Measuring cell adhesion forces of primary gastrulating cells from zebrafish using atomic force microscopy

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Accepted 16 June 2005
Journal of Cell Science 118, 4199-4206 Published by The Company of Biologists 2005
doi:10.1242/jcs.02547

Summary

During vertebrate gastrulation, progenitor cells of different germ layers acquire specific adhesive properties that contribute to germ layer formation and separation. Wnt signals have been suggested to function in this process by modulating the different levels of adhesion between the germ layers, however, direct evidence for this is still lacking. Here we show that Wnt11, a key signal regulating gastrulation movements, is needed for the adhesion of zebrafish mesendodermal progenitor cells to fibronectin, an abundant extracellular matrix component during gastrulation. To measure this effect, we developed an assay to quantify the adhesion of single zebrafish primary mesendodermal progenitors using atomic-force microscopy (AFM). We observed significant differences in detachment force and work between cultured mesendodermal progenitors from wild-type embryos and from *slb/wnt11* mutant embryos, which carry a loss-of-function mutation in the *wnt11* gene, when tested on fibronectin-coated substrates. These differences were probably due to reduced adhesion to the fibronectin substrate as neither the overall cell morphology nor the cell elasticity grossly differed between wild-type and mutant cells. Furthermore, in the presence of inhibitors of fibronectin-integrin binding, such as RGD peptides, the adhesion force and work were strongly decreased, indicating that integrins are involved in the binding of mesendodermal progenitors in our assay. These findings demonstrate that AFM can be used to quantitatively determine the substrate-adhesion of cultured primary gastrulating cells and provide insight into the role of Wnt11 signalling in modulating cell adhesion at the single cell scale.

Key words: AFM, Gastrulation, Cell adhesion, Fibronectin, Zebrafish

Introduction

During vertebrate gastrulation an intricate set of cellular rearrangements leads to the formation of the three germ layers: the ectoderm, mesoderm and endoderm. Gastrulation starts with the internalization of mesodermal and endodermal (mesendodermal) progenitors, a process that separates non-internalizing cells, which give rise to the ectoderm, from internalizing cells that will form the mesoderm and endoderm (Stern, 2004).

Zebrafish are popular for the study of molecular and cellular aspects of tissue morphogenesis during vertebrate gastrulation. In zebrafish, germ layer formation is triggered by the internalization of mesendodermal progenitor cells at the margin of the blastoderm where the embryonic germ ring forms. Once internalized, mesendodermal progenitors migrate both as single cells and in groups of cells between the overlying ectodermal germ layer and the underlying yolk cell away from the margin towards the animal pole of the gastrula (Montero et al., 2005).

The molecular mechanisms that regulate mesendodermal cell internalization and migration have only just started to be elucidated. TGF-β-related Nodal signals are required for mesendodermal cell specification and subsequent internalization (reviewed by Schier, 2003). When Nodal signalling is abolished, no mesendodermal cell fates are induced and consequently no cell internalization is observed (Feldman et al., 1998; Gritsman et al., 1999). Conversely, overactivation of Nodal signalling causes ectopic mesendodermal cell specification and cell-autonomous cell internalization (Carmany-Rampey and Schier, 2001; David and Rosa, 2001). This indicates that Nodal signalling is required and is sufficient to induce mesendodermal cell fate and internalization.

The migration of mesendodermal progenitors away from the margin of the blastoderm towards the animal pole of the gastrula is controlled by the Wnt signal Wnt11 and its presumed receptor Frizzled-7 (Fz-7) (Djiane et al., 2000; Heisenberg et al., 2000; Ulrich et al., 2003; Winklbauer et al., 2001). In zebrafish silberblick *(slb)/wnt11* mutant embryos that carry a loss-of-function mutation in the *wnt11* gene, and in *Xenopus* 'morphant' embryos, in which Fz7 translation is inhibited, mesendodermal progenitors exhibit slower and less-directed cell migration and do not remain separated from the overlying ectodermal germ layer (Ulrich et al., 2003;

Winklbauer et al., 2001). It has been suggested that Wnt11 and Fz-7 control these processes by signalling through a non-canonical Wnt pathway to regulate cytoskeletal and cell adhesion changes (reviewed by Tada et al., 2002; Veeman et al., 2003), although the precise function of Wnt signalling therein has not yet been resolved.

Cell adhesion plays an important role for germ layer formation during vertebrate gastrulation. Both integrins and fibronectin are required for diverse types of gastrulation movements including radial cell intercalations, mesodermal progenitor cell migration and convergent extension (Marsden and DeSimone, 2001; Winklbauer and Keller, 1996). Interestingly, integrin-fibronectin interaction has been suggested to regulate convergent extension by modulating cadherin-dependent cell-cell adhesion (Marsden and DeSimone, 2003). Furthermore, integrin-fibronectin-mediated binding between gastrulating cells is accompanied by a recruitment of the intracellular Wnt signalling mediator Dishevelled (Dsh) to the plasma membrane (Marsden and DeSimone, 2001; Marsden and DeSimone, 2003), a key step associated with the activation of Wnt signalling (Rothbacher et al., 2000). These findings indicate a close crosstalk between integrin, cadherin and Wnt signalling at this developmental stage.

Although these findings point to the possibility that Wnt signalling controls gastrulation movements by interfering with the activity of cell adhesion molecules, its relative effect on adhesion compared to intracellular signalling mediated by cell adhesion molecules is still largely unclear. In order to measure the effect of Wnt11 on the adhesiveness of cultured zebrafish gastrulating cells, we used atomic force microscopy (AFM) to measure adhesion forces of single cells. In addition to its imaging capabilities (reviewed by Fotiadis et al., 2002; Lesniewska et al., 2002), this technique has been used successfully in recent years to study single molecule adhesion of isolated proteins (reviewed by Hinterdorfer, 2002) and cell adhesion in cell lines expressing a specific subset of adhesion molecules to a predefined substrate (Benoit and Gaub, 2002; Wojcikiewicz et al., 2004). However, in most of these cases, the analysed cells merely served as carriers for adhesion molecules and the biology of the cells was not directly addressed.

In this study, we adapted AFM to measure endogenous adhesive properties of single cells from zebrafish embryos to coated substrates. At the same time, the functional potential of the cells was analysed genetically in a developmental context. We found that mesendodermal progenitor cells from *slb/wnt11* mutant embryos that carry a loss-of-function mutation in the *wnt11* gene, exhibited reduced detachment force and work to a substrate coated with fibronectin when compared to wild-type cells. This suggests that Wnt11 signalling is involved in modulating the adhesion of these cells to fibronectin, providing novel insight into the role of Wnt signalling in regulating cell adhesion and morphogenesis during vertebrate gastrulation.

Materials and Methods

Embryo maintenance

Fish maintenance and embryo collection were carried out as described (Westerfield, 2000). Embryos were obtained from zebrafish Gol, TL, Tü, Wik or AB backgrounds; for the mutant analysis, homozygous slb^{tx226} carriers in a TL background were used. Embryos were staged during the early cleavage period, grown at 28°C or 31°C and manipulated in E3 zebrafish embryo medium.

Cell culture

Cell culture was done as previously described (Montero et al., 2003). Dissociated cells were adjusted to a concentration of 2×10^5 cells/ml. 100 μl cell suspension were plated on plastic wells coated with 35.4 ng fibronectin/mm^2 (Invitrogen, Germany). Cells were kept for 3 hours to overnight at 25°C. For DIC image acquisition, a Zeiss inverted microscope with a CCD camera and MetaMorph software was used; pictures from random regions were acquired.

AFM setup

We used a JPK Nanowizard atomic-force microscope (JPK Instruments, Berlin) on top of an Axiovert 200 inverted microscope (Carl Zeiss, Jena). The AFM stand-alone head has a linearized piezo-electric ceramic with a 15 μm range and is equipped with an infra-red laser. We used the softest cantilevers available (320 μm long, nominal spring constant 10 mN/m; Microlevers, MLCT-AUHW, Veeco). Sensibility and spring constant of each cantilever were calibrated prior to each experiment using built-in routines of the JPK software.

Cantilever and surface coating

To attach a single mesendodermal progenitor cell to the cantilever, we adapted described procedures (Wojcikiewicz et al., 2004). Cantilevers were cleaned either by using detergent and/or plasma cleaning. They were then treated with biotinylated BSA, followed by incubation in streptavidin and biotinylated ConA. During the mounting procedure, the cantilevers were always kept wet to ensure the integrity of the surface. Clean glass slides were plasma-activated for at least 1 minute and then incubated overnight at 37°C with 50 μg/ml fibronectin in PBS. Prior to use, the non-bound protein was removed by extensive washing first with PBS, then with fresh DMEM.

RGD control

Cells were incubated 30 minutes prior to use with 250 μM RGD peptide (G1269, Sigma-Aldrich). During all force measurements, the cells were kept in medium containing the blocking peptide.

Cell capture, adhesion experiment and data processing

Single cells plated on fibronectin were captured by lowering a calibrated ConA-coated cantilever towards a suitable cell, pressing the cantilever on the cell with a given force (~350-450 pN) and then letting the adhesion set for 1 second (Fig. 2). The cell-cantilever couple was subsequently lifted from the surface (several tens of μm) and the cell was allowed to establish a firm adhesion on the lever for 5-15 minutes under constant observation. After this, the approach and retraction speeds were set to 4.5 μm/second, the pulling range to 4.5 μm, the contact time between 1 and 5 seconds and the maximal pushing force to ~250-400 pN. The complete procedure is summarized in Fig. 3. Five to ten force curves were acquired for each cell for a given contact time, and the cell was subsequently allowed to recover far from the surface for several minutes, before testing a different spot on the surface and/or different contact times. The captured cells always detached from the fibronectin-coated substrate and never from the cantilever during the pulling experiments and resisted detachment forces up to several nN in agreement with previously published results (Wojcikiewicz et al., 2004). Typically, several tens of force curves were acquired per cell and forces did not show clear tendencies depending on the substrate spot tested. In

particular, testing the same spot twice or more with the same cell gave similar force curves, indicating that the fibronectin was not peeled off the surface. To extract the relevant parameters such as detachment force, detachment work, number and magnitude of small force steps along the retrace curves (Fig. 4), post-processing with home-programmed procedures using Igor Pro 4.09 (Wavemetrics) was used. We also used this software to extract the means and s.d. of the data sets. We used built-in procedures of KaleidaGraph for running Student and ANOVA-Bonferroni tests.

Results

Wnt11-mediated cell reaggregation of mesendodermal progenitors cultured on fibronectin

We have previously shown that directed migration and orientation of cellular processes in mesendodermal progenitors depends on Wnt11 during early zebrafish gastrulation (Ulrich et al., 2003). To test if Wnt11 controls these processes by modulating cell adhesion, we characterized the reaggregation of mesendodermal progenitors from wild-type and *slb/wnt11* mutant embryos when cultured on fibronectin. Primary cultures of mesendodermal progenitors were prepared as previously described (Feldman et al., 1998; Montero et al., 2003; Rebagliati et al., 1998) and the reaggregation behaviour of the cells was observed for the first 3 hours in culture. During this time, wild-type mesendodermal cells efficiently formed distinct cell aggregates with an average size of ~3 cells/aggregate (Fig. 1A,B,G). In contrast, *slb/wnt11* mutant mesendodermal cells formed significantly smaller aggregates with an average size of ~2 cells/aggregate (P=1.7×10^{-3}; Fig. 1D,E,G). This difference in cell reaggregation between wild-type and *slb/wnt11* cultures was already clearly detectable after 1 hour in culture (Fig. 1G) suggesting that Wnt11 influences the adhesive properties of mesendodermal progenitors at early stages of reaggregation.

In order to test whether fibronectin adhesion is involved in this process, we performed the same cell reaggregation assay, but this time added 250 μM RGD peptide to the culture medium to effectively block integrin-fibronectin binding (Ruoslahti and Pierschbacher, 1987). When RGD was added to the medium, the average size of wild-type cell aggregates was significantly increased (~4 cells/aggregate; P=0.004 after 1 hour in culture) whereas *slb/wnt11* aggregates remained largely unchanged (~2 cells/aggregate; P=0.782 after 1 hour in culture; Fig. 1C,F,G). This shows that the reaggregation of wild-type cells but not *slb/wnt11* mutant mesendodermal cells is sensitive to fibronectin binding and also that Wnt11 function in promoting reaggregation of mesendodermal cells is enhanced rather than reduced when binding to fibronectin is blocked. We therefore suggest that Wnt11 in wild-type cells is involved in the binding of mesendodermal cells to fibronectin and that fibronectin acts as an inhibitor rather than effector of Wnt11-mediated reaggregation of mesendodermal cells.

Using AFM to measure cell adhesion of cultured mesendodermal progenitors

Although the cell reaggregation experiments are indicative of a role for Wnt11 in determining the adhesion of mesendodermal progenitors to fibronectin, direct evidence for such a function is still lacking. To determine if fibronectin binding is affected in *slb/wnt11* mutant cells, we measured the adhesion of cultured mutant and wild-type cells to fibronectin using AFM. Primary cultures of mesendodermal progenitors were prepared as previously described (Montero et al., 2003) and a ConA-coated cantilever was then used to pick a single cell plated on a fibronectin-coated glass surface (Fig. 2). After this, the captured cell was allowed to settle on the cantilever for 5-15 minutes away from the surface. Then the cantilever/cell was lowered towards the substrate until a maximal force of 250-400 pN was reached, and pressed onto the substrate for 1-5 seconds (Fig. 3A,B and red curve). The detachment of the cell was recorded during retraction of the cantilever at a prescribed speed (Fig. 3C,D). The retraction force curves (green curve in Fig. 3) typically showed maximum detachment forces (F_{detach}) of several hundred pN followed by a finite number of step-like unbinding events, which were either preceded ('T' events) or not preceded ('J' events) by a force plateau (Fig. 3), which probably represented the rupture of single adhesion units (see below). The T events have

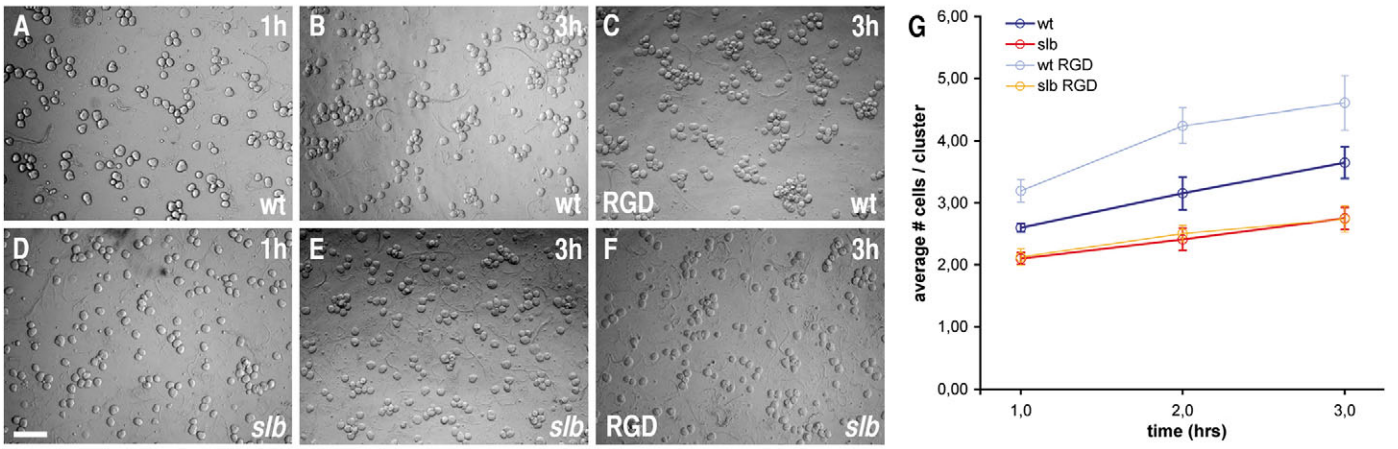

Fig. 1. Reaggregation of mesendodermal progenitors cultured on fibronectin. Images of cultures of wild-type (A-C) and *slb/wnt11* mutant mesendodermal progenitors (D-F) after plating for 1 hour (A,D) and for 3 hours (B,C,E,F) in control medium (A,B,D,E) or after adding RGD peptides to the culture medium (C,F). (G) Average number of mesendodermal progenitors/aggregate for wild-type and *slb/wnt11* cultures in the absence and presence of RGD peptides at different time-points after plating. Bar, 50 μm.

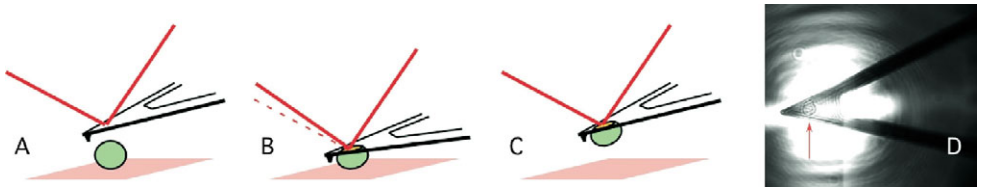

Fig. 2. Schematic diagram illustrating the capture of a single primary gastrulating cell from zebrafish using a ConA-coated cantilever. Using an inverted optical microscope we placed the cantilever on top of a cell (A) and pressed for 1-2 seconds with a force of ~350-500 pN (B). (C) The cantilever together with the adhering cell was then withdrawn several micrometers from the surface and the cell was allowed to settle at this position for 5-15 minutes. (D) Optical image of such a captured cell (arrow) taken 50 μm above the fibronectin surface. The full length of the cantilever is 320 μm.

previously been described as membrane tether extrusion (Benoit and Gaub, 2002). To quantify the adhesion, we recorded the magnitude of the maximum detachment force and the magnitude and number of the small detachment events. We also calculated the so-called 'work of de-adhesion' (the shaded area in Fig. 3) that contains an adhesive element due to the rupture of formed complexes and a mechanical component due to cell deformation.

In contrast to cell lines, cultures of mesendodermal progenitors do not necessarily represent a homogeneous population of cells. To make sure that only cells with similar morphology were compared between wild-type and *slb/wnt11* mutant cultures, we categorized their shape into different morphological groups and determined their apparent diameters when freshly plated on glass. Both wild-type and mutant cells had an average diameter of 17-18 μm (Fig. 4D,E) and the majority of cells displayed short, round and often mobile cellular protrusions (Fig. 4A-C). We also determined the average local elastic constant, k (by setting $F = k \times$ indentation), of wild-type and mutant cells using a non-sharpened AFM tip similar to those used in the force measurements as a nano-indentor. The k values were around 0.5 mN/m for the two genotypes under a moderate indentation force of several hundred pN resembling the k values obtained for MDCK cells (Hoh and Schoenenberger, 1994). The Young's modulus for the two cell types was ~0.5-1.0 kPa, being compatible with previously published values (Radmacher, 2002). No gross difference was detected between the two genotypes (Fig. 4F) in terms of cell shape and elastic properties, indicating that cultured primary mesendodermal progenitors from zebrafish represent rather homogenous populations with no apparent large differences between wild-type or mutant cells. In all AFM experiments, we discarded cells that were damaged during the cell culturing procedures and only compared cells with similar morphology and size between the different genotypes (Fig. 1A, top row and caption).

Influence of Wnt11 on detachment of mesendodermal progenitors from fibronectin

To assess whether there are measurable differences in the adhesion of mesendodermal progenitors to fibronectin between wild-type and *slb/wnt11* mutant cells, we recorded detachment curves for both cell types (Fig. 5A). Histograms for detachment force and work for the different evaluated contact times exhibit large distributions (Fig. 5B and data not shown). To evaluate the results obtained from the different experiments recorded, we calculated the mean and s.d. of the measured parameters and applied statistical tests to examine the relevance of the observed differences (see Materials and Methods, Table 1). Wild-type cells typically showed average detachment forces ranging from 198-405 pN and average detachment work ranging from 1.07×10^{-16} to 2.80×10^{-16} J (Fig. 6). In contrast, *slb/wnt11* mutant cells exhibited average

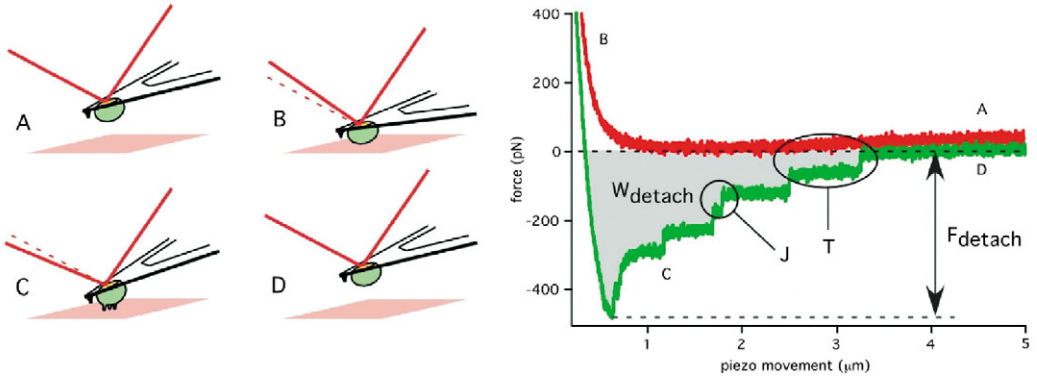

Fig. 3. Schematic diagram of an adhesion experiment and the corresponding typical force curves for approach (red) and retraction (green) from the surface. The cantilever/cell is moved towards the surface and no force is detected (A). When the cell is pressed on the fibronectin surface, the force increases until it reaches a pre-set level (B). After a given contact time, the cantilever/cell is withdrawn from the surface and the bonds that have been formed break sequentially (C) until the cell has completely separated from the surface (D). To quantify the adhesion, one can measure the maximal force needed to separate cell and surface F, the detachment work W, and the number and magnitude of detachment events along the curve (J, T; see also text).

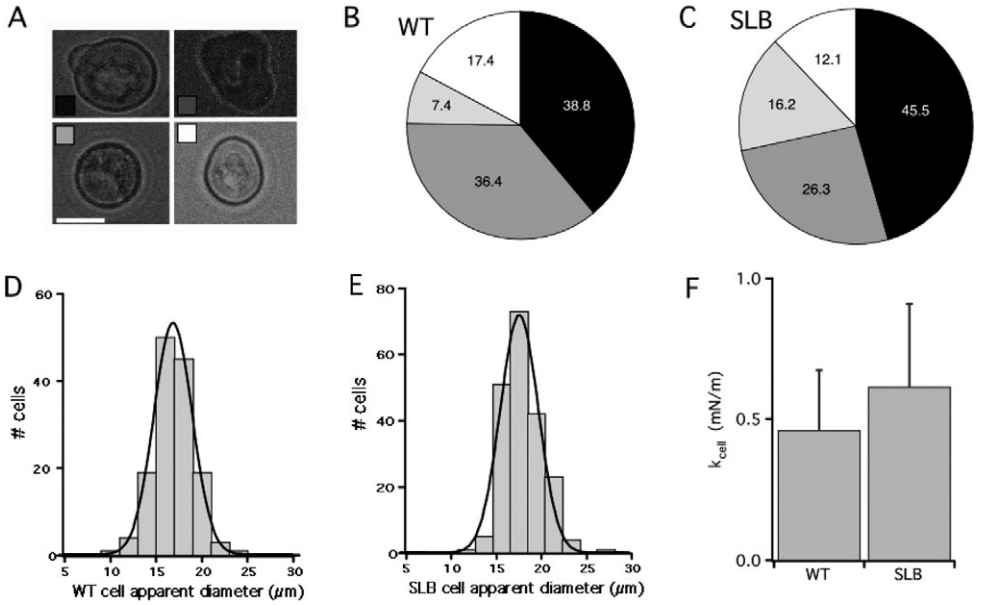

Fig. 4. Mesendodermal progenitor cell shape and elasticity. (A) The most common cell shapes in a wild-type cell culture. (B,C) Percentage distribution in of the different cell shapes for wild-type cultures (B) and *slb/wnt11* mutant cultures (C). Shading in the diagram corresponds to the inset labelling of the different cell shapes in A. The cells used for force measurement were of the two most frequent types. (D,E) Distribution of cell diameters for cells resting on glass in wild-type (D) and *slb/wnt11* cultures (E). Gaussian fits give mean±width of 17.0±3.0 μm for the wild type and 18.0±3.1 μm for *slb/wnt11* mutant cells. (F) Evaluation of the spring constant k (mean±s.d.) of wild-type and *slb/wnt11* mutant cells (>20 cells/genotype; ⩾3 curves/cell) resting on glass measured by using the AFM tip as an indentor. There is no gross difference in k between the wild type (0.46±0.21 mN/m) and *slb/wnt11* mutant (0.61±0.30 mN/m) cells. SLB, *slb/wnt11*; WT, wild type. Bar, 10 μm.

detachment forces ranging from 123-156 pN and work ranging from 7.80×10^{-17} J to 1.3×10^{-16} J. Moreover, although the detachment force and work from wild-type cells increased with contact time (1-5 seconds), no such correlation was observed in *slb/wnt11* mutant cells (Fig. 6). These findings suggest that Wnt11 modulates the adhesion of mesendodermal cells when tested on substrates coated with fibronectin.

The difference in the adhesion forces recorded between wild-type and *slb/wnt11* mutant mesendodermal progenitors is probably due to their differences in specifically adhering to fibronectin. However, potential differences in unspecific substrate binding and/or visco-elastic properties between wild-type and mutant cells might have also an influence on the results. To test if fibronectin adhesion is the primary source for the adhesion forces recorded and to identify potential ligands expressed in mesendodermal progenitors that bind to fibronectin, we added 250 μM RGD peptides to the culture medium in order to specifically compete with integrin-fibronectin binding (Ruoslahti and Pierschbacher, 1987). When RGD peptides were added, wild-type mesendodermal progenitors exhibited strongly reduced adhesion forces and work (Fig. 6), indicating that the detachment parameters recorded were specific for fibronectin and that integrins expressed in mesendodermal progenitors are involved. Moreover, the observation that the average number (Table 2A), but not the average force (Table 2B), of small unbinding events within single force curves (Fig. 3J) differed between mutant and wild-type cells and also was effectively reduced when RGD peptides were added, indicates that Wnt11 is influencing the number rather than the type of adhesion complexes involved in the binding to fibronectin.

Taken together, the results of our experiments show that Wnt11 is involved in the specific binding of mesendodermal progenitors to fibronectin, and that integrins are likely to be involved in this process.

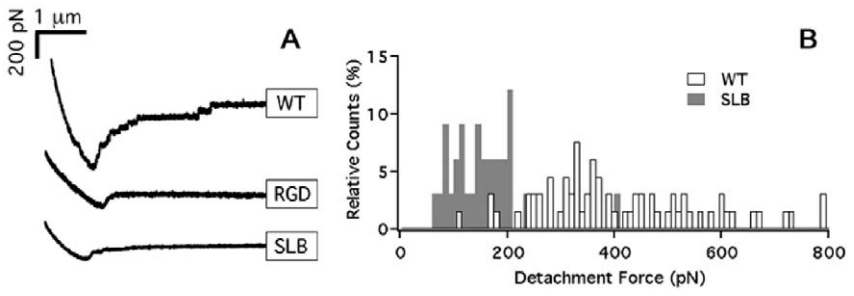

Fig. 5. Typical force curves and histograms for the different cell types and experimental conditions. (A) Force curves for wild-type cells, wild-type cells plus RGD peptide and *slb/wnt11* cells adhering to a fibronectin substrate for a contact time of 5 seconds. (B) Corresponding relative count histograms of the detachment forces for wild-type and *slb/wnt11* cells on fibronectin. As explained in the text and in the legend to Fig. 6, these data were evaluated by extracting the mean and the s.d. SLB, *slb/wnt11*; RGD, RGD peptide; WT, wild type.

Table 1. Number of force curves processed for histograms in Fig. 6

Time (seconds)	WT (*n*=16*)		WT + RGD (*n*=10*)		SLB (*n*=13*)	
	F	W	F	W	F	W
1	103	103	–	–	27	27
2	130	129	147	147	184	178
5	66	64	96	96	33	30

All cells were grown on glass surfaces coated with fibronectin.
*Number of single cells pooled for statistics.
WT, wild-type cells; SLB, *slb/wnt11* cells; +RGD, 250 μM RGD peptides added to the cultures; F, detachment force; W, detachment work.

Table 2. Quantification of small detachment events in the processed force curves and mean force jump for all 'J' and 'T' events recorded

A

Time (seconds)	WT		SLB	WT + RGD
	*n**	Force (pN)†	*n*	*n*
1	2.5±0.3	36.9±26.1 (280)	1.3±0.5	–
2	2.0±0.3	42.7±29.6 (151)	1.1±0.2	0.8±0.1
5	3.5±0.8	35.2±26.8 (135)	1.3±0.5	0.5±0.1

B

Force (pN)‡	WT	SLB
'J'	34.5±27.0 (666)	29.5±18.9 (221)
'T'	39.0±14.0 (163)	–

All cells were grown on glass surfaces coated with fibronectin.
*mean±s.e.m.
†Gaussian fit (mean±width) with the total number of such events given in brackets.
‡Mean force jump (in pN) for all 'J' events recorded and for tethers ('T' events in Fig. 3). The comparison of wild-type and *slb/wnt11* (SLB) average data over all the recorded 'J' events showed that the number but not the force associated with these events is affected in mutant cells. The RGD blocking experiment also showed a decrease in the number of these events.
WT, wild-type cells; SLB, *slb/wnt11* cells; +RGD, 250 μM RGD peptides added to the cultures.

Discussion

In this study we used AFM to measure the adhesion of primary gastrulating cells from zebrafish to coated substrates. We show that Wnt11 modulates the adhesion of mesendodermal progenitors to fibronectin providing the first direct and quantitative evidence that Wnt signalling modulates adhesive properties of mesendodermal cells during gastrulation.

We have previously shown that Wnt11 signalling controls directed migration along with the orientation of cellular processes in axial mesendodermal progenitors moving from the germ ring towards the animal pole of the gastrula (Ulrich et al., 2003). However, the underlying cellular and molecular mechanisms that mediate the function of Wnt11 in these processes are still unknown. Studies in *Xenopus* have shown that fibronectin constitutes a key extracellular matrix component that is required for the migration of involuting mesodermal progenitors at early gastrulation (Winklbauer and Keller, 1996). When adhesion to fibronectin is blocked, mesendodermal cells fail to spread on the blastocoel roof, fail to form lamelliform cytoplasmic protrusions and do not migrate towards the animal pole (Winklbauer and Keller, 1996). Similarly in zebrafish, maternal-zygotic mutants for the fibronectin-1 gene (*natter*) display severe morphogenetic defects at the onset of gastrulation, suggesting that mesendodermal progenitor internalization and migration are affected (Trinh le and Stainier, 2004).

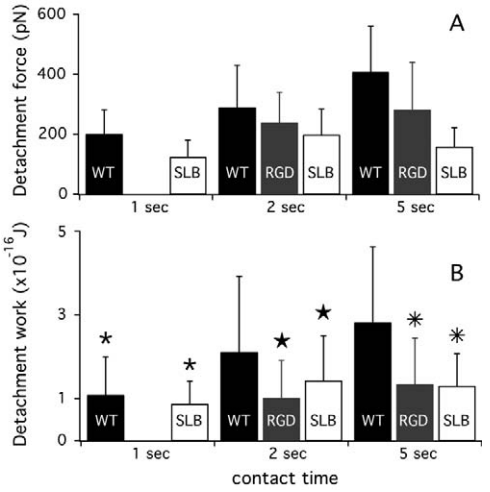

Fig. 6. Statistics of *slb/wnt11* mutant and wild-type mesendodermal progenitor cell adhesion to fibronectin. (A) Detachment force. (B) Detachment work. Data are mean±s.d. The difference between the various conditions becomes more apparent for the detachment work, which takes into account the small unbinding (J and T) events (see Table 2). For 1 second contact time and the RGD experiments, the data sets were compared using the Student's *t*-test. For 2 and 5 second contact times, wild-type and *slb/wnt11* data sets were compared using ANOVA followed by a Bonferroni test. Labels indicate the pairs of data sets with no significant differences (P>0.05). FN, fibronectin; SLB, *slb/wnt11*; RGD, RGD peptide; WT, wild type.

There has been no direct evidence that Wnt signalling can interfere with fibronectin-mediated cell adhesion. Reports in *Xenopus* have indicated that fibronectin-integrin interaction in gastrulating cells is accompanied by localization of the intracellular Wnt signalling mediator Dishevelled (Dsh) to the plasma membrane (Marsden and DeSimone, 2001). As Dsh also translocates to the plasma membrane when Wnt signalling is activated (Rothbacher et al., 2000), these observations have been interpreted as evidence that fibronectin-integrin binding is linked to Wnt signalling activity. Our finding that Wnt11 modulates the adhesion of mesendodermal progenitors to fibronectin provides direct experimental support for Wnt signalling modulating fibronectin adhesion.

To assess the influence of Wnt11 on the adhesion of mesendodermal progenitors to fibronectin, we used AFM as a sensitive and quantitative method to measure single cell adhesion forces. A number of advanced techniques have been developed to measure receptor-ligand interactions, mainly at the single molecule scale, such as the biomembrane force probe (Evans et al., 1995), AFM (Florin et al., 1994; Moy et al., 1994), optical tweezers (Litvinov et al., 2002) and the parallel plate flow chamber (Chen and Springer, 2001). The biomembrane force probe, AFM and the parallel plate flow chamber have been used to analyse ligand-receptor systems that primarily operate in the immune system, such as selectin-mediated adhesion (Chen and Springer, 2001; Evans et al., 2001; Fritz et al., 1998), whereas laser tweezers and AFM have been used to analyse integrin binding to RGD-containing

peptides including fibronectin (Lehenkari and Horton, 1999; Li et al., 2003; Litvinov et al., 2002). However, up to now, AFM is the only technique that offers the possibility of easily measuring the adhesion of intact cells under a large range of physiological conditions. It furthermore provides the ability to address cell adhesion by controlling contact force, contact time and speed of pushing/pulling the cell towards and away from the surface.

The adhesion forces obtained for mesendodermal progenitors to fibronectin are in good agreement with recent AFM studies of integrin-fibronectin binding in human leukaemia cell lines (Li et al., 2003). Both the rupture force and work of de-adhesion to fibronectin fragments observed for leukaemia cells specifically expressing integrin α5/β1 resembled the average binding forces measured for mesendodermal progenitors on fibronectin although the exact values differed depending on the experimental parameters and cell types tested. The similarities between these measurements, together with our observation that RGD peptides can significantly block the adhesion of mesendodermal progenitors to fibronectin, suggest that mesendodermal progenitors bind to fibronectin via RGD-dependent integrins.

When determining the unbinding force profile of wild-type mesendodermal cells we observed multiple small detachment events ('J' and 'T' events; Fig. 3) with some of them preceded by a force plateau (T events). These T events are probably due to the pulling of membrane tethers from the cell surface to which they adhere. This view is supported by studies showing that such membrane tethers or 'tubes' can be extracted over long distances (up to several μm) in model systems (Rossier et al., 2003) or from cellular membranes (Dai and Sheetz, 1999). The existence of membrane tethers underlines the fact that adhesive properties are closely linked to the local (membrane and cortical cytoskeleton) and global elastic properties of the cells. The exact nature of the tethers, whether purely membranous or coupled to the cytoskeleton, is still unclear and probably depends on the function associated with them and to the adhesive molecules that are involved (Evans et al., 2005). The exact shape of the curves before the separation force jump depends on the local physical properties of the membrane (Cuvelier et al., 2005).

The number of small detachment events (J and T events) increased for wild-type cells with contact time but stayed rather constant for *slb/wnt11* mutant and RGD control cells (Table 2A and data not shown). Moreover, the numbers of small detachment events in these latter cases were always smaller than those for wild-type cells. These observations indicate that the small detachment events increase in number with time, are specifically mediated by integrins and are modulated by Wnt11. Notably, the small detachment events together with the magnitude of the first large peak of several hundreds of pN, contribute to the work of de-adhesion. Considering that there are more J and T events in the wild type than in *slb/wnt11* mutant and RGD control cells (Table 2A and data not shown), and taking into account that the work is the integral of the force over the distance, this explains why the difference in the detachment work between wild-type and mutant/control cells was enhanced compared to the difference in the detachment force. Obviously, the difference in detachment work is also affected by the visco-elastic properties of the different cell types, because the work of de-adhesion includes both adhesion and mechanical components that are often difficult to separate. In particular, the elastic component influences the shape of the first large peak (global elastic properties), but also the deformation of the membrane (local elastic properties), leading to the extension of membrane tethers and thereby increasing the distance at which the cell completely detaches from the substrate. Further experiments will be required to dissect the specific contributions of adhesion compared to mechanics in our measurements.

Several questions remain: (1) the molecular mechanism by which Wnt11 controls the adhesion of mesendodermal progenitors to fibronectin; (2) which ligands fibronectin binds to in these cells; and (3) the precise role of fibronectin in controlling mesendodermal progenitor cell migration and polarization in zebrafish. It is conceivable that Wnt11, for example, regulates the expression and/or localization of integrins in mesendodermal progenitors. Future studies will reveal the type of integrins expressed in mesendodermal progenitors and the potential regulatory mechanisms by which Wnt signalling controls fibronectin-integrin adhesion.

The authors thank P. Bongrand, C. Franz, L. Rhode and K. T. Sapra for fruitful discussions, H. Janovjak for valuable input at the beginning of this project and K. Poole, H. Haschke, D. Knebel, C. Loebbe, G. Behme and T. Jaehnke from JPK Instruments for their help in developing the software and hardware used in these experiments. This work was supported by grants from the Emmy-Noether-Program of the German Research Foundation (DFG), the German Volkswagen-foundation, the Bundesministerium für Bildung und Forschung (BMBF) and the Max-Planck-Society (MPG).

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