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PAR-1 promotes primary neurogenesis and asymmetric cell divisions via control of spindle orientation

Jacqueline M. Tabler*, Hiroaki Yamanaka*,† and Jeremy B. A. Green‡

SUMMARY

In both invertebrate and vertebrate embryonic central nervous systems, deep cells differentiate while superficial (ventricular) epithelial cells remain in a proliferative, stem cell state. The conserved polarity protein PAR-1, which is basolaterally localised in epithelia, promotes and is required for differentiating deep layer cell types, including ciliated cells and neurons. It has recently been shown that atypical protein kinase C (aPKC), which is apically enriched, inhibits neurogenesis and acts as a nuclear determinant, raising the question of how PAR-1 antagonises aPKC activity to promote neurogenesis. Here we show that PAR-1 stimulates the generation of deep cell progeny from the superficial epithelium of the neural plate and that these deep cells have a corresponding (i.e. deep cell) neuronal phenotype. We further show that gain- and loss-of-function of PAR-1 increase and decrease, respectively, the proportion of epithelial mitotic spindles with a vertical orientation, thereby respectively increasing and decreasing the number of cleavages that generate deep daughter cells. PAR-1 is therefore a crucial regulator of the balance between symmetric (two superficial daughters) and asymmetric (one superficial and one deep daughter) cell divisions. Vertebrate PAR-1 thus antagonises the anti-neurogenic influence of apical aPKC by physically partitioning cells away from it in vivo.

KEY WORDS: Cell polarity, Neurogenesis, PAR-1 (MARK, EMK), Spindle orientation, *Xenopus*

INTRODUCTION

In both the invertebrate and vertebrate embryonic central nervous system (CNS), deep cells differentiate while more superficial (ventricular) epithelial cells remain in a proliferative, stem cell state (Götz and Huttner, 2005; Knoblich, 2008; Wodarz and Huttner, 2003). The regulation of the balance between deep and superficial cells is extremely important in determining the proper rate of neuronal differentiation, as is the partitioning of deep and superficial cell-type determinants in the asymmetric cell divisions that generate these populations. However, in the vertebrate CNS, the role of spindle orientation in this balance has been controversial (Cabernard and Doe, 2009; Chenn and McConnell, 1995; Konno et al., 2008; Kosodo et al., 2004; Morin et al., 2007; Zigman et al., 2005). The very small size of the apical domain of the columnar neuroepithelial cells of the mammalian CNS confuses the issue: cleavages that appear to be symmetrical rarely bisect it symmetrically (Kosodo et al., 2004). Here we examine the primary neurogenic regions of the early *Xenopus* embryo, where the large apical domains of the cuboidal neuroepithelium avoid such ambiguity.

PAR-1 (also known as MARK) is a serine-threonine kinase with diverse substrates, many of which are associated with cell polarity, including non-muscle myosin (NMY1), microtubule-associated proteins (e.g. Tau), Oskar, Exuperantia and Dishevelled (reviewed by Hurov and Piwnica-Worms, 2007; Matenia and Mandelkow, 2009). PAR-1 is basolaterally localised in epithelia by atypical protein kinase C (aPKC), which phosphorylates PAR-1 to remove it from the apical membrane, thereby restricting PAR-1 to the basolateral membrane (Hurov et al., 2004; Suzuki et al., 2004). We previously showed that PAR-1 in ectoderm drives ciliated cell specification in the deep layer of the *Xenopus* epidermis, that it antagonises Delta-Notch lateral inhibition of deep cell differentiation (Ossipova et al., 2007), and that PAR-1 is necessary and sufficient for deep layer neurogenesis (Green and Ossipova, 2006; Green et al., 2009; Ossipova et al., 2009) (see Fig. S1 in the supplementary material). However, whereas Delta is expressed only in the deep cells in *Xenopus* (Deblandre et al., 1999), PAR-1 is generally thought to function in polarised epithelia, such as the superficial layer of the ectoderm. It has been speculated that PAR-1 acts at cell junctions to regulate both neuronal specification and cell ingression (Krahn and Wodarz, 2009). Here, we show that PAR-1 drives neurogenesis from the superficial layer not by ingression, but by a spindle-orientation mechanism, partitioning daughter cells away from the apical inhibitory effects of aPKC and increasing the population of deep cells competent to become neurons.

MATERIALS AND METHODS

Plasmids and morpholino oligonucleotides

Plasmid constructs, morpholino (MO) oligonucleotides and *Xenopus* embryo methods were as previously reported (Ossipova et al., 2005). The N-tubulin probe was provided by N. Papalopulu (Chitnis et al., 1995). Injection doses per embryo in layer grafting and spindle orientation experiments were as follows: GFP-CAAX (mRNA), 400 pg; Myc-PAR-1TA (mRNA), 1 ng; PAR-1A MO, 80 ng; control MO, 80 ng. For unilateral gain- and loss-of-function experiments, 200 pg GFP-CAAX mRNA, 400 pg Myc-PAR-1TA mRNA, 80 ng PAR-1A MO, or 80 ng standard control MO were injected.

Layer grafting experiments

Superficial layer grafts were made by mechanically peeling this layer from host and donor embryos at stage 9.5-10.0 using No. 5 watchmaker's forceps (Dumont) and placing the outer layers on the peeled host embryos before stage 10.5. After grafting, embryos and grafts were held in place with glass coverslip fragments in clay wells to heal. Embryos were grown

King's College London, Department of Craniofacial Development, Floor 27, Guy's Tower, London SE1 9RT, UK.

*These authors contributed equally to this work
†Present address: Laboratory of Pattern Formation, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan.
‡Author for correspondence (jeremy.green@kcl.ac.uk)

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to tailbud stages, fixed using buffered formaldehyde (MEMFA) (Kay and Peng, 1991) and imaged in whole-mount using a Leica SP5 laser-scanning confocal microscope. Neurite-like cell processes were traced in *z*-stacks of images so that true processes could be distinguished from filopodia and labelled cell edges.

Spindle orientation experiments

For spindle orientation measurements, embryos were injected with mRNAs and MOs in the animal pole at cleavage stages, fixed at early gastrula stage (stage 10) in Dent's Fixative (20% DMSO in methanol) at –20°C overnight and stained in whole-mount with anti-tubulin (mouse monoclonal anti-alpha-tubulin, Sigma DM1A; 1/200) and anti-GFP (rabbit polyclonal, Abcam ab290; 1/200) antibodies. Animal caps were dissected out, cleared and imaged with a confocal microscope, taking *z*-stacks through the superficial ectoderm. Spindle angles were measured relative to the *xy* plane using ImageJ (NIH), employing a macro that we modified from Johannes Schindelin's 3D Angle Tool (modified macro available on request). In brief, the macro allows the user to click a point at one end of a spindle, then scroll to the section containing the other end and click on it, whereupon the angle between the *xy* plane and the line between the two points is calculated.

RESULTS AND DISCUSSION

To test the effect of PAR-1 on layer organisation in the neuroectoderm, we traced the fate of superficial epithelial cells following the expression of activated PAR-1. To do this, we overexpressed PAR-1TA, an aPKC-resistant form of PAR-1 that localises to both apical and basolateral domains in embryonic ectodermal epithelia and behaves as an activated PAR-1 (Ossipova et al., 2007). PAR-1TA was introduced into *Xenopus* embryos together with a GFP lineage label. At stage 10, the outer ectodermal layer was peeled from PAR-1-activated or control-injected embryos. The outer layer was then homotopically grafted onto an untreated stage 10 sibling (early gastrula) and the embryo allowed to develop to later stages (Fig. 1A-C). Embryos in which the graft remained intact and healthy and had healed-in well were fixed and the location and fate of the labelled graft cells examined. At early neurula stage (stage 14, Fig. 1B), sagittal confocal sections revealed that in control grafts, most cells remained in the superficial layer of the neural plate (Fig. 1D). By contrast, activation of PAR-1 caused graft-derived cells to appear in the deep layer of the ectoderm (Fig. 1E), with almost half of the graft-derived cells being in the deep, non-epithelial layer of the ectoderm (Fig. 1F). Thus, PAR-1 activation drives the generation of deep layer progeny from the superficial layer.

After neurulation (up to stage 19), cells from the deep and superficial layers of the neural plate radially intercalate to form a single pseudostratified epithelium. At tailbud stage 25, control grafts had produced superficial-layer-derived neuroepithelial cells showing no signs of differentiation (Fig. 2A). By contrast, PAR-1-activated grafted embryos contained significant numbers of labelled superficial-layer-derived cells with long processes (Fig. 2B,C), suggesting neuronal differentiation, concomitant with the ability of PAR-1 to enhance the expression of neuronal marker genes (see Fig. S1 in the supplementary material) (see Green and Ossipova, 2006; Green et al., 2009; Ossipova et al., 2009). After stage 26, both control and PAR-1TA-expressing grafts produced labelled process-bearing cells (see Fig. S2 in the supplementary material), indicating that secondary neurogenesis, which generates neurons from both deep and superficial layer progeny, begins at this stage. This is much earlier than the stage 35 previously reported (Hartenstein, 1989). PAR-1 activation thus changes not only the destination of superficial layer progeny in the neural plate, but also drives them to differentiate.

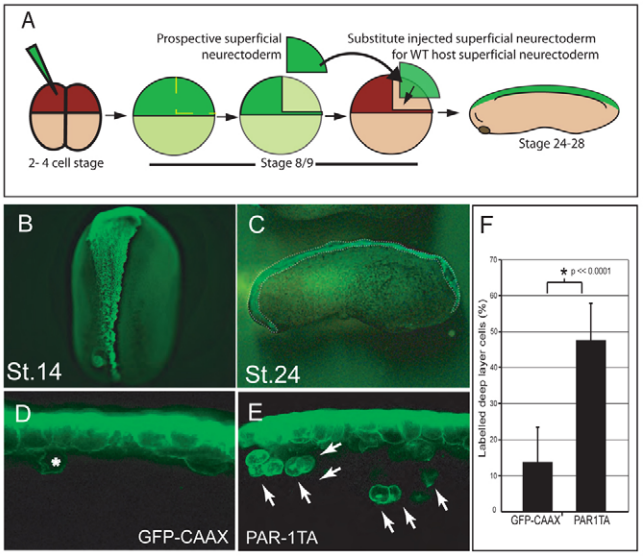

Fig. 1. PAR-1 promotes the generation of deep cells from the superficial epithelium. (**A**) Schematic of layer-grafting experiment showing homotopic, homochronic transplant to an untreated host of manually dissected superficial ectodermal epithelium from a *Xenopus* embryo injected with GFP-CAAX plus or minus PAR-1TA mRNA. (**B**,**C**) Whole-mount live fluorescence images of GFP-expressing superficial layer grafts in the dorsal ectoderm at stage 14 (B) and stage 24 (C), revealing graft integrity and integration into the elongating and neurulating host embryos. (**D**) GFP-labelled control grafts at stage 14, showing that nearly all superficial-layer-derived cells remain within the superficial epithelial layer. Infrequently, individual cells outside the layer could be seen (asterisk). (**E**) GFP-CAAX-labelled, PAR-1TA-expressing grafts at stage 14 (open neural plate stage) showing numerous labelled deep layer cells (arrows). (**F**) Quantification of the highly significant increase in the number of labelled deep cells derived from superficial layer grafts with PAR-1 activation. Cells were scored as deep if they were not part of the superficial layer.

Promotion of deep cells, as seen in Fig. 1, could occur by two types of mechanism: ingression (delamination) of epithelial cells to deeper layers, or an increase in cell divisions with a horizontal cleavage plane in which one of the two daughters lies below, rather than within, the epithelium. Cell ingression from the superficial epithelial layer has previously been seen upon overexpression of the basolateral polarity protein Lgl2 (Sabherwal et al., 2009). PAR-1 perturbation produced by expression of a dominant-negative PAR-1 causes cells to be expelled from cultured mammalian epithelia (Bohm et al., 1997), suggesting that this could be occurring in the superficial ectodermal epithelium. However, upon direct observation of injected embryos by time-lapse photography, we observed that, unlike Lgl2, PAR-1 does not appear to cause significant ingression from the superficial layer. We estimated that between the gastrula and open neural plate stages, less than 6% of injected cells showed any perturbation of behaviour. This perturbation consisted of loss of pigment, transient apical constriction and, occasionally, ingression, but too little of the latter to account for the nearly 50% of graft-derived cells that ended up in the deep layer.

To test the second possibility, that PAR-1 generates deep daughter cells from the superficial layer by vertically orientating mitotic spindles to give horizontal cleavage planes, we measured the effect of PAR-1 activation on spindle orientation. Embryos

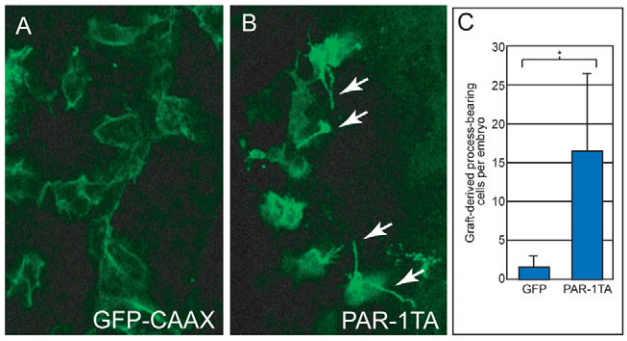

Fig. 2. PAR-1 promotes neurite differentiation in superficial layer progeny. (**A**) Control-grafted stage 25 *Xenopus* embryos showing that labelled cells lack neurites. (**B**) Lineage-labelled cells from PAR-1TA-expressing superficial layer grafts at stage 25 showing numerous cells bearing long neurite-like processes (arrows). (**C**) Quantification of superficial layer graft progeny showing significant induction of differentiation in PAR-1TA-expressing, but not control-grafted, embryos. Neurite-like processes were scored conservatively as labelled cell extensions that extended at least 5 μm from the cell body. *, P=0.025; Student's t-test.

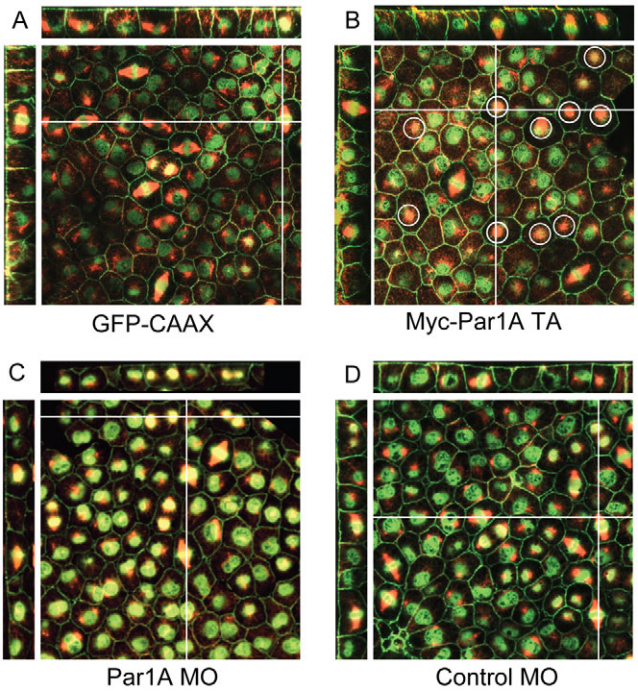

Fig. 3. PAR-1 is necessary and sufficient to promote vertical spindle orientation in the early neuroectoderm. (**A-D**) Maximum z-projections (main panels) and selected orthogonal yz (left) and xz (above) sections (with the plane of yz and xz sections indicated by the vertical and horizontal white lines, respectively) of confocal image stacks. Superficial neuroectoderm at early gastrula stage was stained for alpha-tubulin (spindles, red) and GFP (membranes and nuclei, green). Tissues injected with GFP-CAAX only (A), PAR-1A MO (C) or control MO (D) show numerous metaphase spindles in the xy plane, whereas with Myc-PAR-1TA-injection (B), many spindles are seen end-on (circled).

were injected with PAR-1TA, PAR-1A morpholino antisense oligonucleotide (MO) or a control MO. The MO against PAR-1A has been validated previously for efficacy and specificity (Ossipova et al., 2005). Embryos were fixed at gastrula stage and stained for alpha-tubulin, which reveals the mitotic spindles. Confocal image stacks were obtained and spindle orientation was quantitated with respect to the plane of the epithelium in the three-dimensional datasets (see Materials and methods). At gastrula stages, there are very few cell divisions in which spindles are aligned vertically, i.e. perpendicular to the epithelium (Kieserman and Wallingford, 2009). We observed that PAR-1TA dramatically increased the number of vertical spindles, as is readily apparent from the numerous 'end-on' spindles seen in the planar (xy) view of the PAR-1TA-expressing epithelium (Fig. 3B) as compared with controls (Fig. 3A). We quantified the angle distribution of spindles for cells in anaphase/telophase (indicative of ultimate cleavage orientation) (Fig. 4) as well as all mitotic spindles (see Fig. S3 in the supplementary material) and found the PAR-1 effect to be highly statistically significant for both. A 15-fold increase in the proportion of spindles with an angle greater than 45° to the plane of the epithelium was seen with PAR-1 gain-of-function (Fig. 4). Interestingly, the resulting angle distribution was not random, but rather bimodal, with maxima at horizontal and vertical orientations. PAR-1A MO decreased the number of vertical spindles to zero and produced a significant shift of the spindle angle distribution (Fig. 4E and see Fig. S3 in the supplementary material). Neither gain- nor loss-of-function of PAR-1 appeared to affect spindle morphology or the number of mitoses (Fig. 3). Thus, PAR-1 is both necessary and sufficient in gastrula neuroectoderm to drive cleavage spindle orientation from planar towards vertical.

This novel effect of PAR-1 on spindle orientation provides a mechanism for two aspects of neurogenesis. First, promotion of vertical spindle orientation increases the number of cells that lack apical membrane. Since apical membrane contains aPKC, which is an inhibitor of neurogenesis (Sabherwal et al., 2009), partitioning a daughter cell away from apical membrane is a mechanism for relieving this inhibition and is thus a novel form of aPKC antagonism. It is important to note that physical displacement of superficial cells into deep positions, either by dissection or by overexpression of Lgl2, is not sufficient for neurogenesis (Chalmers et al., 2002; Sabherwal et al., 2009; Strauss et al., 2006), and so it is this partitioning rather than the deep non-epithelial microenvironment that is important. Second, PAR-1 loss-of-function shows that its effect on the cleavage plane is normally required to maintain the total number of deep cells that are generated, and it is only deep cells that are competent to become neurons (Chalmers et al., 2002). Consistent with this, we observed that the total thickness of the neuroectoderm by neurula stages is significantly increased by PAR-1 gain-of-function (see Fig. S4 in the supplementary material), although additional effects, such as reduced radial intercalation, could also be involved. Thickening of the animal cap at gastrula stages was also observed (data not shown), although in this case it was not possible to distinguish thickening by cleavage division changes from changes in epiboly or even slight developmental delay.

Our findings are consistent with the models of mammalian cortical neurogenesis in which physically asymmetric cleavage drives neuronal differentiation (Chenn and McConnell, 1995; Gotz and Huttner, 2005). However, mouse *Par-1* (*Mark2*, *Emk*) knockouts reported to date do not show any obvious signs of loss of neurogenesis or cortical architecture (Bessone et al., 1999; Hurov et al., 2007; Hurov et al., 2001) [although they do display learning and memory defects (Segu et al., 2008)]. Whether this is because of redundant *Par-1* genes (of which there are four in

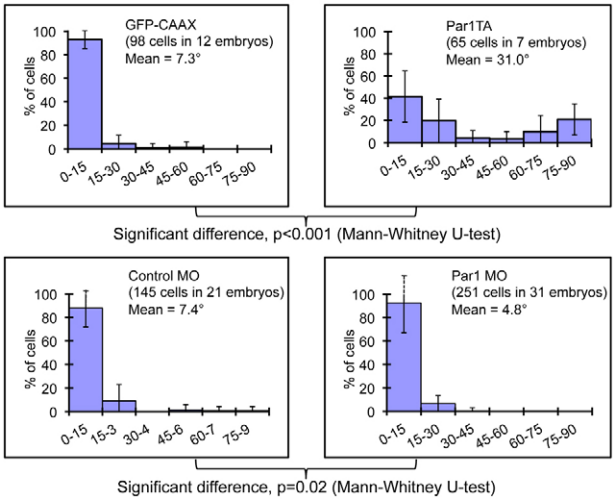

Fig. 4. PAR-1 loss- or gain-of-function has a significant effect on the distribution of anaphase-telophase spindle angles. Bar charts showing quantification of anaphase-telophase spindle angle measurements in control and PAR-1-perturbed *Xenopus* embryos. Angles were measured relative to the plane of the epithelium so that an angle of 0° is in the plane and would generate side-by-side daughter cells. Gain-of-function PAR-1 (PAR-1TA expression) shows a highly significant increase in the number of oblique and orthogonal (vertical) spindles. Loss-of-function [PAR-1A MO and kinase-dead-PAR-1 (not shown)] treatments reduce the already low proportion of orthogonal spindles still further.

mammals), or because mammalian neurogenesis differs from *Xenopus* primary neurogenesis, is unknown. However, it has been shown in slice culture experiments that PAR-1 has a role in directing the persistent vertical migration of neurons in the mammalian cortex (Sapir et al., 2008a; Sapir et al., 2008b) and this has not been reported for the knockouts, suggesting that analyses of the knockouts to date might not have revealed all the roles of PAR-1 in the mammalian CNS.

In *Drosophila*, neuronal specification and lateral inhibition by Delta-Notch precede delamination of neuronal cell precursors. We speculate that in early vertebrate neurogenesis, unlike in *Drosophila*, the partitioning of deep cells from their superficial sisters precedes neuronal specification. PAR-1 would then act first in apicobasal polarity, then spindle orientation (this work), and finally as a deep cell determinant acting on Delta-Notch signalling (Ossipova et al., 2007; Ossipova et al., 2009). As an effector protein for epithelial polarity, cleavage orientation and differentiation, PAR-1 thus couples these processes so that not only epithelial polarity, but also multicellular tissue polarity, in the apical-basal dimension of the CNS can be established. This role extends even further with the action of PAR-1 in directing the above-mentioned radial migration of neurons (Sapir et al., 2008a).

Whether spindle orientation is coupled to the decision in the vertebrate CNS between functionally symmetric cleavages (self-renewing stem cell amplification) and functionally asymmetric (non-self-renewing, basal progenitor-generating) cleavages remains unclear for amniotes and for later stages in *Xenopus*, with the consensus leaning towards little or no coupling (Knoblich, 2008). However, the data we present in a large-celled system in which the physical asymmetry of the cleavage plane is unambiguous, suggest that this coupling can be tight and that higher resolution analysis in other systems and at different stages is warranted.

We conclude that PAR-1 serves at early developmental stages not only to supply cells to the deep layer of the neuroectoderm, but also, by doing so, to select for neuronal competence by partitioning daughter cells from inhibitory aPKC. Rather than driving ingression, i.e. an epithelial-to-mesenchymal transition in which cell junctions are altered as occurs in *Drosophila* neurogenic delamination, this tissue and cellular remodelling is achieved by regulation of spindle orientation.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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