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MEETING REVIEW

Exciting times to study the identity and evolution of cell types

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ABSTRACT

The EMBO/EMBL Symposium on 'The Identity and Evolution of Cell Types' took place in Heidelberg, Germany, on 15-19 May 2019. The symposium, which brought together a diverse group of speakers addressing a wide range of questions in multiple model systems, provided a platform to discuss how the concept of a cell type should be considered in the era of single cell omics techniques and how cell type evolution can be studied.

KEY WORDS: Cell fate, Computational analysis, Evolution, Single cell sequencing

Introduction

Organised by Detlev Arendt (EMBL Heidelberg, Germany), Oliver Hobert (Columbia University, USA), Henrik Kaessmann (Heidelberg University, Germany), Nicole King (University of California, Berkeley, USA) and Gunter Wagner (Yale University, USA), the EMBO/EMBL Symposium 'The Identity and Evolution of Cell Types' was predominantly dedicated to the discussion of the nature of cell types in the evolution of animals. The rapid development of novel single cell genomic and transcriptomic technologies has paved the way for the emergence of a new field dedicated to the study of cell type identity and evolution. The avalanche of newly available single cell sequencing data from a variety of different biological model systems (Fig. 1) has started to provide important insights into the biology of cell types. However, it also imposes fundamental questions, such as: (1) How can we define a cell type? (2) What are the molecular mechanisms of cell identity? (3) Can we compare cell types across species and phyla? (4) How did cell types first originate? (5) What computational methods can be used to study cell type biology? Key topics discussed at the symposium, and summarised here, were the origins of cell types in the evolution of animal multicellularity, their specialisation in different animal lineages, and the gene regulatory networks (GRNs) that may underlie the specification of various animal cell types.

What is a cell type?

Traditionally, a cell type is considered as a morphologically distinguishable entity that performs a specific function within an organism. Cell type identification methods have typically been based on a limited number of cell type-specific markers. However, with the development of single cell sequencing techniques it has become clear that, at the molecular level (usually transcriptomics), individual cells apparently of the same type are quite diverse. Moreover, it is not clear how to distinguish bona fide cell types from temporary cell states (e.g. differentiation or metabolic states). A striking example of this was shown by Leonid Moroz (University of Florida, USA), who presented preliminary single cell RNA sequencing (scRNAseq) data in the placozoan *Trichoplax adhaerens*. He showed that around 50 cell types can be distinguished transcriptionally, but only six cell types have been morphologically identified (Smith et al., 2014; Varoqueaux et al., 2018). There is thus a disconnect between the morphological and transcriptional data; resolving this represents an ongoing challenge for the field.

Importantly, conclusions about cell type identity based on transcriptomics data strongly depend on the computational methods used to analyse the sequencing data. As Amos Tanay (Weizmann Institute of Science, Israel) reported, many variations occur in scRNAseq data due to differences in transcript levels between single cells caused by the stochastic nature of transcription and variation of cell states (e.g. Stapel et al., 2017); therefore, a significant number of cells needs to be sequenced. Tanay proposed the concept of 'metacells' (Baran et al., 2019 preprint), which captures cells that likely come from similar statistical distribution, thus helping to avoid erroneous trajectory inferences. Metacells can reflect cell types or cell states, small variation in secondary gene modules within bigger clusters, or transcriptional gradients. Stein Aerts (KU Leuven, Belgium) presented recent computational approaches to identify transcription factors (TFs), gene networks and cell states from single cell data: describing SCENIC as a method to infer gene networks from scRNAseq (Aibar et al., 2017), cisTOPIC as a method to predict co-regulatory enhancers from scATAC-seq data (Bravo González-Blas et al., 2019), and Scope as a tool to visualise single cell atlases (Davie et al., 2018).

It is well known from developmental studies that GRNs control cell differentiation and might therefore be considered to define cell types (Davidson, 2010). Oliver Hobert discussed the availability of molecular maps defining neuronal identity in *Caenorhabditis elegans*, enabling the study of regulatory factors (terminal selectors) specifying the identity of individual neuron types. He presented a model in which individual neuron types acquire their identity via master terminal selectors that coordinate the expression of distinct identity features, including neurotransmitter identity. Hobert and colleagues have been able to identify terminal selectors for most neuron classes, and found that homeobox genes dominate in the specification of neuronal identity even though they represent only 10% of all the TFs encoded in the genome: every neuron class is identified by a unique homeobox code. Interestingly, members of the same neuronal class often do not belong to the same developmental lineage and different TFs may regulate the same phenotype. Intriguingly, it appears that re-usage of the same TF in different neuron classes is associated with those neurons being synaptically connected.

Cell types are not static: they go through developmental processes such as differentiation or may be re-defined in regeneration and reprogramming. Barbara Treutlein (ETH Zürich, Switzerland) studies these processes in human hepatic organoids (human hepatocytes embedded into 3D extracellular matrix) and in axolotl (*Ambystoma mexicanum*) regeneration models. She showed that the 3D microenvironment (including endothelial and mesenchymal cells)

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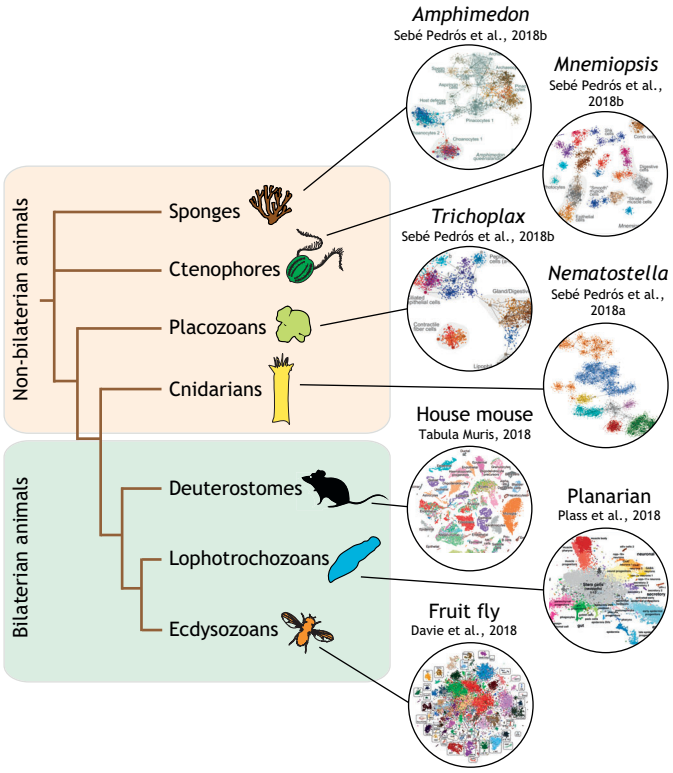

Fig. 1. Animal phylogenetic tree with special emphasis on non-bilaterian phyla and newly available single cell sequencing data from a variety of animal species/taxa. Illustrations of animals were reused with modifications from phylopic.org; t-SNE plots modified from previous publications (Davie et al., 2018; Plass et al., 2018; Sebé-Pedrós et al., 2018a,b; Tabula Muris Consortium, 2018).

in liver bud organoids leads to the differentiation of hepatic endoderm cells into cells that resemble fetal hepatocytes. Thus, cellular interactions through ligand-receptor networks are important for proper cell differentiation (Camp et al., 2017). Through scRNAseq analyses at different time points during axolotl limb regeneration, Treutlein and colleagues have compared regenerating blastema cells with those of the developing limb bud, and can track *in vivo* dedifferentiation and reprogramming events (Gerber et al., 2018).

How do we define cell type homology and study evolution of cell types?

Detlev Arendt proposed an evolutionary definition of cell types to enable comparisons between species: a set of cells accessing the same regulatory programme driving differentiation (Arendt et al., 2019). He discussed how cell types may evolve through mutations leading to two regulatory identities followed by mutual repression of alternative identities and distinct regulatory programmes controlling division of labour. Similarly, as Gunter Wagner noted, cell type function and shape are not directly connected to evolutionary origin. He suggested that one should consider the evolutionary origin of cell types as the evolution of a molecular mechanism that instantiates a cell type in development. This implies not only internal cellular factors but also the specific context in which cell types differentiate.

Arendt also highlighted that whole-body scRNAseq in marine animals from diverse taxa enables the discovery of apomeres, derived cell type-specific traits. He emphasized that it is of great importance to reconstruct the topology of cell types to explore their signalling environment and the structure and function of apomeres. To address this point, Arendt and colleagues have constructed a full-body cell atlas of the annelid worm *Platynereis dumerilii*. It currently combines whole-body imaging at the resolution of electron microscopy with single cell RNAseq data, with the potential to include ATACseq data, mapping of protein-protein interactions and co-binding of TFs as well as cellular metabolomics in the future. This comprehensive cell atlas is able to connect cellular transcription activity to the phenotype in the context of intercellular interactions.

Within mammals, it has been possible to trace changes that led to the origin of a new cell type. Decidual stromal cells (DSCs), essential for implantation and the maintenance of pregnancy, are an evolutionary innovation in placental mammals. As Gunter Wagner discussed, DSCs differentiate from endometrial stromal fibroblasts (ESFs), which are also found in marsupials. The regulatory network controlling DSC differentiation appears to have evolved through modifications of the cellular apoptotic and oxidative stress response found in marsupials (Erkenbrack et al., 2018). There is no evidence for a novel core regulatory network arising with DSC evolution. On the contrary, ancestrally expressed TFs have evolved novel trans-regulatory activities necessary for DSC differentiation. In comparing DSCs and ESFs, the autocrine signalling networks are more different than the transcriptional networks of TF genes. Thus, integration and modification of signalling pathways into a cell type-specific autocrine regulatory network is an important mode of gene regulatory evolution. These findings are consistent with the core regulatory complex model of cell type identity (Arendt et al., 2016).

In her talk, Mihaela Pavlicev (Cincinnati Children's Hospital Medical Center, USA) focused on how ESFs evolved from skin fibroblast-like cells, and showed that the two cell types differ in the requirement for stable oestrogen receptor expression for ESF differentiation. Her work points to the importance of extrinsic factors (such as oestrogen) in controlling cell identity. Interestingly, she suggests that cell types tend to depend on internal cellular factors to define their identity in highly variable environments, whereas cell type identity relies on external input in a stable context.

Several talks focussed on cell type evolution in vertebrates. Through comparisons between sea lamprey (*Petromyzon marinus*), skate (*Leucoraja erinacea*), zebrafish (*Danio rerio*) and chicken (*Gallus gallus*), Marianne Bronner (California Institute of Technology, USA) showed that the regionalisation of neural crest – and specifically the GRN defining the amniote cranial neural crest – emerged via gradual addition of network components (Martik et al., 2019). Gerhard Schlosser (NUI Galway, Ireland) highlighted that (1) duplication and divergence of core GRNs and (2) recombination/co-option of core GRNs are among important mechanisms in cell type evolution. In vertebrates, for example, hair cells and sensory neurons are specified by different but related GRNs as they both likely evolved from a primary mechanosensory cell. Merkel cells (which develop from the epidermis but have a TF signature similar to that of hair cells) might have evolved by recombination of epidermal and hair cell identities, i.e. recruitment of the sensory gene battery into epidermal cells. Convergence in cell type evolution may also be an important mechanism. Using scRNAseq in the vertebrate dorsal pallium, Maria Antonietta Tosches (Max Planck Institute for Brain Research, Germany) showed that GABAergic interneurons are conserved between reptiles and mammals, whereas pallial glutamatergic neurons diversified independently in these animals. As a result, the mammalian cortex is made of evolutionarily new glutamatergic cell types (Tosches et al., 2018).

Clare Baker (University of Cambridge, UK), who studies the vertebrate lateral line system, reported that non-teleost electroreceptors appear to have evolved from ancestral vertebrate

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lateral line mechanosensory hair cells. Both cell types develop from embryonic lateral line placodes. Paddlefish electrosensory organs express TFs essential for hair cell development and genes required for synaptic transmission specifically at hair cell ribbon synapses, supporting the idea of homology between electroreceptors and hair cells (Modrell et al., 2017). Furthermore, only a few developmental genes have so far been identified as specific either to electrosensory organs or to neuromasts (lateral line organs containing hair cells), highlighting their close relationship.

Another example of cell type evolution was reported by Martin Cohn (University of Florida, USA). He first described that collagen-based cartilage is conserved across extant vertebrates (e.g. jawed vertebrates, lampreys and hagfishes) (Zhang et al., 2006; Zhang and Cohn, 2006), which then led him to look at invertebrates to investigate the evolutionary origin of chondrocyte formation. His work on chondrocytes showed that collagen cartilage predates the origin of vertebrates (Tarazona et al., 2016): even in invertebrates like cuttlefish, chondrogenesis is similar to vertebrates. Collagen A is an invertebrate pro-orthologue of the vertebrate collagen 2α1, and a core ColA-SoxD-SoxE network marks chondrocytes in cuttlefish and horseshoe crab. Using a transgenic approach, he showed that horseshoe crab SoxE can activate expression of human COL2α1. Thus, the GRN for making a chondrocyte is conserved in vertebrates and invertebrates and was likely present in the common ancestor of bilaterians.

Allon Klein (Harvard University, USA) studies orthology between cell types through comparisons in pairs of model systems with different divergence times. Here, he presented data on two different comparisons: mouse versus human immune cells (~80 million years apart) and developmental cell states between teleost fish and frog (~435 million years apart). He highlighted that, to study the evolution of cell states, it is essential to measure their similarity accurately. Expression of genes with one-to-one orthologues can be used to estimate distances between the cell types and consequently between major cell lineages. For such estimations, it appears more convenient to consider gene modules re-used between the cell types rather than whole-cell state transcriptomes. However, this approach is more efficient in closely related species (e.g. when comparing mouse and human immune cells) because orthologous genes may be lost as species diverge. Thus, cell type phylogenies may be reconstructed in a stepwise way starting from comparisons between closely related species. In longer evolutionary distances, it might be difficult to compare cell types between species; therefore, comparison between full cell differentiation dynamics may be helpful in cell type phylogenetic studies (work on zebrafish *Danio rerio* and frog *Xenopus tropicalis*). Importantly, expression patterns of orthologous genes do not always reflect homology of cell states because some orthologues may change their expression patterns. Additionally, Klein's work shows a disconnect between sequence similarity and expression similarity, suggesting that evolution may work independently on protein sequence and on expression.

Cong Liang (Tianjin University, China) highlighted that inference of cell type homology and evolution from transcriptomes needs to account for correlated evolution. Because expression of different genes may undergo correlated changes (for example, due to pleiotropic effects of mutations) cell types may not evolve independently. Hierarchical clustering and phylogenetic reconstruction methods have been applied to transcriptomic data to infer cell type evolution; however, the clustering patterns might be affected by correlated evolution. Liang introduced a model to estimate levels of correlated transcriptome evolution (LCE) and it shows that tissues with similar morphology or developmental lineage share higher LCE compared with distantly related tissues (Liang et al., 2018).

However, although within relatively short evolutionary distances (for example, within mammals or vertebrates) it is possible to identify conserved components of GRNs, in larger evolutionary distances the homology of GRN components might be hindered by high sequence divergence. For example, Amos Tanay reported that in early branching animals, it is hard to find conserved TFs and therefore gene module relationships are important (Sebé-Pedrós et al., 2018a,b). Promoters show strong motif enrichment, enabling prediction of gene expression (which is not always possible in mammals that have multiple enhancers which might be distantly located). Heather Marlow (Institut Pasteur, France) reported that homologies between cell types are difficult to detect across phyla. Within phyla, when comparing members of different cnidarian classes, such as the anthozoan *Nematostella vectensis* and the hydrozoan *Hydra vulgaris*, apart from a few specific cell types such as nematocytes, relationships between many cells, for example subtypes of neurons, are difficult to detect. Such difficulties in detection could result from real biological differences or from technical challenges. Leonid Moroz also highlighted that neurons should be considered a functional category rather than necessarily representing a set of cells with a single evolutionary origin. He discussed the putative absence of classical pan-neuronal markers and suggested that, during evolution, neuronal centralisation may have happened 9-12 times independently. Maria Antonietta Tosches also reported that transcriptional factor combinatorial codes are not conserved between the glutamatergic cell types of the mammalian and reptilian cortex (Tosches and Laurent, 2019).

On the other hand, effector modules of muscle cells appear to be largely conserved between *Nematostella* and bilaterians, as Ulrich Technau (University of Vienna, Austria) reported. Distinct transcriptomic profiles reflect morphologically defined muscle cell populations (tentacle, mesentery, parietal, circular muscles). Both pan-muscle and cell type-specific TFs are found in these muscle populations. All four cell types overlap only partially with bilaterian muscle signatures. Similar to vertebrates, anthozoan endodermal retractor muscles express acetylcholine and GABA receptors [but not inhibitory glutamate receptors (iGluRs), which are seen in invertebrates]. iGluRs are expressed only by tentacle retractor muscle of ectodermal origin. These data not only shed light on the evolution of muscle cell types, but also challenge our classic notions of germ layer derivatives (Steinmetz et al., 2017).

It is not only components of GRNs that are important for evolution of cell types in a multicellular organism. Manuel Irimia (Centre for Genomic Regulation, Spain) reported that gene duplication and alternative splicing have enabled the evolution of variants optimised for cell type-specific functions. After whole-genome duplication in vertebrates, the vast majority of gene families have at least one member that have lost expression domains, many of which became specialised for brain expression. Interestingly, the more restricted a gene's expression is, the higher number of ATAC-seq peaks can be found (Marlétaz et al., 2018). Irimia also discussed microexons (very short exons, sometimes encoding only one or two residues). These are highly neuron specific in nearly all bilaterians, and their inclusion is regulated by the brain-specific splicing factor SRRM3/4 (Torres-Méndez et al., 2019). He proposed that, in many cases, the inclusion of the microexon might change protein function by modulating binding domains.

How did animal cell types originate?

As well as in-depth discussion of how cell types evolved in diverse animal groups, the symposium also included a number of talks that considered how specialised cell types – and multicellular organisms – arose in the first place. Nicole King introduced choanoflagellates,

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the closest unicellular relatives of animals, as a model system for reconstructing animal origins. Although all choanoflagellate species share a unicellular life history stage, some can switch between single and colonial stages. In addition to the two already sequenced genomes, King presented 19 more transcriptomes from diverse choanoflagellates. This valuable resource now allows us to distinguish between animal-specific innovations and genes inherited from the last common ancestor of choanoflagellates and animals (Richter et al., 2018). In addition, new genetic tools in choanoflagellates, such as stable transformation (Booth et al., 2018) and Crispr/Cas9-mediated genome editing, were presented, which allow the underlying genetic mechanisms of choanoflagellate colony formation to be studied. In the last part of her talk, King presented a newly discovered choanoflagellate species that forms large cup-shaped colonies (Brunet et al., 2019 preprint) and can invert the curvature in response to light. This behaviour has similarities with concerted movement and morphogenesis in animals.

Pawel Burkhardt (University of Bergen, Norway) used serial section electron microscopy to reconstruct in 3D the subcellular composition of unicellular and multicellular choanoflagellates as well as the collar cells from a sponge. He described differences between uni- and multicellular choanoflagellate life stages in structures associated with cellular energetics, membrane trafficking and cell morphology (Laundon et al., 2019). Surprisingly, cells with significantly different morphologies and probably representing different cell types were found in choanoflagellate colonies. These findings suggest that spatial cell type differentiation was likely present in the stem lineage leading to animals (Fig. 2A). Burkhardt also presented recent discoveries on synaptic protein homologues found in choanoflagellates. Biochemically and structurally characterised synaptic protein homologues from choanoflagellates are strikingly similar to those found in vertebrate synapses (Burkhardt and Sprecher, 2017).

Iñaki Ruiz-Trillo's (Institut de Biologia Evolutiva, Spain) research aims at reconstructing how the unicellular ancestor of animals may have looked. He presented data on 11 genomes of the closest relatives of animals – choanoflagellates, filastereans and ichthyosporeans – which were analysed using comparative genomics. Many genes previously considered to be animal specific are also encoded in the genomes of their unicellular relatives (Suga et al., 2013; Grau-Bové et al., 2017). In addition, Ruiz-Trillo presented the different temporal cell types of the closest relatives of animals in great detail. The ichthyosporean *Creolimax fragrantissima* comprises amoeboid and coencoytic (multinucleated) life stages, the filasterean *Capsaspora owczarzaki* can switch between filopodial amoeba, cystic and aggregative life stages, and the choanoflagellate *Salpingoeca rosetta* between different single (thecated and free swimmers) and colonial (chain and rosette colonies) life stages. Thus, the last common ancestor of animals likely possessed a complex life cycle involving both temporal and spatial cell differentiation (Fig. 2A). Ruiz-Trillo showed preliminary evidence of spatial cell differentiation in the aggregative stage of the filasterean *Capsaspora owczarzaki*.

Perspectives

The symposium underscored the power of single cell transcriptomics to enable cell-to-cell comparisons between species, thus pushing research of cell type evolution forward. However, there are still

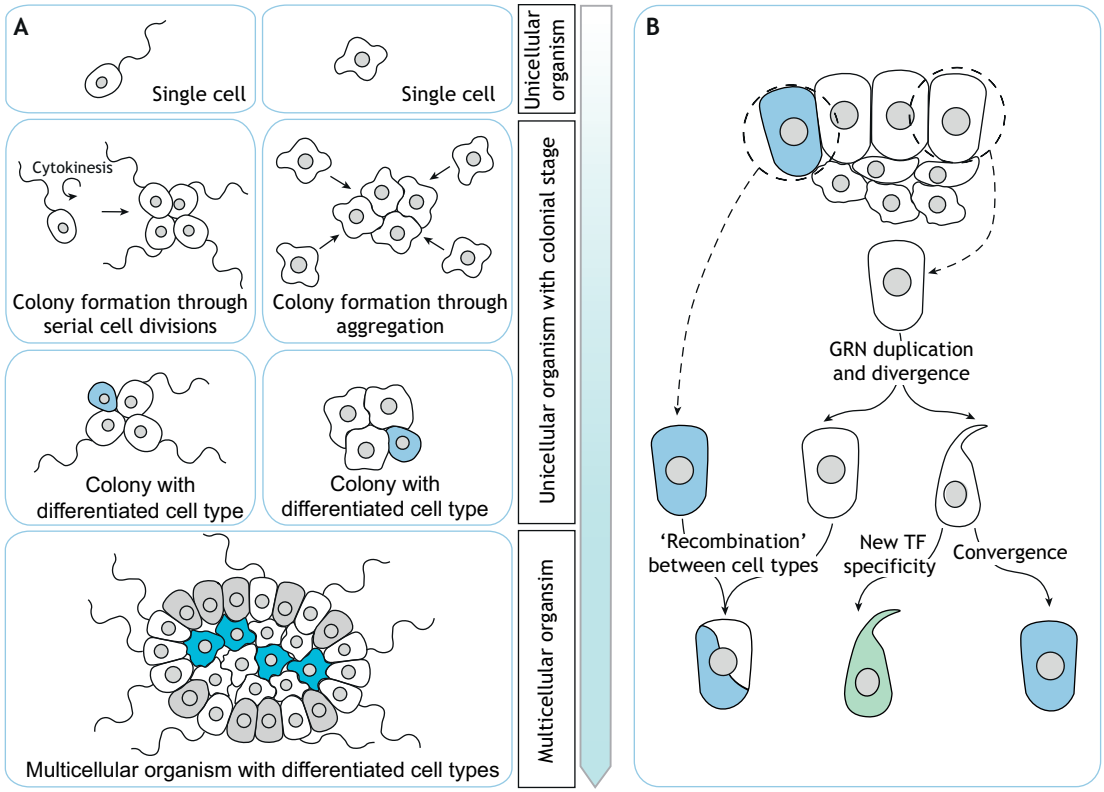

Fig. 2. Mechanisms of cell type evolution discussed during the symposium. (A) Two different hypothetical evolutionary scenarios from a unicellular organism to a multicellular organism with multiple differentiated cell types (last common ancestor of animals). (B) Key mechanisms leading to the origin of new differentiated cell types within animals discussed during the symposium. Different colours and shapes of the cells represent different cell types. The blue arrow represents evolutionary time.

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several challenges to overcome, especially in terms of how we can define homology between cell types. Comparisons between closely related species with known cell type homologies will likely help to understand how homology is defined at the level of single cell transcriptomes. However, as discussed above, homologous cell types might not show highest mutual transcriptome similarity (Liang et al., 2018) and conserved GRNs might be a better proxy to identify cell homology – at least within evolutionary distances where orthology between GRN components is still detectable.

One other important point to emerge from the symposium was whether to consider cells expressing the same set of effector genes (and having the same function/morphology) but regulated by different TFs as homologous. TFs may evolve new specificity and acquire the ability to induce expression of new effector genes (for example, McKeown et al., 2014). In contrast, effector genes may evolve new cis-regulatory elements and start being induced by new TFs (for example, Ataman et al., 2016) and thus acquire new expression patterns. Importantly, cell type evolution occurs through mechanisms different from the evolution of species where genetic isolation is one of the key factors (Fig. 2B). Within a species, cell types carry the same genetic programme and therefore are not isolated.

Overall, the meeting provided a stimulating environment for discussion of cell type identity and evolution, bringing together a diverse group of researchers and showcasing innovative technologies and approaches to the questions outlined in the introduction to this report – what do we mean by a cell type, how did they arise and diversify, and what are the molecular mechanisms underlying cell identity?

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Competing interests

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