, CA, USA) described their separate strategies to address this conundrum.

It has previously been established that WNT signaling is crucial for the development of both AT1 and AT2 cells during alveologenesis. Both groups reasoned, therefore, that AT2 stem cells might also remain WNT responsive. To test this hypothesis, each group used a different reporter for WNT transcriptional activity and lineage tracing: CreERT2 knocked into the WNT target gene *Axin2*. Nabhan also used a powerful new *in situ* hybridization technique called PLISH to visualize gene expression in single cells (Nagendran et al., 2018). Both groups observed $Axin2^{+}$ cells within the steady state alveolar epithelium that express the AT2 marker surfactant protein C (Sftpc). The Morrissey group calculated their frequency as 20% of all AT2 cells and designated them as alveolar epithelial progenitors (AEPs) (Zacharias et al., 2018). Their AEPs are enriched for the expression of WNT target genes, appear to be the only AT2 cells to proliferate after injury and robustly form alveolar organoids *in vitro*. In contrast, Nabhan found that only 1% of AT2 cells were $Axin2^{+}$ at steady state but many more cells become positive after injury (Nabhan et al., 2018). One explanation for the discrepancy may lie in the two distinct Axin2-CreERT2 drivers used, an idea based on differences recently observed between the widely used $Lgr5^{EGFP\text{-}ires\text{-}CreERT2}$ allele that undergoes transgene silencing and variegated expression (Barker et al., 2007), and a new allele ($Lgr5^{2A\text{-}CreERT2}$) that reveals previously unknown $Lgr5^{+}$ chief cells in the oxyntic stomach (Leushacke et al., 2017). Irrespective of the 20-fold discrepancy in frequency, both groups established that $Axin2^{+}$ AT2s respond to constitutive WNT signals, with Nabhan narrowing the signal source to fibroblasts that lie in close proximity to AT2s. In her presentation, Brigid Hogan (Duke University, Durham, NC, USA) suggested that these fibroblast-like cells, which her group has termed TASCs (type 2 associated stromal cells), have a very distinct morphology, with long cellular extensions, and are the source of other regulatory factors such as

Development (2018) 145, dev163550. doi:10.1242/dev.163550

BMP antagonists important for modulating the proliferation and differentiation of AT2s (Chung et al., 2018). Final resolution of the issue of whether there are distinct subpopulations of AT2 cells with intrinsically different *in vivo* stem cell potential must await the development of a robust engraftment assay in which transplanted cells repopulate the lung to effect long-term repair.

Making mutants: words of caution

In 2015, Didier Stainier and colleagues reported that zebrafish morphants with decreased levels of the endothelial extracellular matrix (ECM) protein Egfl7 display severe vascular defects, whereas *egfl7* zebrafish mutants generated by TALEN gene editing display no obvious phenotypes (Rossi et al., 2015). An explanation for these perplexing phenotypic differences emerged by comparing the proteomes and transcriptomes of the morphants and mutants: in *egfl7* loss-of-function mutants but not in *egfl7* morphants, members of the Emilin family of ECM proteins were upregulated. At the conference, Stainier offered insight into how this regulatory mechanism – which he terms 'genetic compensation' – might be triggered (El-Brolosy and Stainier, 2017). Ongoing work from his lab, both in zebrafish and mammalian cells, is now focusing on a specific form of genetic compensation termed transcriptional adaptation, which, as the name indicates, involves an increase in transcript levels of related genes in specific mutants. He provided several lines of evidence that transcriptional adaptation is not caused by the loss of protein function. Rather, the increase is due to upregulation of transcription, a phenomenon that appears to be dominant because it can also be observed in heterozygous embryos. His lab is currently investigating whether transcriptional adaptation is triggered by the DNA lesions themselves or by the mutant mRNAs. In the future, these data might guide the design of mutant alleles that do not exhibit transcriptional adaptation. In the CRISPR/Cas9 era of rapidly introducing mutations into one's favorite gene, this and other cautionary tales prompt careful interpretation of any resulting phenotypes, including those observed when analyzing RNA-seq data.

Concluding remarks

In summary, the meeting emphasized how significantly our understanding of the endoderm – its developmental origins, regionalization and ability to repair – has advanced in recent years. We urge the organizers of the next Keystone Endoderm Symposium to adopt a similarly broad, multi-systems approach, showcasing a diversity of organ systems and state-of-the-art technologies that will hopefully inspire and energize scientists tackling important biological and medically related problems from different perspectives.

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Competing interests

The authors declare no competing or financial interests.

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