Cadherin-dependent differential cell adhesion in *Xenopus* causes cell sorting in vitro but not in the embryo

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Summary

Adhesion differences between cell populations are in principle a source of strong morphogenetic forces promoting cell sorting, boundary formation and tissue positioning, and cadherins are main mediators of cell adhesion. However, a direct link between cadherin expression, differential adhesion and morphogenesis has not yet been determined for a specific process in vivo. To identify such a connection, we modulated the expression of C-cadherin in the *Xenopus laevis* gastrula, and combined this with direct measurements of cell adhesion-related parameters. Our results show that gastrulation is surprisingly tolerant of overall changes in adhesion. Also, as expected, experimentally generated, cadherin-based adhesion differences promote cell sorting in vitro. Importantly, however, such differences do not lead to the sorting of cells in the embryo, showing that differential adhesion is not sufficient to drive morphogenesis in this system. Compensatory recruitment of cadherin protein to contacts between cadherin-deprived and -overexpressing cells could contribute to the prevention of sorting in vivo.

Key words: Cadherin, Adhesion, Cell sorting, Boundary formation, *Xenopus laevis*, Gastrula

Introduction

The notion that cell sorting, tissue boundary formation and tissue positioning is based on graded, quantitative differences in cell–cell adhesion was introduced 50 years ago (Steinberg, 1963), and is now widespread in cell biology. The identification of cadherins as major cell adhesion molecules has provided a molecular basis for this concept, and differential cadherin expression has been linked to sorting and boundary formation (Gumbiner, 2005; Nelson, 2008; Shapiro and Weis, 2009; Stepniak et al., 2009). Early on, the concept of tissue surface tension was applied to explain effects of differential adhesion (Steinberg, 1970). By definition, surface tension is equal to half the work required, per unit area, to separate a liquid into two parts, and tissue surface tension is thus a measure of tissue cohesion, which in turn is related to the strength of cell–cell adhesion. For the case of adhesion solely mediated by cadherin, direct proportionality between cadherin expression and cell aggregate surface tension has been demonstrated (Steinberg and Takeichi, 1994; Foty and Steinberg, 2005).

Recently, renewed interest in tissue surface tension has led to its reinterpretation in terms of cell cortical tensions. In fact, the regulation of mechanical tension in the cortical cytoskeleton is suggested to be an integral part of cell–cell adhesion. In an isolated cell, cortical tension minimizes the surface area of the cell by rounding it up into a sphere. For two cells to attach, tension has to be lowered in the contact area to allow the cells to flatten against each other. This difference between tensions at free and contacting surfaces is directly related to tissue surface tension of cell aggregates (Harris, 1976; Brodland, 2002; Krieg et al., 2008; Manning et al., 2010; Maitre and Heisenberg, 2011). Importantly, the basic role of surface tension in sorting and boundary formation is not affected by this interpretation (Brodland, 2002; Krieg et al., 2008). However, a full correlation between cadherin expression, differential adhesion as expressed in surface tension differences, and sorting has not been demonstrated yet for an in vivo process. To provide such a connection, we combined the knockdown of a main adhesion molecule of the Xenopus *laevis* gastrula, C-cadherin (also termed EP-cadherin) (Choi et al., 1990; Ginsberg et al., 1991), with measurements of adhesion-related parameters. Although other mediators of adhesion are present in the gastrula (Winklbauer, 2009), C-cadherin modulation effectively changed these parameters and is therefore sufficient to test their role in morphogenesis. We found that differential adhesion and corresponding differences in surface tension do not lead to cell sorting in the intact *Xenopus* embryo.

Results and Discussion

Moderate attenuation of C-cadherin function affects cell adhesion but not gastrulation

Initial blastomere adhesion is mediated by maternal C-cadherin (Heasman et al., 1994). As this supply is replaced by zygotic protein, adhesion becomes sensitive to C-cadherin morpholino

antisense oligonucleotide (C-cad-MO). To examine the effects of aberrant differential adhesion, we first characterized the consequences of C-cadherin knockdown. After injection of 40 ng C-cad-MO, gastrulation became abnormal and embryos disintegrated during the neurula stage. Development was rescued by co-injection of C-cadherin mRNA (supplementary material Fig. S1C–E). In C-cad-MO-injected ectoderm, C-cadherin expression was diminished (supplementary material Fig. S2B–B″). Co-injection of C-cadherin mRNA rescued expression (supplementary material Fig. S2C–C″), and mRNA alone increased membrane staining (supplementary material Fig. S2A–A″). Classic cadherins bind to β-catenin, and as expected (Schneider et al., 1993; Fagotto and Gumbiner, 1994; Kurth et al., 1999), membrane β-catenin paralleled C-cadherin expression (supplementary material Fig. S2E–G″), indicating that other cadherins do not compensate for changes in C-cadherin levels. Also, expression of E-cadherin, XB/U-cadherin, cadherin-11 and protocadherins PAPC and PCNS (Winklbauer, 2009) (also known as protocadherin-8-like) were not altered by C-cadherin knockdown or overexpression (Fig. 1I). A cytoplasmically deleted EPΔC construct (Wacker et al., 2000) caused disintegration of the gastrula (supplementary material Fig. S1A,B). It led to increased C-cadherin staining, but because of the lack of the cytoplasmic domain, not to increased β-catenin

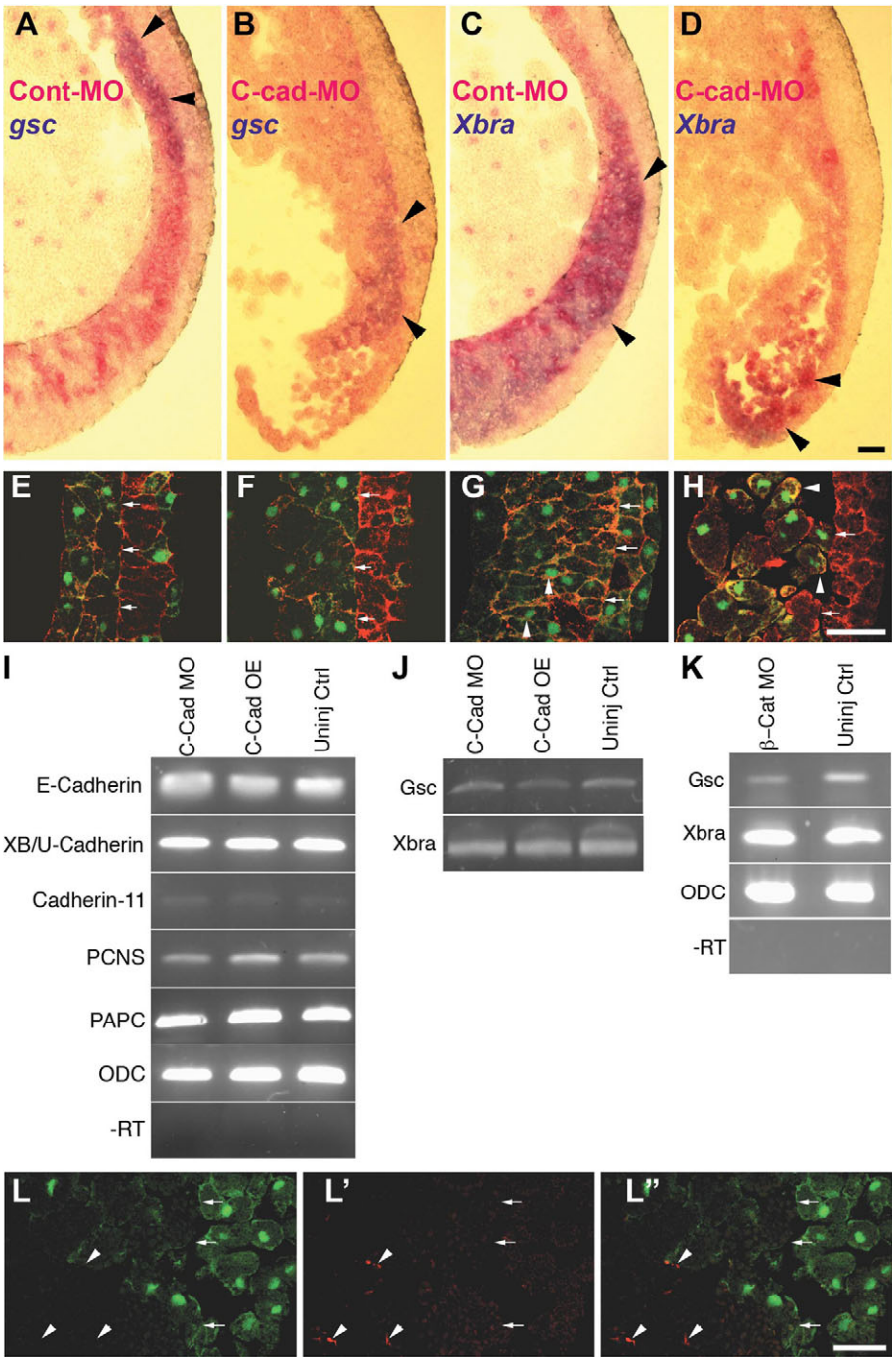

Fig. 1. Effects of C-cad-MO. (**A**–**H**) Late gastrula sagittal sections, after dorsovegetal injection of control MO or C-cad-MO (40 ng/embryo). (A–D) In situ hybridization. *gsc*-expressing anterior mesoderm (arrowheads, A,B, dark purple); *Xbra*-expressing chordamesoderm (arrowheads, C,D). Injected cells, magenta. (E–H) High magnification, injected mesoderm cells (green) counterstained for actin (red). Brachet's cleft (arrows) separates mesoderm (left) and ectoderm (right). Tightly packed cells in control MO (E) and C-cad-MO (F) anterior mesoderm. Chordamesoderm cells are spindle-shaped in controls (arrowheads, G), but rounded in C-cad-MO embryos (arrowheads, H). (**I**) RT-PCR for cadherins and protocadherins in embryos injected with C-cad-MO (40 ng) or C-cadherin mRNA (2 ng). (**J**,**K**) RT-PCR to show *gsc* and *Xbra* expression after C-cad-MO or C-cadherin mRNA (J) or β-catenin MO (K; 6 ng/embryo) injection. (**L**–**L″**) β-catenin membrane localization is reduced by C-cad-MO (10 ng) in anterior mesoderm. β-catenin staining (L′, red, arrowheads) in C-cad-MO cells co-injected with GFP mRNA (L, green; L″, merged). Anterior mesoderm is to the left of Brachet's cleft (arrows). Scale bars: 50 μm.

staining (supplementary material Fig. S2D–D″,H–H″). No effect of C-cad-MO or EPΔC on gastrula epithelial structure was observed (supplementary material Fig. S2I–Q).

The chordamesoderm of controls, marked by *Xenopus brachyury* (*Xbra*; also known as *t*), elongated by convergent extension (Keller, 2002), whereas the *goosecoid* (*gsc*)-marked prechordal mesoderm spread on the ectoderm (Damm and Winklbauer, 2011) (Fig. 1A,C,E,G). In C-cad-MO embryos, the markers were still expressed (Fig. 1B,D), but chordamesoderm formed a loose cell mass that did not undergo convergent extension and thus lacked the dorsal accumulation of *Xbra*-expressing cells (Fig. 1D,H). Although mesoderm positioning was altered, neither *gsc* nor *Xbra* overall expression was changed by C-cadherin knockdown or overexpression (Fig. 1J), consistent with an absence of cell fate changes. However, *gsc* expression was reduced by β-catenin knockdown (Fig. 1K), as expected (Heasman et al., 2000). Injected ectoderm cells were also loosely arranged (supplementary material Fig. S2B,F). Such cell detachment is expected for very low adhesion when tissue begins to dissociate. By contrast, prechordal mesoderm formed a compact layer (Fig. 1B,F), and leading edge mesendoderm (Fig. 1) and vegetal endoderm (not shown) were likewise little affected. Thus, C-cadherin knockdown visibly affects tissue cohesion in the animally derived ectoderm and chordamesoderm, but has no histologically discernible effect on vegetally derived tissues. Consistent with this, head induction by anterior mesendoderm was not affected by C-cad-MO (supplementary material Fig. S1G–K).

Despite the lack of visible effects, cadherin expression was diminished in anterior mesoderm by C-cad-MO (Fig. 1L–L″). This is seen from staining for β-catenin, indicating again that other classic cadherins are not expressed to compensate. To see whether functional compensation by other adhesion molecules took place, we determined the surface tensions of whole tissue explants (Luu et al., 2011). In anterior mesoderm and vegetal cell mass, surface tension was indeed lowered (Table 1), as expected from reduced cell–cell adhesion. Thus, a high dose of C-cad-MO affected C-cadherin function throughout the embryo, although histological effects of lowered tissue cohesion were seen only in ectoderm and chordamesoderm. Different degrees of redundancy of C-cadherin with other adhesion factors could explain this differential sensitivity.

Our results suggested that morphogenesis is affected when tissue cohesion is visibly impaired, but is normal when adhesion is reduced moderately. We used a lower dose of C-cad-MO to examine this further. At 10 ng/embryo (instead of 40 ng/embryo), the most conspicuous effect was a separation of inner and epithelial ectodermal layers, which was rescued by co-injection of C-cadherin mRNA (supplementary material Fig. S1L–N). Otherwise, almost normal larvae formed with only a moderate shortening of the body axis (supplementary material Fig. S1O–Q). Dissociated C-cad-MO cells showed reduced adhesion to C-cadherin protein (Fig. 2A–C). Adhesion was rescued by C-cadherin mRNA, and binding to fibronectin was not affected by C-cad-MO (Fig. 2C). C-cadherin overexpression barely increased adhesion. Tissue surface tension, measured in explants where cells were left in their normal tissue context (Luu et al., 2011), was also strongly diminished by C-cad-MO, rescued by co-injection of C-cadherin mRNA, and slightly increased by C-cadherin overexpression (Fig. 2D–G; Table 1). Thus, single cell and whole tissue measurements yielded similar results. By contrast, EPΔC expression did not diminish adhesion to cadherin, confirming earlier results (Brieher et al., 1996; Seifert et al., 2009), but it reduced surface tension as strongly as C-cad-MO (Table 1). In summary, tissue cohesion is two- to fourfold reduced by a moderate reduction of cadherin function, but development is only affected when very low adhesion causes tissues to dissociate.

Differential adhesiveness does not lead to cell sorting in the embryo

Although gastrulation tolerates overall changes in tissue cohesion, differences between regions could nevertheless be crucial. Cell sorting in response to differential adhesion, based on a quantitative difference in cadherin expression, has been demonstrated in vitro (Friedlander et al., 1989; Steinberg and Takeichi, 1994; Foty and Steinberg, 2005). We examined whether experimentally induced differences in cadherin expression and adhesion can also prompt cell sorting in the intact embryo.

We first confirmed that adhesion differences drive sorting in vitro. When mixed, GFP- and RFP-labeled ectoderm cells became randomly dispersed in reaggregates (Fig. 3A). Robust sorting occurred when C-cadherin mRNA-injected cells and C-cad-MO cells were mixed (Fig. 3B). The less adhesive C-cad-MO cells sorted to the surface, as expected (Steinberg, 1970). However, sorting boundaries were not distinct despite a fourfold difference in tissue surface tension (Table 1). When C-cadherin- and EPΔC-injected cells were mixed, no sorting occurred (Fig. 3C), at variance with the finding that tissue surface tension is lowered upon EPΔC expression. Also, EPΔC cells sorted from C-cad-MO cells (Fig. 3D), suggesting that during in vitro sorting, as in the single cell adhesion assay, EPΔC behaves like full-length C-cadherin. This discrepancy cautions against the use of such constructs as ‘dominant-negative’ cadherins.

We next examined the encounter of C-cadherin-overexpressing and C-cad-MO-injected cells in the intact embryo (Fig. 3E–G). Surprisingly, no sorting was observed in ectoderm of mosaic

Table 1. Surface tension values for *Xenopus* gastrula tissues (mJ/m^2)

	Ectoderm (*n*)	Anterior mesendoderm (*n*)	Vegetal endoderm (*n*)
Cont-MO	0.29±0.21 (9)	0.23±0.14 (7)	0.072±0.057 (20)
C-cad-MO[a]	0.11±0.05[b] (10)	0.060±0.017[b] (9)	0.044±0.018[b] (23)
C-cad mRNA	0.38±0.26 (9)		
C-cad-MO + mRNA	0.39±0.29 (9)		
EPΔC	0.12±0.10[b] (14)		
M-PAPC	0.21±0.12 (10)		

[a]10 ng C-cad-MO was used for the ectoderm assay and 40 ng for the anterior mesendoderm and vegetal endoderm assays.
[b]Values significantly different from cont-MO ($P<0.05$).

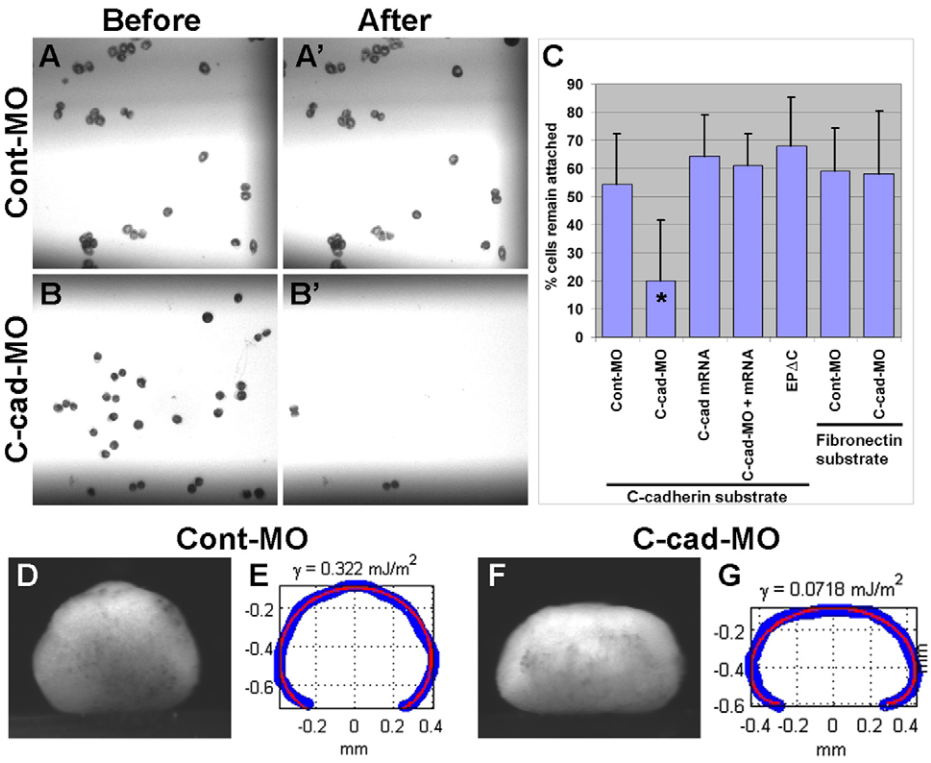

Fig. 2. Adhesiveness of ectoderm cells. (**A**–**C**) Cells on C-cadherin-Fc substratum before and after dish inversion: (A,A′) control MO-injected cells; (B,B′) C-cad-MO cells (10 ng/embryo); (C) fraction of cells remaining attached. *Ratio significantly different from control condition ($P<0.05$). (**D**–**G**) Surface tensions of ectoderm. (D,F) Side view of aggregates after 2 hours. (E,G) Drop shapes (red lines) were fitted to scanned outlines of aggregates (blue) by axisymmetric drop shape analysis (ADSA). (D,E) Control; (F,G) C-cad-MO aggregates.

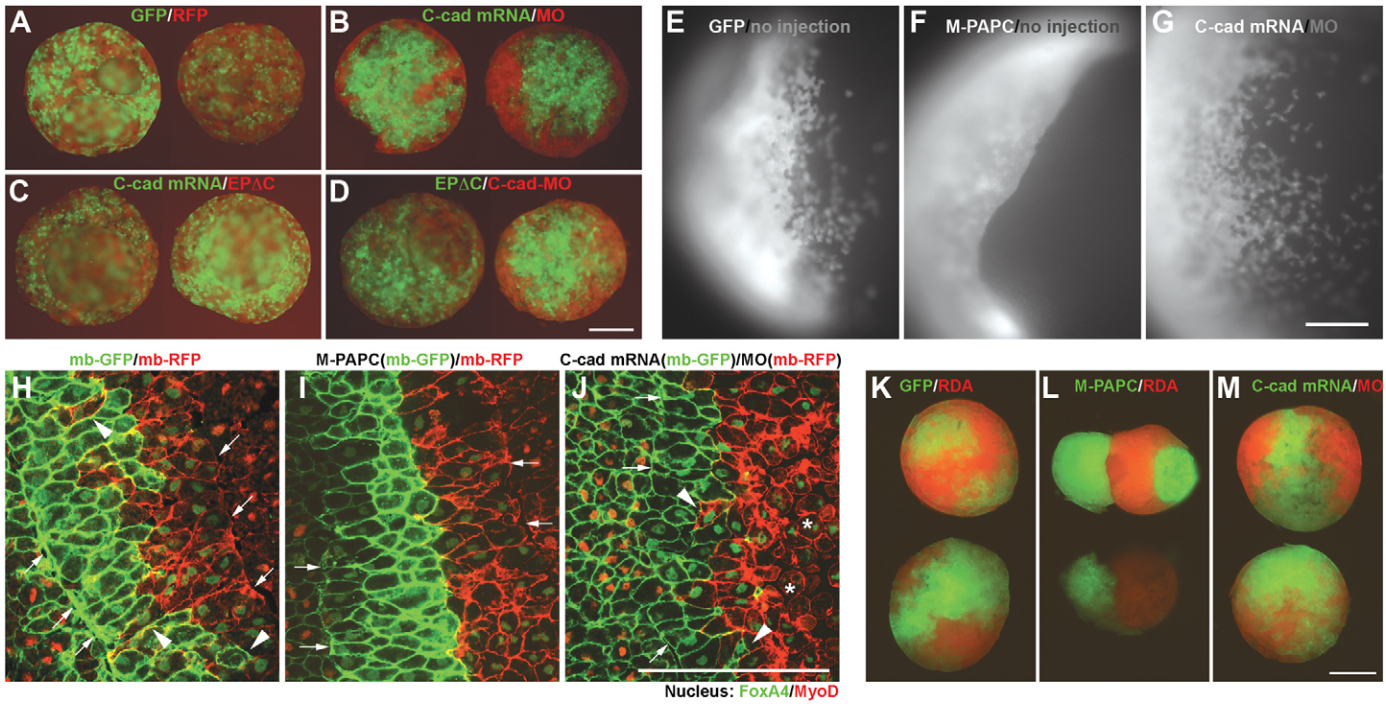

Fig. 3. Cadherin expression and sorting. (**A**–**D**) Mixed and reaggregated ectoderm cells at the neurula stage. C-cad-MO (10 ng/embryo) cells sort from C-cadherin or EPΔC cells (B,D); C-cadherin and EPΔC cells do not segregate (C), similar to controls (A). (**E**–**G**) Ventral ectoderm C-cadherin and C-cad-MO cells do not segregate in vivo (G), similar to controls (E); M-PAPC-injected cells separate from normal cells (F). (**H**–**J**) Chordamesoderm cell intercalation in the late gastrula. C-cadherin and C-cad-MO cells intercalate (J, arrowheads) as in controls (H, arrowheads). C-cad-MO cells are abnormally round (asterisk). Intercalation does not occur between M-PAPC-expressing and uninjected tissue (I). Nuclei were immunostained with FoxA4 (chordamesoderm) and MyoD (paraxial mesoderm) antibodies. Arrows indicate the notochord–somite boundary. (**K**–**M**) Explantation does not induce sorting. Inner ectoderm isolated from early gastrulae, cultured to the neurula stage. The C-cadherin–C-cad-MO boundary (M) resembles that of the negative (K), rather than positive (M–PAPC injection, L) control. Scale bars: 200 μm.

embryos. By contrast, cells expressing the extracellular domain of the paraxial protocadherin, M-PAPC (Kim et al., 1998), sorted completely from uninjected cells (Fig. 3F), although M-PAPC barely affected tissue surface tension (Table 1). The sorting observed with reaggregating cells is not due to in vitro conditions, as can be seen when explants from mosaic embryos are cultured in vitro (Fig. 3K–M).

Whereas cells in the ectoderm disperse randomly, chordamesoderm cells intercalate actively (Keller, 2002) (Fig. 3H). This interdigitation of cells continued when C-cadherin was overexpressed on one side of the tissue and knocked down on the other; expression of notochord and somitic mesoderm markers was not affected. On the C-cad-MO side, cells are abnormally rounded, indicating that the tissue was on the brink of dissociation (Fig. 3J). These observations confirm, first, that morphogenetic processes can operate close to the point of cohesion failure; and second, that differences in cohesion do not induce sorting in vivo. Again, sorting was induced by M-PAPC (Fig. 3I). Lack of in vivo sorting is also seen when mutant cadherins are use to interfere with endogenous cadherin function (Lee and Gumbiner, 1995; Broders and Thiery, 1995). However, the effects of such constructs can be ambiguous, as seen for EPΔC. By contrast, C-cadherin knockdown consistently reduces adhesion to cadherin, lowers surface tension, and prompts in vitro cell sorting.

In vitro, sorting occurs during initial cell contact at reaggregation (Fig. 4A,A′; supplementary material Movie 1). C-cadherin-overexpressing cells attach to each other within minutes and form aggregates of polygonal cells. By contrast, C-cad-MO cells remain round for prolonged times and become progressively excluded from the growing aggregate, until they eventually also attach to each other and to C-cadherin-overexpressing cells. When the interior of the aggregate was exposed by cutting it in half, 2 hours later, some C-cad-MO cells were still surrounded by C-cadherin-overexpressing cells, but no evidence for cell sorting after aggregation was seen (Fig. 4B,B′; supplementary material Movie 2). Thus, sorting in vitro occurs by differential aggregation. In vitro, cadherins are lost from the cell surface after dissociation (Tao et al., 2007), and the redeployment of cadherins at cell contacts, which is probably sensitive to cadherin expression levels, could limit the reaggreation rate. Once cell contacts are well established, sorting ceases, consistent with the absence of sorting in the embryo.

Putative mechanisms that prevent sorting due to differences in cadherin density

Differential adhesion-driven sorting assumes stable differences in adhesiveness between cells. The absence of sorting in the embryo could be due to an adjustment of cadherin densities of adjacent cells upon contact. YFP-tagged β-catenin was used to visualize cadherin levels at heterotypic contacts in a cell autonomous manner (supplementary material Fig. S3). Levels of YFP–β-catenin were chosen that did not lead to nuclear localization, avoiding activation of Wnt signaling.

In the ectoderm of mosaic embryos, β-catenin–YFP was increased in normal cells at contacts with EPΔC cells (Fig. 4D). Thus, cells locally upregulate cadherin in response to cadherin density on adjacent cells. Because EPΔC lacks a cytoplasmic domain, no increase of β-catenin–YFP density was expected on this side of the contact (Fig. 4C). In C-cadherin-overexpressing cells, the β-catenin–YFP signal was stronger at contacts with like cells compared with C-cad-MO cell contacts (Fig. 4E,F). In particular, a cell that touched a C-cad-MO cell on one side and a C-cadherin-overexpressing cell on the other showed low and high β-catenin–YFP densities, respectively, at the different contacts. Apparently, cadherin density within a cell can be locally up- or downregulated. In C-cad-MO cells, membrane β-catenin–YFP density was generally low, but there was nevertheless a slight upregulation at points of contact with overexpressing cells versus contacts with C-cad-MO (Fig. 4F).

Thus, cadherin density is not constant over the surface of a cell, but adjusts at heterotypic contacts. Qualitatively, this would be consistent with a redistribution of pre-existing membrane cadherin by diffusion, followed by the trapping of molecules by trans-binding (Kusumi et al., 1993). However, recent findings show that cadherins are mainly redistributed through a combination of endocytosis and exocytosis (reviewed in Delva and Kowalczyk, 2009; Harris and Tepass, 2010; Green et al., 2010), suggesting a complex mechanism of cadherin density adjustment. Adjustment of cadherin density at heterotypic contacts should tend to reduce the efficiency of cell sorting. If sufficiently slow, adjustment would not be effective during the rapid initial phase of in vitro sorting, but would be significant in vivo, where C-cadherin expression differences develop gradually as the maternal pool becomes depleted.

Another mechanism to diminish sorting is suggested by the ability of cadherin-depleted cells to move normally between cadherin-overexpressing as well as cadherin-depleted cells. A similar phenomenon has been observed at the *Xenopus* notochord–somite boundary (Reintsch et al., 2005). Possibly, a minimum of cadherin-dependent adhesion is sufficient for intercellular migration. Above a certain level, cells might not discriminate between cadherin levels, because active attachment and detachment due to regulated adherens junction turnover could be independent of cadherin density (Delva and Kowalczyk, 2009; Harris and Tepass, 2010; Green et al., 2010). Moreover, when densely packed, as in the embryo, adhesion of transiently detached cells can be re-established before a cell moves any appreciable distance. In the gastrula, intercellular migration occurs at 0.2–0.4 μm/minute (Damm and Winklbauer, 2011) (unpublished observations). It would take a cell 100–200 minutes to move one cell diameter, whereas firm contacts are formed at times an order of magnitude lower, even when cells are loosely arranged, as during reaggregation.

Cells transfected to express different amounts of cadherin sort out in vitro, but boundaries are fuzzy (Steinberg and Takeichi, 1994; Duguay et al., 2003; Foty and Steinberg, 2005). By contrast, when cells from different tissues are mixed, sorting boundaries can be sharply delineated (Townes and Holtfreter, 1955; Steinberg, 1970; Foty et al., 1996). This is also apparent in the gastrula. Fuzzy boundaries form in vitro between cells differing in cadherin expression, but the in vivo ectoderm–mesoderm boundary is smooth, and is in fact maintained by cell repulsion based on Eph receptor signaling (Rohani et al., 2011; Park et al., 2011) and PAPC signaling (Medina et al., 2004); M-PAPC generates a distinct boundary without much reduction in surface tension. Thus, our results challenge the proposed role of cadherin-based differential adhesion in cell sorting and boundary formation in vivo. We propose that mechanisms beyond differential adhesion, such as active cell repulsion, i.e. the induced complete detachment of cells, are required for this function.

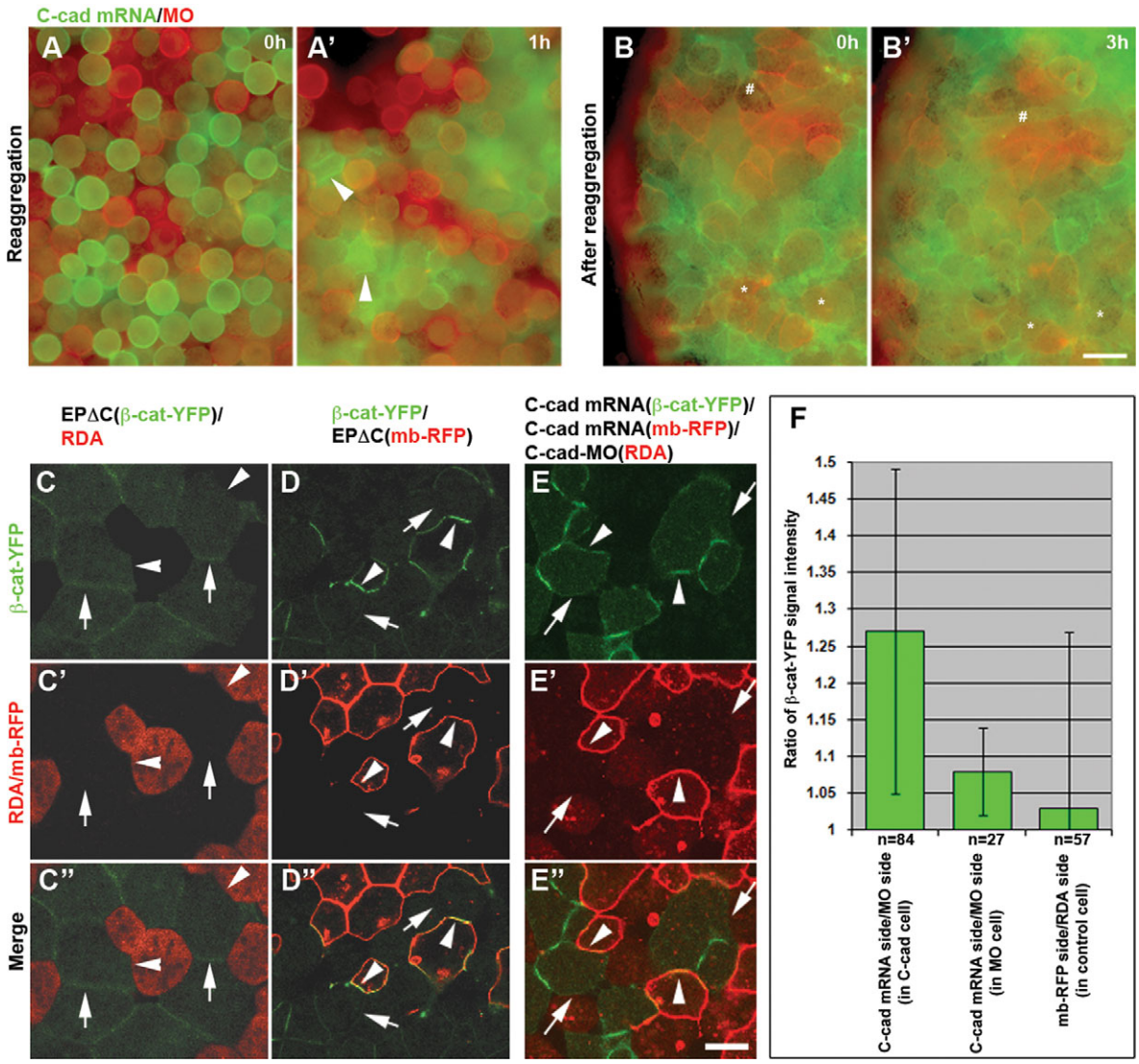

Fig. 4. Cellular response to cadherin modulation. (**A**–**B′**) C-cad-MO (10 ng/embryo) and C-cadherin cells segregate during reaggregation (A,A′), but not afterwards (B,B′). (A) C-cadherin cells (green) establish early contacts (arrowheads, A′), MO cells (red) remain round (A′). (B,B′) Random cell rearrangements, for example, deep C-cadherin cell moves to the surface (#) and surface C-cad-MO cells move inside (*), contrary to what would be expected from continued sorting. (**C**–**F**) β-cat–YFP at the cell membrane varies with the amount of C-cadherin in adjacent cells. (C–D″) EPΔC cells increase β-catenin when adjacent to normal cells (D–D″, arrowheads and arrows), but not when adjacent to EPΔC cells (C–C″, arrowheads and arrows). (E–E″) In C-cadherin cells,β-catenin is more prominent at contacts with like cells (mb-RFP, arrowheads) than at those with C-cad-MO cells (RDA, arrows). Scale bars: 50 μm (B′), 20 μm (E″).
(F) Quantification of β-catenin localization. Average pixel intensity was measured, and the ratio of intensities at contacts to high and to low cadherin cells was calculated for single cells for C-cadherin (left) and C-cad-MO (middle) cells. Ratio in controls shows no preference for sides (right). Left and middle bar, ratios significantly >1 ($P<0.0001$).

Materials and Methods

Embryo manipulations

To trace cells, *Xenopus laevis* embryos were injected with fluorescein or Rhodamine dextran amines (Molecular Probes) or β-galactosidase mRNA, green fluorescent protein (GFP), membrane-tethered GFP (mb-GFP) or red fluorescent protein (mb-RFP; 200 pg/blastomere). C-cadherin (500 pg) or EPΔC (500 or 1000 pg) mRNA, control or C-cad-MO (5′-CCACCGTCCCGAACAGAAGC-CTCAT-3′), β-catenin MO (5′-TTTCAACCGTTTCCAAAGAACCAGG-3′) (Heasman et al., 2000) and β-catenin–G-linked yellow fluorescent protein (YFP) mRNA (100 pg) were injected at the 4-cell stage. Stage 10 ectoderm was prepared in Steinberg's solution, and dissociated in 50% Ca^{2+}- and Mg^{2+}-free PBS and 0.1% BSA. The substrate adhesion assay (Reintsch and Hausen, 2001) used C-cadherin-Fc (0.2 μg/ml) (Zhong et al., 1999) or bovine fibronectin (2 μg/ml). The cell-sorting assay was performed as described previously (Ninomiya and Winklbauer, 2008). For β-catenin localization, fluorescence was detected using a Zeiss LSM510 scanning laser confocal microscope. Pixel intensity was determined using the Carl Zeiss Axiovision program (pixel size 625 nm^2). Tissue surface tension was measured using axisymmetric drop shape analysis (ADSA) (Luu et al., 2011).

Histology

Cell sorting was examined in formaldehyde-fixed ectoderm explants. In situ hybridization on sections was performed as described previously (Ninomiya et al., 2004). Injected cells were labeled with anti-fluorescein alkaline phosphatase antibody and Red substrate. Whole mount in situ hybridization was performed according to the method of Harland (Harland, 1991). Immunohistochemistry on cryosectioned specimens used anti-GFP (Invitrogen), 6B6 anti-*Xenopus* C-cadherin (Hybridoma Bank) (Choi et al., 1990), anti-β-catenin (Santa Cruz), or anti-actin (Cedarlane, Burlington, ON, Canada) antibodies. To observe intercalating chordamesoderm cells, the archenteron roof was dissected from

fixed gastrulae, and epithelium was removed after treatment with protease K. Immunostaining was then performed with anti-GFP, D7F2 anti-MyoD (Hybridoma Bank) (Hopwood et al., 1992) and anti-FoxA4 (Reintsch et al., 2005) antibody.

RT-PCR

Four-cell stage embryos were injected with 40 ng C-cad-MO or 2 ng C-cadherin mRNA. mRNA was extracted at stage 11 using Trizol (Invitrogen), and cDNA was synthesized using Superscript III (Invitrogen). Primers for PCR reactions are listed in supplementary material Table S1. Reaction conditions were 25 cycles for *Xbra* and *gsc*, 28 cycles for all others; annealing temperature was 55°C.

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