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Before programs: The physical origination of multicellular forms

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ABSTRACT By examining the formative role of physical processes in modern-day developmental systems, we infer that although such determinants are subject to constraints and rarely act in a "pure" fashion, they are identical to processes generic to all viscoelastic, chemically excitable media, non-living as well as living. The processes considered are free diffusion, immiscible liquid behavior, oscillation and multistability of chemical state, reaction-diffusion coupling and mechanochemical responsivity. We suggest that such processes had freer reign at early stages in the history of multicellular life, when less evolution had occurred of genetic mechanisms for stabilization and entrenchment of functionally successful morphologies. From this we devise a hypothetical scenario for pattern formation and morphogenesis in the earliest metazoa. We show that the expected morphologies that would arise during this relatively unconstrained "physical" stage of evolution correspond to the hollow, multilayered and segmented morphotypes seen in the gastrulation stage embryos of modern-day metazoa as well as in Ediacaran fossil deposits of ~600 Ma. We suggest several ways in which organisms that were originally formed by predominantly physical mechanisms could have evolved genetic mechanisms to perpetuate their morphologies.

KEY WORDS: *self-organization, canalization, differential adhesion, epigenetic determinant, generative entrenchment*

Introduction

The 20th century biologist Theodosius Dobzhansky, a key figure in the modern synthesis of Darwinism and Mendelism, famously stated "Nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1973). Our point of departure in this paper is to suggest that like all generalizations in biology Dobzhansky's tenet is not universally true, or rather, is true in unanticipated ways. Indeed, we suggest that it may mislead in exactly the domain set out by the editors of this Special Issue, subtitled: "bridging the gap between the genome and embryo physics." Since modern-day embryos, in their molecular complexity, are products of several hundred million years of evolution, our view that Dobzhansky's statement may be misleading has nothing to do with skepticism about the reality of gene change over time, the acquisition of new molecular pathways by this process and the fact that much, if not most of this was due to natural selection. The question we raise here and in earlier work (Newman and Müller, 2000; Müller and Newman, 2003) rather, is whether an understanding of the forms assumed by multicellular organisms might not more productively be analyzed by conceptually stripping away the overlay of stabilizing and fine-tuning genetic circuitry accumulated over the last half-billion years so as to better see the originating physical and otherwise non-programmed determinants of multicellular form.

Plasticity of form and developmental trajectory in many modern-day organisms provide an entry into our perspective. Organisms including protists such as *Dictyostelium discoideum* (Bonner, 1967), fungi such as *Candida albicans* (Magee, 1997), plants (Hutchings and de Kroon, 1994) and animals such as arthropods (Emlen and Nijhout, 2000) and molluscs (Trussell, 2000), may exhibit radically different forms in different microenvironments or ecological settings. Even in vertebrates organism-environment interactions can play a decisive role in morphological development. Amphibian body shape and tail length, for example, can be influenced at the tadpole stage by changes in the predation environment (Van Buskirk, 2002, LaFiandra and Babbitt, 2004). In mice, the number of vertebrae can depend on the uterine environment (McLaren and Michie, 1958).

Neo-Darwinian interpretations of these phenotypic polymorphisms hold that they represent specifically-evolved adaptations and are therefore sophisticated products of evolution. The differ-

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ent phenotypes consistent with a given genotype are thus considered to be programmed subroutines that have evolved as a result of distinct sets of selective pressures acting at different life-history stages (Stearns, 2000) or the outcomes of evolution for "evolvability" (Gerhart and Kirschner, 1997). While the concept of environment-dependent reaction norms has both adaptive and nonadaptive aspects (Schlichting and Pigliucci, 1998), discussion of plasticity in this framework has centered on selection and genetic mechanisms (Van Tienderen and Koelewijn, 1994, Pigliucci, 1996). Our view is that rather than being the result of evolutionary adaptation, much morphological plasticity reflects the influence of external physico-chemical parameters on any material system and is therefore an inherent, inevitable property of organisms.

Nonliving viscoelastic materials such as clay, rubber, lava and jelly, for example, are subject to being molded, formed and deformed by the external physical environment. Such materials have been called "soft matter" by the physicist Pierre-Gilles de Gennes (de Gennes, 1992). Most living tissues are soft matter and all of them are also what physicists term "excitable media" (Mikhailov, 1990); (Winfree, 1994, Winfree, 2002), materials that respond in active and predictable ways to their physical environments. It is clear that some, if not much of organismal plasticity results from such material properties.

Non-programmed plasticity of phenotypic characters of the sort found in contemporary organisms has been proposed to be a source of evolutionary change (West-Eberhard, 2003). But it is likely that in earlier multicellular forms morphological plasticity based on an interplay of intrinsic physical properties and external conditions was even more prevalent. This is because ancient organisms undoubtedly exhibited less genetic redundancy and metabolic integration and homeostasis, than modern organisms and were thus more subject to external molding forces. This provides the basis for our proposal that morphological variation in response to the environment is a primitive, physically-based property, carried over to a limited extent in modern organisms from the inherent plasticity of the viscoelastic cell aggregates that constituted the first multicellular organisms.

The inference that ancient metazoa were even more developmentally plastic than modern ones implies that the general correspondence of a given genotype to one morphological phenotype is a product rather than a precondition of evolution. Such close mapping can result from an evolutionary scenario in which the developmental mechanism by which a phenotype is generated changes from being sensitive to external conditions to being independent of such conditions (Newman, 1994, Newman and Comper, 1990, Salazar-Ciudad *et al.*, 2001a, Salazar-Ciudad *et al.*, 2001b). If modern organisms are "Mendelian," in the sense that genotype and phenotype are inherited in close correlation and that morphological change is most typically dependent on genetic change, then our hypothesis can be encapsulated in the postulate that there was a "pre-Mendelian world" of polymorphic organisms at the earliest stages of metazoan evolution whose genotypes and morphological phenotypes were connected in only a loose fashion (Newman, 2005, Newman and Müller, 2000).

In this exploratory period of organismal evolution the mapping of genotype to morphological phenotype would therefore have been one-to-many, rather than one-to-one. With the subsequent evolution of genetic redundancies (Nowak *et al.*, 1997, Pickett and Meeks-Wagner, 1995, Tautz, 1992, Wagner, 1996, Wilkins, 1997) and other mechanisms supporting reliability of developmental outcome (e.g., Rutherford and Lindquist, 1998), a closer linkage between genetic change and phenotypic change was established. In particular, with evolution under selective criteria favoring the maintenance of morphological phenotype in the face of environmental or metabolic variability (see Baldwin, 1896, Schmalhausen, 1949, Waddington, 1942, Riedl, 1978, Salazar-Ciudad *et al.*, 2001a) organisms would come to be characterized by a closer mapping of genotype to phenotype, giving rise to the familiar Mendelian world. But even as body plans and other major morphological features, such as the bauplan of the vertebrate limb, became locked in by the accumulation of reinforcing genetic circuitry, fine-tuning of details of organismal and particularly organ morphology continued (and continues) to occur through an interplay of genetic and nongenetic factors.

By considering the origination and transformation of developmental mechanisms as an evolutionary problem in its own right (Müller and Newman, 2003, 2005), we have arrived at the view that epigenetic mechanisms, rather than genetic changes, are the major sources of morphological novelty in evolution. In our usage "epigenetic" refers to the context-dependence of developmental mechanisms, not to DNA-associated mechanisms of inheritance, such as methylation and chromatin assembly (see for a review, Müller and Olsson, 2003). The epigenetic mechanisms that we consider are *conditional, non-programmed determinants of individual development*, of which the most important are (i) interactions of cell metabolism with the physicochemical environment within and external to the organism, (ii) interactions of tissue masses with the physical environment on the basis of physical laws inherent to condensed materials – what we have termed "generic" processes (Newman and Comper, 1990) and (iii) interactions among tissues themselves, according to an evolving set of rules. We suggest that different epigenetic processes have prevailed at different stages of morphological evolution and that the forms and characters assumed by metazoan organisms originated in large part by the action of such processes.

While the standard neo-Darwinist account of the evolutionary generation of novel phenotypes focuses on *contingency*, we, along with other investigators (e.g., Ho and Saunders, 1979, Goodwin, 1994, Kauffman, 1993) emphasize the *inherency* of morphogenetic and patterning mechanisms. Eckstein (1980), writing in a different context, has provided a useful formulation of the distinction between contingency and inherency in conceptualizing a complex developmental process: "Something is contingent if its occurrence depends on the presence of unusual (we might say aberrant) conditions that occur accidentally, conditions that involve a large component of chance", while "something is inherent either if it will always happen (e.g., entropy) or if the potentiality for it always exists and actuality can only be obstructed."

In what follows we will provide examples that show that the inherent properties of metazoan organisms and the tissue masses they comprise extend beyond their genomes to encompass their physical identity as semi-solid to solid excitable materials. Because the inherent physical properties, in their self-organizing capacities, but also conditioned by external parameters and extrinsic forces, can act as morphogenetic determinants, the dynamical, constraining and environmental aspects of develop-

mental causation can productively be analyzed in the framework of *inherency* and *interaction*, i.e., epigenesis.

Generic physical mechanisms in development

Modern-day developing systems utilize a number of basic physical mechanisms that are common to non-living and living materials soft and excitable materials and have thus been termed "generic" (Newman and Comper, 1990). While there is debate around each of these as to its efficacy on its own in determining developmental patterns and transitions, they are all experimentally confirmed. Here we will list them with brief descriptions and citations. More information can be found in Müller and Newman (2003) and Forgacs and Newman (2005).

Diffusion

Because of "molecular crowding," free diffusion plays only a limited role within individual cells (Ellis, 2001, Hall and Minton, 2003, Shav-Tal *et al.*, 2004). There is good quantitative evidence, however, for its involvement in setting up gradients on the scale of multicellular embryos and organ primordia (Green, 2002, Gurdon and Bourillot, 2001, Lander *et al.*, 2002). Lander and coworkers showed that for the *Drosophila* wing imaginal disk morphogen Decapentaplegic (Dpp), plausible rates of extracellular diffusion and kinetics of receptor binding and occupancy were more consistent with measured transport rates than alternative non-diffusion models (Lander *et al.*, 2002). Active mechanisms in addition to diffusion also appear to be involved in Dpp transport (Kruse *et al.*, 2004).

Another case where diffusion plays a role in early development (although in a syncytial, rather than cellular context) is in the establishment of a gradient of the maternal protein Bicoid (Bcd) in the early *Drosophila* embryo (Driever and Nüsslein-Vollhard, 1988a, Driever and Nüsslein-Volhard, 1988b). Gene expression patterns downstream of and dependent on Bcd are required to be much more precise than the distribution of Bcd itself, which indeed exhibits the variability expected for a diffusive distribution mechanism (Houchmandzadeh *et al.*, 2002). The Bcd signal seems to be "rectified" by other factors, most likely including the product of another maternal gene, *staufen* (Houchmandzadeh *et al.*, 2002).

Differential adhesion

It has long been recognized that cell aggregates, particularly those derived from embryonic tissues, round-up like liquid droplets (Foty *et al.*, 1994, Steinberg and Poole, 1982). When pairs of tissues differ in cohesivity, based on spreading behavior on a common substratum and response to compressive force (Forgacs *et al.*, 1998), they correspondingly behave like immiscible liquids, forming interfaces across which cells do not mix, or in heterotypic mixtures of cells they phase separate. Each tissue in a given pair will engulf the other or become engulfed according to relative cohesivity predictable from physical measurements (Forgacs *et al.*, 1998, Steinberg, 2003).

While these behaviors are generic in the sense that they can be attributed entirely to quantitative differences in cell adhesivity (Foty and Steinberg, 2005, Steinberg and Takeichi, 1994), it has been controversial as to whether differential adhesion actually plays a determining role in embryonic development. That it does in some cases has been demonstrated in a series of *in vivo* experiments on the arrangement of the oocyte relative to the follicular cells during oogenesis in *Drosophila* (Godt and Tepass, 1998, Gonzalez-Reyes and St Johnston, 1998). Analogous studies of the development of the *Drosophila* retina similarly demonstrate the role of differential adhesion in cell patterning (Hayashi and Carthew, 2004). Boundaries of immiscibility within otherwise uniform tissues (i.e., "compartment" boundaries) form in systems ranging from *Drosophila* imaginal disks (Garcia-Bellido *et al.*, 1976) to the mammalian hindbrain (Guthrie and Lumsden, 1991). In many of these systems differential adhesion has been found to be an important, although not always exclusive, determinant of boundary formation (reviewed in Forgacs and Newman, 2005).

Differential adhesion across the individual cell surface is also employed during development. Epithelioid tissues are formed by cells bearing uniformly distributed adhesive molecules. Such tissue masses become epithelial by expression of proteins that mediate or regulate adhesion in a polarized fashion (Rodriguez-Boulan and Nelson, 1993). As a result of random cell movement, or death of cells that have become detached from their neighbors, cell regions of lower affinity will automatically come to adjoin one another and interior cavities or lumens will form. In mammals, for instance, blastocyst formation is driven by the expression of specific sets of gene products (e.g., E-cadherin and catenin) that direct the acquisition of cell polarity within the trophectoderm, which is both the first epithelium to form during development and the cell layer encircling the blastocoel and inner cell mass (Watson and Barcroft, 2001).

Biochemical oscillation

Temporally-periodic generation of functionally active protein complexes, or expression of genes, play important roles in development (reviewed in Forgacs and Newman, 2005). In the cleavage stage *Xenopus* embryo the 14 cell divisions that produce the blastula are triggered by M-phase promoting factor (MPF), a protein kinase consisting of two subunits: Cdc2 (the catalytic subunit) and cyclin B (the regulatory subunit). MPF phosphorylates an array of proteins involved in nuclear envelope breakdown, chromosome condensation, spindle formation and other events of meiosis and mitosis. Whereas Cdc2 is present at a constant level throughout the cell cycle, the concentration of cyclin B and thus MPF, varies in a periodic fashion, rising to a peak value just before M-phase and dropping to a basal value as cells exit M-phase. No transcription is required to produce this oscillation. In nucleus-free cytoplasmic extracts of immature frog eggs there are spontaneous oscillations of MPF with a period of about 60 min (Murray and Hunt, 1993). Cyclin protein is periodically degraded in the extract and resynthesized in a manner that depends upon the presence of its mRNA (Murray and Kirschner, 1989).

Oscillations in the expression of components of the Notch-Delta juxtacrine cell-cell signaling pathway and associated transcriptional regulators (c-hairy in chicken; Her1 and Her7 in zebrafish) are responsible for progressive formation of somites from the segmental plate in vertebrates (Giudicelli and Lewis, 2004). This appears to also involve a gradient of FGF8 (Dubrulle *et al.,* 2001) with its high point at the tail tip of the embryo and its low end providing a "gate" beyond which cells are respecified by the oscillating determinants (Pourquié, 2003). The oscillations controlling both the cleavage-stage cell cycle and somitogenesis

are "generic" network properties. The dynamical mechanisms proposed to account for these biochemical clocks - see Novak and Tyson (1993), Borisuk and Tyson (1998) and Novak and Tyson (2003) for the cell cycle and Lewis (2003), Monk (2003) and Pourquié and Goldbeter (2003) for the somite oscillator - do not depend on the unique molecular identities of the gene products involved so much as on formal relationships among them: positive and negative feedback, time lags, etc. (Goldbeter, 1996).

Multistability of biochemical state

Biochemical networks within cells, in addition to being capable of oscillatory behaviors under specific conditions, are also potentially capable of switching between distinct, stable compositional states. Unlike closed chemical systems which always evolve toward a unique state of chemical equilibrium, living cells are open systems. With sufficiently complex dynamics such systems can exhibit multiple dynamical "attractors" whereby the system will evolve toward one or another distinct state. The biochemical oscillations of the cleavage cell cycle and somite clocks are both attractors of this sort: small alterations in the system parameters (rate constants, time lags) can suppress the oscillation. For multistable systems, alternative states, oscillatory or non-oscillatory, can potentially be achieved by differences in the system preparation (initial conditions). However, where multistable dynamics is used in modern-day organisms, as in the eukaryotic cell cycle, stabilization of various cell states (i.e., robustness, see below) is achieved by additional biochemical complexity (Novak and Tyson, 2003).

Dynamical multistability, which has been demonstrated experimentally in the lactose utilization network of *E. coli* (Ozbudak *et al.*, 2004) has been proposed to also underlie eukaryotic cell differentiation (Keller, 1995, Laurent and Kellershohn, 1999, Cinquin and Demongeot, 2005). The dynamical phenomenon of "isologous diversification" (Kaneko, 2003, Furusawa and Kaneko, 2006), in which systems (model cells) exhibit alternative compositional states only when in communication with other copies of the same system, provides a model for the "community effect" seen during muscle development in *Xenopus* (Buckingham, 2003, Standley *et al.*, 2002).

Reaction-diffusion coupling

In complex dynamical systems of the sort that exhibit biochemical oscillation and multistability and permit the diffusion of released factors (e.g., the embryonic tissues described above), there is a generic propensity to form complex spatial patterns of one or more of the diffusible factors, or morphogens. "Complex" here means more elaborate than the simple gradients that diffusion alone can produce. The basis for such pattern formation - reaction-diffusion coupling - was described by Turing more than half a century ago (Turing, 1952) and is the subject of several recent accessible presentations (Forgacs and Newman, 2005, Meinhardt and Gierer, 2000, Miura and Maini, 2004). The basic notion is that a diffusible, positively autoregulatory "activator" (e.g., the Even-skipped transcription factor in the syncytial *Drosophila* embryo (Harding *et al.*, 1989), or TGF-β in limb bud mesenchyme, Miura and Shiota, 2000b) will tend, if unconstrained in its action, to create an explosive, spreading front of its own production and any downstream effects of its activity. If, however, the activator also induces in the same population of cells an inhibitor of its action that diffuses or otherwise spreads faster than the activator itself, there will be a zone around any peak of activation within which no activation can occur. New peaks of activation will only form sufficiently far from other peaks such that the effect of the inhibitor has faded. Such systems thus have an intrinsic "chemical wavelength."

Pattern formation by reaction-diffusion mechanisms was a theoretical curiosity for several decades after Turing's paper, until the mechanism was demonstrated unambiguously in several nonliving physicochemical systems (Castets *et al.*, 1990, Ouyang and Swinney, 1991). Since then evidence has accumulated for a role for this class of mechanism in vertebrate axis formation (Meinhardt, 2001), formation of pigment stripes on the skin of fish (Kondo and Asai, 1995), formation of feather patterns in avian skin (Jiang *et al.*, 1999), breaking of left-right symmetry in the vertebrate embryo (Solnica-Krezel, 2003) and generation of the skeletal pattern in the vertebrate limb (Hentschel *et al.*, 2004, Miura and Shiota, 2000a, Miura and Shiota, 2000b, Moftah *et al.*, 2002). The seven stripes of Even-skipped in the *Drosophila* embryo have the appearance of a reaction-diffusion pattern but are actually generated in a more complex fashion (Akam, 1989, Clyde *et al.*, 2003, Small *et al.*, 1991). This may be the result of evolution for developmental stability (Newman, 1993, Salazar-Ciudad *et al.*, 2001b), as we will discuss below.

Mechanochemical excitability

The tissue systems described above are examples of excitable media (Mikhailov, 1990, Winfree, 1994, Winfree, 2002); they store or generate energy in various forms and can react to stimuli by continuous production of a characteristic activity. The positive autoregulation discussed in relation to reaction-diffusion processes is an example of this and generation of chemical oscillations also depends on such excitability. In addition to biochemical excitability such materials may exhibit mechanical excitability, whereby a stimulus evokes an active mechanical response. This is not a major effect in the liquid-like tissues described above in relation to differential adhesion; the mobility of cells within those aggregates dissipate most mechanical perturbations. However, the complex basement membranes (Lindblom and Paulsson, 1996) of epithelia confer stiffness to these tissues (Danielsen, 2004), which facilitate the storage of mechanical energy (Tidball, 1986). In combination with the biochemically excitable cellular component and the mechanical continuity fostered by cell surface-cytoplasmic linkage (Ingber *et al.*, 1994), embryonic epithelia become capable of exhibiting tension-dependent collective cell movement (Beloussov *et al.*, 2000) leading to complex folding, branching and bucking behaviors (Beloussov, 1998).

The first metazoa

Multicellular organisms first arose more than 1.5 billion years ago (Knoll, 2003). The earliest of these were filamentous algae, plants whose rigid cell walls would have provided a somewhat different set of physical capacities and constraints from those that pertain to animal morphogenesis. Despite this, several of the processes and mechanisms considered here pertain to the development of plants as well (Nagata *et al.*, 2003). The first "metazoa" (multicellular animals) appear in the fossil record earlier than 700 million years ago (Ma) (Brasier and Antcliffe, 2004). The most

extensively-studied of these fossils, dating from 580-543 Ma, have been described as relatively simple, flat, quilt-like creatures, probably without body cavities (Brasier and Antcliffe, 2004, Seilacher, 1992). Modular body subdivisions often exhibited a fractal branching pattern instead of the segmentation seen in modern organisms (Narbonne, 2004). By approximately 540 Ma the "Cambrian explosion" had occurred, a term denoting the fact that virtually all the general categories of body organization seen in modern organisms had burst into existence in the preceding 25-30 million years, a brief instant, geologically speaking (Conway Morris, 2003).

Metazoan bodies are characterized by axial symmetries and asymmetries, multiple tissue layers, interior cavities, segmentation and various combinations of these properties. Each species can be assigned to one of approximately 35 body plans (Arthur, 1997, Raff, 1996) or organizational categories (Minelli, 2003). These are essentially the same as the phyla of the standard taxonomic system (Valentine, 2004). The organs of an animal are constructed using similar morphological motifs as the body plans. While the early world contained many unoccupied ecological niches within which new organismal forms could flourish, this alone cannot account for the rapid profusion of body plans once multicellularity was established, nor for the particular forms bodies and organs assumed.

As we have seen above, generic physical mechanisms play an active, albeit constrained role in modern-day embryonic development. The most ancient cell aggregates, lacking highly integrated program-like hierarchical genetic interactions to control their morphogenesis, would inevitably have been even more susceptible to molding and patterning by physical forces and determinants inherent to their material properties and scale. Physical considerations therefore permit us to hypothetically reconstruct the forms likely to have been assumed by these ancient cell aggregates.

Scenario for the origination of body plans

As described above, many forms that arise during early stages of embryogenesis are either produced by mechanisms common to cell aggregates and nonliving materials (generic mechanisms), or resemble the outcomes of such generic mechanisms. Here we present a scenario by which the physical attributes and behaviors of tissue masses can have led to the generation of the basic features of modern metazoan body plans.

The scenario can be summarized as follows: the single-cell organisms that existed before the emergence of multicellularity must have been metabolically active, thermodynamically open systems, much like present-day cells (evolution of eukaryotic cells had been underway for more than a billion years before metazoans appeared; Knoll, 2003). Since the pre-metazoan aggregates would thus have been composed of chemically active and responsive cells, they were not only viscoelastic and chemically heterogeneous, they were also excitable media. This means they would have had the potential to elaborate self-organized spatial and temporal patterns of cells with different biochemical states. And in cases where these incipient cell types exhibited different amounts of adhesive molecules, adhesion-based sorting-out into distinct tissue layers and tandemly arranged segments would have been inevitable. Polarization in the expression of adhesive proteins would lead, as a physical side-effect, to aggregates with lumens; similarly, production of a stiff extracellular layer by an epithelium would cause it to act as a viscoelastic sheet with the morphological properties associated with such materials (Mittenthal and Mazo, 1983; Beloussov, 1998; reviewed in Newman, 1998). These features,

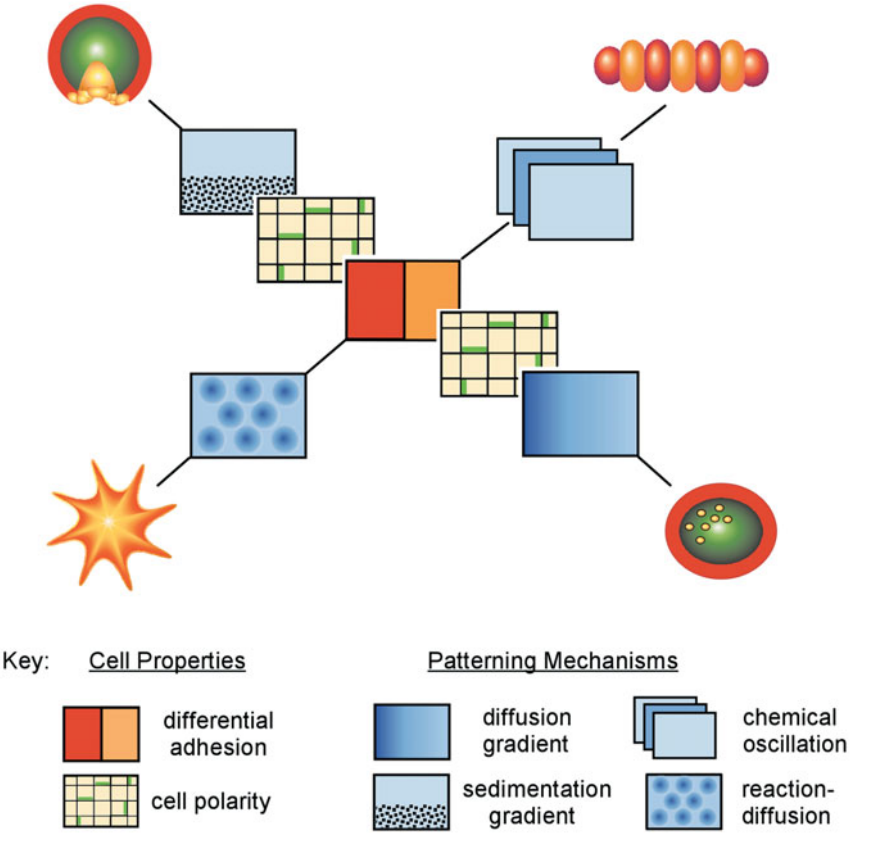

Fig. 1. Schematic representation of hypothesized origination of body plans via the morphogenetic consequences of linking regulation of cell-cell adhesion to various physical and chemical pattern-forming mechanisms. *The central box denotes the effects of differential adhesion in causing the formation of boundaries within a tissue mass, across which cells will not mix. Polarized expression of adhesion molecules leads to cavities and other lumenal structures. All of the peripherally arranged boxes denote pattern-forming mechanisms which, when deployed in conjunction with differential adhesion, can lead to standard body plan organizational motifs. Sedimentation of a dense cytoplasmic component, or diffusion of a morphogen, are ways in which an egg or morula can become spatially nonuniform; if these nonuniformities become coupled to the expression of differentially adhesive cell populations, gastrula-like structures will form. Similarly, chemical oscillations or prepattern-generating reaction-diffusion processes, if they come to regulate adhesive differentials between cells, can lead to segmentation or other periodic structures. Buckling of epithelial sheets can lead to invagination or other folding processes (After Newman, 1994.)*

simple consequences of generic physical processes acting on excitable viscoelastic materials, can account for virtually all the major structural motifs in bodies and organs.

The advent of multicellularity opened up possibilities for the molding of biological form that were unavailable to single-celled organisms. Diffusion of typical biomolecules, for example, is rapid over the ~10 μm distances spanned by a single cell; in the absence of special docking or compartmentalization mechanisms (Agutter and Wheatley, 2000) intracellular molecules would be well-mixed within minutes. In contrast, on the >100 μm size scale of a cell aggregate, over a time-scale of minutes to hours, the formation of gradients of released molecules is fostered, rather than undermined, by diffusion (Crick, 1970). The capacity of cells to secrete products, which must have predated multicellular organisms, acquired a new meaning once multicellularity arose - it provided a means for establishing differences across an otherwise undifferentiated population of cells.

Let us imagine an ancient aggregate of cells before the emergence of true metazoa. If a group of cells in one region of this cluster released a protein or other product at a higher rate than their neighbors - either randomly or because some external cue stimulated them to do so - the aggregate would thereby have become chemically nonuniform from one end to the other. Let us further assume that in some of these cases the cells happen to react differently to different amounts of the molecule in question (we will call it a "proto-morphogen") and thus assume different states in a fashion that depended on its concentration. Under these circumstances, a spatially non-homogeneous distribution of a chemical (e.g., a gradient) would have fortuitously given rise to a nonhomogenous distribution of cell states (Fig. 1). In this fashion, "generic" physics (i.e., diffusion) acting on a scaled-up biological system (i.e., a multicellular aggregate) could have given rise to an incipient developmental process.

But how could such a haphazardly-determined effect be perpetuated from one generation to the next? In present-day embryos the position of the embryonic "organizer" (i.e., a cell or group of cells that is a unique source of a diffusible morphogen) is often determined by maternally deposited cues, or some other genetically-influenced process, in conjunction with external cues, such as the sperm entry point. In such cases, hereditary transmission of the relevant genes or gene variants creates reproducible conditions for the recapitulation of the event from one generation to the next. In our hypothetical ancient form, containing numerous genes specifying cellular proteins, but lacking such a genetic "program" for pattern formation, recurrence of the developmental event could have been perpetuated by less formal means. If, for example, the cells in the primitive aggregate had a 1% chance of randomly producing and secreting the proto-morphogen, then half of all 50-cell aggregates (or each 100-cell aggregate) would have a (proto-) organizer cell. These variants would "develop," that is to say, they would self-organize a nonuniform distribution of cell states.

In this scenario, if there were a selective advantage to having a phenotype containing nonuniformly distributed cell types, cell clusters whose genotype inclined them to produce proto-organizer cells at a higher frequency would become more prevalent. This tendency would be balanced by the fact that if all cells became organizers there would be no gradient. And this, in turn, would put a premium on genetically-variant clusters in which proto-organizer cells limit the appearance of other proto-organizer cells, that is, produce a lateral inhibitory factor simultaneously with the proto-morphogen.

We described above how dynamical multistability could lead to generation of alternative cell types. Single-cell organisms can use this capacity as a physiological alternative to genomic evolution to function in environmental niches with different biosynthetic demands. If any genetic change did occur that locked the cell into one of the alternative types, the original uniform population would simply break into subpopulations of distinct kinds of single-cell organisms. Once multicellularity arose, however, there was the possibility of having the alternative cell types present simultaneously in the same organism. This would have created a premium on retaining plasticity (i.e., condition-dependent reversibility) of cell type switching (see the discussion in Furusawa and Kaneko, 2006) and of utilizing physical mechanisms such as diffusion (see above) for reliably determining where in the organism the different cell types arise.

The use of a diffusible signaling molecule with an externally-determined gradient as the sole cue causing switching between alternative cell types is a simple, hierarchical "feed-forward" mechanism of pattern formation. ("Hierarchical" here means unidirectional determination in the way the individual cells acquire their identity, without any feedback on the cue or each other; Salazar-Ciudad *et al.*, 2001a). An even simpler way is to form an intracellular gradient by sedimentation of dense cytoplasmic materials. Once division occurs across the cell's "equator" the progeny will be biochemically distinct; Fig. 1). If, alternatively, the responding cells or nuclei (in a syncytium) themselves participate in the formation of the gradient, pattern formation can be "emergent," that is, the pattern arises from self-organizing dynamics (Salazar-Ciudad *et al.*, 2001a).

Oscillation in chemical composition is another physical process that took on novel functions in the multicellular context (Fig. 1). The cell division cycle is a temporally-periodic process driven by a chemical oscillation (Murray and Hunt, 1993). In a world of free-living cells it leads to generation of more of the same; it has no special *morphological* consequence. Even in a multicellular aggregate the division cycle typically acts only to increase the number of cells. In a multicellular aggregate that contains a "gating" chemical gradient (Dubrulle *et al.*, 2001, Hentschel *et al.*, 2004, Pourquié, 2003) or an additional biochemical oscillation with a period different from the cell cycle (Holtzendorff *et al.*, 2004, Newman, 1993), populations of cells can be generated periodically, with distinct, recurrent, states. The developmental consequences of such oscillatory mechanisms must have appeared early in the history of multicellularity.

How, then, could spatial patterns of cell state or type resulting from diffusion gradients and excitable (e.g., biochemically oscillatory) properties of cell aggregates have led to the evolution of new organismal shapes and body plans? They likely did so by mobilizing cell adhesion. This, of course, is the defining condition of multicellularity. That is to say, organisms became multicellular by evolving cell surface molecules that either prevented them from separating after division, or caused individual cells to aggregate (Bonner, 1998). Modern-day organisms have multiple highly-evolved gene network-based regulatory mechanisms devoted to controlling the precise strength of intercellular adhesion (Braga, 2002, Buckley *et al.*, 1998). In contrast, cell stickiness is likely to

have been less stringently regulated in the earliest metazoa than it is at present.

Just as organizer cells secreting a proto-morphogen could have arisen randomly in primitive cell aggregates, it is reasonable to envision that some cells with distinct adhesive properties - either more or less adhesive than the parental cells - could have emerged randomly in these ancient multicellular masses. We have just seen how the existence of a proto-morphogen-secreting cell has developmental consequences in a tissue mass of a certain scale as a result of the physical process of diffusion. In a similar fashion, differential adhesion within cell mixtures, mobilizes its own physically-based morphogenetic effects; sorting-out, establishment of boundaries of immiscibility, engulfment, etc. (see previous section). In other words, the presence of two or more differentially adhesive cell populations within the same tissue mass immediately establishes the conditions for the formation of multiple non-mixing layers or compartments.

Although compartment boundaries in the developing embryos of modern organisms are typically allocated with precision by spatially distributed cues based on juxtacrine signaling (e.g., the Notch-Delta couple, Artavanis-Tsakonas *et al.*, 1999) or paracrine-type pattern-forming mechanisms (Green, 2002), even random assignment of cells to distinct adhesive states can result in a compartmentalized tissue. This is because the sorting-out process will eventually bring the cells of similar adhesive state to one side or another of a common boundary.

Let us recall the discussion above regarding the proto-organizer and various physically-instigated pattern-forming processes. If the alternative cell states produced in that example happened to be adhesively different, the primitive pattern forming mechanism would provide a way to regulate the generation, number and position of differentially-adhesive cells in a somewhat reliable, rather than random, fashion. Furthermore, for the reasons stated in the previous section, evolutionarily ancient metazoan organisms which were made up of adhesively polar cells would have taken the form of hollow sacs (Fig. 1). Mechanical instabilities in the resulting epithelial sheets (Beloussov, 1998, Drasdo and Forgacs, 2000) would have led to folding inward or outward when they reached a certain size.

Most of the new forms generated by these processes would have been indeterminate and unstable, but in those cases in which any of the primitive physically-based pattern forming mechanisms mentioned above caused adhesive or mechanical properties to become nonuniform across the cell mass, new stable forms would have emerged by invagination or ingression of part of the hollow sac. The linking (as an outcome of gene evolution) of distinct physical mechanisms - differential adhesion, chemical gradient formation (by sedimentation, diffusion or reaction-diffusion), or chemical oscillation - would thus produce primitive but authentic developmental mechanisms for body plan generation (Fig. 1).

The implication of the foregoing discussion is that long before complex, modern-type body morphologies emerged in the course of evolution, simpler forms resembling gastrulae and budding and segmented tubes, (i.e., the forms that can be generated by relatively simple physical mechanisms acting on cell aggregates), should have arisen. Despite their appearance, these would not have been embryonic stages of more complex organisms, but rather the morphologically most advanced organisms of their

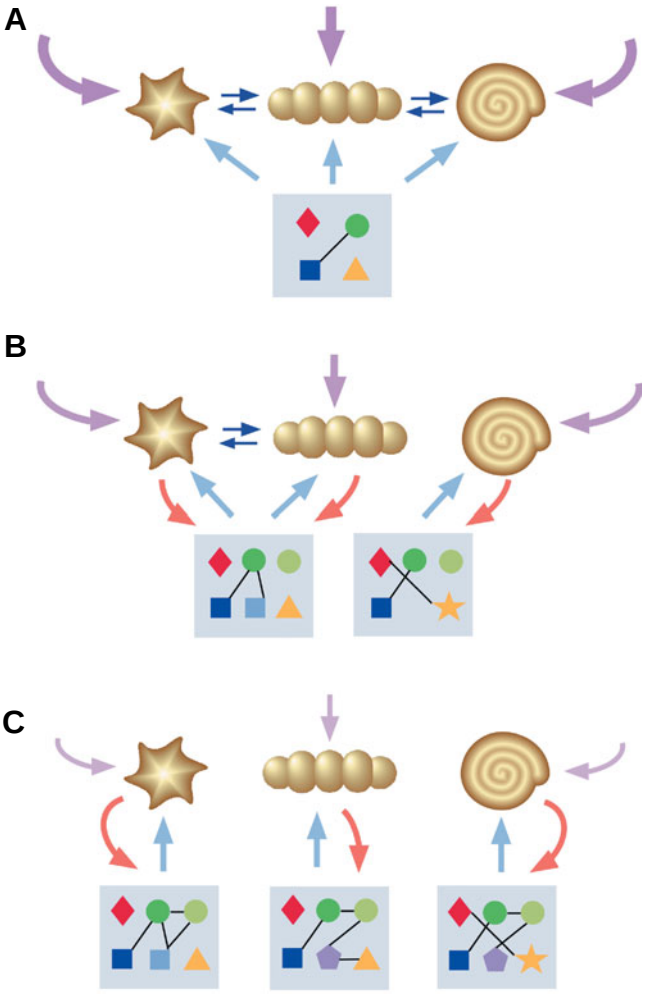

Fig. 2. Schematic representation of evolutionary partitioning of a morphologically plastic ancestral organism into distinct morphotypes associated with unique genotypes. (A) *A hypothetical primitive metazoan is shown with a schematic representation of its genome in the box below it. Specific genes are shown as colored geometric objects; interactions between them by lines. Determinants of the organism's form include the materials provided by expression of its genes (light blue arrows) and the external environment, including physical causes (purple arrows) acting on its inherent physical properties. At this stage of evolution the organism is highly plastic, exhibiting several condition-dependent forms that are mutually interconvertible (dark blue arrows).* **(B)** *Descendents of organism in (A) after some stabilizing evolution. Gene duplication, mutation, etc. have led to non-interbreeding populations that are biased toward subsets of the original morphological phenotypes. Determinants of form are still gene products and the physical environment, but the effect of the latter has become attenuated (smaller, lighter purple arrows) as development has become more programmatic. There is also causal influence of the form on the genotype (orange arrows), exerted over evolutionary time, as ecological establishment of forms filters out those variant genotypes that are not compatible with the established form. Some morphotypes remain interconvertible at this stage of evolution.* **(C)** *Modern organisms descended from those in (B). Further stabilizing evolution has now led to each morphotype being uniquely associated with its own genotype. Physical causation (faint purple arrows) is even more attenuated. Note that in this idealized example the forms have remained unchanged while the genes and mechanisms for generating the forms have undergone extensive evolution.*

time. It is significant, therefore, that such structures have been found in Ediacaran period sediments dating from ~600 Ma, unaccompanied by the complex metazoan forms characteristic of the later Cambrian period (Chen *et al.*, 2004, Xiao and Knoll, 2000, Xiao *et al.*, 2000).

Back to the present: canalization of morphological outcome

We have argued that the inherent material properties of organisms and their tissues, in interaction with the physical environment would have led to stereotypical outcomes that are reflected in structural similarities in body plans across all metazoan taxa (Moore and Willmer, 1997, Sanderson and Donoghue, 1989, Wake, 1991). Similar considerations hold for the forms of organs such as blood vessels (Merks *et al.*, 2006, Serini *et al.*, 2003), glands (Lubkin and Li, 2002) and the vertebrate limb (Hentschel *et al.*, 2004, Newman and Müller, 2005) (reviewed in Forgacs and Newman, 2005).

An expectation of this scenario is that the body plans of contemporary organisms, for all their variety, would be produced more or less with the same "genetic toolkit." Their morphological variety would, by our hypothesis, have originated by conditional physical determinants acting on viscoelastic, chemically excitable materials, not primarily by genetic evolution (Newman, 2006). Consequently, those transcription factors (homeobox, paired box, T-box, etc.), cell attachment proteins (e.g., cadherins) and signal transduction proteins and modules (e.g., Notch-Delta, Wnt, Sonic hedgehog) that were in place at the origin of the metazoa would be used for similar purposes in widely divergent taxa (arthropods, echinoderms, chordates). The genetic toolkit comprising these components is indeed conserved across all metazoan phyla (reviewed in Wilkins, 2002 and Arthur, 2004).

We would also expect that the set of body plans generated early in the evolutionary history of the metazoa would have emerged relatively abruptly and, because there would have been no barrier to exhausting the physical possibilities for generation of form early on, would have not significantly increased in number or changed in overall character despite half a billion years of subsequent evolution. Both rapid origination (Conway Morris, 2003) and stasis (Eldredge and Gould, 1997) of body plans are borne out by the paleontological evidence.

But genes are constantly undergoing mutation and genetically variant organisms are always subject to natural selection. The foregoing discussion suggests that the result of this process is not to generate new members of the molecular toolkit (with rare exceptions such as laminin; Czaker, 2000), body plans (with rare exceptions such as the Bryozoa; Valentine, 2004), or organs (with rare exceptions such as the vertebrate limb; Shubin, 2002). Our proposal that multicellular forms were originally based on the inherent physical properties of tissue masses suggests an alternative interpretation of the impact of genetic change on the evolution of development. If such forms were functionally adaptive or even neutral, they would have served as templates for the accumulation of stabilizing and reinforcing genetic circuitry (Müller, 2003, Müller and Newman, 1999). Over time, stabilizing evolution (Schmalhausen, 1949) would have produced what Waddington (Waddington, 1942, Waddington, 1957) termed developmental canalization: robustness of developmental outcome against genetic variation, biochemical noise, or environmental perturbation.

Theoretical models suggest that evolution of canalization depends both on complexity (Bergman and Siegal, 2003, Proulx, 2005) and topology (Ingolia, 2004, Salazar-Ciudad *et al.*, 2001a, von Dassow *et al.*, 2000) of the genetic networks involved in producing the system's components. Canalization could be accomplished, for example, if genetic circuitry with emergent topological character, associated with a high degree of morphological plasticity and conditional outcomes, were superseded over the course of evolution by circuitry with a hierarchical topological character, associated with less versatile but more reliable outcomes (Salazar-Ciudad *et al.*, 2001a,b).

The effect of such canalizing evolutionary change is not so much to turn organisms into morphologically different ones, but to turn them more into "themselves": types that are less morphologically plastic and therefore less mutually interconvertible, than ones molded by relatively unconstrained physical mechanisms (Fig. 2). This view assigns a different role to natural selection in the process of phenotypic evolution than what is usually portrayed. Rather than being responsible for the origination of novelties it explains their stabilization and spread. This is in keeping with the more nuanced interpretations of this process in the literature of evolutionary biology (Williams, 1966; Endler, 1986; Goodwin, 1994)

The molecular basis of canalizing evolution typically involves genetic redundancies (Nowak *et al.*, 1997, Pickett and Meeks-Wagner, 1995, Tautz, 1992, Wagner, 1996, Wilkins, 1997), including duplication of developmental control genes (Holland, 1999) and multiplication of their regulatory elements (Goto *et al.,* 1989; Small *et al.,* 1991), as well as chaperone proteins acting as "phenotypic capacitors" (Rutherford and Lindquist, 1998). Over time, there would be a tendency to bring particular morphological phenotypes into coordination with particular genotypes (Fig. 2). If this evolutionary outcome is all that is examined and not the process by which it was attained, the false impression can be gained that the genotype *determines* the phenotype (Newman, 2002, Robert, 2004).

Body plans and other constructional motifs, moreover, can attain an autonomy that allows them to persist despite genes or gene networks taking on new roles in the production of conserved forms (Abouheif, 1999, Wray, 1999, Wray, 2001). Such autonomization (Müller, 2003, Müller and Newman, 1999, 2005), in which the more things change (genetically) the more they stay the same (morphologically), casts a new light on Dobzhansky's tenet about evolution, quoted at the beginning of this article. In many respects, it may not be the overall structure and appearance of organisms and their parts that required long periods of genetic evolution. The major role of molecular evolution over the last half billion years, we suggest, has been, rather, the integration and "generative entrenchment" (Wimsatt, 1986) of physically inherent morphological motifs into the developmental repertoire.

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