Differential adhesion in morphogenesis: a modern view

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The spreading of one embryonic tissue over another, the sorting out of their cells when intermixed and the formation of intertissue boundaries respected by the motile border cells all have counterparts in the behavior of immiscible liquids. The 'differential adhesion hypothesis' (DAH) explains these liquid-like tissue behaviors as consequences of the generation of tissue surface and interfacial tensions arising from the adhesion energies between motile cells. The experimental verification of the DAH, the recent computational models simulating adhesion-mediated morphogenesis, and the evidence concerning the role of differential adhesion in a number of morphodynamic events, including teleost epiboly, the specification of boundaries between rhombomeres in the developing vertebrate hindbrain, epithelial–mesenchymal transitions in embryos, and malignant invasion are reviewed here.

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Introduction

If this subject can be said to have sprung from any single source it is the 1955 study by Townes and Holtfreter [1] of the spontaneous self-organization *in vitro* of combinations of amphibian embryonic cells and tissues, itself a continuation of Holtfreter's 1939 investigation of 'tissue affinity' [2]. Combined heterotypic tissue fragments rearranged to adopt combination-specific anatomies. Their dissociated and randomly intermixed cells did likewise, forming the same structures but by an entirely different pathway. When the tissues or cells combined were the components of some normal structure, they re-formed a semblance of that same structure, inspiring confidence that whatever mechanism underlay these anatomically goal-directed rearrangements must underlie the related processes of embryogenesis itself. The underlying mechanism was not resolved but was suggested to be a combination of 'directed movements' and 'selective adhesion.' Since different underlying mechanisms should generate different pathways of rearrangement, it was possible, using marked cells, to compare the patterns observed with those anticipated according to each extant hypothesis. Only the 'differential adhesion hypothesis' (DAH) accounted for both those patterns that were already known and correctly predicted unknown others that were subsequently sought and confirmed (reviewed in [3–5]).

The cell population rearrangement patterns found in this way mimicked those already well known in ordinary liquid systems: rounding up of irregular masses, merging of identical masses, spreading of different kinds of masses one over another, the hierarchical ranking of these mutual envelopment tendencies, the sorting out of mixtures, and the approach toward the most stable configuration by opposite pathways (both mutual spreading and sorting). The rationale for this behavioral mimicry was that both early stage embryonic tissues and ordinary liquid droplets consist of a large number of mobile, cohesive subunits: cells versus molecules. The subunits of any such system will spontaneously tend to rearrange so as to maximize their mutual adhesive bonding and the relative bonding energies of the various possible interfaces determine whether the 'phases' will be miscible or immiscible (forming a mutual boundary) and what the precise equilibrium configuration (anatomy) will be. The DAH proposes that cell populations showing these behaviors do so as a consequence of maximizing the 'strength' of mutual binding within them, minimizing the adhesive free energy of the system.

Although the DAH was formulated to explain the sorting out and mutual spreading behaviors of vertebrate embryonic cells and tissues, differential adhesion itself has been implicated in a range of morphogenetic phenomena far wider than liquid-like cell population behavior. The DAH itself has been verified through direct physical measurements, and its predicted behaviors have been confirmed by computer modeling. Differential adhesion has been demonstrated to play a part in a wide range of events in embryogenesis, wound healing, and malignancy, and the underlying principle has been used to guide efforts at tissue engineering [6,7•,8,9]. Here I review some of the main developments in this area in the past few years, reaching back before 2005 only when it seemed necessary to do so. Space constraints prevent the discussion of several relevant systems, which can be cited here only for reference. These include the compound eye of the insect, which is treated elsewhere in this volume,

many examples of cell intercalation [10], involvement of a protocadherin in zebrafish somite segmentation and movements of paraxial cells [11], establishment of the notochord–somite boundary [12], the layering of granule cell precursors and inhibitory interneurons in the developing cerebellar cortex [13], the stratification of endocrine cells in pancreatic islets [14•], and the positioning of zebrafish germ layers (E-M Schötz et al., unpublished).

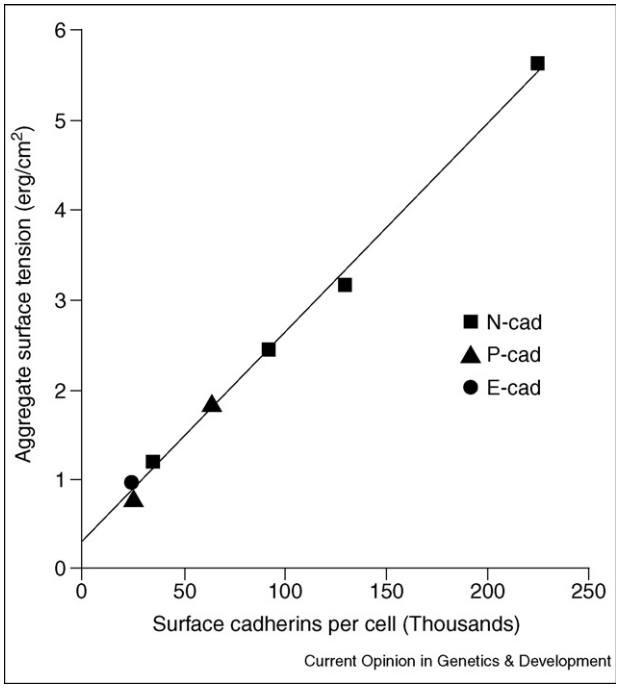
Experimental verification of the DAH

The segregative and mutual spreading behaviors of immiscible liquids are governed by surface and interfacial tensions that themselves arise from the forces of attraction between the constituent molecules. The DAH proposes that multicellular systems displaying liquid-like behavior should possess aggregate surface tensions sufficient to generate that behavior, arising from the mutual adhesive interactions of the constituent cells. Following the earlier demonstrations that rounding up cell aggregates do indeed possess true, force-independent surface tensions that, without exception, predict the direction of mutual aggregate spreading, Foty and Steinberg [15••] transfected originally nonadhesive L cells to express surface E-cadherin, P-cadherin or N-cadherin in amounts accurately measured by flow cytometry. Clones of these cells were allowed to aggregate, and their surface tensions were measured and plotted against the cells' surface cadherin expression levels. Aggregate surface tension was found to be a linear function of cadherin expression level (Figure 1). Moreover, even a modest difference in the level of expression of a given cadherin was sufficient to cause two otherwise identical L cell populations to sort out, with the lower expression (and lower surface tension) population enveloping the higher expression one (Figure 2). This has also reconfirmed that not only differing recognition specificities but even mere quantitative differences in the expression levels of cell adhesion molecules suffice to render two cell populations immiscible. These findings have verified the DAH over 40 years after it was proposed.

Computational modeling of adhesion-mediated multicellular assembly

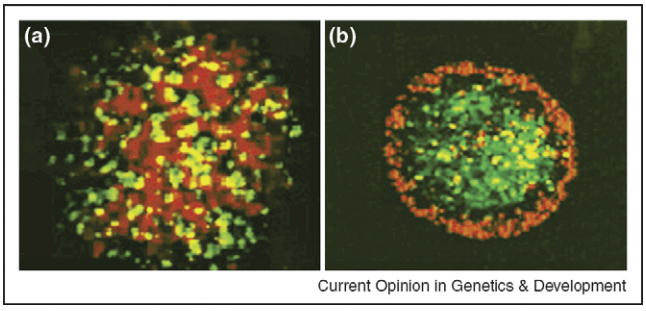
Multicellular rearrangement caused by differential adhesion, alone or together with other guiding principles, has been modeled using both discrete [16] and continuous [17] computational models. Glazier and colleagues have developed a three-dimensional lattice-based simulation known as the Cellular Potts or Glazier–Graner–Hogeweg model in which spatially extended 'cells' rearrange as a result of membrane fluctuations to minimize their effective free energy, augmented, if the user chooses, by terms that imitate other morphogenetic agents. The latter include chemotaxis, haptotaxis, changes in cell shape and size, cell division, cell death, and changes in cell response due to differentiation. They also permit the introduction of Turing-like reaction–diffusion-mediated mechanisms. This

Figure 1



Aggregate surface tension arises from cell–cell adhesion. Data points expressing the relationship between cadherin expression level and aggregate surface tension fall almost exactly on a straight line that passes very close to the graph's origin, intersecting the *Y*-axis (representing zero cadherin expression) at the very low surface tension value of 0.32 erg/cm^2. This demonstrates that aggregate surface tension is a linear expression of cell–cell adhesiveness (reprinted from [15••], with permission from Elsevier).

Figure 2



Sorting out of subclones differing only in expression level of a given cadherin. Two N-cad-transfected L cell subclones, expressing N-cad at their surfaces in the ratio of 2.4:1, were stained with red and green fluorescent membrane-intercalating dyes, mixed in equal proportions and cultured as hanging drops. **(a)** Confocal optical section through an aggregate after 4 hours of incubation, showing initial cell mixture. **(b)** Confocal optical section through another aggregate after 24 hours of incubation. As predicted by the DAH, the cell line expressing the lower level of N-cad (surface tension ~2.4 erg/cm^2), labeled red, segregates from and envelops the cell line expressing higher amounts of N-cad (surface tension ~5.6 erg/cm^2), labeled green. This demonstrates that cell sorting does not require (although it does, of course, permit) qualitative differences in cell–cell 'recognition specificity' (reprinted from [15••], with permission from Elsevier).

modeling technique is available as an open-source modeling environment, CompuCell3D, which allows users to specify complex developmental-biology simulations using a simple XML syntax, without the need for complex computer programming. The program has been tested in a variety of simulations, for example skeletal patterning in the avian limb bud [18•,19]. The source code, operation instructions, and simulation-development tutorials for CompuCell3D are available on its web site http://simtk.org/home/compucell3d.

Kafer et al. [20] formulate a model directed toward *Dictyostelium* morphogenesis incorporating both differential adhesion and chemotaxis. They show that the latter factor can speed up cell sorting and that the direction of cell motion can depend upon such parameters as cell size, adhesive strengths, and other system properties. Mombach et al. [21] simulate the rounding up of both chick embryonic and *Hydra* cell aggregates, focusing on the effect of aggregate size upon relaxation time, whereas Moreira and Deutsch [22] present a cellular automaton simulation showing that 'differential cellular adhesion together with an appropriate mechanism of stem cell regulation are able to reproduce the main characteristics of (pigment) pattern formation in the wild type zebrafish and mutants.'

E-cadherin in teleost epiboly

Before the onset of gastrulation movements, the zebrafish blastula consists of a large group of blastomeres — the blastoderm — perched upon the syncytial yolk cell at the animal pole. Gastrulation begins with *epiboly*, by which the blastoderm spreads over the yolk cell toward the vegetal pole. The blastoderm itself is subdivided into a superficial 'enveloping layer' (EVL) covering the deep cells (DCs). Within the yolk cell are recognizable the yolk syncytial layer (YSL), over which the blastoderm spreads, and the nonnucleated yolk cytoplasmic layer (YCL). During epiboly, the thinning-out EVL, DCs, and YSL all spread over the yolk cell until the latter is completely enveloped (reviewed in [23]). Kane et al. [24•] and Shimizu et al. [25•] have both produced evidence for a role of E-cadherin in teleost epiboly through the use of mutants that inhibit the later stages of epiboly. Most of these mutants map to a single locus, *hab*, encoding a homolog of E-cadherin, expressed both maternally and zygotically. *In situ* hybridization at late epiboly showed *hab* expression to be highest in the epithelioid EVL, grading radially to lower levels in the DCs [24•]. Thinning and spreading of the exterior layer was found to be associated with radial intercalation of subsurface cells into the superficial layer, where they then flatten. Wild-type deep cells entering the superficial layer remain there, whereas mutant cells often recross the interface back into the subsurface layer. It is tempting to speculate that wild-type cells enter the surface layer by upregulating E-cadherin to the level of their new neighbors, thereby becoming miscible with the latter and immiscible with their old neighbors. Antisense oligonucleotides that produce specific splicing defects in the *hab* mRNA phenocopy the defective epiboly [24•] as do antisense morpholino oligonucleotides against E-cadherin [25•]. These and other observations indicate that intercalation of originally lower-cadherin-expressing deep cells into the higher-cadherin-expressing enveloping layer, necessary for the blastoderm to spread over the yolk, is an E-cadherin-mediated morphogenetic movement.

Rhombomere domain boundaries

Differential cell–cell adhesion provides a simple mechanism for producing and maintaining boundaries between different cell populations. This is not to say, of course, that any particular compartment boundary is actually specified through differential adhesion; that must always be a matter for empirical investigation. A case in point is that of rhombomere boundaries in the developing vertebrate hindbrain, in which neighboring members of a metameric series of hindbrain segments resist cellular intermixing (reviewed in [26,27]). Alternating rhombomeres differ in their expression of members of the Eph family of receptor tyrosine kinases and of ephrins, their cell surface ligands. In the mouse, for example, EphA4 is expressed in forming rhombomeres #3 and #5 while its ligand, ephrin-B2, is expressed in rhombomeres #2, #4, and #6. At nascent rhombomere boundaries, cells expressing receptor versus ligand are apposed. Moreover, elimination of EphA4 and ephrin-B2a with antisense morpholinos (MO) causes disruption of rhombomere boundaries in zebrafish [28••]. Because ephrins and Eph receptors function as repulsive guidance molecules for migrating axon growth cones and neural crest cells, mutual repulsion (as opposed to differential adhesion) between cells bearing them at rhombomere boundaries has been considered as a likely mechanism governing their segregation. An argument raised against the operation of differential adhesion in this instance was that 'an adhesion protein with alternating segmental expression has not been discovered.' This argument, however, has been nullified by the demonstration that even a moderate difference in the expression level of a single kind of cell–cell adhesion molecule is sufficient to render two otherwise identical domains immiscible (reviewed in [4]; see Figure 2). Moreover, it has been shown that Eph receptor activation can increase cell adhesion to extracellular matrix via integrins. There is evidence that Eph receptor activation restricts growth cone movement by causing a local depolymerization of the actin cytoskeleton, leading to a growth cone collapse. Depolymerization of junction-associated actin also greatly weakens cadherin-mediated cell–cell adhesions, pointing to the possibility that modulation of adhesion might play an important role in the Eph-ephrin-mediated establishment of domain boundaries. This possibility has received support from the observation [28••] that, in mosaic embryos, EphA4-expressing cells and EphA4MO cells segregate from one another, the latter sorting out to the periphery

of rhombomeres #3 and #5 after transplantation into a wild-type host. In the reciprocal transplantation experiment, wild-type cells transplanted into an EphA4MO host formed cohesive clumps with sharp boundaries within rhombomeres #3 and #5. Both of these results are those to be expected if the EphA4MO cells have significantly lower cohesivity than their wild-type equivalents and neither result would be expected on the premise that these Eph-ephrin-based segregations are specified by repulsive interactions alone. Cooke et al. propose that ''EphA4-dependent adhesion contributes to the cell sorting process that underlies rhombomere-boundary formation.''

Epithelial–mesenchymal transitions and malignant invasion

What initiates an EMT?

In an epithelial–mesenchymal transition (EMT), an epithelium loses its characteristic intercellular junctions and its apicobasal polarity. Its cells adopt a fibroblast-like form, become motile and move away. Examples are cellular gastrular ingression at the amniote primitive streak and emigration of the vertebrate neural crest. At the molecular level, epithelial markers such as E-cadherin and cytokeratins are downregulated and β-catenin is relegated to the nucleus, whereas mesenchymal markers such as vimentin, fibronectin, and smooth muscle actin are upregulated (reviewed in [29,30]). Pleiotrophin, a ligand for the receptor protein tyrosine phosphatase βζA, inactivates the latter, allowing increased phosphorylation of its many substrates. This removes cadherin function, reorganizes the actin cytoskeleton, and brings about an EMT in U373 human glioblastoma cells [31•].

A number of growth and transcription factors can also initiate an EMT. Fibroblasts resulting from an EMT produce fibroblast-specific protein 1 (FSP1), also known as S100A4, encoded by a gene whose promoter contains an element called fibroblast transcription site-1 (FTS-1). Using a mouse kidney proximal tubular cell line, Venkov et al. [32••] have reported that the binding of a complex of two proteins, CArG-box binding factor-A (CBF-A) and KRAB-associated protein-1 (KAP-1) to this site initiates transcription of the FSP1 gene and initiation of an EMT with all its molecular concomitants. Moreover, the FTS-1 response element is also found in the promoter regions of E-cadherin, β-catenin, desmoplakin, vimentin, ZO-1, snail, twist, rho, α-smooth muscle actin, and many other genes upregulated or downregulated in EMT. The authors conclude that 'the finding that the CBF-A/KAP-1/FTS-1 complex is an activator of the genes encoding the EMT proteome suggests that it is an early proximal regulator, if not a candidate master gene' in the EMT program. Unlike increased cell motility, the morphological changes characteristic of EMT, including cytoskeletal reorganization and disruption of cell junctions are, however, independent of cadherin switching [33••].

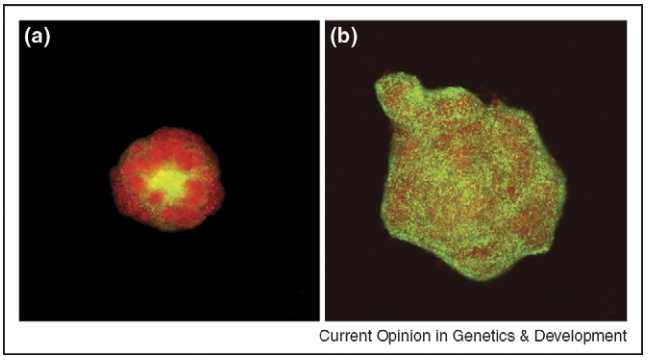
Malignant invasion: cell sorting in reverse?

The morphogenetic changes of EMT have invited comparison with malignant invasion, not without objection [34], and it has been asked whether malignant invasion can be seen as a case of sorting out in reverse, in which the intercellular adhesive differentials favor intermixing rather than segregation (Figure 3) [4,35]. Indeed, the E-cadherin gene has been characterized as a tumor suppressor gene and low or absent E-cadherin expression is considered to be a hallmark of malignancy in carcinomas of many kinds (reviewed in [4]).

Tissue cohesivity and invasiveness

The relationship between invasive potential and cell–cell cohesivity has begun to be examined. Winters et al. [36••] measured the surface tensions of aggregates of three malignant astrocytoma lines of different *in vitro* invasive potentials, the latter measured by Matrigel transfilter invasion. A strong inverse correlation was found between aggregate cohesivity and invasion into Matrigel, but evidence was not found to attribute these cell–cell adhesions to cadherins. Using nine brain tumor cell lines, Hegedus et al. [37] compared the pattern of invasion into type 1 collagen gels with aggregate surface tension, cadherin expression level, aggregation rate in stirred suspension, malignancy grade, content of matrix metalloproteinase (MMP-1, MMP-2, MMP-3 and MMP-9), and of tissue inhibitor of metalloproteinase (TIMP)-1. No simple correlation was found between invasion pattern (which was complex) and either the magnitude of aggregate surface tension or MMP expression, although it was concluded that 'a consistent interpretation of the observed invasion patterns can be given by simultaneously considering both tissue cohesivity and cell–matrix interactions.'

Figure 3



Cell sorting versus intermixing in prostate cancer. Normal Rat 2 fibroblast cells were co-aggregated for 24 hours in hanging drop cultures with rat prostate cancer cells of two kinds. When combined with the noninvasive Dunning rat prostate cancer AT-2 cell line, they sorted out into sharply demarcated phases, with the fibroblasts occupying the internal position **(a)**. When combined with the highly invasive MLL rat prostate cancer cell line, the two cell populations intermixed to a great degree **(b)** (reprinted from [4], with permission from the publisher).

Conclusion

Following Holtfreter's work in the mid-20th century [1,2], there was a period of struggle to make sense of the phenomena like the sorting out of embryonic cells to reconstruct life-like structures [38,39]. One popular paradigm took sorting out to be a kind of rejection of nonself, invoking the operation of recognition factors unique to each kind of tissue. Investigators anxious to test that hypothesis, the DAH and others improvised various bootstrap assays of 'cell adhesiveness.' Even some currently cited examples of homotypic cadherin specificity are based upon kinetic measurements in which shear forces discourage the initiation of more slowly formed adhesions, presenting a false impression of disaffinity (discussed in [40]). However, the term 'adhesiveness' is not a single, rigorously defined entity like 'mass' or 'velocity' but is used to describe many different aspects of material association. Widely employed assays of cell–cell 'adhesiveness' have measured cell aggregation rates in sheared suspensions or forces required to separate cohering cells, neither of which measures the equilibrium binding energies of mature intercellular adhesions invoked by the DAH. The latter are simulated in the successful Cellular Potts models discussed above and quantified in the aggregate surface tensions measured in [15••,21,36••,37]. These accurately reflect intercellular adhesive intensities, correlate perfectly with tissue layering patterns [41], and are increasingly adding to the understanding of a range of morphogenetic phenomena.

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