Cell state switching factors and dynamical patterning modules: complementary mediators of plasticity in development and evolution†

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Ancient metazoan organisms arose from unicellular eukaryotes that had billions of years of genetic evolution behind them. The transcription factor networks present in single-celled ancestors at the origin of the Metazoa (multicellular animals) were already capable of mediating the switching of the unicellular phenotype among alternative states of gene activity in response to environmental conditions. Cell differentiation, therefore, had its roots in phenotypic plasticity, with the ancient regulatory proteins acquiring new targets over time and evolving into the "developmental transcription factors" (DTFs) of the "developmental-genetic toolkit." In contrast, the emergence of pattern formation and morphogenesis in the Metazoa had a different trajectory. Aggregation of unicellular metazoan ancestors changed the organisms' spatial scale, leading to the first "dynamical patterning module" (DPM): cell-cell adhesion. Following this, other DPMs (defined as physical forces and processes pertinent to the scale of the aggregates mobilized by a set of toolkit gene products distinct from the DTFs), transformed simple cell aggregates into hollow, multilayered, segmented, differentiated and additional complex structures, with minimal evolution of constituent genes. Like cell differentiation, therefore, metazoan morphologies also originated from plastic responses of cells and tissues. Here we describe examples of DTFs and most of the important DPMs, discussing their complementary roles in the evolution of developmental mechanisms. We also provide recently characterized examples of DTFs in cell type switching and DPMs in morphogenesis of avian limb bud mesenchyme, an embryo-derived tissue that retains a high degree of developmental plasticity.

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1. Introduction

Plasticity (i.e. stochastic or condition-dependent variability) of developmental outcome in multicellular organisms is due to two principal mechanisms. The first is largely, though not entirely, a function of differential gene expression and is based on the capacity of individual cells to switch between alternative states under different environmental conditions, which had its roots in the unicellular biological world predating the existence of multicellular organisms. In multicellular organisms, conditional cell type switching (including stable differentiation), is employed not only during development, but also during tissue repair and regeneration, and, according to some accounts (Bissell *et al.* 2003; Soto and Sonnenschein 2005), in neoplasia, where its role as a mechanism of plasticity is particularly evident.

The second phenomenon underlying phenotypic plasticity in complex organisms is the array of pattern forming mechanisms that operate during their development. These mechanisms promote cell rearrangement and (utilizing the above-mentioned inherent propensity of cells to switch between alternative types), transform populations of cells into new ones of increasing spatiotemporal heterogeneity. Unlike the cell switching mechanisms, which

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Abbreviations used: DPMs, dynamical patterning modules; DTFs, developmental transcription factors; ECM, extracellular matrix; GRNs, gene regulatory networks; PCP, planar cell polarity; PPARγ, Peroxisome proliferator-activated receptor gamma; UCP1, uncoupling protein 1

pre-existed multicellularity (*see* below), these mechanisms of developmental pattern formation seem to have arisen simultaneously with, and indeed are the distinguishing properties of multicellular life.

Here we will consider the complementary roles of cell type switching and cell pattern formation in the Metazoa, the ancestral and modern forms of the multicellular animals. Other taxonomic groups with multicellular members – prokaryotes, protists, plants – will almost certainly all have analogous stories. The metazoans, however, have some unique characteristics. Their evolutionary history is relatively well described. Essentially all their modern body plans emerged within a time span of no more than 20 million years (Rokas *et al.* 2005) during the Cambrian period, more than 500 but less than 600 million years ago (Conway Morris 2006). Simpler sheet-like and hollow spherical forms, and budding and segmented tubes (Droser and Gehling 2008), are seen in fossil beds of the Ediacaran period spanning about 635 to 542 million years before the present.

The relatively sudden ("compressed in time"; Rokas *et al.* 2005) phylum-scale emergence of complex metazoan forms that occurred in the early Cambrian was not anticipated by classical neo-Darwinian evolutionary mechanisms of natural selection acting on random genetic variation (Müller and Newman 2005; Müller 2007). Equally surprising ("amazingly parsimonious"; Duboule and Wilkins 1998) relative to the standard evolutionary model has been the discovery that a common set of highly conserved gene products, the so-called developmental-genetic toolkit (which includes determinants of both cell type switching and multicellular pattern formation), has been used to generate animal body plans and organ forms for the more than half billion years since the inception of the Metazoa (Wilkins 2002). The combination of rapidity of morphological diversification and lack of substantial change in the identity of the molecules that mediate development over the entire range of metazoan taxa raises the possibility that plasticity-promoting factors beyond genes and their products were involved in the origination of metazoan forms and might continue to participate in their development.

We have suggested that this additional causal agency in metazoan evolution is the physics of viscoelastic, chemically excitable materials operating on the spatial scale of small cell clusters (Newman and Comper 1990; Newman *et al.* 2006). In particular, we hypothesize that a subset of the "toolkit" gene products harnessed or mobilized physical processes and forces which were irrelevant (for reasons described below) to the scale of individual cells so as to form "Dynamical Patterning Modules" (DPMs; Newman and Bhat 2008, 2009). The DPMs, functioning separately and in combination, but without extensive evolution at the gene level, transformed simple, spherical, topologically solid, cell clusters into hollow, multilayered, elongated, segmented, folded and appendage-bearing structures, e.g. embryos. Both upstream and downstream of the DPMs a different subset of the toolkit gene products, the developmental transcription factors, or DTFs, and intracellular gene regulatory networks (GRNs) composed of and associated with them, mediated switching between the dynamical states available to cells in these clusters.

In section 2 we will describe the dynamical basis of cell type switching and review only briefly (since it has been written about extensively, e.g. Davidson 2006), the role of DTFs and their associated GRNs. Then in section 3, we will discuss the less familiar concept of DPMs and explain how their collective action constitutes a "pattern language" for the generation of metazoan form (Newman and Bhat 2009). In section 4, recognizing that it is impossible to perform experiments on ancestral metazoans, but that it is nonetheless feasible to examine the variable activities and roles, both of DTFs and their associated intracellular GRNs, and DPMs, on tissue primordia of present-day metazoan embryos, we will present some recent experimental results on the differentiative and morphogenetic properties of the limb bud mesenchyme of the avian embryo. Finally, in section 5 we will attempt to draw some conclusions from all this concerning the role of plasticity of cell state and tissue form in the evolution of development.

2. Dynamic multistability, developmental transcription factors and cell differentiation

Since each cell of a multicellular organism (with a few exceptions, such as the egg and sperm and their immediate precursors, and some cells of the immune system) contains an identical set of genes, a fundamental question of development is how the same genetic instructions are compatible with many different types of cells. Multicellular organisms solve the problem of specialization by activating type-specific subsets of genes, and inactivating other subsets, in each cell type, but the ability to do so is based on properties generic to complex gene regulatory systems, and thus predated multicellularity.

The biochemical state of a cell can be characterized by a list of all the different types of molecules contained within it, along with the set of reactions that determine their interconversions and regulatory interactions. And since the cell's molecules can be transported across its membrane or converted from one to the other, the biochemical state is also a dynamical state. As with any dynamical system, the system's state resides in a multidimensional space, the "state space," with a dimensionality equal to the number of system variables (e.g. chemical components) (Strogatz 1994).

Cells of any kind, including unicellular organisms, can switch between alternative stable or metastable states of differential gene expression because the subregion of state space defined by the molecules that specify cell determination

and differentiation exhibits a complex dynamical structure, including oscillatory orbits and multiple steady states (Strogatz 1994; Goldbeter 1996; Kaneko 2006). The regions of state space in which dynamical trajectories tend to converge are called "attractors". And although cells contain molecules of various categories and sizes – water, ions, sugars, amino acids – the *differentiated state* or *type* of a cell, whether free-living or part of a multicellular organism, can generally be identified with the collection of RNAs and proteins it is capable of producing. The cell's differentiated type thus differs from its biochemical state. In particular, not every cell state possible within an organism can be accessed within a given type, and not all states a given cell type is capable of assuming will be realized at any given time.

RNAs and proteins are specified by a cell's genes, and genes, in turn, are initially regulated by transcription factors, a subset of the cell's protein products. The genes that specify transcription factors are controlled by the products of other such genes. Because of this autoregulatory architecture (Keller 1995), transitions of a cell between the states of differentiation available to it can be driven by changes in the relative levels of a fairly small number of key transcription factors (Davidson 2006).

To a great extent, then, we can understand the dynamical basis of cell type switching (i.e. determination and differentiation) by focusing on molecular circuits, or networks, consisting solely of transcription factors and the genes that specify them. Using such circuitry, cells can pass on not only "informational" macromolecules such as DNA and RNA to their progeny, but also epigenetic states, including specific patterns of gene expression. The ability of cells to undergo transitions among a limited number of discrete, stable epigenetic states and to propagate such decisions from one cell generation to the next is clearly essential to metazoan development. But even organisms that have traditionally been considered unicellular, including bacteria, fungi, protists and algae exhibit alternative states of differentiation due to stochastic effects or as functions of particular conditions, which can be environmental, social, a combination of both (Pan and Snell 2000; Kaiser 2001; Ryals *et al.* 2002; Blankenship and Mitchell 2006; Süel *et al.* 2006, 2007; Vlamakis *et al.* 2008). The role of transcription factor networks in mediating condition-dependent switching between or among alternative cellular phenotypes in single-celled organisms (Papp and Oliver 2005) strongly suggests that cell-state switching networks had their origins not primarily as regulators of multicellular development, but rather as mediators of unicellular plasticity.

Some of the toolkit gene products that we refer to as DTFs are used to mediate general, or early-acting, cell lineage choices. Several of these factors are involved in inducing "homologous" tissues and cell types in widely divergent taxonomic groups. (See the papers in Bock and Cardew 1999 for discussion of definitions of, and controversies around, the concept of homology.) Members of the Forkhead, Sox and Runx families, for example, are variously involved in mesoderm specification, neurogenesis and skeletogenesis across all or most metazoan groups (Coffman 2003; Oliveri *et al.* 2006; Kiefer 2007). A point of reference in the following discussion is the unicellular protozoan *Monosiga brevicollis*, a choanoflagellate, which is the group considered to contain the closest living relatives of the Metazoa (Wainright *et al.* 1993; King *et al.* 2003; Snell *et al.* 2001; Lang *et al.* 2002; Phillipe *et al.* 2004). Both Forkhead and Sox genes appear in the *M. brevicollis* genome (King *et al.* 2008), although none of the cell types associated with the transcription factors they specify in metazoans appear to be in the choanoflagellate repertoire.

Others of the DTFs are used during metazoan development to bring about lineage choices that result in terminally differentiated states. For example, the gene networks that control differentiation of cardiac muscle in insects and mammals, and pharyngeal muscle in nematodes, employ the homologous (i.e. evolutionarily related) NK-class homeobox proteins tinman, Nkx-2.5 and CEH-22 (Haun *et al.* 1998). In addition, the formation of eyes in cephalopods, vertebrates and insects all use homologous factors of the eyeless/Pax6 class (Gehring 2002), and jellyfish, earlier-diverging cnidarians, also use a Pax gene, although more distantly related, for this purpose (Kozmik 2008). The NK family appears to have arisen in the last common ancestor of sponges (the most primitive metazoans) and eumetazoa (cnidarians and bilaterians) after the sponge-eumetazoan divergence (Larroux *et al.* 2007, 2008), while an eye-pathway related Pax gene is present even in sponges (Kozmik 2008).

In still other cases, DTFs, rather than being involved in establishing different cell types, operate within populations of cells of a single type to elicit subtype differences. This functionality can be used during metazoan development to establish both spatial uniformity and distinctions within tissue domains. For example, the products of the hairy/enhancer of split (Hes) family, help regulate positional identity during segmentation in arthropods, annelids and vertebrates (Howard and Struhl 1990; Song *et al.* 2004; Damen *et al.* 2005; Giudicelli *et al.* 2007) by its involvement in global spatial coordination of cell state (Ozbudak and Lewis 2008). The Pbx1 (Gonzalez-Crespo and Morata 1996; Selleri *et al.* 2001) and Dlx families (Panganiban and Rubenstein 2002), mark proximal and distal regions, respectively, of insect and vertebrate appendages. Also, members of the Hox class of transcription factors serve as determinants of regionalization of the ectodermal, mesodermal and endodermal germ layers during development of all bilaterian metazoans (reviewed in Wilkins 2002). Table 1 provides a list of selected DTFs and their taxonomic associations.

Table 1. Names, cell-function- and clade-associations of some developmental transcription factors (DTFs)

DTF	Cell association	Taxonomic association
Hox	regional variation of differentiated state	all eumetazoans
Hes	alternating variation of differentiated state	annelids; arthropods; chordates
ey/Pax6	eye/sensory cells	nematodes; arthropods; mollusks; chordates , etc.
extradenticle/Pbx1	proximal portions of appendages	arthropods; chordates
distal-less/Dlx	distal portions of appendages	arthropods; chordates
grainyhead	cuticle/epidermis	nematodes; arthropods; chordates
ceMyoD/nautilus/MyoD	skeletal muscle cells	nematodes; arthropods; chordates
ceh-22/tinman/Nkx2.5	cardiac/pharyngeal muscle cells	nematodes; arthropods; chordates
atonal/neurogenin	neurons	arthropods; chordates
PPARγ	white adipose	chordates
PPARγ + PGC-1α	brown adipose	chordates
Runx2	bone	chordates
PGC-1α + Sox9	cartilage	chordates
Sry + Sox9	testis	chordates

Note: The DTFs shown are used individually or in combination to elicit the indicated cell types in members of the respective clades. The same factors may mediate other differentiation functions in other clades. DTFs separated by a slash are homologues; those linked by a plus are used combinatorily to bring about the indicated effect. Hox: Kappen and Ruddle (1993); Hes: Howard and Struhl (1990); Damen *et al* (2005); Song *et al* (2004); Giudicelli *et al* (2007); ey/Pax6: Gehring (2002); extradenticle/Pbx1: Gonzalez-Crespo and Morata (1996); Selleri *et al* (2001); distal-less/Dlx: Panganiban and Rubenstein (2002); grainyhead: Stramer and Martin (2005); ceMyoD/nautilus/MyoD: Zhang *et al* (1999); ceh-22/tinman/Nkx2.5: Haun *et al* (1998); Schwartz and Olson (1999); atonal/neurogenin: Quan *et al* (2004); PPARγ: Gesta *et al* (2007); PPARγ + PGC-1α: Gesta et al (2007); Runx2: Franceschi *et al* 2007; Marie (2008); PGC-1α + Sox9: Kawakami *et al* (2005); Sry + Sox9: Sekido and Lovell-Badge (2008).

Any notion of evolution based on descent with modification should be able to accommodate the cooptation of pre-existing genes to new functions. However, metazoans of wide morphological disparity (which, by the expectations of Darwinian gradualism, would have required very long periods of time to diverge), use homologous (and often interchangeable; Gehring 2002), gene products for similar purposes. This implies either that the common uses of the transcription factors were locked into place before substantial morphological evolution took place, and that few genetic changes in the most widely used factors occurred during the major morphological transitions (a truly perplexing form of genetic stasis for the gradualist Darwinian model), or that (as we will suggest below), initial morphological diversification of the premetazoa took place quickly, by mechanisms that did not require extensive genetic change.

How was this complexity achieved? As described in the following section (see also Newman *et al.* 2006; Newman and Bhat 2008, 2009), we attribute the rapid emergence (Rokas *et al.* 2006) of metazoan morphological complexity to the DPMs. These patterning modules use the capacity of a class of toolkit gene products distinct from the DTFs to mobilize the physics of condensed materials to communicate the effects of cell state changes between cells and across cell aggregates.

3. Dynamical patterning modules and the origins of metazoan embryogenesis

Development of metazoan embryos involves a number of transformational processes, some of which are used in all taxonomic groups and some of which are used in most of those groups. These include, as a required first step, the formation of a multicellular cluster. Within such a cluster any or all of the following can occur: the local coexistence of cells of more than one epigenetic state or type, the formation of distinct cell layers, the formation of an internal space or lumen, the elongation of the cluster, the formation of repeated metamers or segments, the change in state of the cells of one region of the cell cluster due to local or long-range signals from another region, the change in stiffness or elasticity of a cell layer and the dispersal of cells while they continue to remain part of an integral tissue. Ancient DPMs implemented all of the above transformations, beginning with the most fundamental of them – the mediation of cell-cell adhesion by cadherins and lectins. They were able to do so because unlike the "toolkit" gene products comprising the DTFs, which perform only one function, regulation of transcription, those associated with the DPMs mobilize physical forces and processes characteristic of viscoelastic, chemically active materials on the spatial scale of cell

aggregates and tissues, that is, "soft matter" (de Gennnes 1992) which is simultaneously an "excitable medium" (Ivanitsky *et al.* 1987; Mikhailov 1990).

In the following subsections we briefly summarize the properties of the most prominent DPMs. Along with describing each DPM's effects on cells and cell clusters we propose scenarios for their origination based on the acquisition of novel developmental roles by molecules that pre-existed the emergence of multicellularity due to their mobilizing one or another basic "mesoscale"[1] physical force, effect, or process. We also show how DPMs may combine spatiotemporally so as to embody more complex physical phenomena (e.g. biochemical oscillation, reaction-diffusion patterning instabilities) that also play developmental roles.

A schematic representing the distinct roles proposed for DTFs and DPMs in evolution and development is provided in figure 1. Each DPM is given a three-letter designation (table 2). Additional details of the molecular and physical aspects of the DPMs are provided in Newman and Bhat (2008, 2009).

3.1 *Adhesion and differential adhesion*

Multicellularity requires cell-cell adhesion. We now know that homologs of the homophilic adhesion molecules, the cadherins, and the sugar-binding C-type lectins, which are also employed as cell attachment proteins in multicellular organisms (Kaltner and Gabius 2001), are present in the genome of the choanoflagellate *M. brevicollis* (King *et al.* 2008). Since both types of adhesion proteins require Ca^{2+} for their attachment function, the evolutionary innovation of metazoan multicellularity could have emerged with changed external conditions, such as alteration in the ionic content of the ambient medium (Kazmierczak and Kempe 2004).

Therefore, while cadherins, which are as abundant genetically in the genome of *M. brevicollis* as in morphologically complex metazoans (Abedin and King 2008), as well as lectins, likely evolved in unicellular organisms to serve functions different from cell-cell adhesion, they took on a new developmental role as changed conditions led to their mobilizing the physical effect of adhesion. The origination of the DPM[2] we designate as ADH (table 2) can thus be considered a "preadaptation" or, in the terminology of Gould and Vrba (1982), an "exaptation."

A second DPM that inevitably follows from the existence of ADH is differential adhesion, or DAD (table 2). If subsets of cells within an aggregate contain sufficiently different amounts of cell adhesion molecules on their surfaces, the subpopulations will sort out into islands of more adhesive cells within lakes composed of less adhesive ones (Steinberg and Takeichi 1994). Eventually, random cell movement will cause the islands to coalesce and an interface to be established, across which cells will not intermix (Steinberg 2003). The sorting-out behavior of cell populations is physically similar to the phase separation of two immiscible liquids, like oil and water (reviewed in Forgacs and Newman 2005). Tissue multilayering will thus arise whenever there are quantitatively distinct levels or qualitatively different types of cell adhesion molecules on the surfaces of cells in a common aggregate. Whereas in the embryos of modern organisms differentially adhesive cell populations are generated by precise spatiotemporal regulation (e.g. Godt and Tepass 1998; Gonzales-Reyes and St Johnson 1998; Damon *et al.* 2008), the imprecisely controlled gene expression that may have existed in the earliest metazoans could also have given rise to adhesive differences sufficient to cause multilayering.

3.2 *Lateral inhibition and choice between alternative cell fates*

One of the most ubiquitous mechanisms of modern metazoan development is lateral inhibition, whereby early differentiating cells signal to cells adjacent to them to take on a different fate (Rose 1958; Meinhardt and Gierer 2000). The Notch signalling system, an ancient transduction pathway, found in sponges (Nichols *et al.* 2006) but not in choanoflagellates, functions mainly to mediate lateral inhibition in metazoan organisms (Simpson 1997). Notch signalling depends on the interaction of the cell surface receptor Notch with members of a class of other integral membrane proteins that act as ligands for the receptor and modulators of Notch activity: Delta, Serrate (or Jagged) and Lag2 (the DSL-class proteins) (Ehebauer *et al.* 2006). Activation of Notch results in an intracellular portion of it translocating to the nucleus where it acts as a transcriptional co-regulator of certain dual-action transcription factors, changing them from repressors to activators. Since Notch's effects are entirely dependent on which of the dual-action

[1]Although this term is used in different ways in the physical sciences, depending on the context, we take it to pertain to the scale of cell aggregates and embryonic primordia, ~0.1–1 mm.

[2]This example and the others we discuss all involve the harnessing by an early-evolved molecule or pathway of a physical force, effect, or process that became newly applicable in the multicellular context. All these associations then maintained quasi-autonomous (i.e. modular) roles as determinants of morphogenesis and pattern formation during subsequent evolution of the metazoa. Although there are many different senses of (and disagreements about) the term in the biological literature, following the usage of some investigators (e.g. von Dassow *et al.* 2000; Winther 2001), we refer to them as (functional or developmental) "modules".

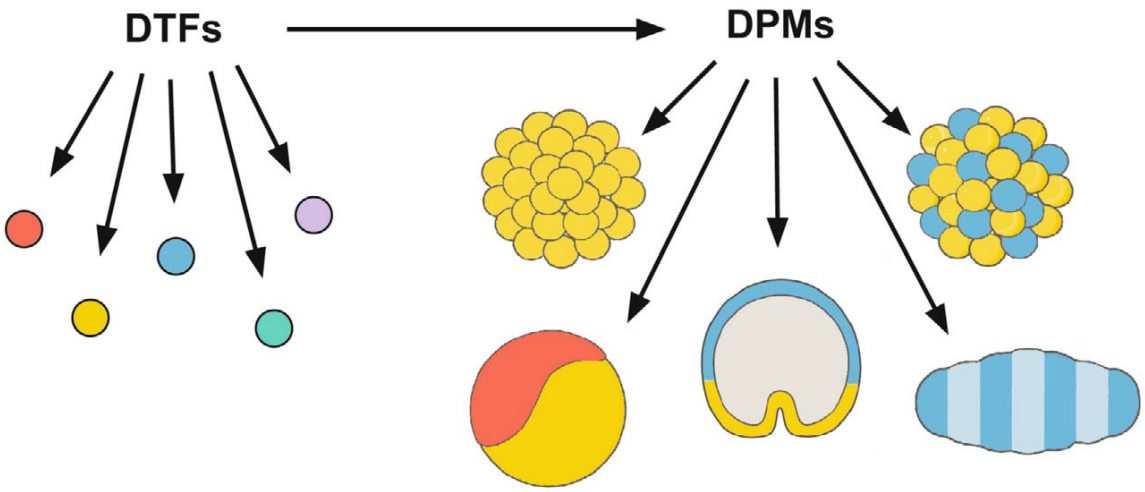

Figure 1. Schematic representation of role of developmental transcription factors (DTFs) and dynamical patterning modules (DPMs) in evolution and development. DTFs, which in some cases pre-existed multicellularity, are transcription factors that function within multistable gene regulatory networks to cause cells of a given genotype to switch between distinct types (indicated by different colours). DPMs, which define (in the case of adhesion), or depend on, the multicellular state, comprise molecules, many of which pre-existed multicellularity, capable of mobilizing one or another mesoscale physical force, effect or process, to transform uniform aggregates of cells into patterned and shaped tissue masses. Several DPMs employ DTFs in generating nonuniform patterns of cells.

Table 2. Names, components and roles of major dynamical patterning modules (DPMs)

DPM	Characteristic molecules	Physical effect	Role
ADH	cadherins	adhesion	multicellularity
DAD	cadherins	differential adhesion	multilayering
LAT	Notch	lateral inhibition	coexistence of alternative cell types
POL_a	Wnt	cell surface anisotropy	lumen formation
POL_p	Wnt	cell shape anisotropy	tissue elongation
OSC	Wnt + Notch	synchronized biochemical oscillation	morphogenetic fields; segmentation
MOR	TGF-β/BMP; Hh, FGFs	diffusion	pattern formation, induction
TUR	MOR + Wnt + Notch	chemical waves	periodic patterning
ECM	collagen; chitin; fibronectin	stiffness; dispersal + cohesion	epithelial elasticity; skeletogenesis; epithelial-mesenchymal transformation

factors are present in a given cell type, the pathway does not determine the specific fate of a cell, but rather causes the cell to choose one of two of its potential fates, whatever those may be.

The Notch pathway operates in a juxtacrine fashion. That is, the Notch receptor on one cell interacts with a DSL-class protein on an adjacent cell, and the two cells, though initially equivalent and therefore expressing both Notch and DSL, come to assume different fates.

Protein modules found in Notch receptors are present in *M. brevicollis* (King *et al.* 2008) and a Notch receptor, ligands, and an intracellular mediator are present in *Ministeria vibrans*, a unicellular representative of the phylum Choanozoa, which also includes the choanoflagellates (Shalchian-Tabrizi *et al.* 2008). As we have seen in section 2, the propensity to exhibit alternative cellular phenotypes is an ancient one, based on dynamical properties of gene regulatory networks (GRNs) that predate the common ancestor of Choanozoa and Metazoa. Sponges, which also express Notch receptors (Nichols *et al.* 2006), have several cell types, one of which, the choanocyte, resembles the cells of choanoflagellates (Funayama *et al.* 2005; Nielsen 2008). It is reasonable to hypothesize that a major step toward complexity of metazoans was taken when, in the new multicellular context, juxtacrine-mediated lateral inhibition (i.e. the LAT module), enabled more than one

differentiated cell state to reside in colonies of choanozoan-like ancestors.

3.3 *Induction of apical-basal and planar cell polarity*

Because of the propensity of cell aggregates to behave like viscoelastic liquid droplets (reviewed in Forgacs and Newman 2005), the default morphology of a cell aggregate held together by cadherins (or any other uniformly distributed cell-cell adhesive protein) is solid ("simply connected" in the topological sense; in the biological sense, having no lumen) and spherical. In most metazoan phyla, secreted factors of the Wnt family interact in a paracrine fashion with receptors of the Frizzled family, and depending on the presence of different accessory proteins, induce cells to become polarized along their apical-basal (A/B) axis (Karner *et al.* 2006b), or oriented in a plane perpendicular to this axis (planar cell polarity; Mlodzik 2002; Karner *et al.* 2006a). These are behaviours of individual cells, but in a multicellular context they permit multicellular aggregates go beyond the default morphologies of solidity and sphericality.

Although Wnt genes and their cognate secreted proteins are not present in choanoflagellates (King *et al.* 2008), they are found, along with their Frizzled receptors, in sponges (Nichols *et al.* 2006) and in *Trichoplax adhaerens*, the single known member of the phylum Placozoa, a very early diverging clade within the Metazoa (Srivastava *et al.* 2008). Certain components of the Wnt intracellular pathway are even more ancient, appearing in fungi such as *Schizosaccharomyces pombe*, where they also mediate polarization along the A/B axis during cell division (Mendoza *et al.* 2005). In multicellular aggregates the same pathway can mediate the formation of an interior space or lumen. In particular, if cells are polarized in their distribution of surface attachment proteins they will not form a solid mass when aggregated; rather the more adhesive portions of the cell membranes will bind to each other while the less adhesive regions are freed up to enclose an interior lumen (Newman 1998). Another potential configuration that A/B polarization can mediate (if more than one attachment protein is involved), is multiple non-mixing cell layers.

Small hollow cell clusters identified in the Pre-Cambrian Doushantuo Formation, China have been referred to as "embryos" (Chen *et al.* 2004; Hagadorn *et al.* 2006), but these may have been the definitive forms of the earliest metazoans and metazoan-like organisms (Newman *et al.* 2006). Along with Notch-associated LAT, which permitted coexistence of multiple cell types (see above), the canonical Wnt pathway would have led to cell aggregates developing interior spaces. It is plausible that the origination of these hollow forms at the transition between the Ediacaran biota and those of the Cambrian explosion depended on A/B polarization induced by the mobilization of an ancient intracellular pathway by the more recently evolved Wnt-Frizzled couple. We designate this module POL_a (table 2).

In terms of the DPM model presented here, the labyrinthine interior spaces of sponges should depend on the products of the Wnt and Frizzled genes seen in these organisms (Nichols *et al.* 2006). Although the placozoan *T. adhaerens* also contains the Wnt-pathway components of the POL_a module (Srivastava *et al.* 2008), it lacks a lumen of any sort. Nonetheless, they are unusual (for such morphologically simple organisms) in having three adjacent non-mixing layers (Miller and Ball 2005), an arrangement only possible with a sophisticated system of A/B cell polarization.

The train of events leading to planar cell polarity (PCP) is also initiated at Frizzled receptors, but the intracellular pathway employed is only partly similar to that involved in induction of A/B polarity. In particular, β-catenin is not involved, leading this Wnt pathway to be referred to as noncanonical. Like A/B polarization, PCP can occur with or without the involvement of Wnt ligand (Veeman *et al.* 2003) and thus could be based on a cellular mechanism that predates multicellularity or even cell-cell communication.

In the multicellular context of metazoan embryos the consequences of PCP are as follows: cells elongate in the plane orthogonal to the A/B axis and acquire, by mechanisms not well-understood, anisotropic adhesive properties. Both observationally (Keller *et al.* 2000) and as predicted on the basis of physical principles (Zajac *et al.* 2003; Keller *et al.* 2008), the polarized cells spontaneously align and intercalate among one another, leading to the tissue mass narrowing in one direction and elongating in the orthogonal one. The cellular rearrangements and tissue reshaping effects induced by this DPM, termed POL_p (table 2), are seen in "convergent extension," which establishes the elongated body axis during gastrulation, and in related phenomena throughout the Metazoa (Keller 2002).

3.4 *Oscillatory cell states and their synchronization*

An appropriate balance of positive and negative feedbacks in any dynamical circuit can lead the system to exhibit temporal oscillations in its characteristic state variables. Given the complexity of metabolic control and gene regulatory networks, biochemical oscillations within the confines of single cells are all but inevitable (Goldbeter 1996; Lewis 2003; Reinke and Gatfield 2006). When coordinated across cell boundaries by juxtacrine (e.g. Notch pathway) and short-range paracrine (e.g. Wnt pathway) signalling (Kageyama *et al.* 2007; Riedel-Kruse *et al.* 2007; Ozbudak and Lewis 2008) and gated by a spatially nonuniform signal such as a morphogen (Pourquié 2003; see below), such oscillations have the potential to drive morphogenetic change. We designate the associated DPM OSC (table 2).

The role of temporal oscillation in the generation of morphological periodicity was suggested by William Bateson as early as 1891 (Bateson and Bateson 1928; Newman 2007a), proposed as the basis of vertebrate segmentation in a mathematically rigorous fashion 85 years later (Cooke and Zeeman 1976), and demonstrated experimentally in the same system beginning two decades after that (Palmeirim *et al.* 1997). The temporally periodic alteration of adhesion in a growing system is, by itself, sufficient to cause the formation of segmented or partly segmented forms (Newman 1993), and this may explain the apparently independent emergence of segmentation in distinct metazoan lineages.

Somitogenesis is the process by which blocks of tissue, the primordia of the vertebrae and associated muscles, form in a progressive spatiotemporal order along the central axis of vertebrate embryos. In the presomitic mesoderm of chicken and other vertebrate embryos, the expression of certain genes (particularly the Notch pathway mediator Hes1), undergoes temporal oscillation with a period similar to the formation of the somites (Pourquié *et al.* 2003). These oscillations then become synchronized by juxtacrine signalling via Notch and one or more of its receptors (Giudicelli *et al.* 2007; Kageyama *et al.* 2007; Riedel-Kruse *et al.* 2007; Ozbudak and Lewis 2008). In conjunction with an FGF8 morphogen gradient (see below) having its source at one end of the extended embryo, Hes1- and associated oscillations provide the basis for the well-regulated generation of somites in vertebrate embryos (Pourquié 2003).

Whereas biochemical oscillation can occur in individual cells, synchronized oscillations can only occur in interacting populations of cells (Garcia-Ojalvo *et al.* 2004; Masamizu *et al.* 2006), hence the novel identity of the OSC DPM that results from the change in scale from single to multiple cells. The importance of this for multicellular development can be appreciated by considering the concept from the older embryological literature of the "morphogenetic field" (reviewed in Haraway 1976; Gilbert 2006). This phenomenon is characterized by the coordination of state and activity of cells across a spatially extended domain and has long eluded mechanistic explanation. It is quite clear that the synchronization of cell oscillators can produce fields such that the cells within them are poised to mount identical responses to identical signals, and different responses to different signals, even over durations shorter than the period of the oscillation.

As the case of somitogenesis illustrates, if a synchronized oscillatory state interacts with a graded spatial signal a spatially periodic pattern can result. And because there is not a large distance (in the space of relevant parameters) between complex dynamical systems that do not oscillate and those that do, the OSC module and the morphogenetic fields and segmental patterns that may be associated with it, would be expect to arise readily and repeatedly over the course of evolution.

3.5 *Morphogen gradients and activator-inhibitor systems*

Single-celled organisms have the ability to change their physiological state in response to molecules secreted into the microenvironment by other such cells (Luporini *et al.* 2006), and indeed the genome of the choanoflagellate, *M. brevicollis*, the closest extant relative of the metazoa, encodes several Hedgehog-like proteins and their receptors (Nichols *et al.* 2006). Secreted molecules of this class induce concentration-dependent responses in cells, and constitute one of several categories of morphogens (MOR; table 2) in metazoan embryos. The genome of the marine sponge *Oscarella carmela* contains genes specifying Hedgehog and Wnt morphogens and their receptors (Nichols *et al.* 2006), and receptor tyrosine kinases (Sudhop *et al.* 2004; Nichols *et al.* 2006), which in eumetazoa transduce the effects of morphogens like FGF and EGF. Morphogens of the FGF class exist in arthropods and chordates, as well as in echinoderms (Lapraz *et al.* 2006) and cnidarians (Restzsch *et al.* 2008). Although components of the intracellular pathway that mediates signalling by the TGF-β class of morphogens in eumetazoa are present in *Oscarella carmela*, no morphogens or receptors of this type have been identified in animals less complex than the Cnidaria (Holstein *et al.* 2003).

The ability of one or a small group of cells to influence other cells via morphogens enables the generation of heterogeneous cellular patterns. Based on the time-distance-concentration relationships inherent to macromolecular diffusion (Crick 1970) such patterns would form over tens of hours on a spatial scale of 100 μm-1 mm.

The function of morphogens is inextricably tied to the physical process of diffusion, or formally similar distribution mechanisms (Lander 2007). And because setting up molecular gradients across a cluster of initially equivalent but responsive cells can lead to patterns of nonuniformly distributed cell types within a unitary organism, diffusion of a given signal has an entirely different significance in the multicellular context than in the unicellular one. It is also important to recognize that the use of a morphogen gradient in pattern formation does not require that there be a graded response by the target cell population. This is the case (contra Bolouri 2008) even when the response is all-or-none, as in the Wnt-dependent spatiotemporal differentiation of endoderm and mesoderm in the sea urchin (Smith *et al.* 2007).

The emergence of a multicellular aggregate composed of cells in more than one state would have represented an evolutionary novelty: a primitive "body plan." As with the DPMs described earlier, the generation of morphological variation by MOR is tied to the inherent material properties of the system. A morphogen gradient can make a relatively sudden appearance evolutionarily and is capable of inducing

an extensive reorganization of developmental outcome. Like the incremental changes posited as typical by the Darwinian evolutionary model, whether or not MOR-induced variants survive, however, is a matter ultimately decided by natural selection.

When morphogens are positively autoregulatory, that is, directly or indirectly stimulating their own synthesis in target cells, they tend not to be maintained as gradients, since all cells eventually become morphogen sources. If, however, a positively autoregulatory morphogen becomes linked to a mechanism of lateral inhibition (such as the LAT module associated with Notch signalling), a zone will be induced around any peak of morphogen activity within which activation will not spread (Gierer and Meinhardt 1972; Meinhardt and Gierer 2000; Nijhout 2003; Meinhardt 2008). Peaks of activation in such systems can only form at distances from one another at which the effects of the inhibitor are sufficiently attenuated. This arrangement, which is closely related to the chemical pattern-forming systems described by Turing (1952) (who, however, did not formulate it in terms of lateral inhibition), can produce regularly spaced spots or stripes of morphogen concentration (TUR; table 2). The TUR DPM has been proposed to underlie pattern formation of the vertebrate limb skeleton (Newman and Frisch 1979; Hentschel *et al.* 2004; Newman 2007b; Newman and Bhat 2007), dentition (Salazar-Ciudad and Jernvall 2002), feather germs (Jiang *et al.* 2004), and hair follicles (Sick *et al.* 2006).

3.6 *Extracellular matrix in epithelial elasticity, epithelial-mesenchymal transformation and global organization of cell polarity*

The cell aggregates and tissues subject to the DPMs ADH and DAD contain cells that are directly attached to each other and are termed "epithelioid." Such aggregates exhibit viscosity, a measure of the ease with which cells slip past one another while maintaining their often transient attachments. They also possess elasticity, which is primarily a function of the cytoskeleton, since this makes up the bulk of the tissue, and cohesivity, which is determined by the force (dependent on cell adhesion molecules), required to separate the cells. The other major cell aggregate or tissue type in metazoan forms is termed "mesenchyme," and its viscosity, elasticity and cohesivity are largely determined by a secreted macromolecular microenvironment, the extracellular matrix (ECM) (Comper 1996). ECMs, by mobilizing additional physical processes, endow mesenchymal aggregates with different morphogenetic capabilities and varieties of plasticity from those of epithelioid clusters and thus constitute a novel DPM (table 2).

Most metazoans produce fibrillar (e.g. type I) collagen, the most prominent interstitial (i.e. between mesenchymal cells) component of the ECM, and network (i.e. type IV) collagen and laminin, which are components of the basement membrane that attaches epithelial sheets to mesenchymal tissue. ECM proteins of all types bind to cell surfaces via transmembrane proteins known as integrins. Although some eumetazoan groups, like Cnidaria, appear to lack interstitial ECMs, genes specifying all of these components are found in sponges (Nichols *et al.* 2006) and there are even homologues in the choanoflagellate *M. brevicollis* (King *et al.* 2008). Since, unlike some choanoflagellates, *M. brevicollis* does not form colonies, it is possible that the secreted ECM material and receptors was used for substratum attachment in the earliest forms in which it appeared (King *et al.* 2008). Indeed, an integrin homologue is employed in the free living amoeba of *Dictyostelium discoideum* in substratum adhesion but not multicellular development (Cornillon *et al.* 2006).

ECMs exhibit greater molecular variety and are less constrained in their composition, consistency and texture than the cytosol. The rheological (e.g. viscoelastic, cohesive) properties of tissues or organisms that are rich in ECMs are more diverse than those of epithelioid tissues.

A comparison between sponges and cnidarians highlights some of the morphogenetic opportunities afforded by, and limitations consequent on, the use of the ECM DPM. Most sponge cells reside within an ECM called the "mesohyl" to which they bind via integrins (Wimmer *et al.* 1999). Sponges actively remodel the branched skeletal structures that constitute their internal anatomy by the continuous movement of their cells (Bond 1992), and their morphology exhibits environmentally dependent plasticity (Uriz *et al.* 2003). The Porifera are an early-arising morphological dead-end of the Metazoa. One reason for this could be that despite their also containing epithelial cells (Schröder *et al.* 2004) and homologues of basement membrane type IV collagen (Boute *et al.* 1996; Nichols *et al.* 2006), they remained largely mesenchymal organisms, with organizational motifs but not a precisely determined anatomy.

In the eumetazoans ECM is employed in a more limited fashion, and in the most primitive of these, the Ctenophora and Cnidaria (Dunn *et al.*. 2008), it is mainly used to cement together the epithelial sheets which constitute the major portion of these organisms. A basement membrane endows an epithelium with elasticity in the direction perpendicular to the plane (Mittenthal and Mazo 1983; Newman 1998), permitting tissue sheets to exhibit a range of folding, buckling and wrinkling effects seen neither in liquid-like epithelioid tissues nor in mesenchymes (Gierer 1977; Forgacs and Newman 2005). The other DPMs mentioned above (ADH, DAD, LAT, POL, etc.) also have freer play in the epithelial context, making the ctenophores and the cnidarians morphologically more elaborate than the sponges.

However, it is only in the triploblasts (i.e. organisms with three-layered embryos), which have both epithelial

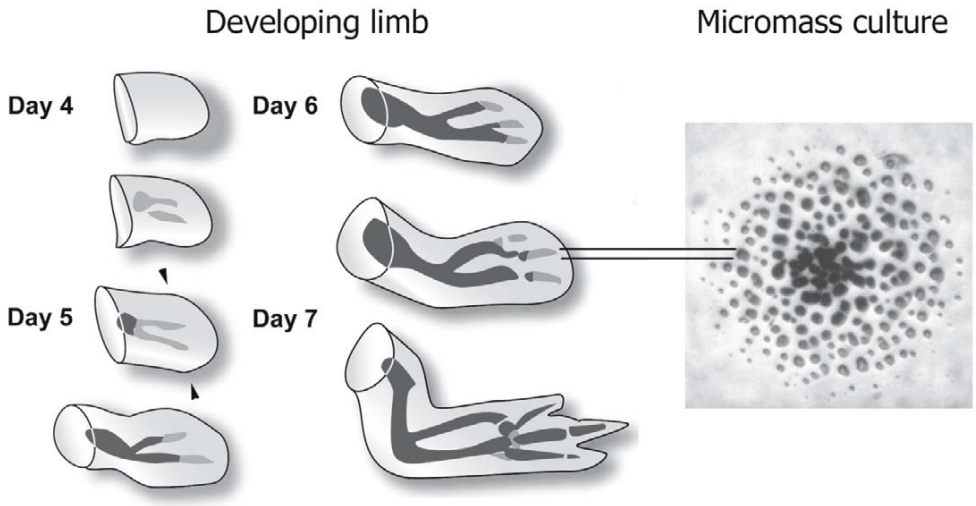

Figure 2. (*Left*) Progress of limb skeletal development in chicken forelimb (wing) between 3 and 7 days of embryogenesis. Gray represents precartilage condensation and black represents definitive cartilage. The developing limb, or limb bud, is paddle-shaped, being flatter in the back-to-front (dorsoventral) dimension than in the thumb-to-little finger (anteroposterior) dimension, or the shoulder-to-finger tips (proximodistal) direction in which it mainly grows. The cartilages that prefigure the bones first arise as stripe-like (e.g. long bones, digits) or spot-like (e.g. wrist bones shown here, or ankle bones in the hindlimb) mesenchymal condensations. The apical zone of the 5 day chicken wing bud (indicated by the arrowheads) or leg bud provide a source of not-yet-condensed mesenchymal cells that when grown in high-density culture will form precartilage condensations. (*Right*) Discrete spot-like cartilage nodules that have formed after 6 days in a micromass culture of 5-day leg bud apical zone limb mesenchymal cells, visualized by staining with Alcian blue. The cells in these cultures are initially plated as a densely packed monolayer and rearrange over short distances in the 2-D plane of the ~3 mm diameter culture over a period of 1–2 days. Each nodule arises from a condensation containing approximately 30–50 cells. Depending on the culture conditions, the nodules remain discrete, as shown here and at higher magnification in figure 4a, b, or fuse, as shown in figure 3a and at higher magnification in 4c, d. As indicated by the parallel lines, the spatial scale of the condensations in the developing limb and in the micromass cultures under the conditions shown are comparable. (Based on Christley *et al* 2007.)

and mesenchymal tissues, that the full range of DPMs exert their effects. These animals, the arthropods, annelids, echinoderms, mollusks, chordates (among others) exhibit all of the morphological motifs capable of being generated by the metazoan "pattern language" (Newman and Bhat 2008).

4. Roles of DTFs and DPMs in the developmental plasticity of limb bud mesenchyme

It is notoriously difficult to test evolutionary hypotheses experimentally. In intact extant organisms it is possible to manipulate genes by inactivating or overexpressing them, throughout the body or in an organ-specific fashion. But phenotypic responses to such alterations in highly organized multi-tissue systems are complex and often compensatory, owing to the pleiotropic and "overdetermined" nature of their genetic architecture. Moreover, as we have seen above, gene products have not been the sole determinants of biological form and function over the course of evolution. A reasonable complementary approach to understanding cell- and tissue-level mechanisms of evolutionary innovation is to study the range of differentiative and morphogenetic behaviours of isolated, relatively uniform populations of embryonic cells under variations in physical and other microenvironmental conditions (the "developmental reaction norm;" Schlichting and Pigliucci 1998). In this section we will describe some experiments on mesenchymal cells of the embryonic avian limb that illustrate the developmental roles of DTF networks and DPMs, and more specifically, the manner in which these two categories of determinants mediate phenotypic and developmental plasticity.

The cells in question are somatopleure (body wall)-derived mesenchymal cells that constitute a portion of the mesoderm of chicken limb buds as the cartilaginous template of the skeleton takes form between 3 and 7 days of development (figure 2). These cells have an entirely non-myogenic fate, because the limb musculature arises from a separate cell population that migrates into the limb bud from its origin in the somites. At 5 days of development the somite-derived myogenic cells have not yet arrived at the distal tip of the wing (pictured) or leg buds (Newman *et al.* 1981; Brand *et al.* 1985). The cells of the limb bud distal tip (whose prospective

fate is the autopod: the skeleton of the hand or foot) can thus be physically isolated from the myogenic population and will differentiate uniformly into cartilage or into a mixture of fibroblasts and apoptotic cells depending on *in vitro* conditions (Newman 1977, 1980; Downie and Newman 1994). The as-yet unpatterned, multipotent somatopleure-derived cell population of 5-day chicken limb bud tips thus constitutes an ideal experimental system for studies of developmental determination and plasticity.

During normal development some of these distal tip mesenchymal cells ("precartilage cells," for short) differentiate into fibroblasts, both of the tendons and the perichondrion (i.e. the connective tissue sheath surrounding each cartilage element). In ducks, the equivalent cells differentiate into the connective tissue components of the interdigital web of the foot, but in the duck's wing, as in chicken, human, and other non-webbed-tetrapod limbs, the interdigital cells assume another differentiated fate, that of apoptosis. The precartilage cell population forms a pattern of skeletal elements consisting of quasi-periodic arrangements of rod-like or nodular elements, separated by soft connective tissue or by the spaces left by the apoptotic cells. Later, another differentiated type arises from additional cells from this population: bone. Bone tissue will typically replace the cartilage primordia entirely except at the articular surfaces, which remain cartilaginous.

In the next two subsections we describe how networks of DTFs regulate switching between the potential cell types of the precartilage mesenchyme, and how DPMs, acting singly and in combination, may establish patterns of cartilage elements in this tissue. Although developmental plasticity can represent condition-dependent switching between alternative, evolved, developmental programs, it can also be a manifestation of non-programmed material responses of living tissues. In the latter case plasticity would be a primitive property rather than a product of selection. The involvement of the second type of plasticity in developmental mechanisms would be confirmed if biological outcomes that are never exhibited in the normal course of development could be induced by external factors. We show experimentally that both DTFs and DPMs can mediate such nonstandard outcomes.

4.1 *DTFs and the fates of limb bud mesenchymal cells*

Of the various choices available to the multipotent (though non-myogenic) limb bud precartilage cell, the best-studied is the cartilage-bone decision. Chondrogenesis and osteogenesis are each associated with expression of a homeobox-containing DTF that serves as a "master regulator" of differentiation: these are, respectively, Sox9 and Runx2 (de Crombrugghe *et al.* 2000; Kawakami *et al.* 2006; Franceschi *et al.* 2007; Marie 2008). While Sox9 is required for the commitment of undifferentiated mesenchymal cells to a bipotential cell type that is both a chondroprogenitor and an osteoprogenitor (Zou *et al.* 2006), with respect to terminal differentiation (in a mesenchymal stem cell system similar to limb bud precartilage mesenchyme), Sox9 and Runx2 are involved in a complex intracellular GRN in which the effects of Sox9 dominate, making cartilage the default cellular phenotype in high-density cultures (Zhou *et al.* 2006), as it is for limb bud precartilage mesenchyme (figure 3a). There is, in general, no simple quantitative relationship between expression levels of these DTFs and generation of the cellular phenotype, high levels sometimes suppressing differentiation.

Apart from cell contact, factors influencing the decision to differentiate into cartilage or bone include components of

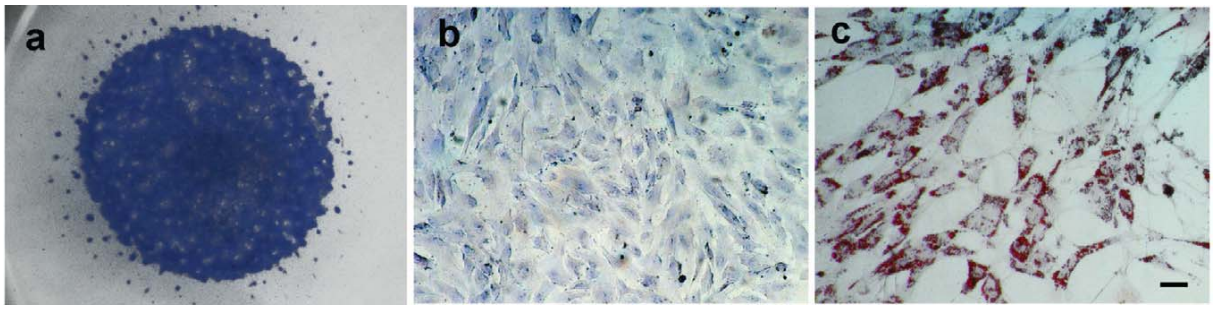

Figure 3. Cell-type plasticity in cell cultures prepared from leg bud distal tip mesenchymal cells isolated from 5 d chicken embryos grown with different densities and sera. (**a**) Alcian blue (pH 1.0; chondrocyte-specific) staining of 6 d high-density cell culture grown in presence of 10% fetal bovine serum. Culture size is the same as on the right of figure 2, but all the condensations have fused under these conditions and all the cells have differentiated into cartilage. (**b**, **c**) Hematoxylin and Oil-red (lipid specific) staining of low density cell culture grown for 8 days in the presence of (**b**) 10% fetal bovine serum or (**c**) 10% horse serum. Cells in (**b**) have all differentiated into fibroblasts and in (**c**) have all differentiated into avian brown adipocyte-like cells. Cells in initially low density culture become confluent by 8 days in fetal Bovine serum. Diameter of stained culture in (**a**) ~3 mm; scale bar (**b**) and (**c**): 50 μm. For details on (**a**), see Downie and Newman (1994); on (**b**) and (**c**), see Newman (1980) and Mezentseva *et al* (2008).

the protein kinase A, ERK/MAPK and Wnt pathways (de Crombrugghe *et al.* 2000; Kawakami *et al.* 2006; Franceschi *et al.* 2007; Marie 2008). The nuclear hormone receptor (NR: a class of ligand-dependent gene regulatory proteins specific to the metazoa Laudet 1997; King *et al.* 2008) known as peroxisome proliferator-activated receptor gamma (PPARγ) is a direct antagonist of Runx2 (Marie 2008). PPARγ, in turn, is a so-called master regulator of the differentiation of the white adipocyte (Lowell 1999), a cell type that (in the form of subcutaneous fat), is expected to be among the repertoire of the limb bud mesenchyme.

It is also of significance that the transcriptional co-regulator PPARγ coactivator 1-alpha (PGC-1α) is a necessary component in the transcriptional complex by which Sox9 induces cartilage in limb bud mesenchyme (Kawakami *et al.* 2005). Although, as its name suggests, PGC-1α was first characterized by its association with PPARγ, the study of Kawakami *et al.* (2005) indicates that it has other functions, and in normal limb development it is probably not expressed other than in coordination with Sox9. In the adipogenic mesenchyme of mammals, the expression of PGC-1α along with PPARγ changes the white adipogenesis pathway mediated by the latter into one that generates the heat-generating tissue known as brown fat (Gesta *et al.* 2007). Brown fat adipocytes appear mainly in neonates in localized depots such as the shoulder girdles and perispinal regions of the upper back, and, unlike white fat adipocytes, would not be expected to be in the differentiative repertoire of the limb bud distal mesenchyme.

We found that culturing limb bud precartilage mesenchyme under sparse conditions where the cells are unable to contact one another, rather than in the high-density conditions that promote chondrogenesis, led to all the surviving cells differentiating into fibroblasts, the characteristic cell type of connective tissues (Newman 1980; Mezentseva *et al.* 2008) (figure 3b). The medium in these cultures contained 10% fetal bovine serum, which is conducive to the formation of cartilage under cell-interactive conditions (figure 3a). When, instead, the medium of the sparsely plated cells was supplemented with 10% horse serum, adipocytes formed (figure 3c). These were not the white fat adipocytes that would plausibly arise from limb bud mesenchyme, however, but cells with the numerous lipid droplets, and mitochondria and biochemical features, of brown fat adipocytes (Mezentseva *et al.* 2008). This was all the more striking because avian species do not have the nuclear gene for mitochondrial uncoupling protein 1 (UCP1), the mediator of thermogenesis in brown fat (Mezentseva *et al.* 2008). At best they produce brown fat-like tissue without heat-generating capability, and definitely not in the limbs. The UCP1 gene, which evolved in cold-blooded animals before the appearance of brown fat, was lost from the tetrapod evolutionary tree no later than the common ancestor of birds and lizards. Therefore, it is likely that no ancestor of birds ever produced thermogenic brown fat. Nonetheless, the avian brown adipose-like cells produced under our unusual culture conditions were perfectly capable of activating the exogenously provided mammalian UCP1 promoter (Mezentseva *et al.* 2008).

What apparently has occurred in this experiment is that a cell type that had evolved to express both PPARγ and PGC-1α was placed under conditions in which the two factors could cooperate (possibly because of the unavailability of PGC-1α's normal partner in this lineage, Sox9), generating a cell type (the brown adipocyte) not characteristic of the tissue of origin (limb bud mesenchyme). This cell type, moreover, has gene regulatory capabilities (induction of UCP1) not characteristic of the clade of origin (birds). One possible implication of this, though one that requires further investigation, is that the striking cell-type plasticity of the kind that is in evidence from the combinatorial use of DTFs in these experiments was employed over the course of evolution to generate novel cell types in a relatively abrupt fashion.

4.2 *DPMs and the patterning of limb bud mesenchyme*

In forming the skeleton of the developing limb, the precartilage mesenchymal cells choose among the various cellular phenotypes available to it in a conditional fashion. According to the description above of the DTFs that mediate the decision between the cartilage or bone fate, the choice is influenced by the protein kinase A, ERK/MAPK and Wnt pathways, and can thus be triggered by factors that originate outside the cell. These include cell contact, shape change and morphogen exposure, all consequences of the DPMs we described in earlier sections. And before the cartilage vs. bone choice is made, the condition-dependent regulation of the choice between cartilage and fibroblast/apoptosis is responsible for generating the pattern of cartilage elements, providing a structural template upon which the later choice is made.

Chondrogenic pattern formation employs several of the DPMs discussed above, with similar processes and events occurring *in vitro* and *in vivo* (reviewed in Newman and Tomasek 1996; Hall and Miyake 2000). The tissue first needs to be physically integral: as mentioned above, sparsely arranged cells differentiate exclusively into fibroblasts or adipocytes. The mesenchyme is initially held together by an ECM rich in the glycosaminoglycan hyaluronan (mediating the ECM DPM). The morphogen TGF-β (mediating the MOR DPM) is produced at low levels throughout the tissue, but because of its positive autoregulation (a general characteristic of TGF-β, demonstrated in limb bud mesenchyme by Miura and Shiota 2000), transient local increases in its level will tend to grow, providing a

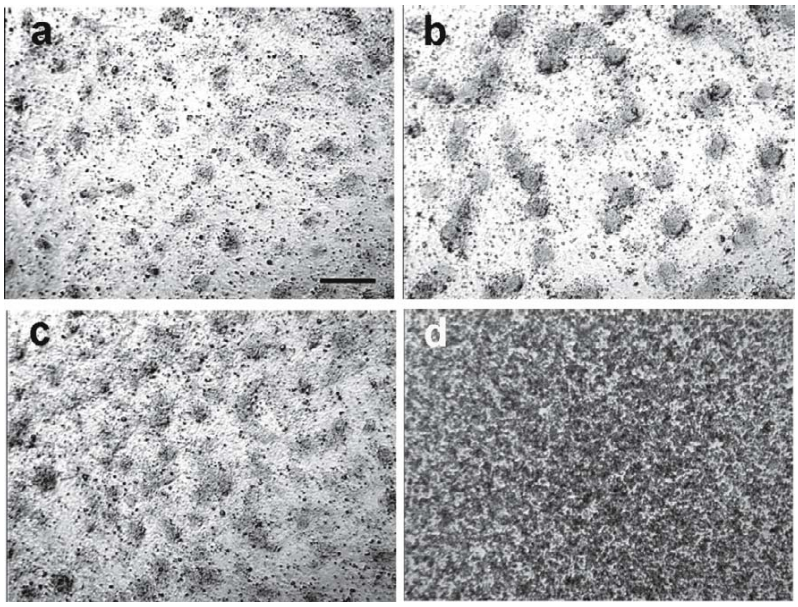

Figure 4. Morphogenetic plasticity in high-density cell cultures prepared from leg bud distal tip mesenchymal cells isolated from 5 d chicken embryos grown for identical periods (46 h) under different growth factor and pharmacological conditions. (**a**) Cultures grown in serum-free defined media (DM; Paulsen and Solursh 1988). Discrete condensations appear as dark patches under Hoffman Modulation Contrast optics (4× objective) (Frenz *et al* 1989b). This control culture is a high-magnification view of an early stage of a culture like that shown in figure 2 (right); (**b**) Culture grown in media containing FGF2 (100 ng/ml). This treatment speeds up the formation of condensations (Miura and Maini 2004), so they are larger and more compact than in (**a**), but it also induces a lateral inhibitory effect (Moftah *et al* 2002; Miura and Maini 2004), leading to larger "halos" of uncondensed mesenchyme surrounding the condensations; (**c**) Treatment with low concentrations of the Notch pathway/γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester) (100 nM) causes an increase in the number of condensations and also recruits uncondensed mesenchyme into the condensations causing neighboring ones to fuse (see also Fujimaki *et al* 2006); (**d**) Treatment with a high concentration of DAPT (20 μM) causes all the mesenchymal cells to fuse into a single large sheet-like condensation and abrogates the spot-like pattern found in the control cultures. Scale bar (**a**-**d**): 20 μm.

component of a LALI-type self-organizing system (i.e. the TUR DPM). TGF-β induces the production of the ECM glycoprotein fibronectin in limb mesenchyme (Leonard *et al.* 1991) and this in turn causes cells to accumulate at the self-enhancing foci in the form of sites of increased density: precartilage condensations (Frenz *et al.* 1989a,b; Downie and Newman 1994, 1995).

Next, the condensed cells acquire epithelioid properties mediated by the ADH module, employing cell–cell adhesion molecules such as endogenous lectins (Matsutani and Yamagata 1982); N-CAM (Widelitz *et al.* 1993) and N-cadherin (Oberlender and Tuan 1994; Delise and Tuan 2002), the expression of the latter being controlled by TGF-β (Tsonis *et al.* 1994). Condensation is followed by chondrogenesis, employing the Sox9/PGC-1α DTF described above.

Theoretical and computational analysis has shown that the quasi-periodic patterns of precartilage condensation and then cartilage that form both *in vivo* (figure 2, left) and *in vitro* (figure 2, right) in high density cultures of limb precartilage mesenchyme (figure 4a) can be generated by a LALI mechanism employing a regulatory network in which TGF-β is the activator (Hentschel *et al.* 2004; Christley *et al.* 2007; Alber *et al.* 2008). The molecular basis of the inhibitory branch of this proposed mechanism has been elusive, however. When cultures are treated with the ectodermally produced morphogens FGF2 or FGF8 the condensations become more tightly confined, with larger areas of uncondensed cells (Moftah *et al.* 2002) (figure 4b). This suggested that FGFs might be inducing a laterally acting inhibitory (condensation-suppressive) effect. Consistent with this, pharmacologic or antisense oligonucleotide blockade of FGF receptor 2 (FGFR2), which is known to localize in the prospective condensations, *in vitro* as well as vivo, abrogated the lateral inhibition (Moftah *et al.* 2002). In this example a morphogen, FGF, does not function as a gradient, but rather by causing subpopulations of cells (the incipient condensations) to transmit a signal (which might be something other that a diffusible molecule, see below) that propagates over a limited distance.

Hours before cells bearing FGFR2 first appear in high-density mesenchymal cultures (~19 h post-plating) a

prepattern of "protocondensations" has already begun to emerge (Bhat R and Newman S A, unpublished data). This spatial organization of cell fate may depend on the capacity of the Notch signalling pathway (described above in relation to the LAT DPM) to suppress chondrogenesis. There are several lines of evidence for this suppressive effect. First, when Hes1, an important downstream transducer of the Notch pathway, was over-expressed in developing chicken limbs there was a reduction in limb size and truncation of skeletal elements (Vasiliauskas *et al.* 2003). Second, inhibiting Notch signalling *in vitro* pharmacologically caused an increase in the number (Williams *et al.* 2006) and size of condensations (Fujimaki *et al.* 2006), the latter effect leading the condensations to eventually fuse (figure 4c,d). This fusion is not characteristic of normally developing skeletal elements *in vivo*, but occurs in the hands and feet of individuals with Apert syndrome, a condition associated with impairment of the FGFR2 gene (Wilkie *et al.* 2002). Third, and of significance with respect to the timing of appearance of prospective condensations, is our recent finding that Hes1 undergoes oscillations in the cell of these cultures (with a period of ~6 h, vs. 1.5 h in the somitic clock described above). By 12 h post-plating there is synchrony of the oscillation in a substantial proportion of the cells (Bhat R and Newman S A, unpublished data). This suggests that the OSC module may be involved in the earliest patterning steps in limb bud mesenchyme (Newman and Bhat 2007), with synchronization being the means (as it is in the somite-forming system; Ozbudak and Lewis 2008) by which the juxtacrine effects of the Notch signal are propagated over broader tissue domains.

5. Conclusion

We have described two categories of developmental determinants, DTFs and DPMs, each of which, by their physical nature, mediate phenotypically plastic outcomes. DTFs and their associated intracellular networks function within cells early in developmental lineages to generate alternative cell states and eventually differentiated cell types (cartilage vs. soft connective tissue, or bone, in the limb mesenchymal system, for example). These cell states are dynamical attractors of systems that are open to the microenvironment and require continuous regulation for their maintenance (Blau and Baltimore 1991). Based on theoretical analyses of gene regulatory networks (e.g. Keller 1995) cells can be tipped between alternative stable states depending on a variety of microenvironmental conditions. Indeed, as the example of the *in vitro* generation of avian brown adipose-like cells from chicken limb precartilage mesenchyme shows (Mezentseva *et al.* 2008; see above), novel differentiated cell types can in principle arise abruptly, in a fashion disconnected from incremental genetic change or gradual selection for function.

While the presence of other cells is among the most important boundary conditions for the cell-type switching functions of the DTFs they act cell-autonomously. The DPMs, in contrast, are inherently multicellular determinants, with sources of plasticity that are more mechanistically varied than those of the DTFs. The reason for this is that, in contrast to the DTFs, which mobilize a single unicellular functionality – transcription – for developmental change, DPMs mobilize a heterogeneous group of ancient functions – adhesion, lateral inhibition, polarity, diffusion, oscillation, and so forth – and employ them in qualitatively new ways on the multicellular scale. The contributions of the ADH, DAD, LAT, MOR, TUR, ECM, and possibly OSC, DPMs to the generation of the limb skeleton demonstrates the ubiquitous but individually discernable (i.e. modular) deployment of these functional complexes and their natural integration in the production of tissue pattern and form. In addition, the variant morphological outcomes seen *in vitro* when components of the DPMs are subject to quantitative modulation (e.g. the fusion of precartilage condensations when Notch signalling is suppressed), indicates that novels morphologies can arise abruptly, also independently of incremental genetic change or gradual selection for function.

We have suggested previously (Newman 1992; Newman and Müller 2000; Newman and Bhat 2008) that plasticity of three-dimensional form (due to the large role of physical processes and effects in primitive developmental systems) was the basis of the Precambrian-Cambrian phylum-grade radiation beginning roughly 600 million years ago. From the examples described above, we can see how rapid morphological change driven, in part, by non-genetic mechanisms, might also provide an explanation for the utilization across the entire range of metazoan phyla of homologous molecules (including differentiation-related transcription factors), for similar roles in organ systems with only tenuous common ancestry. Specifically, once multicellularity and various cell type identities had been achieved, but before morphotypes had been locked into place by "canalizing" evolution, disparate metazoan forms could have inherited cell types and their transcriptional determinants with roles that had become highly specified before standard body plans were (Newman 2006; Newman *et al.* 2006). As the example of the vertebrate limb shows, the same interplay between morphological plasticity and cell type determination continued to prevail as new organs emerged over the course of metazoan evolution. But while the molecular components of the DPMs necessarily came functionally preadapted for their specific roles in morphogenesis and pattern formation, the DTFs, all of which are transcription factors with similar functions, appear to have assumed roles as determinants of particular cell type identities as "frozen accidents" (Newman and Bhat 2009).

Beyond the clear evidence that metazoan DTFs and DPMs remain active determinants of present-day development, performing functions that in many cases are virtually identical to the ones they performed at their inception more than 500 million years ago, is the fact that they carry out these functions in a conditional fashion. Thus, in contrast to the notion that animal cell types and the forms and patterns they assume are rigidly programmed by genetic machines executing computer-like logic (see, e.g. Oliveri *et al*. 2008, for an explicit statement of this view), we propose that they are generated by systems that are inherently plastic, and likely to have been even more so earlier in evolution (Newman and Müller 2000). Between the "pattern language" of the DPMs and the wellspring of cell types potentially generated by the DTFs, the predicted evolutionary outcome is the one that actually exists: a bestiary of incredible variety, with all representatives exhibiting an overlapping array of cell types, in bodies and organs built on a common set of morphological motifs.

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