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Oriented cell divisions asymmetrically segregate aPKC and generate cell fate diversity in the early *Xenopus* embryo

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SUMMARY

A key feature of early vertebrate development is the formation of superficial, epithelial cells that overlie non-epithelial deep cells. In *Xenopus*, deep and superficial cells show a range of differences, including a different competence for primary neurogenesis. We show that the two cell populations are generated during the blastula stages by perpendicularly oriented divisions. These take place during several cell divisions, in a variable pattern, but at a percentage that varies little between embryos and from one division to the next. The orientation of division correlates with cell shape suggesting that simple geometric rules may control the orientation of division in this system. We show that dividing cells are molecularly polarised such that aPKC is localised to the external, apical, membrane. Membrane localised aPKC can be seen as early as the one-cell stage and during the blastula divisions, it is preferentially inherited by superficial cells. Finally, we show that when 64-cell stage isolated blastomeres divide perpendicularly and the daughters are cultured separately, only the progeny of the cells that inherit the apical membrane turn on the bHLH gene, *ESR6e*. We conclude that oriented cell divisions generate the superficial and deep cells and establish cell fate diversity between them.

Movie available online

Key words: *Xenopus*, Superficial cells, Deep cells, Oriented cell division, Neurogenesis, Competence, aPKC, Par proteins, Epithelial polarity, Occludin

INTRODUCTION

The late blastula *Xenopus* embryo is shielded from the exterior by one layer of superficial cells that provide a tight-junction mediated epithelial seal. Underneath this superficial layer, there are several layers of non-epithelial deep cells (Hausen and Riebesell, 1991; Regan and Steinhardt, 1986; Muller and Hausen, 1995; Fesenko et al., 2000). Apart from their role in shielding the embryo from the exterior, superficial cells differ from deep cells in many other ways. Superficial and deep cells do not mix until later in development (Keller, 1978; Smith and Malacinski, 1983; Hartenstein, 1989; Minsuk and Keller, 1997) and they follow different cell fates, express different genes and have different roles in gastrulation (Keller, 1975; Minsuk and Keller, 1997; Keller, 1976; Shih and Keller, 1992; Keller et al., 1985; Glinka et al., 1996; Smith et al., 1995; Sive and Bradley, 1996; Deblandre et al., 1999). Thus, the formation of an outer epithelial cell layer and one or more, internal non-epithelial cell layers, and the developmental distinction between them, is one of the fundamental differentiations in the early embryo.

The deep versus superficial cell distinction is particularly important in the development of the frog nervous system because postmitotic primary neurones, which differentiate early, are derived solely from the deep cells, while the superficial cells give rise to longer dividing precursors (Hartenstein, 1989; Chalmers et al., 2002). We have previously investigated whether the different fate between inner and outer cells is due to an intrinsic difference or is externally imposed. We have shown that the two cell populations are intrinsically different, already at late blastula/early gastrula stage (Chalmers et al., 2002). Specifically, deep-layer cells isolated from a late blastula stage embryo are receptive, while the superficial layer cells are refractory, to signals that promote neuronal differentiation. At the neurula stage, a number of genes are differentially expressed between the two cell types and the expression of at least one gene, the hairy/enhancer of split related *ESR6e*, is restricted to superficial cells from the onset of zygotic transcription (Chalmers et al., 2002; Deblandre et al., 1999). *ESR6* inhibits primary neurogenesis when it is misexpressed in deep cells and may therefore underlie the low differentiation potential of superficial cells (Chalmers et al., 2002).

One mechanism to generate deep and superficial cells would be ingression or delamination of superficial cells to the interior of the embryo. However, timelapse video microscopy has shown that this is not the case in the frog embryo (Keller, 1978). An alternative method would be orientation of the mitotic spindle perpendicular to the surface of the embryo,

thereby generating a superficial cell and a deep cell upon division. Oriented cell divisions, where the spindle is aligned with an axis of cell polarity, play an important role in cell fate diversification in many other systems. Two well-studied examples are the division of *Drosophila* neuroblasts and the *C. elegans* zygote (reviewed by Chia and Yang, 2002; Jan and Jan, 2001; Guo and Kemphues, 1996; Wodraz, 2002). In both cases, cell fate determinants are localised along an axis of polarity in the dividing cell and are differentially inherited by the daughter cells, which then acquire different fates.

In this work, we sought to investigate whether oriented cell divisions play a similar role in the frog embryo. We asked three main questions: do oriented divisions take place, what are their properties and do they underlie the fate diversification of deep versus superficial cells? We show that the divisions of frog blastomeres can be grouped into three classes based on the orientation of the spindle relative to the surface of the embryo and the arrangement of the resulting daughter cells: parallel, oblique and perpendicular. Perpendicular divisions generate inner cells, starting at the sixth cleavage (32-64 cell) and continue to do so until the start of gastrulation. We show that equal numbers of perpendicular divisions occur in each quadrant of the embryo but the spatial distribution of perpendicular divisions is variable between embryos. However, in each embryo, cells with a small apical surface and a long apicobasal axis, show a very high probability of dividing perpendicularly in the next division. This suggests that the choice of division plane orientation correlates with cell shape and tends to bisect the long axis of the cell.

To investigate whether the progeny of perpendicular divisions are molecularly distinct, we examined the distribution of aPKC, an important molecule in oriented divisions of polarised cells in other systems (reviewed by Knoblich, 2001). We show that aPKC is localised to the apical membrane throughout the early cleavage stages and the membrane localised aPKC is inherited only by superficial cells. Finally, to prove that the differences between deep and superficial cells can be traced back to the perpendicular division that generated them, we isolated 64-cell blastomeres, separated their progeny and cultured them in vitro. We show that only clones derived from cells that have inherited the apical membrane express *ESR6e*.

Based on these findings, we propose that orientated divisions generate deep and superficial cells in the *Xenopus* blastula and play a key role in directing these cells towards different fates.

MATERIALS AND METHODS

Embryos culture

Xenopus embryos were obtained using standard procedures, cultured in 0.1× Marc's modified Ringer's solution (MMR) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) or according to the cell number. Dissociated blastomeres were isolated using Ca^{2+}/Mg^{2+}-free medium (CMFM) as described (Muller and Hausen, 1995), transferred into 1×MMR, allowed to divide once and fixed in PBS+0.1% Tween 20+4% formaldehyde. For antibody staining they were isolated and fixed in Dent's fix (80% methanol/20% DMSO). For RT-PCR they were isolated, allowed to divide once in CMFM, transferred to 1× MMR and cultured until the required stage.

Histology

Embryos were fixed in MEMFA [0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde] dehydrated through an ethanol series and embedded following manufacturers instructions in Immuno-Bed a Methoxy-Methylmethacrylate solution, (Polysciences). Serial sections (7 μm) were cut on a Leica rotation microtome, stained with Ehrlich's Haematoxylin and mounted in DPX (BDH Laboratory Supplies).

Immunohistochemistry (wholemount and cryosections)

Albino embryos were fixed at the required stage in Dent's fix with the vitelline membrane removed, unless stated otherwise. Embryos to be wholemount stained were washed in BBT (PBS+1% BSA+0.1% Triton X-100), then BBT+5% heat-treated lamb serum, incubated with the primary antibody overnight at 4°C in BBT+serum, washed four times for 1 hour in BBT and blocked for 1 hour in BBT+serum. They were then incubated overnight in the secondary antibody and washed four times for 1 hour in PBT (PBS+0.1% Tween 20). Embryos that were Cytox Green stained were washed in TBST (TBS+0.1% Tween 20) then incubated overnight in TBST+Cytox Green (1:1000, Molecular Probes), washed four times for 30 minutes in TBST. Embryos were then dehydrated in methanol and cleared in benzylbenzoate:benzyl alcohol (2:1). Embryos that were imaged from the side were cut twice to give two flat surfaces prior to imaging.

Cryosections were prepared as previously described (Fagotto and Gumbiner, 1994). Sections were then washed in acetone, PBS and blocked in PBS+1% BSA+5% serum for 30 minutes incubated with primary antibody for 2 hours, washed three times in PBS, blocked in PBS+1% BSA+5% serum, incubated with the secondary antibody for 1 hour, washed three times in PBS and mounted in Vectashield (Vector Laboratories). Embryos were imaged on a BioRad Radiance confocal microscope.

The following antibody combinations were used: anti α-tubulin 1/2000 (Sigma, DM1A, T9026) with anti-mouse TRITC 1/250 (Sigma, T7782) and Cytox Green (1/1000) (Molecular Probes) (Qian et al., 1998); anti occludin 1/1000 (kind gift from Dr S. Citi) (Cordenonsi et al., 1997) with anti-rabbit cy3 1/1000 (Amersham Pharmacia, PA 43003); anti aPKC 1/200 [Santa Cruz, nPKCζ (C-20) sc-216] with anti-rabbit cy3 or anti-rabbit Alexa 488 1/1000 (Molecular Probes, A-11008) (the aPKC antibody is the same as used previously (Nakaya et al., 2000) and recognises both ζ and λ atypical PKC isoforms, so the staining represents the combined aPKC distribution and is referred to as aPKC); and anti β1 integrin 1/200 (Developmental Studies Hybridoma Bank, 8C8) (Gawantka et al., 1992) with anti-mouse Alexa 488 1/1000 (Molecular Probes, A-11001).

Timelapse video microscopy

Ten embryos from different batches with a clear dorsoventral difference (lighter pigment, smaller blastomeres on dorsal side) were filmed from the eight-cell until the 4096-cell embryo using a Leica MZFL111 microscope, a coolsnap camera (Photometrics) and Openlab software. After filming, each embryo was left to gastrulate to confirm that is was developing normally. Division type was established for each cell by scrolling the movie back and forth, and was marked on a still image of the respective division stage. In the examples presented, the embryo was traced and each cell shaded with a colour representing the type division it is about to undergo in the next cleavage (see Movie at http://dev.biologists.org/supplemental).

For analysis of dorsoventral differences and analysis of the division orientation after an oblique or perpendicular division, embryos were labelled with Nile Blue at the four-cell stage on the pole that contained the two smaller, lighter pigmented blastomeres and filmed until the 1024-cell stage. They were left to develop to stage 10, when the formation of the blastopore lip indicates the dorsal side, and fixed. Thus, the future dorsoventral axis could be unequivocally identified in the five movies analysed. Lineage diagrams were produced for all descendants of each of the four animal blastomeres at the eight-cell stage up to the 1024-cell stage. The division type that each cell is

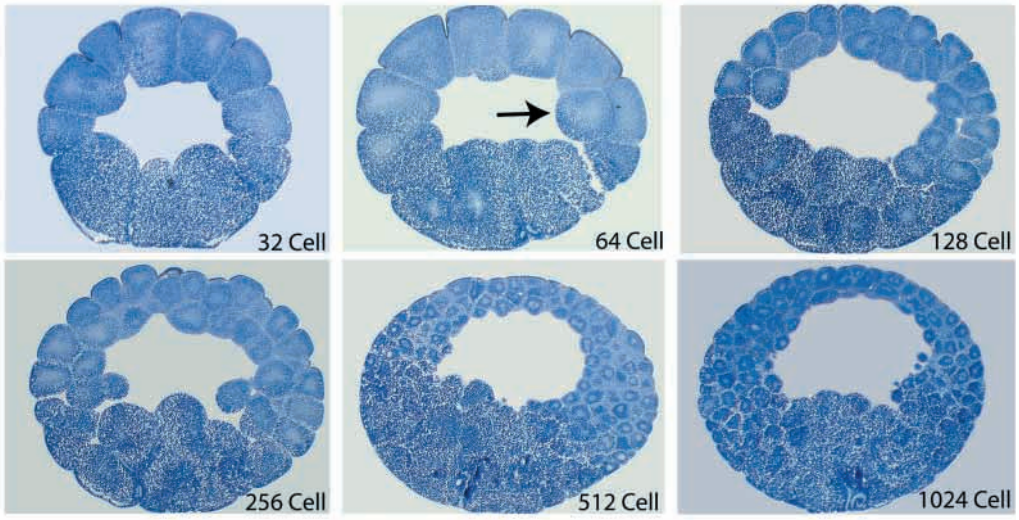

Fig. 1. Generation of the deep cells during development. Histological sections show when the deep cells are generated from the initially single layered embryo. Arrow indicates first deep cell.

about to undergo was recorded on the diagram. For dorsoventral differences, the division type from all the daughters of each of the four blastomeres was calculated as percentages for each blastomere per embryo and averaged for five embryos analysed. For analysis of division orientation after an oblique or perpendicular division, each division of that type (e.g. oblique) in the five movies (eight- to 1024-cell stage) was identified and the division types that followed the division was scored and used to calculate percentages for each orientation of division.

Quantitative real time RT-PCR (QT RT-PCR)

Isolated blastomeres (see above) were allowed to divide. After a perpendicular division the deep and superficial daughters (scored by presence of pigment) were separated and cultured. At stage 10, ~20 deep and 20 superficial clones were pooled (only where both daughters of a division survived) and snap frozen for QT-RT PCR. Fifteen stage 10 caps were also frozen for establishing standard expression levels. Quantitative real-time PCR was carried out using the lightcycler system (Roche) as per instructions and described previously (includes primers and conditions) (Chalmers et al., 2002) except the 1 step RNA kit (Cat no 2 015 137) was used for two-thirds of the independent experiments. When using the RNA kit 6mM magnesium was used. The stage 10 cap sample was used to generate a standard curve and the expression of each experimental sample compared with this sample where 100 is the level in the control cap. The results presented are the mean of three independent experiments.

RESULTS

Histology of the early cleavage stages

To find when the deep cells appear during development, serial sections were prepared of embryos at each division from the 32-cell embryo to the 1024-cell embryo (late stage 8). This was necessary because the commonly used atlas of *Xenopus* histology does not cover the early blastula stages (Hausen and Riebesell, 1991). The sections showed that in the 32-cell embryo all blastomeres are superficial cells and contact both the external surface and the blastocoel (Fig. 1). However, one division later, 64-cell embryos often have at least one deep cell (Fig. 1, arrow). The number of deep cells increases through the next few cell divisions such by the 512-cell stage there is more than one inner layer (Fig. 1) (Hausen and Riebesell, 1991). At late stage 8 (1024-cell stage; Fig. 1), the inner layers are beginning to thin out to one layer by radial intercalation, as described by Keller (Keller, 1980). Thus, the generation of inner cells starts very early and continues over several cell divisions.

Three orientations of cell divisions occur during early development

Timelapse studies investigating the synchrony of early cell division and culture of isolated blastomeres have suggested that perpendicular cell divisions may occur during early *Xenopus* and *Ambystoma mexicanum* (axolotl) development (Sato, 1977; Hara, 1977; Muller and Hausen, 1995). However, oriented cell divisions in the embryo have not been directly demonstrated and even though they are mentioned briefly in some textbooks, their characteristics are unknown (Keller, 1991; Nieuwkoop and Faber, 1967).

In order to establish whether oriented cell divisions are responsible for generating the deep cells, we stained the mitotic spindle and the chromosomes of embryos from a range of cleavage stages (Figs 2, 3). This showed three classes of divisions, which were named parallel, perpendicular and oblique, according to the orientation of the spindle and the arrangement of the resulting daughter cells. Parallel divisions had their spindle oriented in a plane parallel to the surface of the embryo (Fig. 2A,B, blue arrows) and could assume any orientation within this plane (Fig. 2A). A parallel spindle is predicted to lead to a cleavage plane perpendicular to the surface and so give rise to two, parallel, superficial cells (Fig. 2E). Perpendicular divisions had their spindle orientated perpendicular to the surface (Fig. 2A,B; red arrow). These cells are predicted to have a cleavage plane parallel to the surface and so give rise to a superficial and deep cell arranged perpendicularly to the pigmented surface (Fig. 2E). Finally, oblique divisions had the spindle orientated between these two (Fig. 2B, green arrow), giving rise to two superficial cells, one with a small and one with a big external surface (Fig. 2E). To exclude the possibility that the spindles rotate to line up with the plane of the external surface at a later point of cell cycle the spindle orientation in anaphase was examined. The three

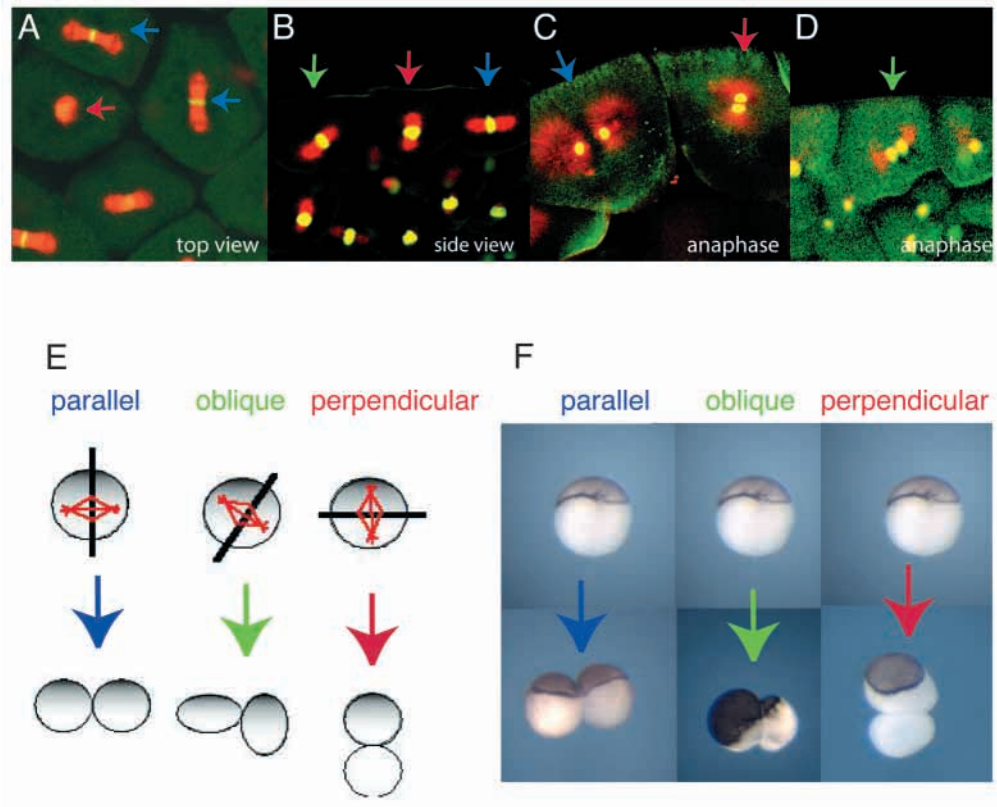

Fig. 2. Three orientations of cell division occur in early *Xenopus* development. (A-D) Stained mitotic spindles (red) of whole-mount embryos showed three orientations of division. The division plane was extrapolated based on the observation that the spindle orientates at 90° to future division plane (Strome, 1993). Chromosomes are shown in yellow. (A) top view of parallel and perpendicular divisions; (B) side view showing parallel, oblique and perpendicular divisions. (C) side view showing parallel and perpendicular divisions in anaphase. (D) Side view showing oblique division in anaphase. Blue arrows, parallel spindles. Red arrows, perpendicular spindles. Green arrows, oblique spindles. (E) Schematic showing the three orientations of division. Parallel divisions generate two superficial daughter cells. Oblique divisions generate one superficial cell with a large and one superficial cell with a small external surface. Perpendicular divisions generate a superficial and a deep cell. (F) The three orientations of division can be seen in isolated blastomeres judged by the distribution of the pigmented, originally external, surface.

different types of spindle orientation were still present in anaphase (Fig. 2C+D).

To confirm using an independent method that these three orientations of divisions do occur, we isolated single blastomeres from 64-cell stage embryos and allowed them to divide once. The orientation of division could then be easily established because the external, apical, surface is pigmented (Fig. 2F). The isolated blastomeres also showed parallel, oblique and perpendicular divisions (Fig. 2F)

Perpendicular divisions start at 32 cell stage and continue up to the start of gastrulation

Perpendicular divisions, which generate the deep cells, may take place during one or several cell divisions and either a few or the majority of the cells may be involved. To distinguish between these possibilities, we examined sequential divisions during early development. At the fourth cleavage (eight- to 16-cell stage) (data not shown) and the fifth cleavage (16- to 32-cell stage) the orientation of the spindle in most cells was parallel or occasionally oblique (Fig. 3B,C). Perpendicular spindles, which generate inner cells, were observed from the sixth cleavage (32- to 64-cell stage) up to stage 9 (Fig. 3). The perpendicular divisions could be seen in metaphase and anaphase spindles (Fig. 3). At stage 10, they were very rare (1/16 embryos) and were not found at stage 11 (0/5 embryos), where all spindles were parallel to the surface (data not shown). Throughout these stages, deep cells continue dividing (dividing deep cells can be seen in Fig. 2B), thereby increasing further the number of inner cells, but no attempt was made to follow their orientation of division. Perpendicularly oriented spindles were also observed in the marginal zone and the vegetal pole but were not characterised further.

The three orientations of division can be identified using timelapse video microscopy

To verify these findings using an independent method and to follow these divisions in vivo, we took advantage of the external development of the frog to analyse timelapse movies of early development. In this analysis, we made the assumption that any cell that appears not to divide when viewed from the outside, while its neighbours divide, must be generating an internal cell and was scored as a perpendicular division. This is based on previous observations that up to the 13th cleavage cell divisions are synchronous in the *Xenopus* embryos (Clute and Masui, 1992; Newport and Kirschner, 1982; Sato, 1977; Wang et al., 2000). Cell counts in other amphibia (Hara, 1977) and the antibody staining shown above also provide independent verification that all early blastomeres divide synchronously. A division that gave rise to two superficial cells of equal external surface was scored as a parallel division, and a division that gave rise to daughters of unequal external surface (more than 1:2 difference) was scored as an oblique one. An example of each kind of division (Fig. 4), the results from one movie (see Movie at http://dev.biologists.org/supplemental/; Fig. 5A) and a quantitation of each division type over 10 embryos (Fig. 5B) are shown. The diagrams presented are tracings of still images from the movies labelled with the type of division the cells are about to undergo (see

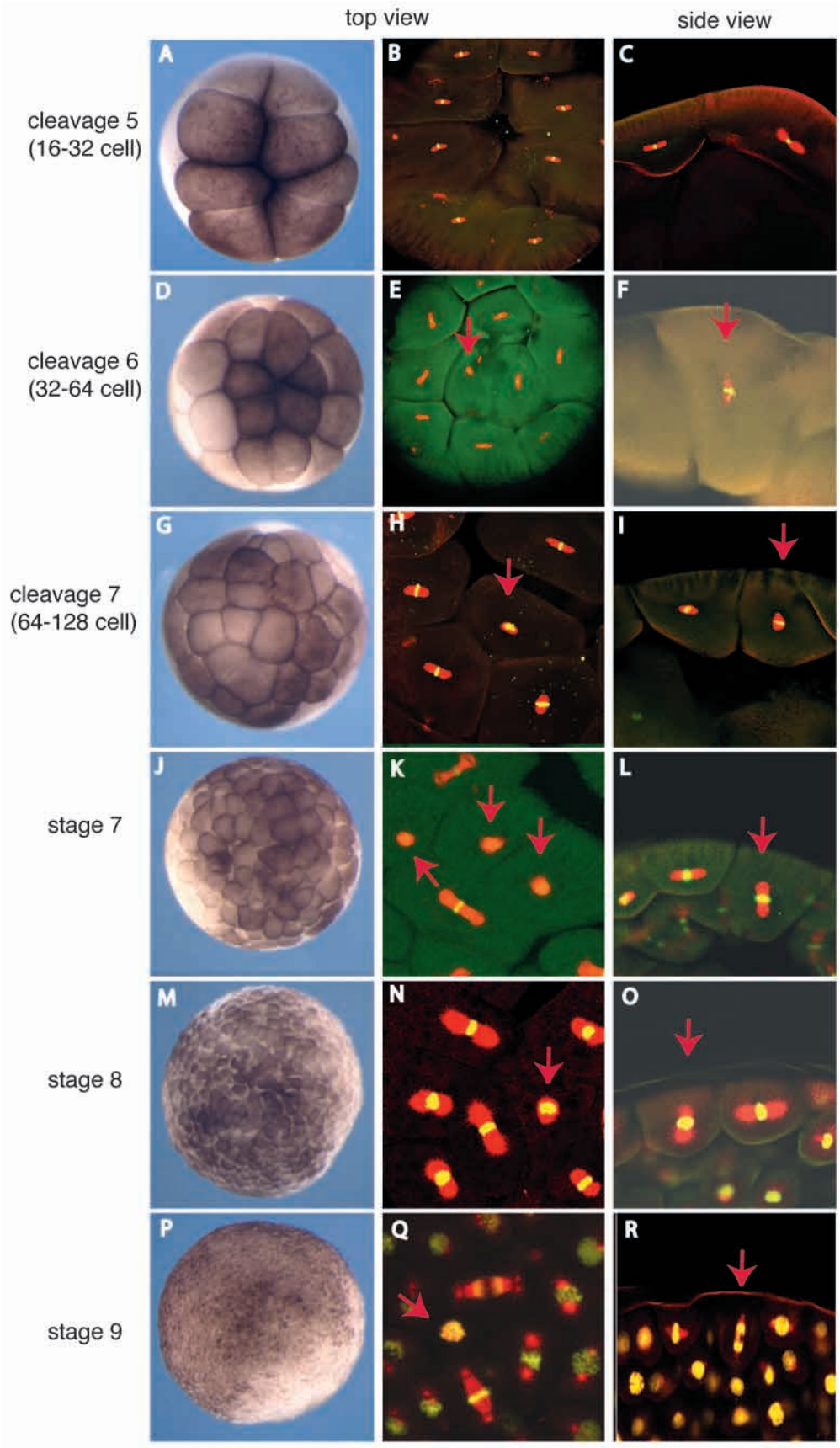

Fig. 3. Perpendicular divisions start in the 32-cell embryo and continue up to stage 9. For each stage, a whole-mount embryo (A,D,G,J,M,P), and a top (B,E,H,K,N,Q) and side (C,F,I,L,O,R) view of whole-mount embryos with stained mitotic spindles (red) and DNA (yellow) are shown. Red arrows indicate perpendicular divisions. Preparations in F, O and R still have their vitelline membrane.

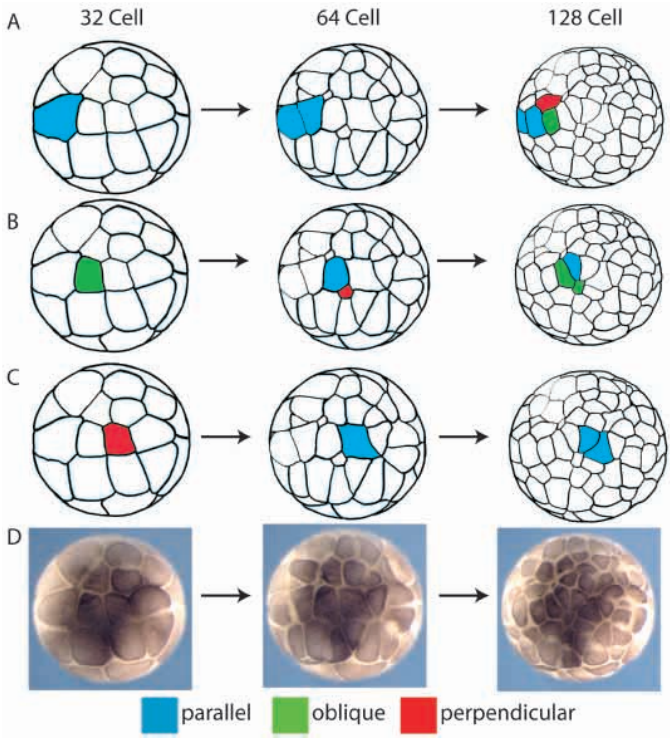

Fig. 4. Three orientations of divisions can be seen in timelapse analysis of cleavage stage embryos. (A-C) Examples of each kind of division are shown. The colour code depicts the type of division that a cell is about to undergo (blue indicates parallel, green indicates oblique and red indicates perpendicular). (D) Corresponding still images from the timelapse movie. All panels are views from the animal pole and dorsal is towards the top.

Materials and Methods). A timelapse movie is also available (see Movie at http://dev.biologists.org/supplemental/).

In agreement with the antibody staining, perpendicular divisions were first seen in the 32-cell embryo (Fig. 5A,B) where 8% of cells divided in a perpendicular orientation. The average percentage of perpendicular divisions rose to 20% at the next division (Fig. 5B), then to 25% in the 128-cell embryo and it stayed remarkably constant through the next four divisions (the last stage where this method can be used because after that the synchrony of division is lost) (Fig. 5B).

The perpendicular divisions do not follow a set spatial pattern

To understand how the choice of division plane might be regulated in the embryo, we examined a number of parameters that might influence it. Perpendicular divisions could preferentially take place in one area of the embryo, perhaps revealing the influence of signalling from that area of the embryo. For example, perpendicular divisions could be biased towards the dorsal or ventral side. Therefore, we calculated the number of perpendicular divisions for the daughters of each of the four animal blastomeres of an eight-cell stage embryo (see Materials and Methods). Equal numbers of deep cells were generated from the four animal blastomeres and therefore there is no bias across the prospective dorsoventral and left/right sides of the embryo (Fig. 5D).

Despite the constant average numbers of divisions seen at different divisions and in different regions of the embryos, different movies showed that the perpendicular divisions were not arranged in a reproducible pattern. Instead there was a different distribution in each embryo (the pattern of division is shown for four embryos at 128-cell stage; Fig. 5C).

Another way of controlling number of perpendicular divisions would be by a process similar to lateral inhibition, i.e. once a cell has been ‘selected’ to divide perpendicular it may inhibit its neighbours from doing so. However, this was not found to be the case as analysis of the timelapse movies showed that cells divide perpendicular in small clusters, doublets or single cells (Fig. 5). Finally, it was possible that once a cell starts to divide perpendicularly, it continues to divide in this orientation. The timelapse data showed that cells can divide perpendicular more than once in a row (29%, n=219 cells); however, they are not fixed in this orientation as they also often divide in parallel after a perpendicular division (57%, n=219 cells). In summary, the distribution of perpendicular divisions appears to vary between embryos and is not fixed through sequential divisions of an individual cell. However, the average numbers of divisions is fairly constant over several divisions and in each quarter of the embryo.

The orientation of division correlates with cell shape

What mechanism could produce this pattern of divisions? A potential answer comes from an observation that cells with a small apical surface had a high probability of dividing in a perpendicular orientation. These cells were usually the result of a previous oblique division that generates two cells with unequal apical surfaces (example of this is shown in Fig. 4B). To confirm and quantitate this observation, the orientation of division for each daughter of an oblique division was analysed (Fig. 6A). The daughter cells with the small external surface (oblique small) were found to divide perpendicular in 88% of cases and parallel in just 6% of cases, while the daughter cells with the big external surface (oblique big) divided perpendicular in only 11% of cases and parallel in 68% of cases.

Why do the two daughters of an oblique division show these predictable and complementary division planes? The daughters of an oblique division have drastically different shapes (see Fig. 2E). Invariably, the daughter with the small apical surface is not a small cell but has an elongated apicobasal axis (Fig. 6B,C). Conversely, the daughter with the large apical surface is usually a flatter cell and is more likely to have the longest axis on the plane of the epithelium (Fig. 6B,C). Therefore, the orientation of the spindle in both cases appears to line up with the longest axis of the cell. Analysis by antibody staining of spindles further supports the idea that cells are dividing bisecting their longest axis (Fig. 6D,E). Therefore, cell shape may be responsible for establishing the orientation of division and the different distribution of perpendicular divisions reflects different distribution of cell shapes.

aPKC is asymmetrically localised during the perpendicular divisions

One could argue that deep and superficial cells differ simply in their location in the embryo (inner or outer). Cells may have initially equal developmental potential but they may follow different fates by virtue of their exposure to different

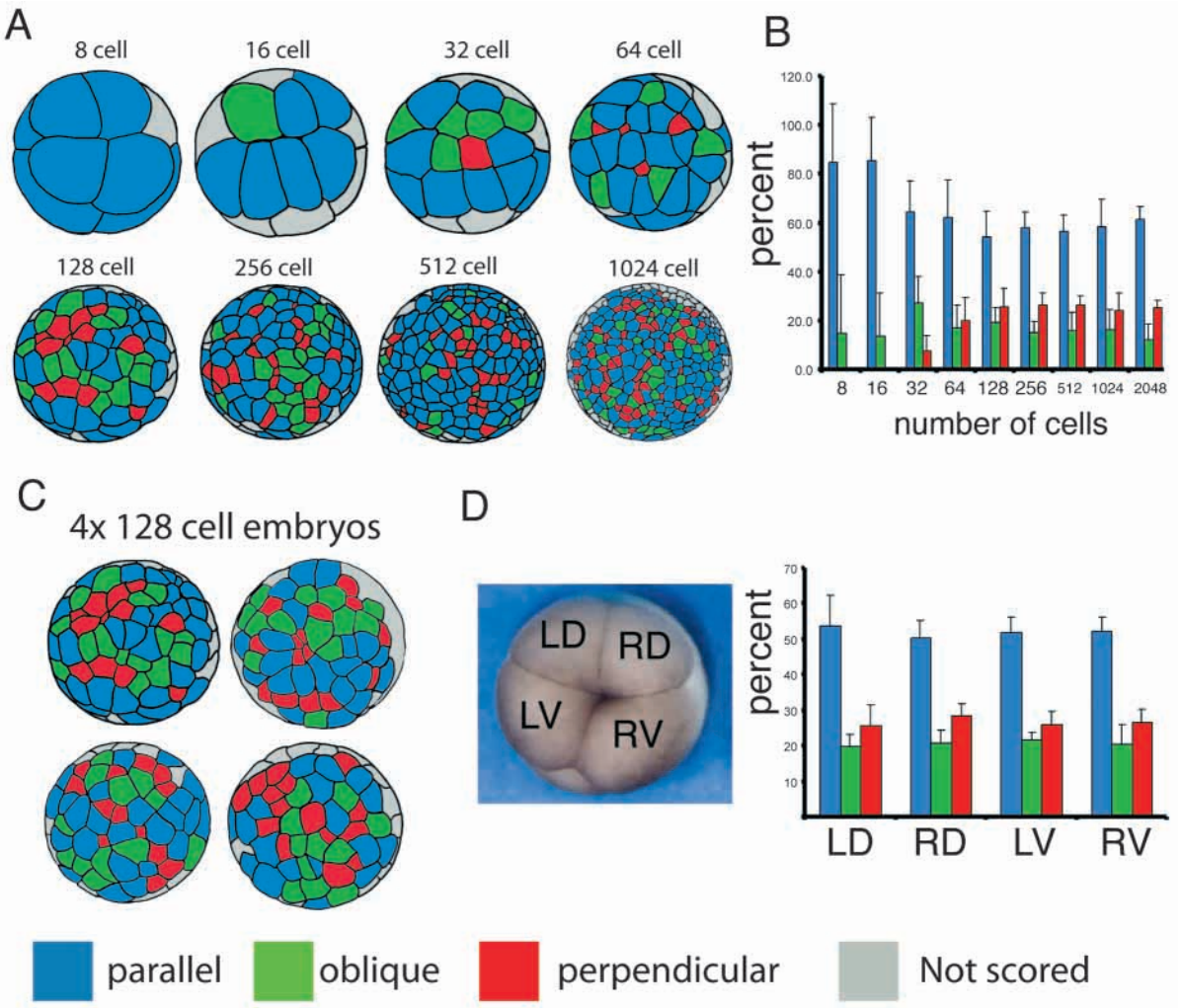

Fig. 5. Distribution and numbers of the three orientations of division shown by timelapse analysis. (A) Still images from one movie are labelled to show the orientation each cell is about to undergo (see Movie at http://dev.biologists.org/supplemental/) (blue indicates parallel, green indicates oblique and red indicates perpendicular). (B) Percent of each orientation of division from the analysis of 10 timelapse movies. Number of cells refers to the cell-stage of the embryos. (C) Variable distribution of different orientations of division in 4× 128-cell embryos. Dorsal is towards the top. (D) Percent of each type of division that occurs in all superficial daughters of the left/dorsal (LD), right dorsal (RD), left ventral (LV) and right ventral (RV) blastomere (left), from eight-cell up to the 1024-cell stage.

environments. Alternatively, differences in the development of deep and superficial cells could be directly linked to the oriented cell divisions that generated them, perhaps by virtue of differential inheritance of cell fate determinants. In *Drosophila* and *C. elegans* a conserved complex of proteins, consisting of Par3, Par6 and aPKC, is asymmetrically localised during oriented cell divisions (reviewed by Knoblich, 2001; Doe, 2001). As these proteins are also present in early *Xenopus* embryos (Choi et al., 2000; Nakaya et al., 2000) they may be asymmetrically localised during the oriented divisions.

In order to test the subcellular localisation of aPKC, cryosections of cleavage stage *Xenopus* embryos were analysed by antibody staining. In blastula embryos, aPKC showed a striking localisation, with high levels in the apical membrane of the superficial cells (Fig. 7A,B,E). As a comparison, embryos were stained for occludin, a component of vertebrate tight junctions. Occludin was localised along the basolateral membrane domains of the superficial cells and all around the deep cells with equal intensity (Fig. 7C,F). The occludin staining along the basolateral membrane is consistent with previous reports of a gradient of occludin protein localisation with a high point at the apical junctions. However, we observed less of a gradient along the basolateral membrane than previously reported (Fesenko et al., 2000) and additional staining around the deep cells. This is most probably due to the use of cryosections for immunohistochemistry in our work. The role of basolateral occludin is unclear but it has been postulated that it might provide Ca^{2+}-independent adhesion or a temporal store of the protein (Fesenko et al., 2000).

To establish when aPKC is first localised, antibody staining was carried out at earlier stages. aPKC was found to be enriched in the membrane of fertilised eggs (Fig. 7J,K) and localised at the apical membrane of the four-cell stage (Fig. 7L,M) where the difference between the apical membrane and the newly inserted basolateral membrane could be seen (Fig. 7M). The apical membrane originates from the egg membrane and, therefore, aPKC is a marker of the future apical domain even before the first cleavage.

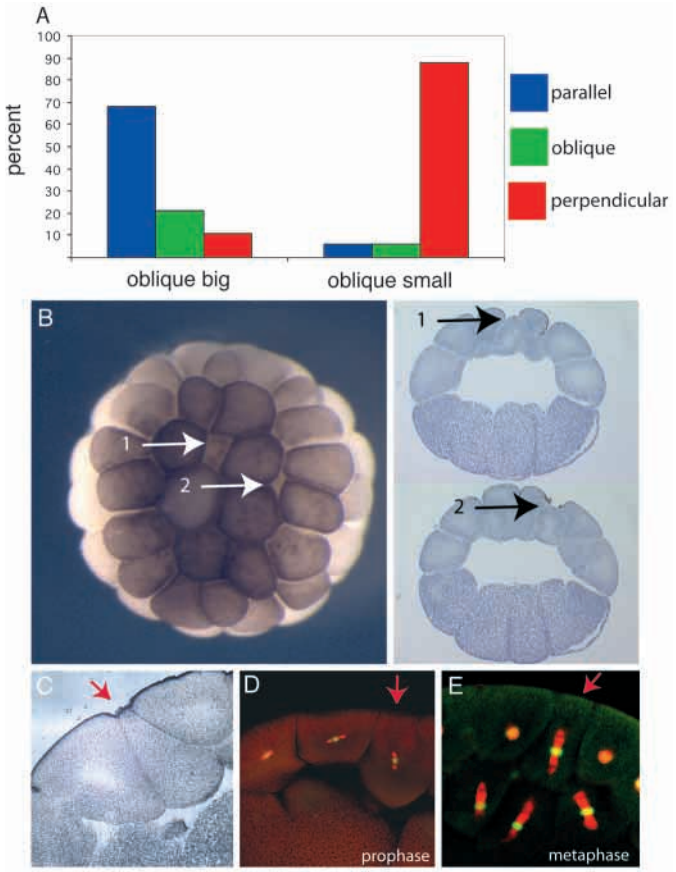

Fig. 6. Orientation of division correlates with cell shape.
(A) Percentage of each type of oriented division that the daughters of an oblique division, one with a large ('oblique big') and one with a small ('oblique small') external surface, will undergo in the next division is shown. A high percentage (88%) of oblique small cells divided perpendicularly in the next division. The percentages are based on *n*=184 'oblique big' cells and *n*=180 'oblique small' cells from five embryos (eight-cell to 1024-cell stage). (B) Wholemount and sections from the same embryo showing that cells with a small external surface (labelled 1 and 2) have a long internal axis.
(C) Unstained section showing that cells with small external surface (arrow) have an elongated apicobasal axis. (D,E) Antibody staining of spindles (red) and DNA (yellow/green) showing that elongated cells with small external surface (arrows) divide in a perpendicular orientation.

Interestingly, in one- and four-cell embryos the animal hemisphere showed enrichment of aPKC compared to the vegetal hemisphere (Fig. 7J,L), consistent with previous reports using whole-mount embryos (Nakaya et al., 2000). However, in sections it was clear that the animal enrichment is due to cytoplasmic aPKC (Fig. 7J,L). By contrast, the apical membrane localisation described here is present in both the animal and vegetal hemispheres.

The apical localisation of aPKC suggests that it would only be inherited by the superficial cells after a perpendicular division. Double staining of blastula embryos with aPKC and tubulin showed that the apical localisation of aPKC is maintained in cells undergoing parallel and perpendicular divisions (Fig. 7G,H). Thus, the superficial but not the deep daughter cell would inherit membrane localised aPKC after a perpendicular division. To confirm that aPKC is asymmetrically inherited after the divisions the localisation of aPKC was established in isolated blastomeres dividing in culture. The isolated blastomeres were double stained with anti aPKC and β1 integrin [which is localised to the basolateral membrane domains (Gawantka et al., 1992)]. The apical localisation of aPKC was found to be present in isolated blastomeres (Fig. 8A) and so does not require cell contact. This is consistent with previous results showing that isolated blastomeres maintain epithelial polarity (Muller and Hausen, 1995; Cardellini et al., 1996; Fesenko et al., 2000). In isolated blastomeres fixed in telophase and immediately after cytokenesis, the division could be seen to generate one polarised daughter with apical aPKC and one non-polarised without any membrane localised aPKC (Fig. 8B,C). Therefore, the oriented cell divisions generate cells with different molecular components.

Perpendicular oriented divisions generate molecular cell diversity

We also wanted to establish if the oriented divisions are responsible for generating differences seen in the later development of the superficial and deep cells. To do this, 64-cell stage blastomeres were isolated and allowed to divide once in culture. The daughters of the blastomeres that underwent a perpendicular division were separated, cultured until stage 10 and assayed for expression of *ESR6e* (Fig. 8D,E). *ESR6e* is a bHLH gene that is zygotically expressed in the superficial cells of the ectoderm (Deblandre et al., 1999; Chalmers et al., 2002). *ESR6e* overexpression suppresses deep layer derivatives in the epidermis (Deblandre et al., 1999) and in the neural plate (Chalmers et al., 2002), and is therefore a good candidate for a gene that specifies superficial cell fate. In this blastomere separation experiment, we found that *ESR6e* was expressed in the cell clones generated by the outer, pigmented, cells but not in the clones generated by the inner cells. By contrast, *odc* and *XSox3*, which are normally expressed in both layers (Chalmers et al., 2002), were expressed by both deep and superficial clones. As the outer and inner cells were exposed to the same environment from the moment they were generated, we suggest that the unequal inheritance of a factor during the perpendicular divisions establishes a different pattern of gene expression in inner versus outer cells.

Together with the aPKC results, these findings show that the deep and superficial cells are molecularly distinct from the outset of their generation and at the start of zygotic transcription, they show at least one differentially expressed gene, in the absence of any other signals from the embryo.

DISCUSSION

Oriented divisions generate deep and superficial cells in early *Xenopus* development

In *Xenopus*, the neural plate consists of a population of deep (inner) and superficial (outer) cells that intercalate during neurulation to generate a monolayered neural tube (Hartenstein, 1989). Deep and superficial cells differ intrinsically in their ability to undergo primary neuronal differentiation (Chalmers et al., 2002), which may explain why primary neurones are derived only from the deep layer (Hartenstein, 1989).

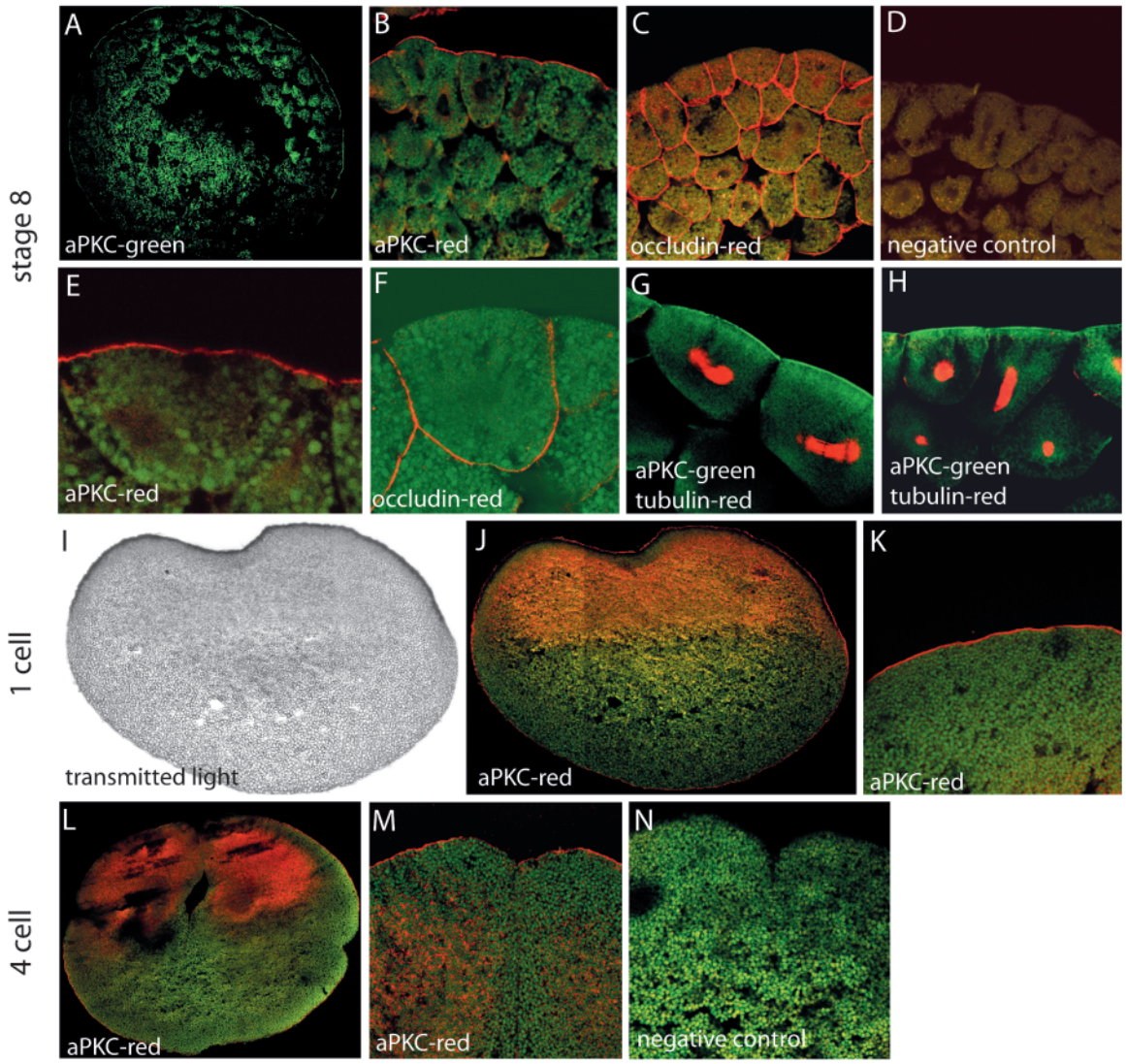

Fig. 7. aPKC is apically localised and asymmetrically inherited during the perpendicular divisions. (A,B,E) Stage 8 aPKC localisation. (C,F) Stage 8 occludin localisation. (D) No primary antibody negative control. (G,H) Stage 8 aPKC and α tubulin double staining. (I) Transmitted light image of fertilised egg. (J,K) aPKC localisation in fertilised egg. In this case, a pigmented embryo was used to allow identification of animal hemisphere. (L,M) aPKC staining at the four-cell stage. (N) No primary antibody negative control at the four-cell stage. (A-H) No vitelline membrane. In I-M, embryos with vitelline membrane are shown (as they were less damaged), but the staining was the same in those without vitelline membrane. All images are from antibody stained sections, except G,H, which are stained as whole-mount preparations.

In this study, we have investigated the origin of these two cell populations in order to understand how this difference in development potential is established. We show that deep and superficial cells are generated by a series of divisions oriented perpendicularly to the surface of the embryo, that take place between the 32-cell stage and the start of gastrulation.

Oriented cell divisions are widely encountered in the development of metazoa as a means of generating cell fate diversity. To date, most of our understanding of oriented divisions comes for work in invertebrate systems, particularly *Drosophila* neuroblasts and *C. elegans* early development (Knoblich, 2001). The model that has emerged from these systems has two main components: the localisation of cell fate determinants along an axis of polarity and the precise alignment of the spindle with this axis. How similar is early *Xenopus* to those other well characterised systems? We argue that early oriented divisions in *Xenopus* show similarities but also some differences from these other systems.

aPKC is a marker of blastomere polarity and is differentially inherited

Similar to other systems that rely on oriented divisions to generate cell diversity, early frog blastomeres are very clearly polarised, along the apicobasal axis. The basolateral domain is inserted during cleavage and expresses a number of known proteins such as B1-integrin, U-cadherin and α catenin proteins (Angres et al., 1991; Gawantka et al., 1992; Schneider et al., 1993). By contrast, the composition of the apical membrane, which is inherited from the egg, has been largely unknown with the exception of the tight-junction protein cingulin, which is

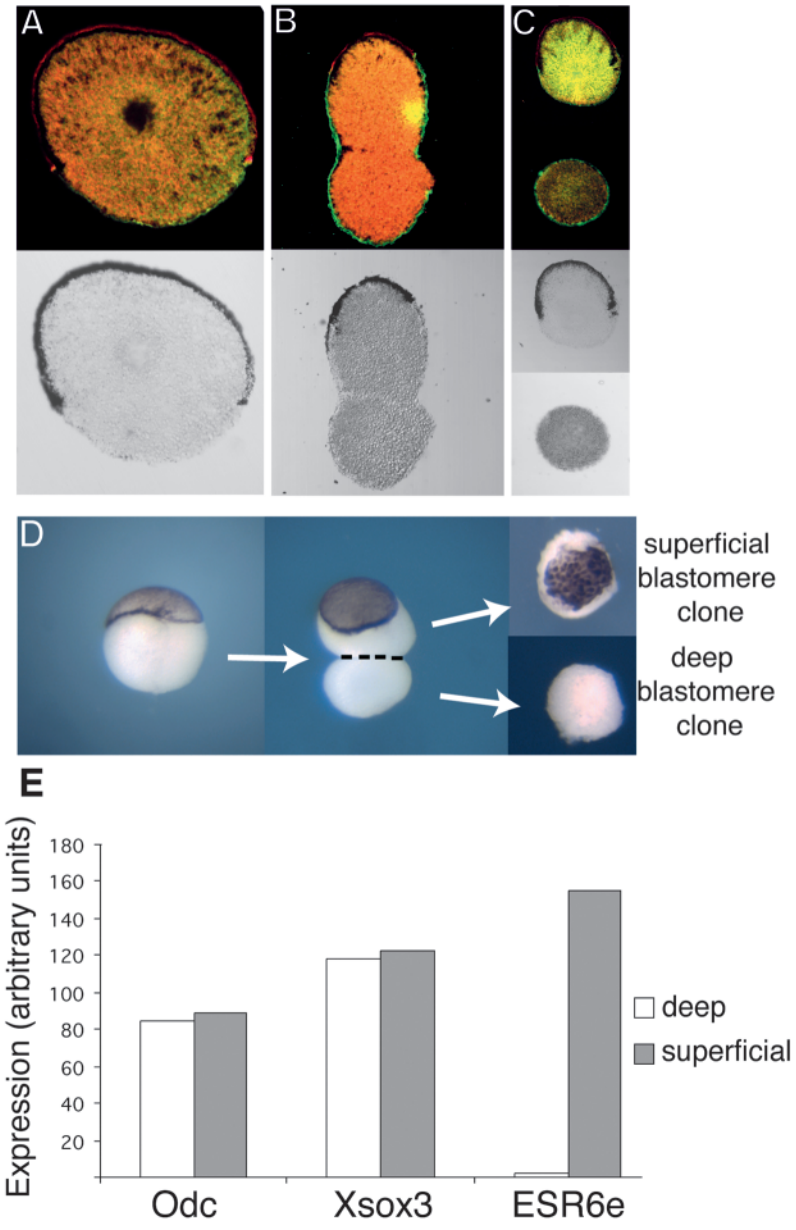

Fig. 8. Perpendicular divisions generate molecularly distinct daughter cells. (A-C) Perpendicular divisions in isolated blastomeres generate one daughter cell with localised aPKC and one daughter cell without. Blastomeres were stained with aPKC (red) and β1 integrin (green). A transmitted light image is shown below the fluorescent image. (A) An isolated blastomere. (B) An isolated blastomere during a perpendicular division. (C) Superficial (top) and deep (bottom) daughters generated after an isolated blastomere completes a perpendicular division. (D,E) The divisions generate differences that lead to later differences in gene expression. (D) An isolated 64-cell stage blastomere was left to divide, separated into a deep and a superficial cell, and cultured until control embryos reach stage 10. (E) Gene expression of the cultured blastomeres was analysed by quantitative real time RT-PCR. The *y*-axis shows expression in arbitrary units, with 100 being the same level as a stage 10 animal cap. *Odc* and *XSox3* are equally expressed, while *ESR6e* is preferentially expressed in outer cell derived clones. The mean of three independent experiments is presented.

recruited from the apical cortex into junctions during early embryogenesis (Cardellini et al., 1996; Fesenko et al., 2000). We have shown here that aPKC localises to the membrane of the fertilised egg and later to the apical side of the blastula stage superficial cells. During perpendicular divisions, membrane-localised aPKC is preferentially inherited by the superficial cells. In many other polarised cells, aPKC is also distributed asymmetrically, often found in the apical domain, and in the cases where it has been mutated, polarity has been disrupted (Horne-Badovinac et al., 2001; Izumi et al., 1998; Tabuse et al., 1998; Wodarz et al., 2000). Thus, the first epithelium in frog development shows molecular features of polarity that are present in later epithelia and are conserved across species.

Previous work on the localisation of aPKC in *Xenopus*, using whole-mount specimens, showed that aPKC is enriched in the animal hemisphere, similar to the localisation in the *C. elegans* embryo (Nakaya et al., 2000). We have confirmed the animal pole enrichment of aPKC but we have found that this enrichment is mainly cytoplasmic. By contrast, we have found membrane-localised aPKC that is present all around the egg and is subsequently inherited by the apical side of blastomeres. The role of the cytoplasmic aPKC is not clear but only the membrane-localised aPKC is clearly differentially inherited during the perpendicular (apicobasal) blastomere divisions. Therefore, we propose that (apical) membrane enrichment, rather than the animal cytoplasmic enrichment, may be more comparable in its potential cell fate determining role to the anterior membrane localisation seen in *C. elegans*.

Oriented divisions diversify deep and superficial cells

An important question is whether the perpendicular divisions that we describe here generate cells that are non-equivalent in their developmental potential. We observed that a single isolated 64-cell stage blastomere can divide perpendicularly in culture and generate one polarised cell (normally a superficial cell) that goes on to express the bHLH gene *ESR6e* and one apolar cell (normally a deep cell) that does not. This finding suggests that the perpendicular division, rather than any external influence, diversifies the progeny of this division. In the embryo, perpendicular divisions precede neural induction; therefore restricted expression of *ESR6e* in outer, polarised, ectodermal cells may simply bias their response to inducing signals later in development.

Differences from other systems

The first four divisions in *Xenopus* show a high degree of reproducibility between embryos. However, perpendicular divisions, which start at the sixth cleavage (32-cell stage) and continue until the blastula stage, do not follow a stereotyped pattern either between embryos or from one division to the next. They show no dorsoventral bias in their distribution and can occur in individual cells or in clusters of cells. Furthermore, cells are not committed to a particular mode of

division and can flip between different orientations in consecutive divisions. These are fundamental differences from the situation in early *C. elegans* development (Guo and Kemphues, 1996) where the division pattern is invariant and from *Drosophila* neuroblasts that, once specified to delaminate, are committed to an asymmetric mode of division (Knoblich, 2001). The lack of stereotyped pattern in the *Xenopus* embryo implies an element of randomness in the selection of cells that divide perpendicularly which may be lacking in the *Drosophila* or *C. elegans* systems. The lack of a stereotyped pattern sharply contrasts with the near constant percentage (25%) of perpendicular divisions, over several consecutive stages, which ensures that each embryo goes into gastrulation with roughly the same number of inner versus outer cells.

How do frog blastomeres decide which way to divide? We have found that the orientation of division for both the parallel and perpendicular divisions appears to correlate with the shape of the cells. In the case of perpendicular divisions, we have shown that cells that are elongated along the apicobasal axis have a very high probability of dividing as to generate an inner cell. These observations provide a model where the orientation of division during early cleavage stages is influenced by cell shape and may be subject to simple geometrical rules. The longest axis model would also be self-regulating, as each division would reduce the length of the long axis and so make the next division less likely to be along the same axis. This situation changes in the late blastula, when both and deep and superficial cells are oriented within the plane of the epithelium (this work) (Marsden and DeSimone, 2001), which, in the deep cells, depends on the presence of a fibronectin-rich extracellular matrix (Marsden and DeSimone, 2001).

In this work, we propose that, in *Xenopus*, the choice of spindle orientation correlates with cell shape, which may also be the case for the mouse blastula stage divisions (Johnson and Ziomek, 1983). The influence of cell shape on the orientation of division has been noted in earlier embryological studies [as for example in Hertwig's rules of cell division (see Wilson, 1987)] but has not received much attention in recent years, presumably because in many instances in development it can be overridden by other cues (Concha and Adams, 1998; Goldstein, 2000). What is interesting here is that a system of oriented divisions that is guided by cell shape is associated with the generation of cell fate diversity. Such a system may be inherently more flexible than the systems found in *Drosophila* and *C. elegans* and may in fact be better suited for early vertebrate development, where the cell fate choices are less hard-wired. Indeed, for early vertebrate development, it may be important to generate sufficient inner and outer cells and to segregate molecules that will bias their developmental potential, but it may be less important to do this with single cell accuracy. This would explain why, as we describe here, the localisation of molecules along an axis of polarity appears to be a highly conserved feature of oriented divisions but the precise alignment of the spindle with this axis of polarity appears not to be so.

Oriented divisions occur during early development of other vertebrates

Oriented divisions that segregate an external polarised cell from an internal non polarised cell, are observed in other vertebrates such as the mouse and the zebrafish. Similar to the situation in *Xenopus*, in these other cases, the outer cells follow different developmental pathways from the inner cells (enveloping layer and embryo proper in zebrafish; trophectoderm versus inner cells mass in the mouse) (Johnson and Ziomek, 1981; Pedersen et al., 1986; Fleming, 1987; Sutherland et al., 1990; Kimmel et al., 1995). However, neither the existence of determinants nor the spatial pattern of these divisions has been established for the mouse or zebrafish early embryo. Oriented divisions have also been reported in the vertebrate cerebral cortex and retina and are thought to asymmetrically localise cell fate determinants (reviewed by Lu et al., 2000; Cayouette et al., 2001). However, the existence of perpendicular (apicobasal) divisions in the vertebrate retina has been disputed and the significance of asymmetric divisions in this system is unclear (Silva et al., 2002; Das et al., 2003)

We would like to suggest that the early oriented divisions in *Xenopus* represent a new system for the study of differentiative cell divisions in vertebrates that is easily tractable and where the two daughters of the division can be experimentally manipulated in vivo and in vitro. Finally, these early oriented divisions in the frog are relevant to the development of the nervous system as they generate cells that are inherently different in molecular terms and in their ability to undergo primary neuronal differentiation.

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