Developmental Mechanisms Controlling Cell Fate, Evolution of

DC Lyons, Duke University, Durham, NC, USA
M Srivastava, Harvard University, Cambridge, MA, USA
DQ Matus, Stony Brook University, Stony Brook, NY, USA

Glossary

Epidermal growth factor (EGF) Secreted growth factor that binds to an EGF receptor.
Epithelial to mesenchymal transition (EMT) Cellular behavior where individual cells lose epithelial characteristics such as adhesion and cell–cell polarity and adopt a mesenchymal phenotype.
Equivalence group Set of cells arising from the same lineage that have equal potential to respond to neighboring inductive cues.
Gene regulatory networks (GRNs) Set of genes and the control elements (*cis*-regulatory elements) that control a specific cell biological process.
Metazoa It is another name for the animal kingdom.
Primary mesenchyme cells (PMCs) Embryonic echinoderm cells located in the vegetal plate that will undergo EMT and ingress giving rise to mesoderm.
Vulval precursor cells (VPCs) Set of ectodermally derived cells that can give rise to the adult nematode vulva, or egg-laying organ.

Introduction

The field of evolutionary developmental biology has matured substantially from its earliest days in the pre-genomic era. We have begun to generate answers to many important outstanding evo-devo questions, and today there are few limits on the questions that can be asked, or the organisms in which to ask them. There are many reasons for this. First, decades of intensive study in a few distantly related 'model' organisms such as the fly, roundworm, and mouse have uncovered an astonishing level of detail about the genetic control of cell function, explaining how their cells build disparate body plans. These studies provide an intellectual framework to generate specific hypotheses about how additional body plans (or cell types, tissues, or processes) may have evolved. Second, a number of new tools are available for perturbing gene function and visualizing cellular behavior, making it possible to investigate the mechanistic basis of development in almost any group of animals (Moczek *et al.*, 2015). Third, the fields of systematics and phylogenetics have established the branching order of most of the major and minor nodes of the animal tree (Aguinaldo *et al.*, 1997; Dunn *et al.*, 2008; Halanych *et al.*, 1995; Hejnol *et al.*, 2009; Moroz *et al.*, 2014; Ryan *et al.*, 2013), a requirement for assessing similarities and differences in animal development in an evolutionary framework (**Figure 1(a)**).

Data from many different organisms and developmental processes clearly shows that complex gene regulatory networks (GRNs) control embryonic development (Davidson *et al.*, 2002; Maduro, 2006; Rottinger *et al.*, 2012). These GRNs ultimately specify the identity and behavior of cells (such as proliferation, migration, shape changes) that determine the adult body plan. This article will focus on the evolution of mechanisms for cell fate specification, a field that has its roots in classical embryology (e.g., Conklin, 1905; Driesch, 1892; Roux, 1888). In that era, any cell or organelle that exhibited a phenomenon of interest was worthy of study, so that, as E.B. Wilson wrote "the problems of evolution have been reduced to problems of the cell" (Wilson, 1911). Today, we are able to be more reductionist, thanks to both our ability to rapidly generate transcriptome data and the increasing ease of functional studies. This allows researchers to characterize the underlying GRNs that, in combination with inductive signaling pathways, specify cell fate. These approaches have expanded our ability to generate comparisons from between homologous cells and cell types to more disparate structures and processes based on changes in gene regulatory network architecture.

Here, we review data on three different aspects of metazoan cell specification (**Figure 1(b)**). The first deals with the behavior of an 'equivalence group' in nematodes. We describe powerful techniques developed in the model nematode, *Caenorhabditis elegans*, which have been used to understand how a group of initially equivalent cells are able to acquire distinct fates through induction from other tissues. These data have been leveraged to study related nematodes, uncovering cryptic variation governing fate specification in a homologous group of cells. A second aspect of specification that we will discuss is the emergence of a novel cell type: the sea urchin skeletogenic mesoderm. A detailed GRN has been established for this cell type, making it possible to compare the network across echinoderms to reveal how this unique cell type arose during evolution. Our third section focuses on the decision between cell differentiation and stemness in the context of adult tissue and how the underlying mechanisms vary over the course of animal evolution. The lessons learned from mature developmental systems are combined with data from emerging models in each case study. In this way we gain a better understanding of the evolution of cell fate specification strategies by examining taxa across the metazoan tree, revealing the 'experiments' that have already occurred over the course of animal evolution. To that end, we begin our discussion of cell fate specification using a study group that allows for functional manipulation of homologous cells at single cell resolution, the rhabditid nematode vulva.

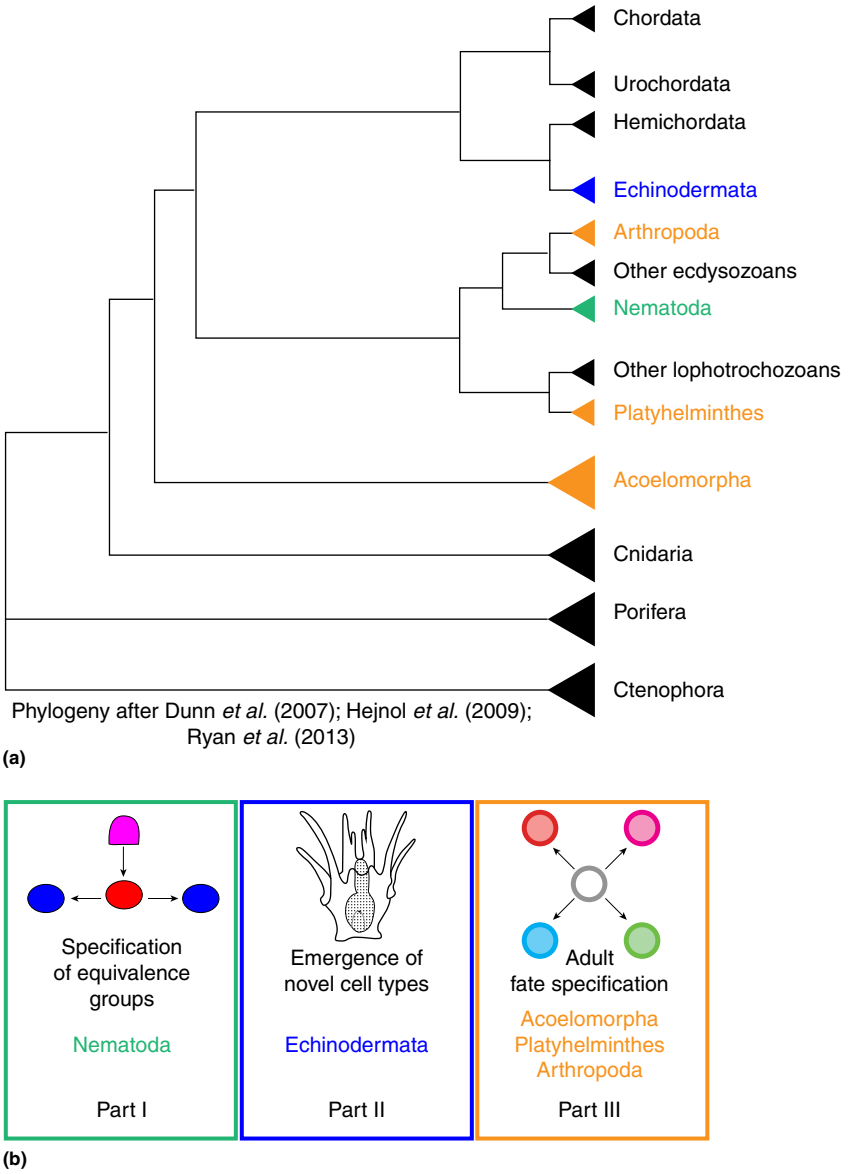

Figure 1 Phylogenetic relationships of animals discussed in this article. (a) Cladogram represents taxonomic relationships. Phylogeny based on recent phylogenomic studies (Dunn *et al.*, 2008; Hejnol *et al.*, 2009; Moroz *et al.*, 2014; Ryan *et al.*, 2013). Colored lineages match taxonomic groups discussed in (b) in each section of this article.

Part I: Competency, Equivalence Groups, and Cryptic Variation

Evolution of Equivalence Groups

A fundamental feature of metazoan cell fate acquisition is the establishment of competency, or the ability of a group of lineage-related cells to respond to inductive signaling. When multiple cells are capable of responding to the same signaling system they are said to form an 'equivalence group' (Kimble, 1981). The discovery of most equivalence groups stems from the ability to map cell lineage patterns during embryogenesis, and thus equivalence groups have been identified in organisms with stereotyped development where individual cells can be both followed over developmental time and destroyed using targeted ablation (e.g., transparent, fast-developing animals, see Table 1). Due to space limitations, in this article, we will focus on the best-studied equivalence group, the rhabditid nematode vulva, or egg-laying apparatus, and refer readers to Table 1 for investigating competence groups in other taxa.

The Rhabditid Nematode Vulva: An Evo-devo Model for Cell Fate Specification

Equivalence groups were first described in nematodes (Kimble, 1981), and due to a wealth of experimental and genetic tools available in *C. elegans*, we have the best molecular understanding of how they function. The most thoroughly characterized equivalence group is the six ectodermal cells (P3.p–P8.p) that

Table 1 Metazoan equivalence groups. Taxonomic examples of specific equivalence groups

Organism	*Phylum*	*Equivalence group*	*Reference(s)*
Mnemiopsis leidyi	Ctenophora	m_1 daughter cells (m_{11}/m_{12})	Henry and Martindale (2004)
Helobdella triserialis	Annelida	O/P ectodermal teloblasts	Weisblat and Blair (1984)
Helobdella robusta			Zackson (1984)
Helobdella stagnalis			Keleher and Stent (1990)
			Huang and Weisblat (1996)
			Kuo and Shankland (2004a,b)
			Kuo and Weisblat (2011)
			Kuo *et al.* (2012)
Drosophila melanogaster	Arthropoda	R7	Greenwald and Rubin (1992)[a]
			Chang *et al.* (1995)
			Dickson *et al.* (1995)
			Crew *et al.* (1997)
			Shi and Noll (2009)
Insects	Arthropoda	Neurogenesis	Doe and Goodman (1985)[a]
			Stollewerk and Simpson (2005)[a]
Caenorhabditis sp.	Nematoda	VPC specification	Sternberg and Horvitz (1986)
Oscheius tipulae			Sulston and White (1980)
Pristionchus pacificus			Kimble (1981)
Other rhabditids			Dichtel-Danjoy and Félix (2004)
			Sternberg (2005)[a]
			Kiontke *et al.* (2007)
			Tian *et al.*, 2008
			Wang and Sommer (2011)
			Penigault and Felix (2011a,b)
			Felix and Barkoulas (2012)[a]
			Kienle and Sommer (2013)
Halocynthia roretzi	Urochordata	Ocellus/Otolith specification	Nishida and Satoh (1989)
			Akanuma *et al.* (2002)
Danio rerio	Chordata	Posterior tailbud progenitors	Martin and Kimelman (2012)
Danio rerio	Chordata	Adaxial cells	Nguyen-Chi *et al.* (2012)

[a]Refers to review article.

will give rise to the adult vulva (Sternberg, 2005). These six vulval precursor cells (VPCs), born in the first larval stage, are all able to generate vulval fates, but under normal (wild-type) conditions only the three inner cells, (P5–7.p) are induced to become vulval cells, adopting either a 1° fate (P6.p) or a 2° fate (P5.p and P7.p). The remaining three cells adopt a default 3° fate and fuse with the epidermis; thus the final pattern is depicted as '3° 3° 2° 1° 2° 3°' (**Figure 2(a)**).

Researchers have spent the past three decades using a variety of experimental and molecular genetic approaches to decode the mechanisms that regulate the fate of these six cells (Sternberg, 2005). Briefly, the VPCs are initially patterned by the expression of a central class Hox5 gene (LIN-39) during the L1 larval stage. Later, in the L3 stage, an EGF signal (LIN-3), secreted by the gonadal anchor cell (AC), which is dorsally situated to the VPCs, induces the 1° fate of P6.p. Upon adopting the 1° fate, P6.p expresses Notch ligands (three delta orthologs, *apx-1*, *lag-2*, and *dsl-1*) (Chen and Greenwald, 2004). Delta ligands activate the Notch receptor (LIN-12) in the neighboring P5.p and P7.p cells which induces the 2° fate. The Wnt pathway functions during VPC specification in a maintenance role to prevent the acquisition of a 3° fate and epidermal fusion (Braendle and Felix, 2008; Eisenmann *et al.*, 1998; Gleason *et al.*, 2002; Myers and Greenwald, 2007).

Comparative work in other nematodes has identified the same signal transduction pathways (Wnt/Notch/EGF) that are used during *C. elegans* VPC specification, although to varying degrees in a taxon-specific fashion (**Figure 2(b)**). For example, data from forward-genetic screens and use of mitogen-activated protein kinase enzyme (MEK) inhibitors in *Oscheius tipulae* has identified a role for EGF/MAPK signaling in VPC induction (Dichtel-Danjoy and Félix, 2004). The initial forward genetic screens to identify vulval development mutants in the diplogastrid nematode *Pristionchus pacificus* led to an unexpected result – rather than a reliance on EGF and Notch/Delta signaling as in *C. elegans*, vulval induction in *P. pacificus* utilizes redundant Wnt signaling from two spatially distinct signaling centers (Tian *et al.*, 2008; Zheng *et al.*, 2005). With many nematode genomes now sequenced and the potential for precise genome engineering offered by new technologies such as CRISPR-Cas9 (Witte *et al.*, 2015) it should now be possible to determine the identity of the signaling pathways that are required to induce VPC fates in different nematode species.

Cryptic Evolution and Developmental System Drift

Not only can we observe striking examples of evolutionary flexibility in signaling pathway usage during nematode VPC induction between related species, but recent work both in *C. elegans* (Barkoulas *et al.*, 2013) and *P. pacificus* (Kienle and

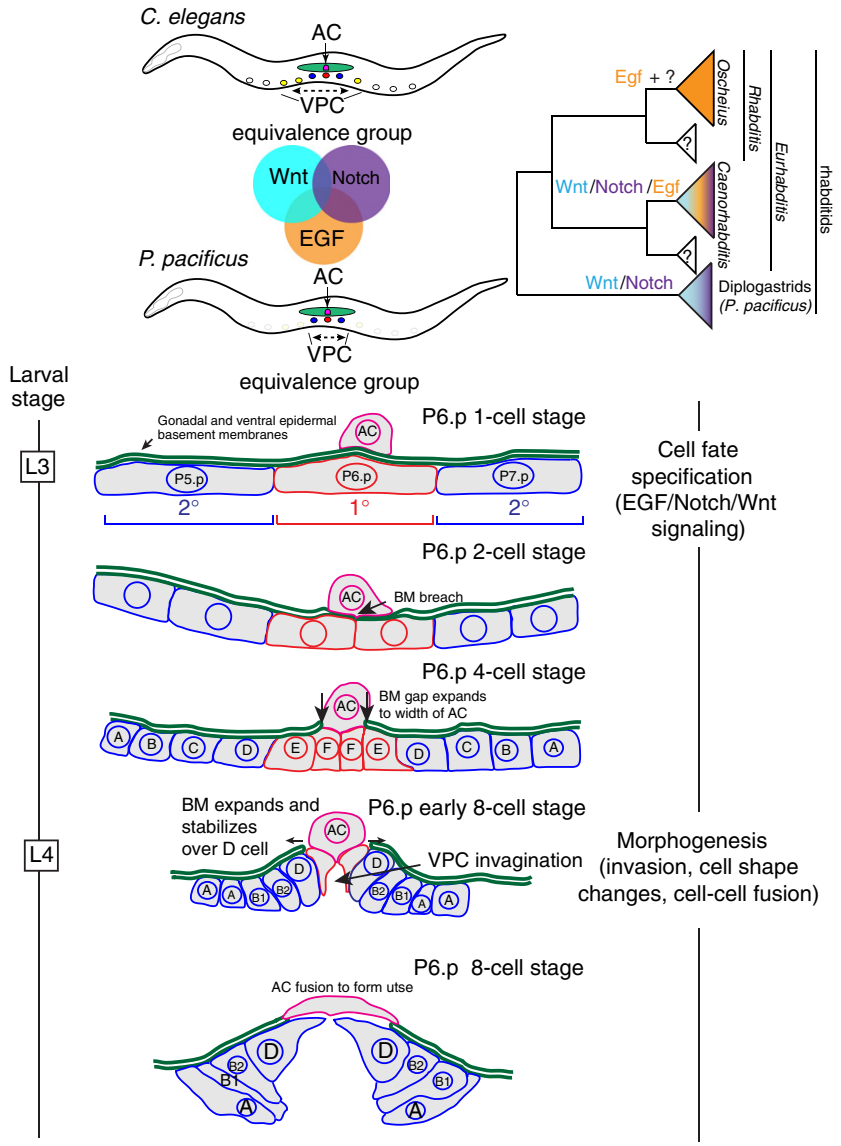

Figure 2 Evolution and development of the rhabditid nematode vulval precursor cell (VPC) equivalence group. (a) Schematics depict VPC fate specification in *C. elegans* (top) and *P. pacificus* (bottom). VPC colors refer to fate (primary, red; secondary, blue; tertiary, yellow (*C. elegans* only)). Venn diagram depicts the contribution of three signaling pathways in VPC specification and corresponds to the colored gradient used in the rhabditid nematode phylogeny shown in (b; phylogeny based on Kiontke *et al.*, 2007). Based on previous research, a role for EGF (orange) signaling (via MEK) has been identified in VPC specification in *Oscheius tipulae*. *P. pacificus* utilizes contributions from both the Wnt (light blue) and Notch (purple) pathway to specify VPC fate, while *C. elegans* receives contributions from all three pathways. It is unknown what signaling pathways specify fate in other rhabditid nematodes (denoted with '?'). (c) Schematic of *C. elegans* uterine–vulval cell specification and morphogenesis (stages defined by division of P6.p and its daughters (e.g., 1-cell stage, 2-cell stage, etc.), with each individual VPC designated by the specific letters, (A)–(F); modified from Matus, D.Q., Chang, E., Makohon-Moore, S.C., *et al.*, 2014. Cell division and targeted cell cycle arrest opens and stabilizes basement membrane gaps. Nature Communications 5, 4184.

Sommer, 2013) highlights cryptic variation that occurs in VPC fate specification within species between natural wild isolates. For example, the Sommer group recently showed that a single *cis*-regulatory change in the conserved Notch ligand, *apx-1*/Delta (one of three Delta ligands redundantly used in 2° fate specification in *C. elegans*) led to gain of a HAIRY binding site in the reference strain of *P. pacificus* (PS312/CA) originally isolated from Pasadena, California. The presence of this HAIRY binding site results in repression of transcription of *apx-1*/Delta in the 1°-fated P6.p cell in PS312/CA. However, most other wild isolates lack this HAIRY binding site and express *apx-1*/Delta in P6.p. Expression of *apx-1* is sufficient to induce 2° fate in the absence of induction from the gonad (Kienle and Sommer, 2013). Their elegant experiments reveal cryptic variation in a core developmental pathway, as a single *cis*-regulatory change can result in the abolition of Notch/Delta signaling as a patterning system in *Pristionchus* VPC specification, and provides a plausible explanation for the diversity in signaling systems that pattern homologous vulval cells between nematode species.

Cell Fate Specification Leads to Differentiation and Morphogenesis

Once the VPCs are properly specified, they execute lineage specific morphogenetic behaviors required to form the adult vulva, including cell division, invagination, and cell fusion (**Figure 1(c)**). *Caenorhabditis elegans* anchor cell (AC) invasion has become a powerful model to understand the genetic control of cell invasive behavior (Matus *et al.*, 2010; Sherwood *et al.*, 2005). Recent work has investigated AC invasion in related nematode species, identifying conserved features: there is only a single AC in all species examined and the AC is required to breach the basement membrane to initiate the uterine–vulval connection (Matus *et al.*, 2014). Following AC invasion, the basement membrane gap expands outward, likely due to forces generated from cell division of the underlying VPCs (Ihara *et al.*, 2011; Matus *et al.*, 2014). The size of this basement membrane gap is tightly regulated, as in all species examined the edges of the gap are stabilized by the same vulval cell, the innermost 2° fated VPC, the D cell. Strikingly, the D cell is the only cell in all nematodes examined to date that never divides (Kiontke *et al.*, 2007). Cell cycle exit of the D cell allows for localization of the extracellular matrix adhesion protein, integrin, to the basal surface of the D cell in response to an increase in the basement membrane component, laminin, at the edges of the basement membrane gap, stabilizing gap expansion (Matus *et al.*, 2014; **Figure 1(c)**). Thus, comparative studies in nematode uterine–vulval development have identified a new mechanism to stabilize basement membrane gaps, a cell biological process that occurs in both developmental contexts and disease pathogenesis (Matus *et al.*, 2014). Connecting cell specification strategies to the cell biology of morphogenetic behaviors across nematode evolution will be informative, especially identifying whether a similar amount of cryptic variation exists between species in executing morphogenetic behaviors as it appears to during cell fate specification.

Part II: Evolution of a Novel Cell Type – Echinoderm Larval Skeleton

The previous section focused on identifying evolutionary changes that alter specification strategies of a homologous group of cells. It is also critical to examine changes in cell fate specification that lead to the origin of new cell types, as they can offer insight into the evolution of novel structures and morphology. To illustrate this point we discuss the echinoderm pluteus larva, which has been a model for developmental, evolutionary, and ecological studies for over a century (Ettensohn, 2009; Lyons *et al.*, 2014; McClay, 2011; Raff and Byrne, 2006; Vaughn and Strathmann, 2008).

The phylum Echinodermata consists of five extant classes: crinoids (sea lilies), asteroids (sea stars), ophiuroids (brittle stars), holothuroids (sea cucumbers), and echinoids (sea urchins, sand dollars). Crinoids are the earliest-branching class, and among the remaining four classes, sea stars and brittle stars are more closely related, forming a clade that is sister to a clade comprised of sea urchins and sea cucumbers (Cannon *et al.*, 2014; Reich *et al.*, 2015; Telford *et al.*, 2014). Members of all five classes develop through an indirect life cycle that includes a planktonic, bilaterally symmetric larva, and a benthic, pentaradial adult. All echinoderms share a homologous calcite endoskeleton at the adult stage, but only ophiuroids and echinoids possess a larval calcite skeleton in the larval stage (**Figure 3(a)**). The skeletonized ophiuroid and echinoid pluteus larva is considered to be a derived form, having evolved from an ancestral auricularia-type larva, shared by the other echinoderm classes (Cannon *et al.*, 2014; Reich *et al.*, 2015; Telford *et al.*, 2014), and their closest out-group, the hemichordates (Rottinger and Lowe, 2012; **Figure 3(a)**). This suggests that the sea urchin and brittle star larval skeleton evolved independently by convergent evolution (or alternatively was lost by crinoids, asteroids, and holothuroids, which is less parsimonious). The development of the sea urchin larval skeleton provides an entry point into understanding how the pluteus skeleton evolved in these two lineages.

The Primary Mesenchyme Cells Build the Pluteus Skeleton in Sea Urchins

The sea urchin skeletogenic lineage arises during cleavage stages as the result of asymmetric cell divisions of vegetal pole blastomeres (Ettensohn, 2009; McClay, 2011). Progeny of these cells undergo an epithelial-to-mesenchymal transition (EMT) and crawl into the blastocoel (the fluid-filled central region of the blastula stage embryo). Once inside the blastocoel, these cells are referred to as primary mesenchyme cells (PMCs). The PMCs then migrate in response to cues coming from the ectoderm/endoderm boundary (Adomako-Ankomah and Ettensohn, 2014; McIntyre *et al.*, 2013, 2014). Although the micromere lineage is autonomously specified at birth (Okazaki, 1975; Oliveri *et al.*, 2008), and will even make skeletal elements *in vitro* (Okazaki, 1975), the pattern of the resulting skeleton is dictated by localized cues emanating from the overlying ectoderm (Armstrong and McClay, 1994; Duloquin *et al.*, 2007; McIntyre *et al.*, 2013; Piacentino *et al.*, 2015; Rottinger *et al.*, 2008). During gastrulation stages, the PMCs fuse to one another and the syncytium forms a ring next to the posterior ectoderm. Soon after ring formation, two aggregates or ventrolateral clusters of PMCs form, where skeletogenesis will begin in response to induction by vascular endothelial growth factor (VEGF) signaling (Adomako-Ankomah and Ettensohn, 2013, 2014; Duloquin *et al.*, 2007; **Figures 3(b)** and **3(c)**). Ventrolateral clusters, expressing the VEGF receptor (**Figure 3(g)**) form directly under signaling centers in the ectoderm expressing the VEGF ligand, where posterior ectoderm and the ciliary band territory intersect (**Figure 3(b)**; McIntyre *et al.*, 2013). When VEGF signaling from the ectoderm is impaired, skeletal patterning within the mesodermal PMCs is perturbed (Adomako-Ankomah and Ettensohn, 2013, 2014; Duloquin *et al.*, 2007). Within the ventrolateral clusters, the PMCs secrete the rudiment of the skeleton, called the triradiate (**Figure 3(d)**). Each prong of the triradiate then grows in a unique and characteristic way to build the mature pluteus skeleton (**Figure 3(e)**; Lyons *et al.*, 2014). The PMC lineage has been used as a model for building GRNs to explain fate specification (Ettensohn, 2013; Oliveri

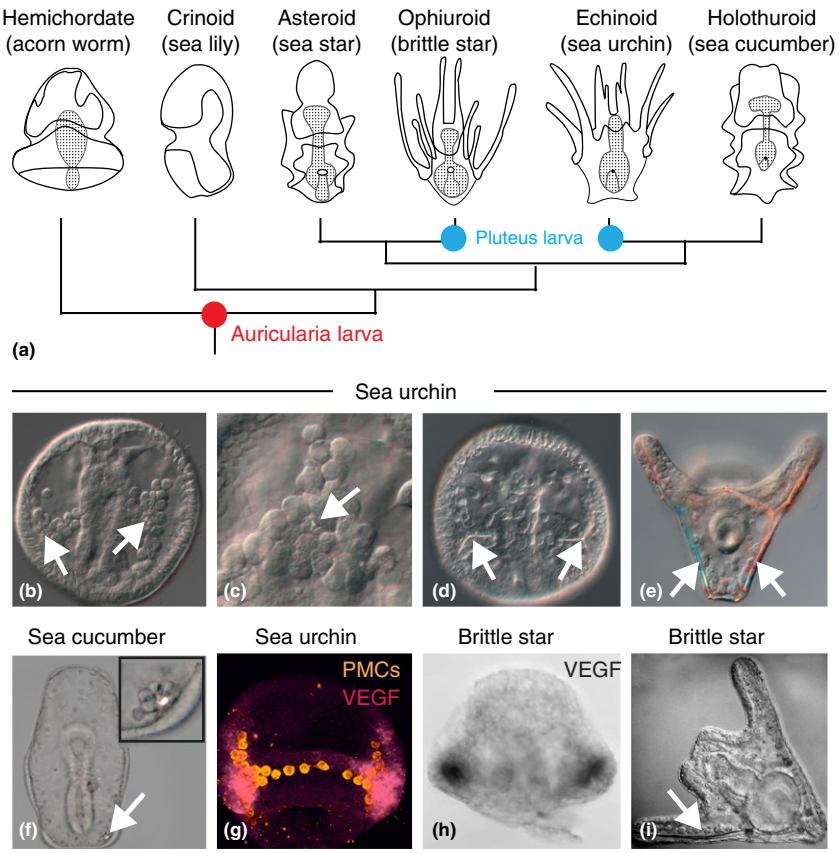

Figure 3 Evolution of larval skeletogenesis in echinoderms. (a) Cladogram showing the evolutionary relationship of echinoderm classes, and their closest outgroup, hemichordates. The auricularia larval form is considered ancestral for echinoderms, making the evolution of the pluteus larva in sea urchins and brittle stars an example of convergence. Cartoons modified from Primus, A.E., 2005. Regional specification in the early embryo of the brittle star *Ophiopholis aculeata*. Developmental Biology 283, 294–309. (b–e) Stages of skeletogenesis in the sea urchin, *Lytechinus*. Arrows point to spicule. (b) Bilateral spicule granules appear within the ventrolateral clusters. (c) Close up of a ventral lateral cluster. (d) The granules grow to form the triradiates during gastrulation stages. (e) Triradiates grow and branch to form the pluteus-stage skeleton. (f) Spicule granule (arrow and inset) in the sea cucumber, *Parastichopus*. Modified from McCauley, B.S., Wright, E.P., Exner, C., Kitazawa, C., Hinman, V.F., 2012. Development of an embryonic skeletogenic mesenchyme lineage in a sea cucumber reveals the trajectory of change for the evolution of novel structures in echinoderms. EvoDevo 3, 17. (g) Double-*in situ* hybridization of *Lytechinus* msp130 transcript (yellow, PMCs) and VEGF transcript (magenta, ectoderm). (h) VEGF transcript is also expressed in the ectoderm overlying the growing arms of the brittle star, *Amphipholis*. Modified from Morino, Y., Koga, H., Tachibana, K., *et al.*, 2012. Heterochronic activation of VEGF signaling and the evolution of the skeleton in echinoderm pluteus larvae. Evolution & Development 14, 428–436. (i) Pluteus of the brittle star, *Ophiopholis*; arrow points to one of the skeletal rods supporting the arms. Modified from Primus, A.E., 2005. Regional specification in the early embryo of the brittle star *Ophiopholis aculeata*. Developmental Biology 283, 294–309.

et al., 2008). This detailed knowledge of PMC specification can be used to ask many evolutionary questions, including how the larval skeleton evolved in echinoids, and what role genes required for PMC specification play in clades that lack a larval skeleton (Hinman and Cheatle Jarvela, 2014).

Co-Option of Adult Skeletogenesis for Larval Skeleton Formation

Genes expressed after metamorphosis, during adult skeletogenesis, appear to have been heterochronically shifted into embryonic stages and expressed in the sea urchin PMC skeletogenic lineage (Gao and Davidson, 2008). Using the sea urchin PMC GRN as a starting point, Gao and Davidson (2008) compared the genetic circuitry upstream of the larval and adult skeleton to identify the points at which the programs overlap, and at which they diverge. They found that whereas the transcription factors *ets1*, *alx1*, and *hex* are expressed in both the micromere lineage and the adult rudiment in sea urchins, *tbr* is expressed only in the PMC lineage. *ets1*, *alx*, and *hex* are also expressed in the rudiment of adult sea stars, but *tbr* is not. These data suggest that the *alx/ets1/hex* node of echinoderm adult skeletogenesis is ancient, and was co-opted for larval skeletogenesis in the sea urchin lineage. *tbr*, which is necessary for larval skeletogenesis, but not adult skeletogenesis, was an independent acquisition into the micromere lineage.

What Can Out-Groups Tell Us About the Evolution of the Sea Urchin Pluteus?

Sea cucumbers are the echinoderm class most closely related to sea urchins and sand dollars (Cannon *et al.*, 2014; Reich *et al.*,

2015; Telford *et al.*, 2014), but they lack a larval skeleton. However, a small spine or spicule granule has been observed in the larvae of some sea cucumber species, but it never grows into a triradiate, or makes arms, as occurs during the development of the sea urchin pluteus skeleton. Recent work has investigated the morphological and molecular basis of granule formation in the sea cucumber *Parastichopus* (**Figure 3(f)**; McCauley *et al.*, 2012). In this species, the granule is made by cells that enter the blastocoel early, and these cells go on to form a dorsal cluster underneath the posterior-dorsal ectoderm. *Parastichopus alx1* is expressed in cells at the vegetal pole, and in the cells that enter the blastocoel early and make the dorsal cluster and spicule granule. Knockdown of *Parastichopus alx1* abolishes the dorsal cluster, and the spicule, suggesting that, as in sea urchins, *alx1* is necessary for specifying cells capable of making skeleton material. In the sea cucumber *Holothuria*, *ets1/2* is expressed in mesodermal cells, including a patch of posterior-dorsal cells that might be the mesodermal cells that secrete the larval spicule granule in that species (Koga *et al.*, 2010). These data suggest that sea cucumber mesodermal cells express some of the same genes when making a spicule granule, as the sea urchin PMCs express when making the pluteus skeleton. Koga *et al.* (2010) propose that ancestrally, *ets1/2* had two functions: one specifying larval mesoderm that was non-skeletogenic, and a second specifying adult skeletogenesis. In the echinoid lineage, the *ets1/2* transcription factor gained the ability to upregulate the skeletogenic program in the larval mesoderm.

In fact, many genes associated with the sea urchin PMC lineage are expressed in the larval mesoderm of species that do not make larval skeletons, such as the sea stars (Koga *et al.*, 2010; McCauley *et al.*, 2010; Morino *et al.*, 2012). These studies support the idea that the genes involved in skeletogenic mesoderm in sea urchins were likely expressed in the larval mesoderm of the echinoderm common ancestor and then later became able to promote skeletogenic cell fate by subtle changes in gene regulation, such as downstream gene target switching (Koga *et al.*, 2010).

Convergent Evolution of the Pluteus in Brittle Stars?

How similar is the development of the larval skeleton between sea urchins and brittle stars? Unlike sea urchins, the brittle star skeletogenic lineage does not arise from a noticeable asymmetric cell division at 4th cleavage (Primus, 2005). Instead, the skeletogenic cells, similarly called PMCs, become obvious inside the blastocoel before gastrulation and form bilateral rudiments of the larval skeleton (Yamashita, 1985). The behavior of the brittle star PMCs suggests that they might also migrate in response to signals from the ectoderm. In fact, in the brittle star *Amphipholis* Morino *et al.* (2012) found that homologs of VEGF are expressed in bilateral patches of ectoderm (**Figure 3(h)**), very much like the pattern in urchins; VEGFR is likewise expressed in adjacent ventrolateral PMC clusters. As in sea urchins (Adomako-Ankomah and Ettensohn, 2013; McIntyre *et al.*, 2013), the expression of brittle star VEGF becomes restricted to lateral ectodermal patches.

This remarkable similarity in expression patterns of VEGF and VEGFR between sea urchins and *Amphipholis* demonstrates that there are fundamental similarities in how the two groups make their larval skeleton. More work on brittle stars will be necessary before we fully understand how deep the similarities go, on a cellular or molecular level. For example, *Amphipholis* (Koga *et al.*, 2010) also expresses the *ets1/2* gene in its PMCs, and a transcriptome of *Ophiocoma* gastrula-stage embryos (Vaughn *et al.* 2012) revealed that many homologs of genes involved in sea urchin PMC specification and skeletogenesis are expressed in this brittle star species. Studies of both the transcripts and proteins made by *Ophiocoma* show that the skeletogenic tool kit is similar, but not identical, to that in sea urchins (Seaver and Livingston, 2015; Vaughn *et al.*, 2012).

The fact that in both sea urchins and brittle stars members of the VEGF signaling pathway are expressed in analogous territories (VEGF ligand in the ectoderm, and VEGF receptor in the PMCs) suggests that communication between mesoderm and ectoderm was critical for the evolution of the pluteus in both echinoderm lineages. Whether sea cucumber or sea star skeletogenic cells are responding to cues from the ectoderm during the larval stage remains to be answered. The expression of *vegf* and *vegfr* has been examined in sea stars (Morino *et al.*, 2012), and no transcripts for either gene were detected by *in situ* hybridization or qPCR during early larval stages. Yet later, *vegf* (expressed in the ectoderm), and *vegfr* (expressed in the mesoderm), were associated with the rudiments of the adult skeleton. Thus VEGF signaling might have been heterochronically activated during larval stages, in ectoderm and mesoderm, in sea urchins and brittle stars independently. How this occurred poses a fascinating open question for future investigations. In order to understand the evolution of a novel cell type, we will need to hone in on the conversation between these two tissues.

Part III: Cell Fate Specification in Adult Animals

Specification of cell fate from undifferentiated cells is essential to the maintenance of adult form, as adult tissue can be lost during homeostatic turnover or due to damage. Some animals (e.g., planarians), have pluripotent adult stem cells that can acquire many distinct cell fates, whereas other animals (e.g., vertebrates) possess lineage-restricted stem cells. Very little is understood about how the mechanisms that underlie stem cell pluripotency and subsequent fate specification compare across these diverse species, but recent studies in previously understudied animals have revealed valuable insight.

Differentiation in Lineage-Restricted Adult Stem Cells

Adult mammals have the capacity to continually replace tissues that are lost to homeostatic turnover or to injury. Pools of lineage-restricted stem cells that are maintained throughout adulthood provide new cells. These include hematopoietic stem cells in the bone marrow, which generate myeloid and lymphoid lineages of blood cells; slow-cycling cells of the intestinal crypt that make transit amplifying cells to replace secretory and digestive cells of the gut; and stem cells located in the bulge regenerate hair follicles (Clevers, 2013; Fuchs and

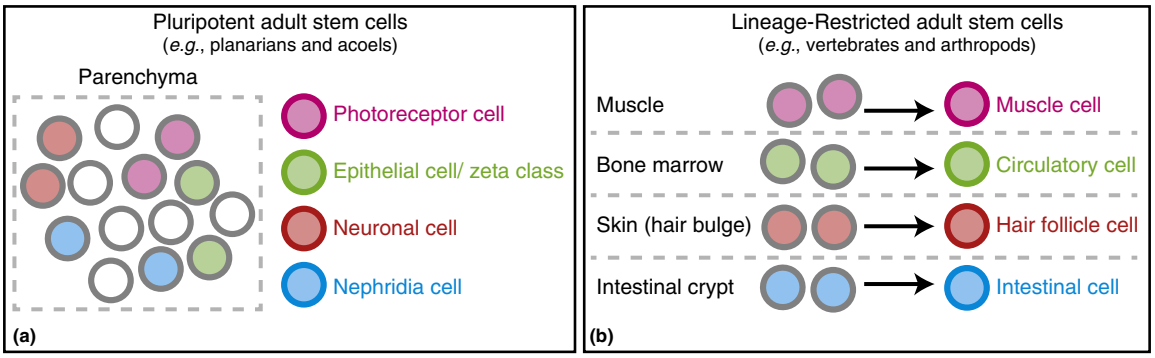

Figure 4 Schematic representation of cell fate specification from planarian adult pluripotent stem cells and lineage-restricted stem cells in vertebrates. (a) Planarian stem cells (neoblasts) are a broadly distributed population of cells that include progenitors that have become committed to specific lineages. The clonogenic-neoblasts (white circles with grey outlines) and progenitors (colored circles with grey outlines) are not separated spatially, but terminally differentiated cell types (colored circles with colored outlines) incorporate into tissues at specific positions in the adult body. Clonogenic-neoblasts are presumed to give rise to the committed progenitors, but this has not been shown directly. (b) Vertebrate organs harbor lineage-restricted stem cells (colored circles with grey outlines) that generate terminally differentiated cells (colored circles with colored outlines) for the specific tissue. These stem cells are spatially restricted to their tissue of origin.

Nowak, 2008; Seita and Weissman, 2010; **Figure 4**). These independent stem cell populations express distinct molecular markers and acquire their restricted cell fates under the control of different factors depending on their varied tissue contexts.

In contrast to blood, intestinal crypts, or hair, which are unique to vertebrates, muscle is present in all bilaterian animals, providing an opportunity to compare specification mechanisms across species. Vertebrates have quiescent stem cells called satellite cells that express *Pax7* protein, may or may not express *Pax3*, and may have sequestered mRNA for the myogenic factor *Myf5* (reviewed in Cerletti *et al.*, 2008; Motohashi and Asakura, 2014). Upon injury, these cells respond to signals from their niche, i.e., the surrounding microenvironment, by dividing asymmetrically to produce a myoblast. This cell downregulates *Pax7* expression, upregulates the expression of *Desmin* and *MyoD*, proliferates, and, with increasing expression of other myogenic factors such as *MRF* and *Myogenin*, differentiates into a muscle fiber.

A recent study on muscle regeneration in a new arthropod model system provides the first opportunity to compare regenerative mechanisms across distantly related bilaterian phyla. Whereas *Drosophila*, the classic model arthropod, cannot regenerate limbs, the crustacean *Parhyale hawaiiensis* is able to regrow its appendages upon amputation. The *Parhyale* ortholog of vertebrate *Pax3* and *Pax7*, *Pax3/7*, labels cells that are present adjacent to muscle fibers and morphologically resemble vertebrate satellite cells (Konstantinides and Averof, 2014). Transgenically-labeled satellite cells were isolated, and upon transplantation into host limbs, contributed to newly formed muscle fibers in regenerating limbs. This finding suggests that arthropod and vertebrate muscles regenerate in a very similar manner, using lineage-restricted stem cells that are labeled by an evolutionarily conserved marker, *Pax3/7* (**Figures 1** and **4**). Moving forward, it will be fruitful to investigate more broadly in cell types besides muscle, commonalities between lineage-restricted stem cells, which are present in many animal phyla, including the early branching non-bilaterians (e.g., ctenophores, cnidarians) (Alie *et al.*, 2011; Plickert *et al.*, 2012).

Specification in Pluripotent Adult Stem Cells

Adult planarians show amazing regenerative abilities. They can regenerate virtually any missing tissue through the activity of a large population of parenchymal cells called neoblasts, which are required for regeneration (Reddien *et al.*, 2005; **Figure 4**). Based on their shared expression of homologs of *piwi*, neoblasts were considered to be a homogeneous population. Single neoblasts transplanted into irradiated animals expand clonally (thus referred to as clonogenic- or c-neoblasts), differentiate into all tissue types of the adult animal, and restore the regenerative capacity of their hosts (Wagner *et al.*, 2011). It is unknown what proportion of the total neoblast population is clonogenic.

Studies of regeneration in planarians of specific organs, such as nephridia and eyes, revealed that transcription factors that are expressed in and required for the regrowth of these structures, also label a small number of *piwi*+ cells (**Figure 4**). These cells are thought to be committed progenitors that begin to express a tissue-specific marker (*Sp6-9*, *Dlx*, *Six1/2*, and *Eya* for pigment cups of the eye; *Six1/2-2*, *Eya*, *Osr*, *POU2/3*, and *Sall* for nephridia), lose *piwi* expression, and become terminally differentiated (Lapan and Reddien, 2011; Scimone *et al.*, 2011). Several progenitor classes for different neuronal lineages have also been recently identified (Cowles *et al.*, 2013; Scimone *et al.*, 2014). Clustering of neoblasts, based on the expression of 96 genes in single cells, revealed progenitor lineages that differentiate into the gut and epidermis (van Wolfswinkel *et al.*, 2014). Thus, neoblasts represent a dynamic population of stem cells that differentiate into varied cell types, presenting a great opportunity to understand the mechanisms of fate specification in adults.

It is unknown whether the mode of adult cell fate specification uncovered in planarians is broadly conserved among animals. Acoel worms diverged from planarians 550 mya and likely represent the earliest-diverging lineage of animals with bilateral symmetry (Hejnol *et al.*, 2009; Philippe *et al.*, 2011; Srivastava *et al.*, 2014). Acoels also have a population of proliferative cells that resemble planarian neoblasts based on

morphology, expression of *piwi*, and sensitivity to radiation (De Mulder *et al.*, 2009). A recent study in *Hofstenia miamia*, a new model acoel species, revealed that expression of *piwi* in neoblasts is required for regeneration (Srivastava *et al.*, 2014). Additionally, Wnt and Bmp signaling pathways are required for correctly regenerating tissues along the anterior–posterior and dorsal–ventral axes respectively in *Hofstenia*, suggesting that the decisions upstream of fate specification are also shared between acoels and planarians. *Hofstenia* is amenable to mechanistic studies of differentiation within the neoblast population which, combined with similar studies in planarians, could inform us on whether fate specification in adult pluripotent stem cells is evolutionarily conserved or independently-evolved.

Many other regenerative animal species have putative pluripotent adult stem cells that express piwi (e.g., sponges, cnidarians, annelids, and ascidians) (Alie *et al.*, 2011; Brown *et al.*, 2009; Funayama *et al.*, 2010; Giani *et al.*, 2011; Juliano *et al.*, 2014; Plickert *et al.*, 2012; Rinkevich *et al.*, 2013), but fate specification mechanisms in these species are currently unknown.

The Evolution of Stem Cell Fate Specification

Given that both lineage-restricted and pluripotent modes of adult stem cells are broadly distributed across animal phylogeny, it is unclear which mode represents the ancestral condition (**Figure 1**). For example, ctenophores and sponges, the two earliest-diverging animal lineages, feature lineage-restricted and pluripotent stem cells respectively (Alie *et al.*, 2011; Funayama *et al.*, 2010).

Fate specification mechanisms may be conserved, regardless of the source of undifferentiated cells. Small populations of lineage-committed progenitors within the total neoblast population in planarians could be analogous to the lineage-restricted pools of stem cells in vertebrates and crustaceans. If the satellite-like cells in *Parhyale* and vertebrates maintain stemness and differentiate into muscle fibers via the same molecular mechanisms, then one would infer that these mechanisms appeared in the bilaterian ancestor. Planarians also evolved from this same ancestor, and one might hypothesize that a subset of neoblasts that are committed to differentiate into muscle would resemble satellite cells, for example requiring the expression of a *Pax3/7* homolog. One *Pax3/7* homolog reported thus far from planarians is required for the formation of progenitors for *dopa-beta-hydroxylase+* cells of the nervous system (Scimone *et al.*, 2014). Recently reported MyoD-expressing *piwi+* cells may represent planarian muscle progenitors (Cowles *et al.*, 2013; Scimone *et al.*, 2014). Thus, a detailed investigation of muscle progenitors within the neoblast population is needed to illuminate this hypothesis.

An alternative explanation for shared mechanisms underlying lineage-committed progenitors could be independent co-option of developmental pathways in the adult. For example, *Pax3* and *Pax7* expressing cells form muscle during mouse embryonic development (Relaix *et al.*, 2005), and if these transcription factors are conserved regulators of muscle cell fate in other animals as well (e.g., in arthropods and flatworms), then their role in adult muscle progenitors would reflect a hard-wired control over downstream genes that mediate muscle cell function. Investigating the details of how *Pax3/Pax7* are regulated to hold progenitor cells in a paused state, and subsequently to release them to acquire muscle fate in distantly related species, will be crucial to understanding the overlap between developmental versus adult muscle specification.

Studies of fate specification in adults not only inform how animal body plans are maintained, but how adult stem cells are regulated. Emerging model systems such as planarians, acoels, and crustaceans offer opportunities for mechanistic investigations of fate specification in a variety of cell types in the context of pluripotent and lineage-restricted adult stem cells. In addition to cell-intrinsic control of fate specification, it will be important to compare niche signals, which are essential regulators of vertebrate adult stem cells.

Conclusions and Future Directions

This is an exciting time to be investigating the molecular basis of cell fate specification in an evolutionary context. We are fortunate to have a wealth of data generated by traditional model systems that provides a framework for empirical testing. The ease of adapting CRISPR/Cas9 to both induce mutations in genes of interest as well as introduce GFP and other fluorescent markers into endogenous loci is enabling researchers to move beyond the descriptive approaches that dominated the field of evo-devo for the past two decades. These new genome engineering techniques will allow us to directly test gene function and visualize cellular behaviors in nearly any taxon of choice. These molecular approaches are critical if we are to understand how alterations in cell fate specification strategies result in evolutionary change. Whether they are represented by cryptic variation in the formation of homologous cell types and tissues, changes in GRNs that result in the formation of novel cell types, or changes in the molecular cascades that maintain the balance between stemness and differentiation in response to injury or environmental stress in adults, the next decade should see huge leaps forward in our understanding of the evolution of cell fate specification in multicellular organisms.

See also: Cellular Behaviors Underlying Pattern Formation and Evolution. Novel Structures in Plants, Developmental Evolution of

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