Sophistications of cell sorting

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The self-sorting of early embryonic cells is mediated not only by pure differential adhesion but also involves other processes. Direct force measurements reveal the role of cell-cortical tension, whereas epithelial-wrapping dominates the sorting of enclosed mesenchymal cells.

Everyone knows that oil and water do not mix. More interestingly, when shaken together, oil and water self-organize into two layers. In 1955, Townes and Holtfreter famously demonstrated a similar 'unmixing' when cells from the three germ layers of the early vertebrate embryo were combined[1]. They hypothesized that embryos rely on differential cell 'affinity' to organize these layers during normal development[1,2]. Using similar assays (Fig. 1), Steinberg proposed that the forces organizing the cells are closely analogous to those organizing the molecules of immiscible fluids[3] and put forward the Differential Adhesion Hypothesis (DAH): in engulfment and unmixing assays of two cell populations, the more cohesive one (with the stronger cell–cell, or homotypic, adhesion) would distribute in the middle and the less cohesive one on the outside. Differential expression of the cell adhesion molecule cadherin is sufficient to drive cell sorting[4], exactly as predicted by the model[5]. However, to some the DAH was only one of the possible mechanisms and, despite acceptance as the conventional wisdom, DAH has remained controversial[6]. In a lucid critique of Steinberg, Harris proposed a Differential Surface Contraction (DSC) model in which cortical tension (the force generated within cells parallel to their surface) rather than adhesion between cells *per se*, could drive cell sorting[7].

On page 429 of this issue, Krieg *et al.*[8] tested these two models by directly measuring the adhesiveness and cortical tension of cells from the three germ layers of zebrafish embryos using an atomic force microscope — a tiny probe mounted and calibrated so that its bending by an object at its tip can be measured and the corresponding bending force determined (Fig. 2). They measured cell adhesion by attaching one cell to the end of the microscope probe and a second cell to a fixed substrate below, bringing the two cells together and monitoring the force required to pull them apart. They also determined cortical tension by measuring the force needed for a hard bead attached to the end of the probe to deform the surface of a cell attached to the surface below. Measurements for cells from the ectoderm (Ec), mesoderm (M) and endoderm (En) showed that homotypic adhesion was stronger in mesoderm than in ectoderm, whereas endoderm values were in between (AdM > AdEn > AdEc). As expected, adhesion was calcium-dependent and correlated with cadherin expression at the surface. The hierarchy of cortical tension values was different: CtEc > CtM > CtEn. In pairwise sorting assays, ectoderm cells were always in the middle, opposite to what would be predicted by DAH, under which their low homotypic adhesion would place them on the outside. The central position of ectoderm cells within the aggregates correlated instead with higher cortical tension. Mesoderm cells were also surrounded by endoderm, extending the correlation. This would seem to be a triumph of DSC (and similar models[6]) over DAH, at least for these cell types, although it fails to reproduce the *in vivo* configuration (ectoderm outside and endoderm innermost) and mechanisms involving differential adhesion and cortical tension are not mutually exclusive.

Apart from requiring a change in what textbooks say about the importance of differential adhesion in cell self-sorting, the work of Krieg *et al.* leads us to reconsider the role of different cell-biological processes in self-sorting. For a start, the cortical actomyosin cytoskeleton becomes more significant. Krieg *et al.* showed that disruption of this network, using blebbistatin (an inhibitor of myosin II activity) and dominant-negative Rho kinase, blocks cell self-sorting. This suggests that sorting is more akin to active migration, in which changes in cell shape are crucial, whereas according to the DAH, cells are, effectively, structureless units. It is no surprise, then, that integrins, adhesion molecules associated with migration, can be crucial for cell sorting[9]. There are also instances when β-catenin regulation

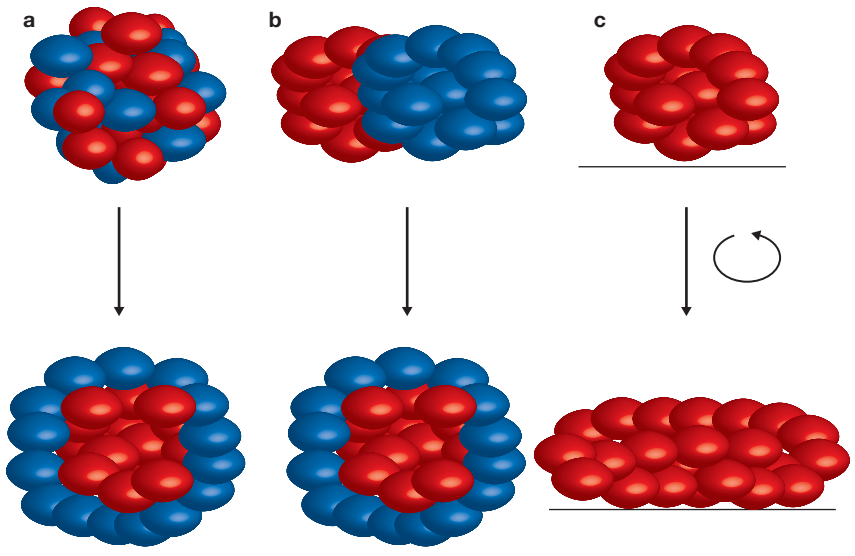

Figure 1 Classical assays for cell sorting and apparent surface tension. (**a**) Cell aggregates made up from two different cell types unmix (self-sort) concentrically. (**b**) Aggregates of two different cell types are juxtaposed and one engulfs the other. (**c**) Cell aggregates of a single type are centrifuged to flatten them and the deformation they undergo provides a measure of apparent aggregate surface tension (summed homotypic cell adhesion and/or cell-cortical tension).

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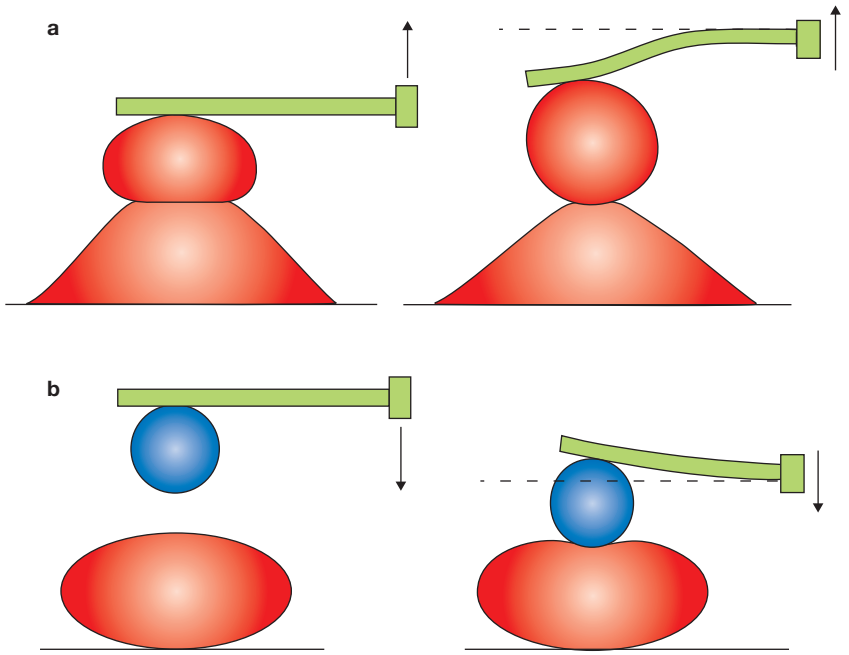

Figure 2 Direct measurement of cell adhesion and cell deformability with an atomic force microscope. (**a**) A cell attached to a probe with Concavalin A is brought in contact with a cell similarly attached to a substrate. The force required to pull cells apart is measured as a function of the deformation of the probe (detected by the angle of deflection of a laser beam, data not shown) and reflects the strength of cell–cell adhesion. (**b**) A hard bead (blue) attached to a probe is slowly brought down onto the surface of a cell until the latter is deformed. Deformation by the probe measures the resistive force, which is a function of the cell-cortical tension.

of cadherin, the adhesion molecule traditionally thought to account for self-sorting is, at most, secondary[10]. Second, the DSC model predicts that contractility must be different and higher at cell-medium interfaces than at internal interfaces[7]. In other words, cortical tension should be localized by cell polarization. Krieg *et al.* addressed this prediction in two ways. First, using computer models of cell sorting with and without localization of cortical tension, they found that sorting operates only when the cortical tension is localized. Second, by examining actin in real-cell aggregates, they showed that it was enriched at the cell-medium interface; however, they did not look at heterotypic cell interfaces, which would have been a useful addition.

Townes and Holfreter[1] had already hinted at the importance of active cell-surface contraction for self-sorting, when they noticed that neurectodermal cells, whether as single cells or organized as sheets, were engulfed by endodermal cells. Neurectodermal sheets penetrated an endodermal mass by infolding or invagination, recapitulating the rolling-up neurulation movements that make a tubular spinal cord. They proposed that the same mechanism may drive both invagination and cell sorting. Indeed, actomyosin-driven apical contraction is now recognized as the main mechanism of epithelial folding in the neural plate and elsewhere, and support for actomyosin-dependent DSC provides a mechanistic link between cell self-sorting and epithelial folding.

Despite the support Krieg *et al.* provide for the DSC hypothesis, how can one explain the peculiar inside-out arrangement observed by the authors in their *in vitro* experiments, in which the ectoderm or the mesoderm are inside the aggregates (one does not need to know much about developmental biology, but merely a little Greek, to know that ectoderm should be on the outside and endoderm on the inside of embryos, with mesoderm in the middle)? To address this issue, the authors attempted to assay cell self-sorting '*in vivo*' using transplantation experiments. They conclude that strong interactions of germ-layer cells with the yolk and the enveloping layer cells invert the inside-out cell sorting seen *in vitro*, thereby producing the endo-in/ecto-out arrangement that we and the fish prefer. Whether this zebrafish-specific dominance of yolk and enveloping-layer cell interactions significantly undermines the authors' case for DSC remains to be seen. Moreover, other challenges should be addressed before applying these findings *in vivo*, when one considers that: first, the quality and strength of adhesions change with contact time (a factor barely taken into account in the authors' adhesion assays); second, cortical tension is only one of several factors determining cell deformability; third, mesodermal cells in zebrafish move as a loose population; fourth, the authors examined the actin distribution at late-differentiating stages rather than during sorting; fifth, that adhesion of sheets of cells may be different from that of individual cells. Krieg *et al.* make a respectable effort, but there remains the inevitable compromise between beautiful measurements on few cells and biological relevance of messy tissues that is the cell biologist's Uncertainty Principle.

This brings us to a recent article by Ninomiya and Winklbauer[11], which extends their previous finding that tissue elongation in mesodermal explants and cell aggregates is enhanced by a wrapping of epithelium[12]. Although quite dramatic elongation is possible without it[13], tissue elongation within an epithelial wrapping occurs normally during development and, of course, contrasts with the rounding-up of cell aggregates *in vitro*. Ninomiya and Winklbauer showed that epithelial wrapping not only enhanced elongation of mesodermal tissue but also increased the centrifugation-induced flattening of rounded-up ectodermal cell aggregates and modified the concentric engulfment and self-sorting of mixed cell types to give a linear arrangement of tissues. They explain their observations as a reduction in surface tension of the aggregates by adhesion to the epithelium. Forces along the surface of cell aggregates resemble fluid surface tension and can be modelled as such, whether driven by adhesion (as with molecules in fluids, and as in DAH) or by cortical tension (as in DSC). However, the DAH-flavoured terminology used by Ninomiya and Winklbauer is secondary to the observation that the epithelium is important and presumably facilitates tissue elongation by reducing the tensions (whether intracellular or intercellular) that drive concentric cell self-sorting and aggregate rounding. Of course, epithelial signalling and polarization of mesenchymal migratory behaviour may also be involved.

In a further instance of how an epithelium breaks the usual sorting rules, Ninomiya and Winklbauer prepared aggregates, in which untreated ectodermal cells were mixed with similar cells, and in which they expressed M-PAPC, a paraxial protocadherin derivative that reduces

cadherin-dependent adhesion[14]. As expected, M-PAPC-expressing cells, with their reduced cohesion, sorted to the outside. Unexpectedly, wrapping with normal epithelial ectoderm sent M-PAPC-expressing cells to the inside, whereas wrapping with M-PAPC-expressing ectoderm kept them on the outside. In short, cells with the same M-PAPC status stick together, suggesting that more complex cell interactions may influence the result of these experiments[15].

The exciting aspect of these papers is that, building on the brilliance of Holtfreter, Steinberg, Harris and others, they represent a new chapter in the analysis of morphogenesis, in which high-resolution force measurement and molecular analysis will be combined with more physiological and multi-component models. Thanks to this type of analysis, the complexity of real embryos is gradually coming within the grasp of hardcore cell biology.

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BRCA1 and stem cells: tumour typecasting

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Phenotypic variation between tumour types is likely to reflect the nature of the cell of origin and the genes involved in pathogenesis. Compared with most sporadic breast cancers, those arising in carriers of *BRCA1* mutations usually have distinctive pathological characteristics. A new study suggests that a role for *BRCA1* in the determination of stem-cell fate may explain this phenomenon.

The ducts and lobules of the human breast are made up of two morphologically distinct cell populations: the inner, milk producing luminal epithelial cells, which express low molecular-weight cytokeratins and the oestrogen receptor (ER), and the outer, supporting basal myoepithelial cells, which express high molecular-weight cytokeratins and smooth muscle markers[1]. There is considerable evidence for a differentiation hierarchy within the breast (Fig. 1) and a common cell of origin of luminal and myoepithelial cells — a mammary stem cell. Similar to the differentiated myoepithelial cells, the stem cell is thought to have a basal phenotype[1,2]. Tumours arising in carriers of germ-line mutations in the breast cancer susceptibility gene *BRCA1* are commonly of the basal subtype, whereas sporadic cases are much more likely to be luminal in nature[2]. Because of this, it has been suggested previously that *BRCA1* may be a stem-cell regulator[3] and that basal-like carcinomas originate from stem cells that have a block in differentiation[4]. Wicha and colleagues now provide support for this notion by showing that loss of *BRCA1* may limit the differentiation potential of mammary stem/progenitor cells, thus preventing formation of ER-positive luminal epithelial cells[5].

Germline *BRCA1* gene mutations cause a significantly increased risk of breast and ovarian cancer. BRCA1 has been implicated in a plethora of cellular processes, including the response to DNA damage, X-chromosome inactivation, ubiquitination and chromatin remodelling[6]. Furthermore, a number of lines of evidence indicate that BRCA1 functions in the regulation of transcription; indeed, BRCA1 seems to regulate the expression of ER α directly[7]. This is consistent with the clinical observation that most cancers developing in carriers of *BRCA1* mutations have an ER-negative basal-like phenotype[2,8]. Moreover, it has been suggested that these tumours may recapitulate some features of breast stem cells[2,8]. However, a causal relationship between BRCA1 dysfunction and the basal-like phenotype remains to be demonstrated, and whether BRCA1 inactivation blocks the differentiation of stem cells or leads to the re-acquisition of stem-cell-like properties has so far been a matter of contention.

Wicha and colleagues used a number of approaches to investigate how BRCA1 may be involved in stem-cell regulation or lineage determination[5]. First, they noted that BRCA1 levels were elevated in mammary cells that were cultured *in vitro* as mammospheres (balls of undifferentiated mammary epithelial cells grown in suspension culture, which can be serially passaged into secondary and tertiary spheres). There is good evidence that only mammary stem cells can generate these structures and that they are composed of both undifferentiated stem and early progenitor cells[9]. Although knockdown of *BRCA1* expression, mediated by RNA interference (RNAi), had no effect on primary mammosphere formation, passaging was severely affected. An assay for stem cells using the Aldefluor stain, which is cleaved by the breast stem-cell marker ALDH1 to generate a fluorescent product[9], indicated that the number of such cells was increased. This was interpreted as an indication that BRCA1 may be involved in mammary stem-cell self-renewal but not progenitor-cell proliferation. Furthermore, RNAi-mediated *BRCA1* knockdown resulted in a 10-fold reduction in cells expressing ER and an increase in the number of cells with

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