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Insight

During embryogenesis primordial germ cells (PGCs) of *Xenopus laevis* migrate from the location of their specification to the site of gonad formation. This transition from a sessile to an actively migrating state requires a number of cellular properties to change.

We found that the level of E-cadherin is reduced in migrating cells leading to a significant reduction of adhesive force between cells and E-cadherin coated surfaces. In essence, the switch from an early, non-migrating state to a late migrating state depends only on a few tenths of piconewtons in adhesion force. We propose that cells move via a ‘chimneying’ mechanism in resemblance of a common climbing technique, using bleb formation in conjunction with non-specific traction forces to propel forward.

Reduction in E-cadherin expression fosters migration of *Xenopus laevis* primordial germ cells

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Abstract

The transition from passive to active migration of primordial germ cells in Xenopus embryos correlates with a reduction in overall adhesion to surrounding endodermal cells as well as with reduced E-cadherin expression. Single cell force spectroscopy, in which cells are brought into brief contact with a gold surface functionalized with E-cadherin constructs, allows for a quantitative estimate of functional E-cadherin molecules on the cell surface. The adhesion force between migratory PGCs and the cadherin-coated surface was almost identical to cells where E-cadherin was knocked down by morpholino oligonucleotides (180 pN). In contrast, non-migratory PGCs display significantly higher adhesion forces (270 pN) on E-cadherin functionalised surfaces. On the basis of these observations, we propose that migration of PGCs in Xenopus embryos is regulated via modulation of E-cadherin expression levels, allowing these cells to move more freely if the level of E-cadherin is reduced.

Introduction

In many species, specification of germ cells occurs in a region away from the site of gonad formation. During embryogenesis, germ cells actively migrate within the embryo, independent of the surrounding tissue. Transition of immobile cells to actively migrating ones correlates with a number of changes in cellular properties originating from a change in gene expression.[1-4]

In *Xenopus laevis*, primordial germ cells (PGCs) are progenitors to the germ cell lines during embryogenesis. Specification of these cells occurs due to the inheritance of maternal factors, including vegetally localized mRNAs and proteins.[5-7] At early stages of embryogenesis, PGCs are tightly associated with their surrounding tissue, and together with somatic endodermal cells they are involuted within the embryo during gastrulation. At the neurula stage, PGCs are clustered within the endoderm, while at the tailbud stage (st.24-25)[8] they initiate active migration within the embryo towards the prospective genital ridges.[9, 10] Although a number of factors were shown to contribute to active migration of PGCs in *X. laevis*, the molecular mechanism of this process remains largely unexplored.[11]

In previous studies, we were able to demonstrate that migratory PGCs do not require specific adhesion to a substrate for their migration *in vitro*.[12] Single cell force spectroscopy (SCFS) analysis of PCGs isolated from the tailbud (st.28-30) and neurula (st.17-19) stage embryos revealed reduced adhesion of migratory PGCs to the surrounding somatic endodermal cells and fibronectin. We could also show that down-regulation of E-cadherin expression on the RNA level takes place in PGCs at the tailbud stage, but not in the surrounding somatic cells. Differential E-cadherin expression was found to be important for proper germ cell development in a number of species.[4] For example, PGC transition to active migration in zebrafish correlates with a slight reduction of E-cadherin levels.[13] Since they migrate as individual cells, down-regulation of E-cadherin was suggested to allow the detachment of PGCs from neighbouring cells. A certain level of E-cadherin, however, is still required for the proper PGC migration to generate the necessary traction force for pushing themselves away from the substrate.[14]

In this study, we analysed the role of E-cadherin in the context of active PGC migration in *X. laevis* embryos. We used single cell force spectroscopy in conjunction with cadherin-coated substrates to measure molecule-specific cellular adhesion down to single molecule level. While overall cell-cell adhesion has been investigated previously, we now focus on the molecular origin of the interaction force measured between two individual cells.[15] While measuring cell-cell interactions only provides a qualitative estimate to which extent adhesion strength has changed, the use of self assembled monolayers with defined molecular composition permits to assign measured force to expression of a specific adhesion molecule.

We found that early pre-migratory PGCs display higher adhesion forces to covalently linked and density-controlled E-cadherin coated surfaces as compared to late migratory PGCs, suggesting that the capability to migrate is fostered by a decrease in E-cadherin mediated adhesion to adjacent cells. We propose that PGCs fine tune the number of adhesion molecules to allow for active migration that requires a certain degree of stickiness to adjacent cells in order to exert enough traction but otherwise should be minimized to produce a decent speed. The remaining traction most likely does not rely on E-cadherin but on nonspecific attraction via van der Waals and electrostatic interaction.

Materials and methods

Ethics statement

All animals were treated according to the German regulations and laws for care and handling of research animals. Experimental manipulations were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, permit number 33.9.42502-05-A-006/08).

Manipulating Xenopus laevis embryos

Embryos were obtained from *X. laevis* females by human chorionic gonadotropin (HCG, Sigma-Aldrich, Munich, Germany) induced egg-laying (800–1000 U HCG injected approximately 12 h before egg-laying). Spawns were *in vitro* fertilized with minced testis in 0.1x MBSH (5x MBSH: 50 mM HEPES, 440 mM NaCl,

10 mM KCl, 10 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.05 mM $CaCl_2$, 1.65 mM $Ca(NO)_3$), dejellied with 2% cystein hydrochloride (pH 7.9) and cultured in 0.1x MBSH at 12.5 °C. Injections were performed in injection buffer (1% (w/v) FICOLL in 1× MBSH) on a cold plate (12.5 °C) vegetally into both blastomeres of two-cell stage embryos. 4 nl of mRNA and/or morpholino oligonucleotide solutions were injected per blastomere. After injections, embryos were kept for at least 1 h in the injection buffer at 12.5 °C. They were then transfered into 0.1× MBSH and cultivated at 12.5 °C until they reach desired developmental stage.

Sense mRNAs and morpholino oligonucleotides

Xenopus laevis E-cadherin ORF (Gene ID: 100337618) was cloned into pCS2+gfpDELE vector[12] to add EGFP ORF on the 5' end and Dead End localization element (DELE) on the 3' end. This plasmid was used to generate *GFP_E-cadherin_DELE* mRNA, while empty vector pCS2+gfpDELE was used to generate *GFP_DELE* mRNA. Sense mRNAs were *in vitro* transcribed from *Not*I (FastDigest, Thermo Scientific Fermentas, Vilnius, Lithuania) linearized plasmids using mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, Texas, USA). These mRNAs were purified by Illustra™ RNA spin MiniRNA Isolation Kit (GE Healthcare, Munich, Germany) according to the manufacturers' protocol. 800pg of *GFP_E-cadherin_DELE* or 400pg of *GFP_DELE* mRNA were injected per embryo as described above. Alternatively, 1.2 nmol of morpholino oligonucleotides specific for *X. laevis* E-cadherin (E-cad MO: 5'-AACCAGGGCCTCTTCAACCCCATTG-3')[16] or unspecific control morpholino oligonucleotides (Contr MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3') were injected alone (for Whole mount *in situ* hybridization), or co-injected with 400pg *GFP_DELE* mRNA (for Force spectroscopy). Antisense morpholinos were purchased from Genetools, LLC (Philomath, USA).

Western blot analysis

Embryos were injected vegetally at the 2-cell stage with 1.2 nmol of control or E-cadherin specific morpholino as described above. At developmental stage 17-19[8] the ventral part was dissected from the embryos. To insure specificity of the signal to the endoderm, epithelial tissue surrounding endodermal cells was manually removed. Obtained endodermal explants were frozen in liquid nitrogen and subsequently

homogenized by use of an insulin syringe (Omnican 40, B.Braun, Bad Arolsen, Germany) in IPP145 buffer [10mM Tris pH=8,0, 145mM NaCl, 0.1% NP-40, 5% (v/v) Glycerol; cOmplete Protease Inhibitor Cocktail Tablets (Roche) was added according to the manufacturer's instructions]. After centrifugation, the supernatant from homogenized samples was used for SDS-PAGE followed by blotting to a nitrocellulose membrane. The membrane was treated with 1:1000 αGAPDH (ab9485, Abcam, Cambridge, UK), together with 1:2500 αE-cadherin [5D3, Developmental Studies Hybridoma Bank, Iowa City, USA; originally developed by Choi and Gumbiner][17] or 1:2500 αC-cadherin [6B6, Developmental Studies Hybridoma Bank, Iowa City, USA; originally developed by Brieher and Gumbiner][18] primary antibody, followed by the treatment with secondary fluorescently labelled antibody (IRDye® 800CW Goat anti-Mouse and IRDye® 680RD Goat anti-Rabbit, LI-COR Biosciences, Lincoln, USA). Analysis was performed using Odyssey® CLx Infrared Imaging System (LI-COR Biosciences).

Whole mount in situ hybridization

Whole mount *in situ* hybridization (WMISH) was performed with *Xenopus laevis* embryos as described previously.[19] Antisense riboprobes were produced by standard *in vitro* transcription from linearized plasmids in the presence of digoxygenin-coupled rUTP. *Xpat* antisense RNA probes were generated as described by Tarbashevich et al.[20] Visualization and calculation of PGC number in *X. laevis* embryos was performed using a LumarV.12 fluorescence stereomicroscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany). In each experiment, the spawn of the same frog was used for the injections of different constructs. Average numbers of PGCs per embryo were calculated for each condition in each experiment. Due to the high variation between individual frogs (Suppl. Fig. 1), for each experiment control injections (control morpholino oligonucleotides or *GFP_DELE* mRNA) were used to normalize E-cadherin overexpression and knock-down phenotypes.

Isolation of primordial germ cells (PGCs) and somatic endodermal cells

PGCs were fluorescently labelled by the injection of *GFP_DELE* mRNA as described above (Fig. 1A). Embryos were cultivated till stage 17-19 and stage 28-30 of development[8] and then used to obtain ventral explants. Dissected explants

containing somatic endodermal cells and PGCs were dissociated in calcium-magnesium free medium (CMFM: 88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 7.5mM Tris-HCl, 1mM EDTA, pH=7.6) for 20-30 min on 30 mm Petri dishes covered with 2% agarose. GFP-positive PGCs and GFP-negative somatic endodermal cells were manually sorted using an in-house made eyebrow hair tool under LumarV.12 fluorescence stereomicroscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) and washed in 1xPBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4).

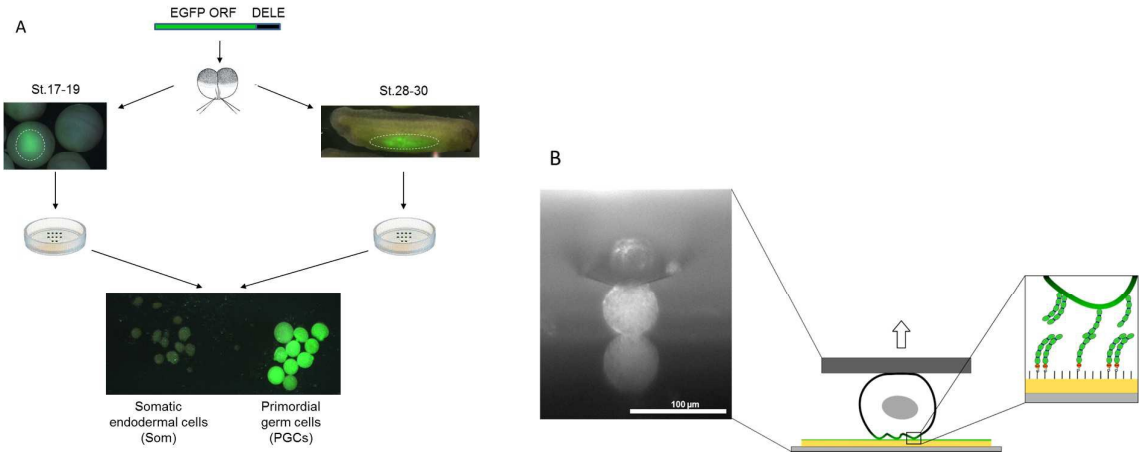

Fig. 1. Principle of the setup. **(A)** Embryos were injected vegetally at 2-cell stage with *GFP_DELE* mRNA. Endodermal explants containing labelled primordial germ cells (PGCs) were isolated from the injected embryos at developmental stages 17-19 and 28-30. These explants were dissociated to obtain individual cells. GFP-positive PGCs can be distinguished from GFP-negative somatic endodermal cells (Som). **(B)** Bright-field side view image of a single cell attached to an atomic force microscope cantilever. The cell is brought into contact with a gold-coated glass cover slip functionalized an E-cadherin (E1-5) layer monolayer. The bottom cell is a mirror image due to the highly reflective gold coating of the substrate. The scheme illustrates the setup in which the cell is pulled back from the E-cadherin (E1-5) layer by the AFM cantilever. The interaction between cell and E-cadherin layer is recorded by force-distance curves. The E-cadherin (E1-5) is attached via SNAP-tag, which is covalently bound to the benzyl guanine (BG) head groups of the thiols organized in a self-assembly monolayer (SAM) formed on the gold surface.

E-cadherin functionalization of gold surfaces

Gold-coated glass cover slips (150 nm gold on 20 nm chromium) were glued onto a petri dish serving as the substrate for the subsequent self-assembly steps. For this purpose, petri dish (TPP, Switzerland) and cover slip were rinsed with

isopropanol, dried, glued together (UHU endfest 300, Bühl, Germany) and cured overnight. After annealing, the petri dish was rinsed with isopropanol, dried and stored. Formation of self-assembly monolayers functionalized with E-cadherin was achieved by following a protocol detailed in Fichtner et al.[21] In brief, a mixture of BG thiol (BGT) and matrixthiol (MT) (1:100) at a total thiol concentration of 100 µM in isopropanol was incubated for 3 h onto the gold-coated glass cover slip. After incubation, the supernatant was removed and the petri dish with the cover slip was rinsed with pure isopropanol. Subsequently, the thiol-surface was exposed to 2 µM E-cadherin fusion proteins dissolved in HBS (1 mM EDTA, 10 mM HEPES, 150 mM NaCl, pH 7.4) for 2 hours at room temperature. The last step was to remove the supernatant E-cadherin solution by rinsing the surface with 1x DMEM (Lonza, Basel, Switzerland) containing 200 µg/mL Penicillin/Streptomycin (GE Healthcare, Freiburg, Germany), 5 µg/mL Amphotericin B (GE Healthcare), 15 mM Hepes (Biochrom AG, Berlin, Germany)). The activity of E-cadherin was confirmed by single molecule force spectroscopy as detailed below.

Sideview imaging

Lateral images of the cantilever in contact with the surface were obtained by using the sideview-setup from JPK Instruments. Notably, mirror images of cells attached to the cantilever occur due to the reflective gold coating of the substrate. Sample preparation and handling was done as described previously by Gonnermann et al.[22]

Single cell force spectroscopy

Single PGCs or somatic endodermal cells isolated either from stage 17–19 (early) or stage 28–30 (late) embryos were used. PGCs were fluorescently labelled by injection of GFP_DELE mRNA, where Dead End localization element (DELE) mediated PGC-specific expression (Fig. 1A). All measurements were performed using a Cellhesion200 AFM (JPK Instruments, Berlin, Germany) mounted on top of an Olympus IX81 microscope (Olympus, Hamburg, Germany). For cell picking, poly-D-lysine coated cantilevers (Arrow-TL2-50, Tipless Silicon SPM-Sensors, Nano World) with a nominal spring constant of 0.03 N/m were used. The cantilever spring

constant was determined before each measurement using the thermal noise method.[23] Before coating with poly-D-lysine, the cantilevers were washed with ethanol, ultra-pure water mixture (1:1), isopropanol and finally cleaned in argon plasma for 1 min. Afterwards, cantilevers were incubated in 100 µg/ml poly-D-lysine (Sigma-Aldrich, Munich, Germany) solution (in 1x PBS) for 15 min. A contact force of 0.5 – 1.0 nN lasting for 30 s was chosen to attach the cells to the cantilever. Petri dishes were separated into two areas using a liquid-repellent slide marker pen (Super PAP Pen Liquid Blocker, mini; Daido Sangyo Co., Ltd, Tokyo, Japan) to investigate the interaction of either primordial germ cells or somatic cells with the E-cadherin layer. One area was coated with bovine serum albumin (BSA) (5% (w/v) in 1x PBS (Biochrom) without Ca^{2+} and Mg^{2+} (PBS^-; Albumin IgG free, Carl Roth GmbH, Karlsruhe, Germany) for 1 h to avoid cell spreading and therefore facilitate picking of the cells, followed by 3x washing with 1x PBS^-. The E-cadherin layer was prepared in the other part of the petri dish. Up to 9 different spots on the E-cadherin layer were probed with 5 force curves using the same cell on the cantilever. At least 5 cells were investigated per category. The whole measurement was performed in measurement buffer (DMEM (Lonza) with 200 µg/mL Penicillin/Streptomycin (GE Healthcare), 5 µg/mL Amphotericin B (GE Healthcare), 15 mM Hepes (Biochrom) at room temperature.

In Figure 1B we show a scheme and sideview micrograph illustrating the measurement principle. A single primordial germ cell is brought into contact with an E-cadherin (E1-5) coated gold layer for a defined time and load force. After pressing the PGC onto the E-cadherin monolayer for a dwell time of 1 s the cell is detached and the resulting attractive forces between the cell and the E-cadherin (E1-5) layer are recorded as force-distance curves. We chose only very short dwell times (1s) since we were mainly interested in mimicking the situation of cell locomotion where short contact times are expected. Moreover, longer dwell times result only in a larger width of the histograms.[12] The adhesion experiments were carried out with an approach/retraction velocity of 5 µm/s, respectively, and at contact forces of 500 pN between the cell, attached to the cantilever, and the E-cadherin layer.

Single molecule force spectroscopy

Single molecule force experiments were prepared and performed as described before in Fichtner et al., 2014.[21] Recording single rupture events allowed us to confirm proper activity of the cadherins and successful surface functionalization. In brief, cantilevers (BioLever, BL-RC150VB, gold-coated on both sides, Olympus) were cleaned in argon plasma for 20 s and were subsequently immersed into the thiol solution for 3 h. Prior to functionalization with cadherin molecules, cantilevers were rinsed with pure solvent and EDTA-buffer (2 mM EDTA in HBS) to remove excess thiols from solution. Cadherins were coupled during incubation with protein solution (1–2 µM in EDTA-buffer) for 2 h. Prior to force spectroscopy measurements, cadherin-coated surfaces were washed with Ca^{2+}-buffer (2 mM in HBS) and activated by incubation in the same buffer for 30 min. The cantilever spring constant (nominal spring constant 6 pN/nm) was determined prior to each experiment by the thermal noise method as described above. Force-distance curves were performed with a pulling velocity of 1000 nm/s. Contact (compression) force was set to 50 pN and the contact time was set to 0 s.

Results

E-cadherin is expressed in the endoderm of X. laevis embryos

Previous immunostaining analysis of the E-cadherin distribution during *X. laevis* development had indicated that endodermal cells up to stage 20 express no or only very low levels of E-cadherin.[24] Our recent results had indicated that E-cadherin mRNA is present in somatic endodermal cells and PGCs at stages 17-19 and 28-30.[12] The presence of E-cadherin protein in the endodermal explants, which includes both PGCs and somatic cells from stage 17-19 embryos, was quantified by Western Blot analysis (Fig. 2). The specificity of the detected proteins was ensured by including controls with E-cadherin knockdown and detection of the closely related C-cadherin into the analysis. Several types of E-cadherin can be detected in *X. laevis* cells, including an E-cadherin precursor (~155kDa), intact E-cadherin (~140kDa), a degradation product (~116kDa) and the trypsin resistant ectoplasmic domain (~100kDa).[17]

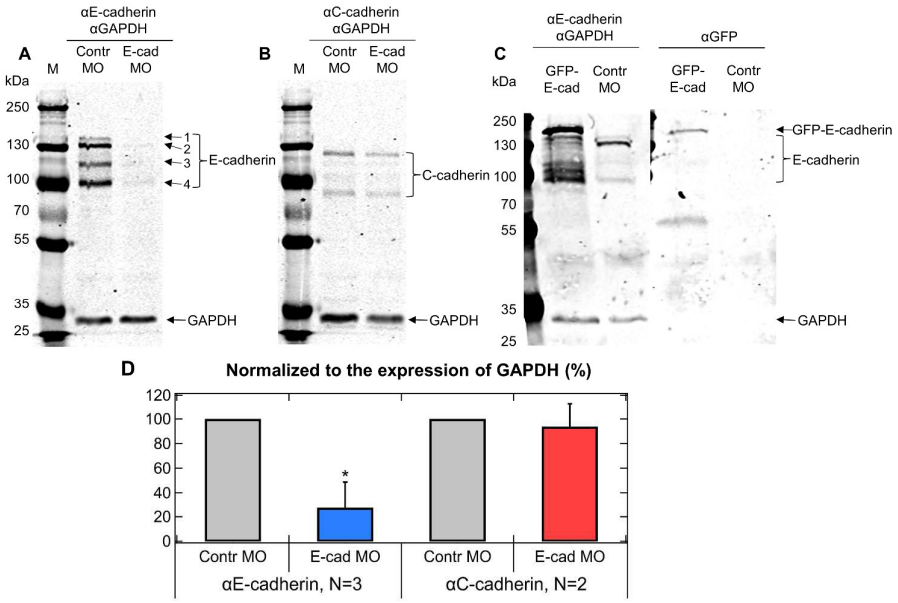

Fig. 2. E-cadherin is expressed in the endodermal cells of *Xenopus laevis*. Embryos were injected at 2-cell stage with control morpholino oligonucleotides (Contr MO), E-cadherin morpholino oligonucleotides (E-cad MO) or *GFP_E-cadherin_DELE* mRNA (E-cad GFP). Endodermal explants were obtained from stage 17-19 embryos and used for Western Blot analysis. Expression of GAPDH was used as a positive control, M correspond to the marker lane. **(A)** Several bands correspond to different forms of E-cadherin[17]: 1 - E-cadherin precursor (155 kDa); 2 - intact E-cadherin (140 kDa); 3 - degradation product (116 kDa); 4 - trypsin-resistant ectoplasmic domain (100 kDa). **(B)** C-cadherin staining was used as a control for E-cad MO specificity. **(C)** GFP-tagged overexpressed E-cadherin can be detected in the endodermal explants. **(D)** Expression of E- and C-cadherin was normalized to the expression of GAPDH and quantified in E-cad MO injected embryos relative to Contr MO. *N* corresponds to the number of experiments. Error bars represent standard deviation. * - $p<0.05$ (Wilcoxon rank sum test).

Each one of these proteins was detected in endodermal explants isolated from stage 17-19 *X. laevis* embryos, and their expression was significantly reduced upon E-cadherin knock-down via morpholino oligonucleotides (E-cad MO) (Fig. 2A). In contrast, C-cadherin expression was not affected by E-cad MO (Fig. 2B).

Interference with endogenous E-cadherin expression leads to a decrease of PGC numbers at the tailbud stage

Maintenance of E-cadherin expression has been shown to be required for proper germ cell development and migration in several species, including *Drosophila*,

zebrafish and mouse.[4] To elucidate whether E-cadherin is involved in PGC development in *X. laevis*, both knock-down and PGC-specific overexpression of E-cadherin were performed (Fig. 3). PGC-specific overexpression was achieved by the injection of chimeric mRNA, containing *X. laevis* E-cadherin ORF fused in frame to GFP ORF and Dead End localization element (DELE). GFP-tag was used to identify overexpressed E-cadherin (Fig. 2C, Suppl. Fig. 2, Suppl. Fig. 3), while DELE insured PGC-specific expression. In comparison to the control injection, embryos with overexpressed E-cadherin showed ~50% reduction in the number of PGCs at the tail-bud stage (st.28-30). Similar results were obtained with E-cadherin knock-down via specific morpholino oligonucleotides (E-cad MO). In the latter case, however, knock-down was not specific to PGCs and led to defects in embryonic development and death of the embryos, especially during neurulation. This resulted in a low number of embryos, surviving until tail-bud stage (Suppl. Fig. 1). Decrease of PGC numbers upon both E-cadherin overexpression and knock-down was observed at tail-bud stage (st.28-30), but not at the neurula stage (st.17-19) of embryonic development (Suppl. Fig. 4).

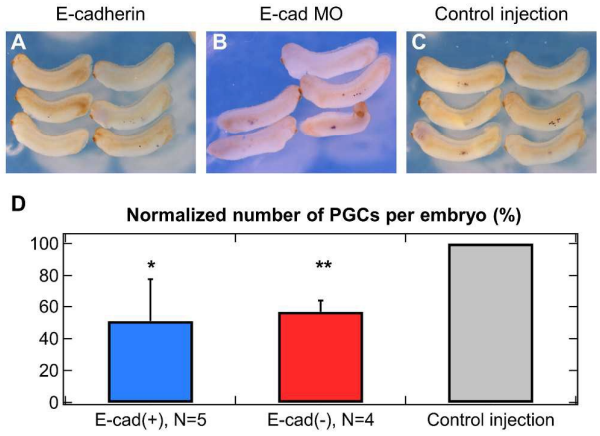

Fig. 3. Overexpression and knock-down of E-cadherin results in a decrease of PGC numbers. Embryos were injected at the 2-cell stage with *GFP_E-cadherin_DELE* mRNA **(A)**, or E-cadherin morpholino oligonucleotides. **(B)**. For each experiment control injections with *GFP_DELE* mRNA **(C)** or control morpholino oligonucleotide were performed. **(A-C)** Embryos were fixed at stage 28-30 and used for whole mount *in situ* hybridisation with antisense *Xpat* RNA as a PGC marker. **(D)** Number of PGCs per embryo was calculated in each experiment and normalised to the control injection. N corresponds to the number of experiments. Error bars represent standard deviation. * - $p<0.05$; ** - $p<0.01$ (2-tailed paired t-test).

E-cadherin-mediated adhesion is reduced in PGCs at the tail-bud stage

Our previous studies had indicated that the E-cadherin mRNA level in late PGCs (but not in somatic endodermal cells) at the tail-bud stage (st.28-30) decreases in comparison to the neurula stage (st.17-19) of *X. laevis* development.[12] Therefore, we decided to investigate the impact of expression level of E-cadherin on the adhesion forces between PGCs at the two different stages and E-cadherin functionalized surfaces using Single Cell Force Spectroscopy (SCFS). Adhesion of somatic cells to E-cadherin monolayers was also investigated.

The SCFS setup allows measuring the adhesive forces between PGCs and artificial E-cadherin (E1-5) layer down to molecular resolution. Using an E-cadherin functionalized substrate instead of living cells permits us to reduce complexity and to focus on a single dedicated non-covalent bond, in our case the homotypic E-cadherin interaction. The force-distance curves (retraction curves) were analysed with respect to the overall dynamic strength, i.e. the largest rupture force, and the occurrence and strength of small unbinding events that can be attributed to rupture of single non-covalent bonds formed between the E-cadherin on the cell's surface and that of the substrate, respectively. The maximum adhesion force represents the maximum dynamic strength of cell-substrate binding[25] including all sources of attractive interactions between cell and surface. Besides specific interactions mediated by cadherins or other intercellular adhesion molecules (ICAMs), adhesion also compiles contributions from electrostatic interactions as well as attractive van der Waals forces partly counterbalanced by repulsive interactions due to steric hindrance of repeller molecules and membrane undulations at low tension.[26]

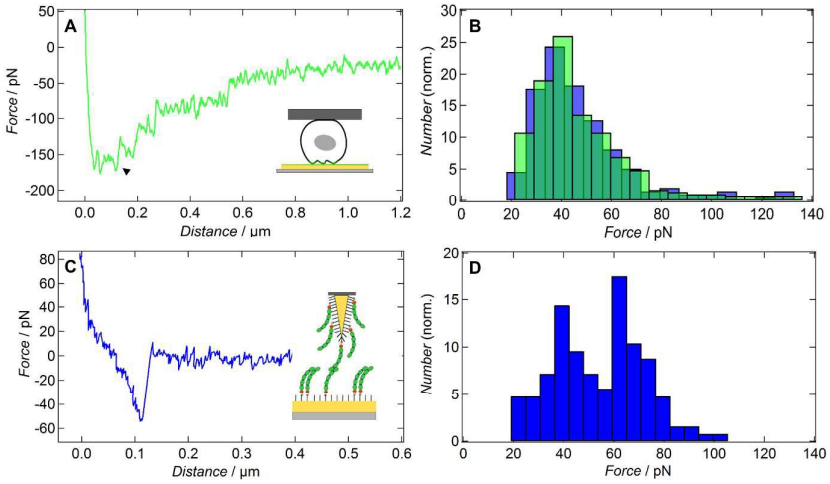

Fig. 4. Comparison SCFS vs SMFS. **(A)** Typical AFM force-distance curve of a primordial germ cell displaying the interaction with the E-cadherin (E1-5) layer. The arrow indicates a single rupture event (SRE) occurring during unbinding of the cell from the E-cadherin coated surface. **(B)** The rupture force histogram (normalized to the sum of 100) shows the distribution of the single rupture events of the PGCs isolated from embryos at stages 17–19 (pre-migratory PGCs/green/*n* (SRE) = 288/ *n* (cells) = 8) and stages 28–30 (migratory PGCs/purple/*n* (SRE) = 197/ *n* (cells) = 8). **(C)** Typical SMFS force-distance curve shows the unbinding of E-cadherin trans-dimers between an AFM cantilever and the gold surface, which are both coated with E-cadherin (E1-5) monomers. **(D)** The rupture force histogram (normalized to the sum of 100) shows the distribution of the single rupture events of the SMFS experiments and reveals two major maxima indicating two types of bonds (*n* (SRE) = 125).

Figure 4A illustrates a typical force-distance curve recorded upon retraction of the cell from the E-cadherin coated surface displaying all relevant features that are usually observed. In addition to the maximal adhesion force measured close to the surface, single rupture events attributed to non-covalent molecular contacts, e.g. homotypic E-cadherin dimers, are also found. The maximum adhesion force sums up non-specific interactions and also the attractive forces exerted by E-cadherin clusters that are not resolved as individual rupture events. Besides, individual rupture events of E-cadherin dimers can frequently be assigned (black arrow Fig. 4A). At larger distance from the surface membrane, tethers are extracted from the cell that eventually detach from the substrate if the lifetime of the bonds is exceeded. The distribution of single molecule forces obtained from rupture events with pre-migratory and migratory PGCs in contact with E-cadherin coated surface is exemplarily shown in Figure 4B. Forces to separate E-cadherin dimers in between 20-80 pN are in very good accordance

with previously published data.[27-29] Importantly, the histogram of rupture forces for migratory PGCs shows the same distribution as the pre-migratory PGCs with one maximum peak at 30 pN. This finding corresponds well with the assumption that differences in adhesion between the two types of PGCs are based on the number of expressed cadherins but not on alteration of the interaction force between two E-cadherins. We also carried out experiments with regular tip-carrying cantilevers equipped with E-cadherin as described recently.[21] The data confirm that the maximum peak at 40 pN can indeed be attributed to specific E-cadherin unbinding events and that specific interactions are probed (Fig. 4C). The histograms for single molecule rupture events reveal the typical bimodal distribution for the unbinding force (Fig. 4D) of the homotypic E-cadherin bond as previously reported.[21] The first maximum lies at 40 pN and the second centres around 65 pN. In comparison to the single cell experiments, the single molecule experiment shows one more maxima, the one at 65 pN, indicating a second type of bond for the E-cadherin interaction that is either not present in the cell-substrate experiments or obscured by a larger spread of loading rates due to the inherent softness of the cell.[28]

In order to compare the adhesiveness between different cell types one can either count the number of single molecule rupture events per cell or measure the overall adhesion force. From a biophysical point of view, the latter strategy might be less molecule specific than counting the number of individual bonds but the overall adhesion force is more decisive for cell locomotion considering the general demands for directional and active cell migration. A certain amount of traction force is necessary for migration since PGCs cannot move without attachment, while on the other hand strong adhesion abolishes movement of cells.

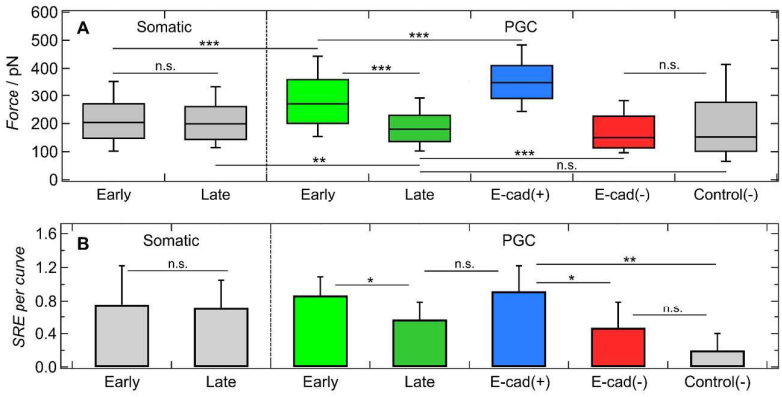

Fig. 5. E-cadherin interaction displayed through maximum adhesion force and single rupture events per curve. (A) Maximum adhesion force of somatic endodermal cells and PGCs both isolated from embryos at stages 17–19 (**early**) and stages 28–30 (**late**). Additionally, the maximum adhesion force of PGCs with E-cadherin over expression (E-cad(+)), E-cadherin knock-down (E-cad(-)) and PGCs in contact with a thiol-monolayer without E-cadherin (Control(-)) is shown. Box-whisker-plots: line denotes the median of the distribution, boxes comprise the 25^{th} and 75^{th} percentile, whisker tops and bottoms are drawn to the 10^{th} and 90^{th} percentiles, respectively. Numbers of curves which were analyzed for each category: somatic endodermal cells early (n (curves) = 320); late (n (curves) = 312); PGC: early (n (curves) = 325); late (n (curves) = 344); E-cad(+) (n (curves) = 135); E-cad(-) (n (curves) = 328); Control(-) (n (curves) = 207) **(B)** Number of single rupture event (SRE) per curve (mean ± SEM) for the same categories as described in **(A)**. At least 5 cells were tested per category. P-values are calculated over the Wilcoxon rank sum test. Not significant (n.s.) - $p>0.05$; * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

Figure 5A shows the maximum adhesion forces of all cell-substrate interactions measured in this study. The plot comprises somatic cells and PGCs harvested at different stages. We also investigated the adhesive behaviour of E-cadherin knock-down (E-cad(-)) and PGCs isolated at the pre-migratory stage (st.17-19) of *X. laevis* overexpressing E-cadherin (E-cad(+)). As an additional control, the adhesion force between PGCs and the self-assembled thiol-monolayer on the gold surface in the absence of E-cadherin proteins (Control(-)) was analysed. This control serves as a measure for the expected nonspecific adhesion comprising van der Waals attraction and electrostatic interaction between cell and the self-assembly layer on the gold surface. The box plots in Figure 5A show that the adhesion force between early (st. 17-19) and late stage (st. 28-30) somatic endodermal cells remains at a constant level. Both median values with 204 pN (early somatic cells) and 199 pN (late somatic cells) are essentially identical.

The primordial germ cells, however, change their adhesive behaviour during the different stages of development (Fig. 5A). Between the early developmental stages 17-19 and the late stage 28-30 the overall adhesion between the cell and the E-cadherin layer is significantly reduced, in fact almost down to the level of non-specific adhesion (152 pN). The median value drops from 270 pN for the early PGCs to 180 pN for late PGCs in accordance with the transition to active migration.

In order to show that indeed E-cadherin is responsible for the drop in maximal adhesion forces we attached both E-cadherin deficient PGCs and PGCs overexpressing E-cadherin to the cantilever. E-cadherin knock-down with morpholino oligonucleotides (MO) resulted in a significant drop of the adhesion force to the level of nonspecific interactions between cells and the substrate (150 pN). In the case of PGCs overexpressing E-cadherin the adhesion force shows the largest values with median value of 350 pN.

Besides measuring the overall adhesion forces we also counted the number of single molecule rupture events (SRE) that match the force jumps found for E-cadherin-E-cadherin interactions in single molecule force spectroscopy experiment. These events are therefore indicative for the presence of E-cadherin molecules on the cell surface (Fig. 5B). We found essentially the same trend for SRE as for the maximum adhesion force. The number of single rupture events (SRE) per force curve remains constant for the somatic endodermal cells at the early and late stage. For the primordial germ cells the number of SRE drops from 0.87 at the early stage (st.17-19) to 0.57 at the late stage (st.28-30) and underlines the reduced number of E-cadherin contacts at the migratory stage. The highest value of SRE per curve is shown by the E-cadherin overexpressing PGCs with 0.92 (E-cad (+)). This means that on average in nearly every force-distance curve at least one E-cadherin rupture event is identified. Note that in some force curves no single events were detected. For the E-Cadherin knock-down PGCs (E-cad (-)) the value is slightly below the one for late stage PGCs (0.47). The control cells in contact with only a self-assembled thiol-monolayer on the gold surface show the lowest number of SRE close to zero, as expected. The values for the control and the knock-down PGCs show that the setup still detects some nonspecific unbinding events between the cell and the artificial system. But these nonspecific interactions occur in every category so that the observed differences are caused by the varying E-cadherin surface concentration and not by nonspecific binding forming the interaction baseline of the experiment.

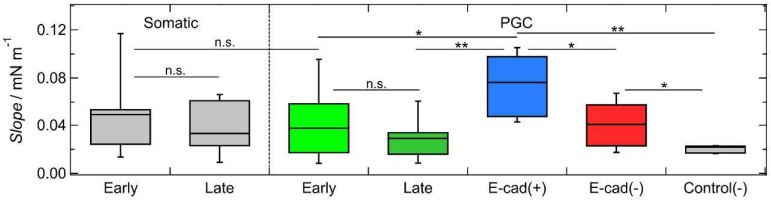

Fig 6. Cortical tension. Cortical tension shown through the slope of the trace curve (compression between parallel plates) as box-whisker-plots. Comparison between somatic endodermal cells and PGCs both isolated from embryos at stages 17–19 (**early**) and stages 28–30 (**late**) is shown. Additionally, the cortical tension of PGCs after E-cadherin over expression (E-cad (+)), E-cadherin knock-down (E-cad(-)) and PGCs in contact with a thiol-monolayer without E-cadherin (Control(-)) is shown. Box-whisker-plots: line represents the median of the distribution, boxes comprise the 25^{th} and 75^{th} percentile, whisker tops and bottoms are drawn to the 10^{th} and 90^{th} percentiles, respectively. P-values are calculated over T-Test/ Wilcoxon rank sum test. Not significant (n.s.) - $p>0.05$; * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

An important prerequisite for the validity of our approach is a maintained cortical tension and thereby conserved adhesion area regardless of the type of cell analysed. A change in adhesion area is likely to occur if the cortical tension is altered by the treatment. Therefore, we measured the cortical tension of each cell type by parallel plate compression (Fig. 6). The slope of the resulting force – compression curves is proportional to the cortical tension of cell.[30] A smaller stiffness of the cells would most likely result in a larger contact area and therefore generate larger adhesion forces. Except for the E-cad (+) PGCs, which were found to be slightly stiffer, we could not detect a systematic change of cortical tension in any of the categories that would explain our differences in cell adhesion. Therefore, it is safe to assume that the change in adhesion force is due to differences in the levels of E-cadherins on the cell surface and not due to an altered cell mechanics. In the case of E-cad (+) PGCs adhesion forces are even underestimated due to the larger stiffness, therefore we can safely assume that indeed E-cadherin overexpression is responsible for the increase in interaction strength. A larger stiffness as observed for E-cadherin overexpressing cells most likely results from a larger cortical tension exerted by the contractile actomyosin cortex. Cells that overexpress E-cadherin are expected to recruit more actin to the plasma membrane attaching to the cytosolic adapter proteins and thereby generate a stronger and thicker cortex, which in turn produces an increased stiffness.[31]

Discussion

Directional PGC migration is highly regulated both in time and space as it follows an intricate pathway in a varying environment. Most of our understanding of PGC migration comes from the model organisms *Drosophila melanogaster*, zebrafish, *Xenopus laevis* and mice. The dynamic changes associated with germ cell migration in *Xenopus* have been classified systematically.[10] Migration of PGCs within the endoderm is coupled to changes with respect to their locomotive and adhesive properties. Prior to the onset of active migration (before stage 25) isolated PGCs show only few protrusions and assume a predominately spherical morphology, similar to somatic endodermal cells. At stage 28 PGCs acquire an elongated shape and start displaying migratory activity. PGCs show a high level of cellular dynamics at stage 33/34 by formation of numerous bleb-like protrusions fostering migratory activity that switches between locomotive and pausing phases. Later, locomotion activity is reduced at stage 41 and subsequent migration of the PGCs to the gonads takes place via the dorsal mesentery. This mesentery is formed by two sheets of splanchnic mesoderm that surround the gut. As these sheets converge at the dorsal crest of the endoderm, PGCs can exit the endoderm and eventually incorporate into the dorsal mesentery (stage 45).

A number of germ cell specific proteins have been identified such as XDead end[32] and XDAZL.[33] XDead and XDAZL are mRNAs encoding RNA binding proteins that are required for dorsal migration since knockdown results in PGCs that are unable to disperse.[6, 32] Therefore, it is assumed that these two proteins are functionally linked to each other as well as to the regulation of adhesive properties. A more recent study has provided further insight into the role of Dead end in the context of bleb-based motility of zebrafish PGCs.[34] Messenger RNAs encoding myosin light chain kinase as well as transcriptional repressor Zeb1 were identified as putative Dead end targets. Zeb1 could be responsible for the downregulation of E-cadherin, as observed during the transition of zebrafish PGCs to their active migratory state.[13] Regulation of cellular adhesion is known to play an important role in the context of germ cell migration in different animal species.[4] Previously, we found that *X. laevis* PGCs isolated from the tail-bud stage (st.28-30) show a decrease in E-cadherin mRNA levels in contrast to somatic endodermal cells and PGCs from the neurula stage (st.17-19).[12] Moreover, a newly established “under-agarose” cell migration assay was

employed to study PGC migration in vitro. PGCs isolated from tailbud stage embryos were placed underneath a layer of agarose on top of bovine serum albumin (BSA)-coated culture dishes to prevent non-specific binding of the cells. The agarose gel on top created a confined environment, which allowed the cells to generate sufficient traction force. Under these conditions, cells migrate actively by producing blebs. The observation that isolated PGCs are able to migrate under these conditions, suggests that they do not require specific cell-cell or extracellular matrix adhesion for motility. This finding is in accordance with our observation that late PGCs essentially display very low E-cadherin mediated interactions. We recently carried out single-cell force spectroscopy (SCFS) to investigate whether cell-cell adhesion is diminished during the transition of Xenopus PGCs to active migration. We found that cell-cell adhesion between migratory PGCs and somatic cells is significantly decreased, as compared to the interactions of PGCs in an earlier state. These experiments suggested that PGCs reduce their adhesiveness to the surrounding somatic cells during development. But in this previous work we could not assign this to the reduced E-cadherin expression. Studies on zebrafish PGCs showed that reduced intercellular adhesion via E-cadherin is important for proper PGC migration.[13, 14, 34] Down-regulation of E-cadherin is also known to result in increased migratory behaviour of germ cells in Drosophila melanogaster,[35] while other cell adhesion molecules such as integrins and selectins are known to be involved in the interactions of mouse PGCs with their surrounding during migration.[36] Altogether, data from different model organisms indicate that lowering of cell adhesion fosters PGC motility. This has also been confirmed by our own qPCR data with *Xenopus* PGCs suggesting that the loss of adhesiveness to the surrounding endodermal somatic cells is mainly due to down-regulation of specific cell adhesion molecules such as E-cadherin.[12] Interestingly, somatic cells retain a similar level of mRNA encoding E-cadherin within the same period of development.

In this study, we quantified the adhesion of PGCs and somatic cells harvested at different stages to E-cadherin coated surfaces to elucidate the role of E-cadherin in migration of PGCs in *X. laevis* embryos. The setup allows us to focus on a particular molecule without interference from other cell adhesion molecules (CAMs). It is clear that besides CAM-CAM recognition, cellular adhesion also results from a competition between attractive van der Waals interactions and electrostatic repulsive mediated

by, for instance, charged (glyco)lipids. In our SCFS experiment not only molecular recognition events between E-cadherin molecules on the cell surface and on the substrate are monitored but also non-specific interactions that appear as a background force. We found that the base level of the interaction was about 150 pN adhesion force. At the other end of the scale overexpression of E-cadherin produces adhesion forces higher than 300 pN for the same dwell time and even slightly elevated cortical tension. In between these two limit we find a significant difference between actively migrating PGCs with low adhesion force and pre-migratory ones that show essentially the identical adhesion force as found for somatic cells. Knock-down of E-cadherin with morpholino oligonucleotides shows only a negligible further decrease in adhesion as compared to late PGCs, suggesting that the number of E-cadherin molecules on the cell's surface is already extremely low. This kind of comparison is possible since the cells display essentially unaltered cortical tension ensuring a constant adhesion area that would otherwise impact the measured force. Importantly, the forces found in our experiments are similar to those probing PGCs and adherent somatic ones,[12] where late PGC also display smaller unbinding forces (< 290 pN) compared to early ones (> 320 pN). Taken together, our approach using defined substrates functionalized with E-cadherin produces more significant results with a smaller spread than cell-cell experiments, which is certainly due to the smaller background noise from other ICAMs and a more broadly varying adhesion area if two soft cells were brought into contact.[12]

Besides the maximum adhesion force that comprises individual molecule pairs as well as clusters and non-specific interactions, we also counted the number of individual rupture events in every force curve. The events were classified as rupture of homotypic E-cadherin bonds based on a comparison with single molecule force spectroscopy experiments. Here, the picture is consistent with the maximum adhesion force. We found a decreasing number of single rupture events for late PGCs in comparison to early ones. While almost every force curve also shows single rupture events, the number is almost cut into half for late PGCs. Altogether, our study confirms that downregulation of E-cadherin is a key step towards active migration of primordial germ cells in *X. laevis* embryos. The necessary traction force to accomplish a directed motion is therefore most prominently provided by non-specific van der Waals forces rather than specialized molecules. It has been proposed that an

independent migration mechanism, referred to as ‘chimneying’ in resemblance of a common climbing technique to conquer rock clefts, can account for 3D migration of cells.[37] Chimneying can be accomplished in the absence of specific adhesion as shown, for instance, by leukocytes, which can migrate by squeezing and exerting pushing forces perpendicular to the cell boundary.[38] Yip et al.[39] showed that chimneying requires a balance between intracellular pressure and membrane cortex strength. Traction is mainly achieved by pushing against the wall. Pushing forces that originate from actin polymerizing against the sides of a cell embedded in confinement also allow for directional cell migration.[40] It has been suggested that cortical flow of actin coupled to friction arising from both nonspecific and specific interactions with the substrate could move cells in confinement forward.[41] Previously, reproducible PGC migration for late stage PGCs was achieved using a so-called “under-agarose” migration assay. There we found that blebbing is strongly enhanced in these migratory PGCs, which together with a reduced adhesiveness allows the cells to propel forward to their destination. Our electric impedance measurements with both early and late single isolated PGCs also suggest that internal cell contractility is not directly coupled to cell-cell adhesion.[12] Similar findings have been reported for zebrafish PGCs.[13,14,34] In conclusion, we claim that a decrease of the E-cadherin expression level is required to change PGCs from a sessile to an actively migrating state. A change of a few tens of Piconewtons decides whether a cell moves or stays in place.

Acknowledgements

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Migratory PGCs from *Xenopus laevis* display smaller adhesion forces in contact with E-cadherin coated surfaces.