Establishment of a relationship between blastomere geometry and YAP localisation during compaction

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SUMMARY STATEMENT

We show that the localisation of YAP, a key factor during the first cell fate decision, is linked to individual blastomere geometry within the 3-dimensional environment of the preimplantation embryo.

ABSTRACT

Precise patterning within the 3-dimensional context of tissues, organs and embryos implies that cells can sense their relative position. During preimplantation development, outside and inside cells rely on apicobasal polarity and the Hippo pathway to choose their fate. Despite recent findings suggesting that mechanosensing may be central to this process, the relationship between blastomere geometry (i.e. shape and position) and the Hippo pathway effector YAP remains unknown. We used a highly quantitative approach to analyse information on the geometry and YAP localisation of individual blastomeres of mouse and human embryos. We identify proportion of exposed cell surface area as most closely correlating with nuclear localisation of YAP. To test this relationship, we developed several hydrogel-based approaches to alter blastomere geometry in cultured embryos. Unbiased clustering analyses of blastomeres from such embryos reveal that this relationship emerges during compaction. Our results therefore pinpoint when during early embryogenesis cells acquire the ability to sense changes in geometry and provide a new framework for how cells might integrate signals from different membrane domains to assess their relative position within the embryo.
INTRODUCTION

Through the course of embryonic development, complex tissues and organs are formed from a single cell. This requires each given cell within the developing embryo to constantly sense its position within its 3D environment in order to make correct fate decisions. Morphogen gradients have long been suggested to play an important role in transmitting positional information across tissues during embryo patterning (Briscoe and Small, 2015; Tickle et al., 1975) though even here, mechanistic details remain unclear (Wolpert, 2016). When small groups of cells are concerned, morphogen gradients are conceptually harder to set up, leading cells to rely on other strategies. In the mouse pre-implantation embryo, around the 16-cell stage, blastomeres differentiate into trophectoderm (TE) or inner cell mass (ICM) cells depending on their outside or inside position respectively. The molecular mechanisms by which outside and inside cells determine their fate has been extensively studied, highlighting the importance of apicobasal polarity and Hippo signalling (Plusa et al., 2005; Sasaki, 2017; White et al., 2018).

In apolar inner cells, AMOT localises at cell-cell junctions where it associates with NF2 and is phosphorylated by the Hippo pathway kinases Lats1/2 (Cockburn et al., 2013; Hirate et al., 2013). In concert with Lats1/2, AMOT is then able to induce the phosphorylation of YAP and its sequestration to the cytoplasm. In inner cells, YAP is consequently unable to bind to TEAD4 and cannot induce the transcription of TE genes such as CDX2 (Nishioka et al., 2007; Nishioka et al., 2009). Instead, genes associated with pluripotency such as SOX2 drive ICM fate in these cells (Wicklow et al., 2014). In outer cells, the establishment of apicobasal polarity generates a contact-free membrane domain where cortical F-actin sequesters AMOT away from cell-cell junctions, preventing its phosphorylation and interaction with Hippo pathway components (Hirate and Sasaki, 2014). This results in the inability of the Hippo pathway to phosphorylate YAP, which can then translocate to the nucleus and interact with TEAD4 to drive the expression of TE-specific genes such as CDX2, consequently inducing TE fate. Together, this body of work, in addition to highlighting the importance of the Hippo pathway and apicobasal polarity in the first cell fate decision, also suggests multiple links between cytoskeletal organisation and Hippo pathway regulation.

It is now widely accepted that forces, related to cell shape changes or cell position within a group of cells, can modulate the localisation and activity of YAP via the actin cytoskeleton (Aragona et al., 2013; Dupont et al., 2011; Halder et al., 2012; Wada et al., 2011). In the preimplantation embryo, although it has been suggested that mechanosensing may occur (Maltré et al., 2016) and despite the multiple links between the Hippo pathway and the actin
cytoskeleton, it remains unclear whether the shape or position of individual blastomeres can directly regulate the subcellular localisation of YAP to modulate cell fate.

To answer this question, we generated a collection of embryos from the 2- to the 64-cell stage, quantifying the subcellular localisation of YAP as well as several descriptors of blastomere shape and position. When considering blastomeres across all these embryonic stages, we find a progressive increase in the proportion of blastomeres with higher nuclear versus cytoplasmic YAP. We used a multivariate analysis on this dataset to test if any of the quantitative descriptors of blastomere shape and position showed a correlation with the ratio of nuclear to cytoplasmic YAP. This revealed that the proportion of exposed surface area of a blastomere had the strongest correlation with differences in YAP localization. Using non-invasive methods to modulate the shape and position of blastomeres within embryos, we uncover that this relationship emerges as early as the 8-cell stage, during compaction.

RESULTS

Cells with high nuclear to cytoplasmic YAP ratio occur prior to the first cell fate decision

In a 2D environment, cellular parameters such as shape and position, that we refer to as cell-geometry, have been shown to affect the localisation (and therefore activity) of YAP through its mechanosensing properties (Aragona et al., 2013; Dupont et al., 2011; Wada et al., 2011). In the pre-implantation embryo, despite the suggestion that mechanosensing may be involved in the regulation of YAP activity (Maitre et al., 2016), it remains unclear whether geometrical properties of blastomeres directly influence the relative distribution of YAP to the nucleus and cytoplasm. To analyse the relationship between the localisation of YAP and the geometry of individual blastomeres during mammalian preimplantation cell fate allocation, we used a highly quantitative approach combining imaging, manual segmentation and image analysis. We collected and stained mouse embryos for YAP, F-actin and E-cadherin at the 2- (n=20), 4- (n=8), 8- (n=10), 16- (n=17), 32- (n=12) and 64-cell (n=2) stages (Figs 1A and S1A).

We next created 3D cellular-resolution volume representations of these embryos by manually segmenting cell membrane and nucleus of individual blastomeres (Fig. 1B and movie 1). This allowed us to accurately quantify the cellular localisation of YAP in the nuclear and cytosolic compartments by determining the ratio of nuclear to cytoplasmic YAP (N/C YAP ratio). We were also able to quantify the area of the blastomere limiting membrane and
categorise it as ‘exposed to the outside’, ‘in contact with other blastomeres’ or ‘junctional’ (Fig. 1C) (Leonavicius et al., 2017). This allowed us to quantify three parameters of blastomere shape (sphericity, oblateness – flying saucer shaped and prolateness – rugby ball shaped), two parameters relating to blastomere position (proportion of surface exposed to outside and proportion of surface in contact with other blastomeres) and six parameters relating to other aspects of blastomere geometry (see methods for a description of all parameters and how they were calculated).

When we considered all blastomeres at each stage we found that, as expected, two populations of blastomeres with distinct N/C YAP ratios seemed to progressively appear from the 16-cell stage onwards (Fig. 1D). In order to quantitatively test this observation, we used K-means clustering of N/C YAP values of all the blastomeres in our dataset, spanning the 2- to 64-cell stage, to unbiasedly separate cells with high versus low N/C YAP ratio. This clustering found a N/C YAP ratio of 1.6 as the threshold separating ‘low’ and ‘high’ nuclear YAP cells, that would be expected to correspond to inside (or ICM) and outside (or TE) cells respectively. To independently test the validity of this threshold in categorising cells, we manually classified cells from 32- and 64-cell embryos as ICM or TE depending on their position. We found that the vast majority of TE cells were correctly classified as having high N/C YAP ratio (111/116, 95.7%) and all ICM cells were classified as having low N/C YAP ratio (Fig. S1B). Using this classification, we observed that, as published before when comparing inside and outside cells, these two populations become strongly separate from the 16-cell stage onwards (Fig. S1C) (Hirate et al., 2015; Nishioka et al., 2009), thereby validating the representativeness of our dataset. However, our detailed quantification further revealed that an increasing proportion of cells with high N/C YAP ratio arises already from the 4- to the 16-cell stage, suggesting that cells with relatively high N/C YAP ratio exist even before the 16-cell stage and the establishment of distinct outside and inside cells (Figs 1D and S1C).

Proportion of exposed surface, as opposed to shape, is strongly associated with N/C YAP ratio

To determine whether geometrical properties of blastomeres may directly influence the relative distribution of YAP to the nucleus and cytoplasm, we interrogated our quantitative data set for the correlation between N/C YAP ratio and the various parameters relating to blastomere shape and position that we had extracted.
Blastomere shape parameters (sphericity, oblateness, prolateness) were relatively poorly correlated with N/C YAP ratio (Fig. 2A,B). However, of the three blastomere shape descriptors, sphericity showed the highest correlation (Fig. 2A,B). Sphericity decreased gradually from the 2- to 64-cell stage, most likely because of the increasingly varied blastomere shapes arising as the embryos developed (Fig. S2A). N/C YAP ratio and sphericity showed a weak negative correlation (R= -0.39; p<2.2e-16) when considering all blastomeres from the 2- to the 64-cell stage (Fig. S2B). This correlation became slightly stronger if only blastomeres of the 32- and 64-cell stages were considered (i.e. as outside cells become more stretched and elongated in shape), but still remained relatively weak (R= -0.54; p<2.2e-16) (Fig. S2B). Together these data show that blastomere shape is a relatively poor predictor of YAP localisation, suggesting that it is unlikely that overall cell shape would directly modulate YAP localisation.

On the other hand, position parameters such as proportion of exposed surface area and its converse, proportion of contact area, showed the strongest correlation with N/C YAP ratio, both across our entire dataset and more so when considering the subset from 16- to 64-cell stage. Interestingly, N/C YAP and A/J ratio (the ratio between blastomere apical surface area and apical junctional interface area, estimating the extent to which the apical surface protrudes outwards) were also closely correlated, suggesting that the shape of the apical domain might be linked to the proportion of YAP in the nucleus and cytoplasm (Figs 2A,B and S2C). Interestingly, at the 16-cell stage, N/C YAP ratio strongly correlated with the proportion of exposed surface and contact areas, A/J ratio and absolute exposed blastomere surface area (Fig. S2D). Unsurprisingly, we found that overall, the proportion of exposed cell surface area continually decreased from the 2- to 64-cell stage, in line with the fact that, as the ICM forms, more cells end up inside. We also saw a dramatic decrease in the overall proportion of exposed cell surface area from the 2- to 8-cell stage, which may be linked to the process of compaction (Fig. 2C). When examining the relationship between the proportion of exposed cell surface area and N/C YAP ratio, considering blastomeres across all stages, we found that they are only moderately correlated (R=0.57; p<2.2e-16). However, this association became strong when considering blastomeres from the 16-cell stage onwards (R=0.7; p<2.2e-16) (Fig. 2D).

To determine if a similar relationship existed in human embryos, we examined the localisation of YAP in human compacted morulae. We found that blastomeres with a high proportion of exposed surface area exhibited high levels of YAP in their nuclei, whereas cells on the outside that were more embedded within the embryo had reduced nuclear YAP and completely inside cells had close to no nuclear YAP. This suggests that cell fate allocation in the human follows the same principles as during mouse preimplantation development (Fig.
Together, our findings suggest that the amount of YAP in the nucleus is proportional to the extent to which cells are outside or inside, based on the proportion of exposed surface. This suggests that the molecular mechanism acting to regulate the localisation of YAP is able to very accurately sense the relative amount of exposed cell surface area.

The relationship between the proportion of exposed surface and N/C YAP ratio is established during compaction

Though the relationship between the proportion of exposed cell surface area and N/C YAP ratio became strong from the 16-cell stage onwards, it was sometimes possible to observe blastomeres already at the 8-cell stage that had a lower amount of YAP in the nucleus and also appeared more deeply embedded within the embryo (Fig. 3A). Furthermore, with respect to the relationship between N/C YAP ratio and proportion of exposed surface area, some blastomeres from 8-cell embryos fell into the regime of the plot occupied by blastomeres from later stage embryos, where a stronger relationship exists between these two parameters (Fig. 2D). This suggests that blastomeres may already be able to sense their position through their proportion of exposed surface area at the 8-cell stage.

To test whether there may already be blastomeres at the 8-cell stage that display a relationship between N/C YAP ratio and the proportion of exposed cell surface area, we first tested whether blastomeres across development could be classified according to just these two characteristics. To do this, we performed unsupervised hierarchical clustering of our entire dataset using N/C YAP ratio and the proportion of exposed cell surface area as variables and found that on the basis of just these two parameters, blastomeres could be grouped into three distinct clusters (Fig. 3B). One was characterised by low N/C YAP ratio and low proportion of exposed surface area, a second by high N/C YAP ratio and intermediate proportion of exposed surface area and the third by intermediate N/C YAP ratio and high proportion of exposed surface area (Fig. S3A-C). Based on these observations, blastomeres from the first two clusters exhibited ICM- and TE-like characteristics respectively, whereas the third cluster potentially represented blastomeres in an ‘undecided’ state, as it contained large numbers of blastomeres from the 2-, 4- and 8-cell stages. We therefore named the clusters ‘inside-like’, ‘outside-like’ and ‘undefined’ respectively. Consistent with developmental trajectories, we could detect a strong positive correlation between N/C YAP ratio and the proportion of exposed surface when just the outside-like and inside-like clusters were considered together, without the undefined cluster (Fig. 3B).
To determine how well the unsupervised clustering performed, we next manually categorised blastomeres from a subset of 32- and 64-cell stage embryos as inside or outside, to generate a ground truth to compare the unbiased clustering against. We found that all blastomeres manually annotated as ICM fell into the inside-like cluster (82/82) and the vast majority annotated as TE fell into the outside-like cluster (108/116, 93.1%). Conversely, the outside-like cluster consisted exclusively of TE cells (109/109), while the inside-like cluster consisted almost exclusively of ICM cells (82/88, 93.2%). Overall, the clustering displayed very few errors, and these lay at the interface of the different clusters (Fig. S3D). The broad accuracy of the unsupervised clustering suggests that the blastomeres clustering as ‘undefined’ might represent a biologically meaningful state.

All 2- and 4-cell stage blastomeres belonged to the undefined cluster whereas only two-thirds of those from the 8-cell stage belonged to this cluster with the remaining one-third falling in the outside-like cluster (Fig. 3B). Thereafter, a much lower proportion of 16- to 64-cell stage blastomeres belonged to the undefined cluster (Figs 3B and S3E). Taken together this again suggested that already at the 8-cell stage, differences were starting to emerge amongst blastomeres, that those first to become different were on a TE trajectory and that ICM cells mostly arise only at the 16-cell stage, when cells start being found inside the embryo.

To verify whether already at the 8-cell stage blastomeres acquire the ability to sense and respond to their position, and whether this is linked to embryo maturation, we examined in more detail 8-cell embryos at different stages of compaction (Fig. 3C). We categorised 8-cell embryos based on their morphology as “Pre-compaction”, “Compacting” and “Post-compaction”. In validation of our categorisation, morphometric parameters of blastomere sphericity and proportion of exposed cell surface were significantly different in embryos post-compaction (Figs S3F and S3G).

Interestingly, the majority of blastomeres from post-compaction 8-cell embryos belonged to the outside-like cluster (14/21, 66.7%). In contrast, all but one blastomere from pre-compaction 8-cell embryos belonged to the undefined cluster (13/14, 92.9%) (Fig. 3D). The allocation of most blastomeres from post-compaction embryos to the outside-like cluster and from pre-compaction embryos to the inside-like cluster could not be explained by changes in YAP localisation alone, as there were no significant differences in N/C YAP ratio between blastomeres depending on the degree of embryo compaction (Fig. S3H). Amongst blastomeres from the undefined cluster, there was only a weak relationship between the proportion of exposed surface area and N/C YAP ratio (Fig. 3E), suggesting that these blastomeres may not yet be able to sense the proportion of cell membrane exposed to the
outside. In contrast, 8-cell blastomeres from the outside-like and inside-like clusters, which come predominantly from post-compaction embryos, exhibited a strong relationship between the proportion of exposed cell surface area and N/C YAP ratio (Fig. 3E). This indicates that the relationship between the proportion of exposed surface and N/C YAP ratio emerges during compaction, suggesting that the position sensing machinery might be in place earlier than previously thought, by the time compaction is completed.

**N/C YAP ratio increase from the 2- to 8-cell stage is dependent on biochemical changes occurring during compaction**

Since our results suggest that the ability of blastomeres to sense their relative amount of exposed surface area is progressively acquired during compaction, we sought to perturb the biochemical events taking place during compaction using Ro-31-8220 (RO), an inhibitor of PKC and other kinases. Treatment of 8-cell embryos with RO leads to reduced phosphorylation of ERM proteins (pERM), likely through the inhibition of aPKC subtypes (Liu et al., 2013). This in turn was shown to lead to decreased apical F-actin (Liu et al., 2013), which would be expected to impair some of the morphogenic process associated with compaction as well as the establishment of blastomere polarity, as the actomyosin network is required for the apical localisation of the Par complex (Zhu et al., 2017).

Embryos treated with inhibitor from the 2- to the 8-cell stage not only exhibited reduced proportion of apical to basolateral pERM (A/B pERM ratio) but were also unable to form apical actin rings, consistent with delayed or perturbed organisation of the apical domain (Fig. 4A,B). RO treatment was also accompanied by a markedly reduced N/C YAP ratio (Fig. 4A,C). Moreover, when we plotted the correlation between N/C YAP ratio and A/B pERM ratio, we could detect a moderate positive correlation (R=0.56, p=6.9e-7), suggesting that N/C YAP ratio was reduced by an amount proportional to pERM inhibition (Fig. 4D). In order to further understand how the RO compound impacted blastomeres from the 2- to the 8-cell stage, we examined the clusters to which RO-treated and control blastomeres belonged. Interestingly, we found a similar proportion of cells between controls (13/26 cells, 50%) and RO-treated embryos (15/29 cells, 52%) outside the undefined-cluster (Fig. S4A, B), suggesting that the RO inhibitor did not prevent blastomeres from maturing out of the undefined-cluster. However, outside the undefined cluster, we could observe two differences between RO-treated and control blastomeres. An increased number of RO-treated blastomeres could be found in the inside-like cluster (5/29 cells, 17.2% of RO-treated blastomeres and 0/26, 0% of control blastomeres). In the outside-like cluster, RO-treated blastomeres exhibited lower N/C YAP ratios in comparison to DMSO-treated blastomeres.
This suggests that disrupting the organisation of the apical domain using RO prevented N/C YAP ratio from increasing in blastomeres with a high proportion of exposed surface area. These results are therefore in agreement with a potential role for the apical domain (as opposed to the maturation of blastomeres out of the undefined cluster) in establishing the relationship between N/C YAP ratio and blastomere proportion of exposed surface area during compaction.

To test the importance of the actin cytoskeleton, we also treated embryos from the 2- to 8-cell stage with cytochalasin D (CCD). CCD treatments resulted in a low proportion of blastomeres being allocated outside the undefined cluster (4/21, 19%) in comparison to DMSO controls (14/25, 56%), suggesting that CCD-treated blastomeres were unable to mature out of the undefined cluster (Fig. S4C, D). Because the majority of CCD-treated blastomeres were also unable to polarise (Fig. S4E), it remains unclear which precise biological process controls the transition of blastomeres from an undefined state to a state in which the proportion of exposed surface and N/C YAP ratio correlate.

Shorter 5-hour treatment with RO inhibitor starting later, at the 8-cell stage, did not result in a significant decrease in nuclear YAP. One potential explanation for this reduced effect on N/C YAP ratio could be that PKC activity started prior to the treatment, giving enough time for YAP to accumulate in the nuclei of blastomeres with high proportion of exposed surface area (Fig. 4E). Interestingly, 5-hour RO treatment resulted in both YAP and F-actin accumulating and colocalising in small cytoplasmic aggregates (Fig. 4E,F). High-resolution imaging showed that even in unmanipulated embryos, YAP can be detected at cell-cell junctions (Figs 4G and S4F) suggesting that YAP can interact, most likely indirectly, with cortical F-actin. Collectively, our data suggest that the organisation of the F-actin cytoskeleton and the apical domain around the time of compaction is required for the nuclear accumulation of YAP observed in a subset of blastomeres with high proportion of exposed surface area, suggesting that this might represent an important step in allowing blastomeres to develop the ability to sense the proportion of their surface that is exposed and, ultimately, their position within the pre-implantation embryo.

**Embryo shape manipulation alters blastomere fate and reveals position sensing at the 8-cell stage**

Our results so far suggest the hypothesis that blastomeres use the proportion of their surface area that is exposed to the outside as a measure of their position within the embryo to modulate the sub-cellular localisation of YAP. A prediction of this hypothesis is that
altering the proportion of exposed surface area will lead to a corresponding alteration in N/C YAP ratio. To test this prediction without resorting to dissociating individual blastomeres that might perturb signals between contacting blastomeres, we developed a non-invasive approach to alter the shape and position of cells in the pre-compaction embryo. Our aim was to modulate the distribution of proportion of exposed surface area amongst blastomeres within each embryo in order to analyse the consequences on N/C YAP ratio. To this end, we used biocompatible hydrogels to create channels of consistent diameters in which to culture embryos. This physical constraint forced embryos to adopt a cylindrical shape leading to blastomere becoming relatively more embedded within the embryos (Fig. 5A,B and movie 2).

As a second approach to modify embryo geometry, we cultured embryos between sheets of hydrogels, which resulted in planar embryos with all blastomeres localised within a single plane and an increased spread in the distribution of exposed surface area (Fig. 5C,D and movie 3). These approaches allowed us to maintain polarity and adhesion between blastomeres.

When cultured within channels for five hours (Fig. 5B), the majority of blastomeres exhibited a reduction in the proportion of exposed surface area. This resulted in overall lower values for proportion of exposed surface in comparison to controls (Figs 5E and S5A). Consistent with the predictions of the hypothesis, shifting the distribution of proportion of exposed surface in this way was accompanied by a significant decrease in median N/C YAP ratio in blastomeres (Figs 5E and S5B). In contrast to cylindrical embryos, in planar embryos, both median N/C YAP ratio and proportion of exposed surface area remained close to the respective values from controls (Figs 5F and S5A,B), supporting the view that it is specifically changes in the distribution of exposed surface area (rather than other factors related to compressing embryos within hydrogels) that led to changes in YAP ratio. A/J ratio, the ratio of apical to junctional area (the ratio between blastomere apical surface area and apical junctional interface area, estimating the extent to which the apical surface protrudes outwards) was also significantly shifted in cylindrical embryos, raising the potential for this being the factor determining YAP localisation. However, arguing against this, A/J ratio was also similarly shifted in planar embryos where there was no overall change in the distribution of N/C YAP (Fig. S5C).

To unbiasedly test whether cylindrical embryos were able to generate a new population of cells simultaneously characterised by lower proportion of exposed surface and reduced N/C YAP ratio, we performed hierarchical clustering of these blastomeres on the basis of N/C YAP ratio and the proportion of exposed cell surface area. This resulted in three clusters (Figs 5G and S5D). Strikingly, cluster 2, containing blastomeres with the lowest proportion of exposed surface area and N/C YAP ratio, was predominantly composed (83.9%) of cells
from cylindrical embryos (Fig. 5H) and close to half (46.4%) of the blastomeres from cylindrical embryos belonged to this cluster (Fig. 5I). When considering blastomeres from cluster 1 and 2 (showing properties similar to the outside-like and inside-like clusters respectively), proportion of exposed surface area had the highest correlation coefficient with N/C YAP ratio, confirming our results in unmanipulated embryos (Fig. S5E). Together, these results suggest that changes to the proportion of exposed blastomere surface area may result in changes in N/C YAP ratio. This tight relationship indicates that blastomeres from as early as the 8-cell stage are able to sense and rapidly respond to subtle changes in their position to modulate YAP localisation.

Though blastomeres from cylindrical embryos show an overall reduction in exposed surface area, they are prevented from becoming completely internalised by the cylindrical confinement. We took advantage of this to test our previous observations suggested that post-compaction, 8-cell blastomeres are on a TE trajectory (Fig. 3B,C). When 8-cell embryos were grown inside channels until the 16-cell stage, in comparison to the controls, fewer cells were internalised and fewer excluded YAP from the nucleus, suggesting that fewer ICM cells were formed (Fig. S5F). To test whether this was indeed the case, we cultured embryos from the 8- to the 32-cell-stage in channels within polyacrylamide of varying stiffness (see methods for details) to subject them to either moderate or extreme confinement. We then stained the embryos for CDX2 to assay blastomere fate. In embryos under extreme confinement, all cells were forced to occupy the outer surface and it was possible to create embryos consisting solely of CDX2-positive cells (Fig. S5G). This effect was titratable – under milder compression, blastomeres managed to move inside and downregulate CDX2, but their numbers decreased with increasing tissue deformation (Fig. S5H). These experiments show that by simply preventing inside cells from arising, it is possible to derive embryos made exclusively of CDX2-positive blastomeres, consistent with 8-cell blastomeres existing on a TE trajectory and with blastomere position and internalisation being the main driving force behind the emergence of the ICM lineage.

**DISCUSSION**

We have discovered that, as early as during compaction at the 8-cell stage, blastomeres start exhibiting a close relationship between their proportion of exposed surface and the proportion of YAP in their nucleus and cytoplasm (Fig. 6). This suggests that following polarisation, blastomeres can sense their proportion of exposed surface area within the embryos and transfer this information to the nucleus by modulating the subcellular localisation of YAP.
To demonstrate the position-sensing ability of blastomeres within the embryo, we used a novel approach that, as opposed to the more traditional use of chimeras or dissociated blastomeres, does not alter the number of cells or the structural integrity of embryos (Leonavicius et al., 2018). By inserting embryos within cylindrical channels of defined diameter, we were able to alter embryo shape and the relative position of blastomeres. Using this method, we also showed that blastomeres of the 8-cell embryo are on a TE trajectory because, when blastomere internalisation was prevented, TE fate was favoured. This confirms the crucial role of blastomere internalisation in the emergence of the ICM lineage.

Following compaction, in the next round of cell divisions, the first inner cells will be formed. However, it remains unclear how precisely this is achieved (White et al., 2018). It has been suggested that cell fate decisions operate differently from the 8- to 16-cell stage (Anani et al., 2014) and from the 16- to 32-cell stage respectively (Hirate et al., 2013), with asymmetric cell divisions making a more important contribution during the 8- to 16-cell transition. However, clear asymmetric divisions rarely occur (Samarage et al., 2015) and in many cases, the apical domain seems to disassemble when blastomeres divide before being re-established de novo after cytokinesis (Zenker et al., 2018). Our results indicate that blastomere position within the embryo, as a result of either the mode of division or movement of cells, is ultimately what defines cell fate, consistent with cell division angle not determining fate (Watanabe et al., 2014). Instead, we propose that changes of global embryo geometry via cell internalisation drive the formation of the ICM, which is consistent with the important role of apical constrictions in cell internalisation to form the ICM (Anani et al., 2014; Samarage et al., 2015). Since we see a relationship between the relative amounts of YAP in the nucleus and the proportion of exposed surface, it raises the question whether nuclear YAP levels themselves can influence cell internalisation. It seems plausible that after cell division at the 8-cell stage, due to increased cell crowding, some cells exhibit a lower proportion of exposed surface by chance, leading to lower relative nuclear YAP levels. It is tempting to speculate that this triggers a positive feedback loop between reduced proportion of exposed surface and the progressive nuclear exclusion of YAP, leading to cell internalisation. This hypothesis is supported by work in Drosophila, showing the importance of the Hippo pathway in regulating apical domain size and apical complexes (Genevet et al., 2009; Hamaratoglu et al., 2009). Ultimately, identifying the genes that are regulated by the Hippo pathway during preimplantation development will help shed light on these challenging questions. Together, our results highlight the truly regulatory nature of the mouse preimplantation embryo to adapt and integrate global geometry changes into cell fate decisions.
What mechanism might blastomeres use to measure the proportion of their surface that is exposed to the exterior? Polarisation at the 8-cell stage is the first event generating asymmetries within blastomeres from a structural point of view (Ducibella et al., 1977; Louvet et al., 1996; Vinot et al., 2005; Ziomek and Johnson, 1980). Recent work has advanced our understanding of how the process of polarisation might be coupled to the morphogentic events of compaction (Zhu et al., 2017). The actomyosin contractility machinery that mediates compaction is recruited to cell contact-free membrane in a chain of events initiated by phospholipase C (PLC) that ultimately, leads to the establishment of apicobasal polarity. Importantly, the organisation of the contractility machinery at the cell cortex enables the generation of the forces driving compaction (Maître et al., 2015). This body of work highlights one of the major outcomes of compaction: the creation of two different membrane domains, apical and basolateral, with different organisation, protein complexes and contractile properties. Several of our observations suggest that YAP may be able to localise to these different membrane domains. First, we can detect YAP at cell-cell junctions, along the basolateral domain, in 8-cell embryos. Second, when the RO compound was used in 8-cell embryos, YAP accumulated in cytoplasmic puncta where it colocalised with F-actin. Since PKC inhibition reduces the phosphorylation of apical ERM and disrupts apical F-actin organisation, we speculate that YAP can interact indirectly with apical actin. Together, these observations suggest a mechanism whereby YAP localised at the apical and basolateral domains are regulated differently. The balance between inhibitory signal at the basolateral membrane and activating signal at the apical membrane might provide the cell with a measure of the proportion of exposed cell surface area. Hippo interactome studies reveal that YAP interacts, directly or indirectly, with various components of not only the apicobasal polarity complexes but also planar polarity molecules and other proteins associated with the plasma membrane (Couzens et al., 2013; Hauri et al., 2013; Wang et al., 2014). It will therefore be important to determine which of those play a role in the preimplantation embryo to regulate the subcellular localisation of YAP in the context of position sensing.

MATERIALS AND METHODS

Mouse husbandry and embryo collection

Mice were housed in a 12-hour dark, 12-hour light cycle. CD1 females (Charles River, England) were crossed with C57BL/6J males (in house) to obtain stage specific embryos. Noon of the day of finding the mating plug was defined as 0.5 day post coitum (dpc). Most experiments were performed using flushed embryos from natural mating. For super-
ovulations, 8-week old CD1 females were given intra-peritoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG) followed by 5 IU of human chorionic gonadotropin (hCG) 48 h later and were mated with C57BL/6J males. Embryos were flushed using M2 medium at the indicated stages (Sigma M7167). All experimental procedures complied with Home Office regulations (Project licence 30/3420) and were compliant with the UK animals (Scientific Procedures) Act 1986 and approved by the local Biological Services Ethical Review Process.

Human embryo collection

Human embryos were donated from patients attending the Oxford Fertility with approval from the Human Fertilization and Embryology Authority (centre 0035, project RO198) and the Oxfordshire Research Ethics Committee (Reference number 14/SC/0011). Informed consent was attained from all patients. Embryos were fixed in 4% paraformaldehyde, washed twice and kept in 2 PBS containing 2% bovine serum albumin (PBS-BSA) at 4°C until they were used for immunostaining.

Wholemount immunostaining

Following fixation with 4% PFA for 15 minutes, embryos were washed twice in 2% PBS-BSA. Embryos were then permeabilized with PBS containing 0.25% Triton X-100 (PBS-T) for 15 minutes and subjected to two washes in 2% PBS-BSA at room temperature. Embryos were then placed in blocking solution for 1 hour (3% BSA, 2.5% donkey serum in PBS containing 0.1% Tween). Incubation with primary antibodies took place overnight at 4°C in a humidified chamber. The following primary antibodies were used in this study and diluted in blocking solution at the indicated concentrations: mouse-anti-YAP, 1/100 (Santa Cruz Biotechnology, sc-101199), rat-anti-E-cadherin, 1/100 (Sigma, U3254), rabbit anti-pERM, 1/200 (Cell Signaling, 3141), rabbit anti-CDX2, 1/100 (Cell Signaling, 3977), rabbit anti-Par6b, 1/100 (Santa Cruz Biotechnology, sc-67393). The next day embryos were washed three times in 2% PBS-BSA for 15-20 minutes. Embryos were then incubated with secondary antibodies and Phalloidin (1/100 in blocking solution for 1 hour). The following reagents were used: Alexa fluor 555 donkey-anti-mouse (Invitrogen, A-31570), Alexa fluor 647 goat-anti-rat (Invitrogen, A-21247, Alexa fluor 488 donkey-anti-rabbit (Invitrogen, A21206), Phalloidin-Atto 488 (Sigma, 49409), Phalloidin–Atto 647N (Sigma, 65906). After another three washes of 15-20 minutes in 2% PBS-BSA, the embryos were mounted in 8-well chambers in droplets consisting of 0.5µl Vectashield with DAPI (Vector Laboratories) and 0.5 µl 2% PBS-BSA. Embryos were transferred between solutions by mouth-pipetting. All incubations took place at room temperature, unless stated otherwise. After mounting the embryos were kept in the dark at 4°C until they were imaged.
Confocal Microscopy

Embryos were imaged on a Zeiss LSM 880 confocal microscope, using a C-Apochromat 40x/1.2 W Korr M27 water immersion objective. Laser excitation wavelengths were 405, 488, 561 and 633 nanometres depending on specific fluorophore. Embryos were imaged using a 1.5x zoom at a resolution of 512x512 pixels and 8-bit depth Z-stacks of entire embryos were acquired at a 1 µm interval using non-saturating scan parameters.

Embryo culture

Embryos were cultured in organ culture dishes in 500 µl pre-equilibrated Evolve medium (Zenith Biotech) at 37°C and 5% CO2 for the indicated amount of time. The PKC inhibitor Ro-31-8220 (Calbiochem, 19-163; RO) was diluted in DMSO and used at 2.5 µM (1/2000 dilution). Cytochalasin D (Sigma-Aldrich, C8273; CCD) was used at 0.5 µg/ml. The same amount of DMSO was used in control cultures and embryos were either cultured from the 2- to 8-cell stage or for 5 hours starting at the 8-cell stage.

For cylindrical embryo cultures, channels were formed by casting a 5% (which corresponded to approximately 4.2kPa stiffness) acrylamide hydrogel (containing 39:1 bisacrylamide) around 25 µm wires within the confinement of a two-part mould (10x10x1mm). In milder compression experiments, the amount of acrylamide/bisacrylamide was reduced to create softer gels of approximately 3.5kPa stiffness (Tse and Engler, 2010). Ammonium persulphate (0.1%) and TEMED (1%) were added to polymerize polyacrylamide. The wires were then removed to form cylindrical cavities within hydrogel pieces, which were cut to roughly 3x3x1mm blocks for easier manipulation during embryo insertion. The hydrogels were carefully washed and equilibrated in embryo culture media at 37°C and 5% CO2 overnight. The embryos were then inserted into the channels using a glass capillary with a diameter slightly larger than the embryo itself. It was used to stretch the hydrogel channel before injecting the embryos and letting the channels relax and deform the embryos. Cell viability in channels had previously been assessed without any noticeable difference with control embryos (Leonavicius et al., 2018). For planar deformations, embryos were overlaid with a sheet of 5% acrylamide hydrogel. Excess media around the embryo was withdrawn with a micropipette to ensure that the acrylamide sheet would force the embryo to adopt a planar configuration. At the end of the experiments, embryos were fixed inside the hydrogels with 4% PFA for 20 minutes. Once fixed, embryos were then removed from the hydrogel channels and immunostained in parallel to the controls.
Segmentation and image analysis

Manual segmentation of confocal data was done using Imaris v6.3 (Bitplane). Cell and nucleus outlines were drawn using a Wacom Cintiq 21UX tablet display to create a 3D surface of each blastomere membrane and nucleus using the contour surface function. Information about geometry (sphericity, total surface area, volume, oblate and prolate) and signal intensity within each compartment could then be exported. Information about blastomere exposed, contact and junctional surface areas was obtained by considering surface proximities and was automated using an in-house developed software (Javali et al., 2017; Leonavicius et al., 2017). Signal intensity around these defined membrane domains could then be extracted. Dividing cells were excluded from the analysis as their geometry parameters were widely different to non-dividing cells and their nuclear envelope disassembled. Imaris files of segmented embryos will be made available on request.

We used the proportion of exposed surface area (and its converse, the proportion of contact area) as a measure of whether a blastomere is embedded within the embryo or is on the surface. The apical surface can be domed or flat, potentially indicative of, or leading to, increased or decreased tensions at the apical junctions. As an estimate of this, we also assessed the extent to which the apical domain was protruding out of the embryo by calculating the ratio between the apical surface area and the apical junction interface area (A/J ratio). The apical junctional interface was represented by a narrow band and was therefore expressed as an area, resulting in the A/J ratio being dimensionless.

Using the approaches above, we categorised the following parameters as relating to blastomere shape: sphericity; oblateness; prolateness; and volume. We categorised the following parameters as relating to blastomere position: Absolute and proportion of contact area; Absolute and proportion of exposed area; Absolute and proportion of junctional area; and Ratio between apical to junctional area.

Clustering and Statistical analysis

Figures and diagrams were assembled and created using the free and open source software, Inkscape and Krita. All statistics and graphs were done using RStudio and R. Graphs were produced using several packages, including ggplot2 and ggpubr. For statistical analysis, normality of the data was first assessed using visualisation tools and statistical tests (Shapiro-Wilk normality test). When the data was normally distributed, we used Analysis of variance (ANOVA), followed by post hoc comparisons using the Tukey HSD test.
when comparing more than two conditions. Otherwise, the Kruskal-Wallis test was used, followed by post hoc comparison using the Dunn test. To test the correlation between two variables, the Spearman method was used when the two variables were not normally distributed. The Corrplot package was used to create a correlation matrix of the different variables in the preimplantation dataset. To define the N/C YAP Ratio threshold between cells with high and low YAP ratio by k-means clustering, the data was standardized, and distance measures were obtained using the Euclidean method. For hierarchical clustering analysis, the dynamicTreeCut function was used to determine the ideal number of clusters. The variables were scaled, and the distance matrix was produced using the Euclidean method. The Ward method was used to perform the clustering.

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Author Contributions

C.R., K.L. and S.S. conceived and designed the experiments. C.R., K.L., A.K., D.F., K.N. and C.G. conducted the experiments. C.R., K.L., A.K. and S.S. analysed the data. C.R. performed the statistical analyses. A.V., C.J., T.C., K.C. and C.G. organised the collection of human embryos. C.R. and S.S. wrote the manuscript.
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Fig. 1. Analysis of N/C YAP ratio across preimplantation development using manual segmentation. A. Immunostaining of preimplantation embryos using antibodies against YAP and E-cadherin. F-actin and nuclei were visualised using Phalloidin and DAPI respectively. B. Example of a manually segmented 32-cell blastocyst showing blastomeres (green and yellow cells) exhibiting different shapes. Part of the cells making the trophectoderm are not displayed to be able to see inside the blastocyst cavity. The ICM is highlighted in cyan. C. Blastomere membranes were segmented to obtain blastomere "exposed", "junctional" and...
"contact" surfaces corresponding to the apical, membrane, apical junction and basolateral membrane. D. Representation of the relative amount of YAP in the nucleus and cytoplasm (N/C YAP ratio) of individual blastomeres at the 2- (n=20 embryos), 4- (n=8 embryos), 8- (n=10 embryos), 16- (n=17 embryos), 32- (n=12 embryos) and 64-cell stage (n=2 embryos). Black dot: median N/C YAP ratio for each developmental stage. E. Representation of the proportion of blastomeres with low and high N/C YAP ratio across developmental stages. Blastomeres from all stages were classified as exhibiting either high (>1.6) or low (<1.6) N/C YAP ratio based on K-means algorithm to separate them into two populations in an unbiased manner. The threshold was also represented in Figure 1C by a dotted line. Scale bar: 20 µm. *** p<0.001, **** p<0.0001 (Fisher's exact test).
Fig. 2. The proportion of exposed surface is associated with the proportion of YAP in the nucleus. A. Correlation matrix between N/C YAP ratio and geometric characteristics of individual blastomeres across preimplantation development. B. Correlation matrix between N/C YAP ratio and geometric characteristics of individual blastomeres from the 16 to the 64-cell stage. Note how the proportion of exposed surface and its converse, the proportion of contact surface correlate the highest with N/C YAP ratio. A. and B. The value of the correlation coefficient (Spearman) between two variables is indicated and also represented by the size and colour of the circles. C. Proportion of exposed blastomere surface area across developmental stages. The median proportion of exposed surface area for each developmental stage is represented as a black dot. ** p<0.01, *** p<0.001 (Kruskal Wallis test).
test followed by Dunn’s test). D. Correlation analysis between the proportion of exposed cell surface area (an indicator of position) and N/C YAP ratio at the indicated stages (Spearman). E. Representative optical sections of human morulae containing the indicated number of cells and immunostained for YAP. White arrowheads point at cells with either low or no exposed cell surface area and low nuclear YAP, whereas green arrowheads point at cells with high exposed cell surface area and high nuclear YAP. F-actin and nuclei were visualised using Phalloidin and DAPI respectively. Scale bar: 20 µm.
Fig. 3. Hierarchical clustering analysis reveals the association between the proportion of exposed surface and N/C YAP ratio in compacted 8-cell embryos. A. images of an 8-cell embryo immunostained for YAP and pERM illustrating variations in N/C YAP ratio at the 8-cell stage. F-actin and nuclei were visualised using Phalloidin and DAPI respectively. White arrowhead points to a blastomere with lower N/C YAP ratio. Green arrowhead highlights the presence of apical pERM. Bottom right panel shows a 3D opacity rendering of the corresponding embryo. Scale bar: 20 µm. B. Hierarchical clustering of blastomeres across preimplantation development into three distinct clusters. Blastomeres with high N/C YAP ratio and intermediate proportion of exposed cell surface area were classified as belonging to the outside-like cluster. Blastomeres with low N/C YAP ratio and low exposed cell surface area were classified as belonging to the inside-like cluster. Finally, the remaining blastomeres, exhibiting high proportion of exposed cell surface area and intermediate N/C
YAP ratio were defined as belonging to an “undefined cluster”. Dot shape indicates stage whereas colour indicates the cluster to which each blastomere belongs (Spearman, R=0.78 p<2.2e-16). Bottom right bar graph represents the distribution of blastomeres across the three clusters for each stage. C. Analysis of N/C YAP ratio and the proportion of exposed cell surface area at the 8-cell stage in pre-compaction, compacting and post-compaction embryos. D. Bar graphs representing the proportion of blastomeres from pre-compaction, compacting and post-compaction embryos in the Undefined, Inside-like and Outside-like clusters (top). The proportion of blastomeres from each cluster found in pre-compaction, compacting and post-compaction embryos is shown at the bottom. E. Correlation between the proportion of exposed surface and N/C YAP ratio in Undefined (top) (Spearman, R=0.52 p=1.4e-04) and Inside- and Outside-like 8-cell blastomeres (bottom) (Spearman, R=0.71 p=5.3e-05).
Fig. 4. Biochemical changes occurring during compaction are required for the nuclear accumulation of YAP in a subset of blastomeres from the 2- to 8-cell stage. A. Representative images of DMSO and RO-treated embryos grown in vitro from the 2- to the 8-cell stage immunostained for YAP and pERM. F-actin and nuclei were visualised using Phalloidin and DAPI respectively. White arrowhead points to a nucleus with high levels of YAP, whereas green arrowhead points to a nucleus with low levels of YAP. Right panel shows 3D opacity renderings of corresponding embryos. Scale bar: 20 µm. B. Boxplot
showing the proportion of pERM at the apical membrane in control (n=4 embryos) and RO-treated (n=4 embryos) embryos. C. Boxplot showing the proportion of YAP in the nucleus in control and RO-treated embryos. *** p<0.001 (Kruskal Wallis test). D. Plot showing the relationship between the proportion of pERM at the apical membrane and N/C YAP ratio in control and RO-treated embryos (Spearman, R=0.56 p=6.9e-07). E. Representative images of embryos cultured for 5 hours at the 8-cell stage in the presence of either DMSO or RO and subsequently immunostained for YAP and pERM. F-actin and nuclei were visualised using Phalloidin and DAPI respectively. Right panel shows magnification of the areas surrounded by dashed outlines. The white arrowhead points to cytoplasmic puncta of F-actin and YAP. Scale bar: 20 µm. F. Representative images of pre- and post-compaction 8-cell embryos immunostained for YAP and showing blastomeres with comparable N/C YAP ratios. The panel at right shows a high-magnification image of the boxed area. The white arrowhead points at YAP localised at cell-cell junctions.
Fig. 5. Embryo shape manipulation reveals position-sensing at the 8-cell stage. A. Diagram representing the experimental design to obtain cylindrical embryos. 8-cell embryos were inserted into 25 μm diameter channels and cultured for 5 hours. B. Representative images of control and cylindrical 8-cell embryos immunostained for YAP and pERM. F-actin and nuclei were visualised using Phalloidin and DAPI respectively. Scale bar: 20 μm. C. Diagram representing the experimental design to obtain planar embryos. 8-cell embryos were covered with a hydrogel sheet and cultured under confinement for 5 hours. D. Representative image of a planar 8-cell embryo immunostained for YAP. F-actin was visualised using Phalloidin. Scale bar: 20 μm. E. Plot showing the proportion of exposed surface and N/C YAP ratio in control (n=6 embryos) and cylindrical (n=7 embryos) embryos. Marginal density plots for control and cylindrical embryos, on the sides of the graph, show a shift in both the proportion of exposed surface and N/C YAP ratio in blastomereres from cylindrical embryos. F. Plot showing the proportion of exposed surface and N/C YAP ratio in
control (n=6 embryos) and planar (n=4 embryos) embryos. Note the absence of changes in the proportion of exposed surface and N/C YAP ratio in the marginal density plots. G. Representation of the proportion of exposed surface and N/C YAP ratio in blastomeres from control and cylindrical embryos and the different clusters obtained by hierarchical clustering. H. Bar graph representing the proportion of blastomeres from control or cylindrical embryos in each cluster. I. Proportion of blastomeres from each cluster in control and cylindrical embryos.
Fig. 6. Summary diagram.