

A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification

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Neural tissue formation is induced by growth factors that activate networks of signal transduction cascades that ultimately lead to the expression of early neural genes, including transcription factors of the SoxB family. Here, we report that fibroblast growth factor (FGF)-induced Erk1/2 (Mapk3 and Mapk1, respectively) mitogen-activated protein kinase (MAPK), but not phosphatidylinositol 3'-OH kinase (PI3K, Pik3r1), signalling is required for neural specification in mouse embryonic stem (ES) cells and in the chick embryo. Further, blocking Erk1/2 inhibits the onset of key SoxB genes in both mouse ES cells (*Sox1*) and chick embryos (*Sox2* and *Sox3*) and, in both contexts, Erk1/2 signalling is required during only a narrow time window, as neural specification takes place. In the absence of Erk1/2 signalling, differentiation of ES cells stalls following *Fgf5* upregulation. Using differentiating ES cells as a model for neural specification, we demonstrate that sustained Erk1/2 activation controls the transition from an *Fgf5*-positive, primitive ectoderm-like cell state to a neural progenitor cell state without attenuating bone morphogenetic protein (BMP) signalling and we also define the minimum period of Erk1/2 activity required to mediate this key developmental step. Together, these findings identify a conserved, specific and stage-dependent requirement for Erk1/2 signalling downstream of FGF-induced neural specification in higher vertebrates and provide insight into the signalling dynamics governing this process.

KEY WORDS: ES cell differentiation, Neural induction, Sustained Erk activity, Sox, MAPK signalling, Chick, Mouse

INTRODUCTION

Neural specification is a fundamental developmental process during which cells embark on the neural differentiation programme. This is indicated by the onset of expression of genes characteristic of neuroepithelium, which include members of the SoxB family of transcription factors (Pevny et al., 1998; Rex et al., 1997; Wood and Episkopou, 1999). There is growing evidence that FGF signalling is a conserved initiator of vertebrate neural development (Bertrand et al., 2003; Delaune et al., 2005; Launay et al., 1996; Streit et al., 2000; Wilson et al., 2000); however, the molecular mechanism underlying this step has yet to be addressed in higher vertebrates. Activation of FGF receptors (FGFRs) can initiate transduction via three major intracellular pathways: classical MAPK (Erk1/2; also known as Mapk3 and Mapk1, respectively), PI3K (also known as Pik3r1 – Mouse Genome Informatics) and phospholipase C gamma (PLCγ; Plcg), the last two of which can activate protein kinase C proteins (PKCs), which can in turn stimulate Erk1/2 signalling (Schonwasser et al., 1998). There is piecemeal evidence that implicates all three of these pathways in the induction of neural tissue in vertebrate embryos. Most of this comes from work in the frog embryo, in which signalling via Ras (upstream of Erk1/2 and PI3K) is required for the induction of posterior neural tissue (Delaune et al., 2005; Ribisi, Jr et al., 2000), whereas PKC activators can also turn on neural genes via an unknown mechanism (Otte et al., 1988).

To monitor the neural specification process in ES cells, we differentiate the 46C line under defined, serum-free, monolayer culture conditions dependent on FGF signalling (Ying and Smith, 2003; Ying et al., 2003). In 46C ES cells, the enhanced green fluorescent protein (EGFP) reporter is targeted to the endogenous

Sox1 locus, which drives expression in neural progenitors and allows rapid and accurate quantification of the differentiation by fluorescence microscopy or flow cytometry (Ying et al., 2003). We cross-reference our in vitro findings in vivo using the chick embryo, a well-studied model organism for the process of neural induction in higher vertebrates (Stern, 2005a; Streit and Stern, 1999). Here, early (preneural) genes that identify potential neural tissue include a different SoxB gene, *Sox3*, whereas, in this vertebrate embryo, the later-expressed *Sox2* gene is a marker of definitive neural tissue (Rex et al., 1997).

MATERIALS AND METHODS

Cell culture

ES cells were grown on gelatine-coated plastics (Greiner and Nunc) in Glasgow modified Eagle's medium (GMEM) containing 10% foetal calf serum, 0.1% modified Eagle's medium (MEM) non-essential amino acids, 2 mM glutamine (all Life Technologies), 0.1 mM 2-Mercaptoethanol (Sigma) and 100 Units/ml recombinant human leukaemia inhibitory factor (LIF; prepared in-house). Differentiation was performed as previously described (Ying and Smith, 2003; Ying et al., 2003) and media was changed every 2 days in all experiments unless stated otherwise. PD173074 (kindly provided by Pfizer) was used at 250 nM and PD184352 (a gift from Philip Cohen, Medical Research Council Protein Phosphorylation Unit, University of Dundee, UK) at 3 μM. For Fig. 1C, 46C cells were differentiated in either N2B27 or N2B27 that also contained: 3 μM PD184352; 0.1 μg/ml noggin/Fc chimera (R&D Systems); PD184352 plus noggin; 10 ng/ml Bmp4 (R&D Systems); or Bmp4 plus noggin, for 3 days before flow cytometric analysis.

Flow cytometry

Cells at the appropriate differentiation stage were trypsinised and transferred into flow cytometry tubes containing phosphate-buffered saline (PBS) +5% foetal calf serum. TO-PRO3 (Molecular Probes) was included to discriminate non-viable cells. For cell cycle analysis, cells were fixed in 70% ethanol, treated with RNase A and stained with propidium iodide.

Western blotting

Cells were differentiated in 9 cm plates in N2B27 media containing 1/5000 dimethylsulfoxide (DMSO; control) or the inhibitors shown (Fig. 1) and lysed daily. Total protein (10–30 μg) was used for western blotting (standard

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protocols) and blots were analysed with phospho-specific primary antibodies (Cell Signalling) at 1/1000 dilution in 2.5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBST) and appropriate peroxidase-conjugated secondary antibodies (Jackson, 1/2500), followed by chemiluminescent detection. Rabbit anti-phospho-Smad1 (S214) antibody was from E. De Robertis (University of California, LA, USA) and used as previously described (Pera et al., 2003).

Embryo manipulations

Chick embryo manipulations and in situ hybridisation were performed as described (Eblaghie et al., 2003). Anti-EGFP antibody was from Jackson (1/100).

PCR

Quantitative PCR (Q-PCR) was performed on a Bio-Rad iCycler with i-Script SYBR green (Bio-Rad) on cDNA synthesised (using Improm-II; Promega) from 1 µg total RNA (Nucleospin RNA II; Macherey-Nagel). The primer sequences and precise conditions for Q-PCR are available on request.

RESULTS AND DISCUSSION

Signal transduction during neural specification

We first established the activation status of the three main signalling cascades regulated by FGF using western blotting on lysates of 46C ES cells grown in the presence of serum and LIF, and of ES cells at different stages of serum-free monolayer differentiation (days 1-4). Over this time period, the majority of ES cells progressively differentiate to Sox1-EGFP-positive neural progenitors under the influence of autocrine and/or paracrine FGF (Fig. 1A) (Ying et al., 2003). After an initial drop in activation following overnight culture in serum-free N2B27 media, phospho-Erk1/2 and phospho-PKB(S473) (PKB is also known as Akt – Mouse Genome Informatics; a key mediator of PI3K signalling) levels increased as differentiation proceeded, while phospho-PLCγ1(Y783) (PLCγ1 is also known as Plcg1 – Mouse Genome Informatics) levels gradually decreased (Fig. 1B). This indicates that all three pathways are active during neural specification and may play a role in the initiation of this process.

Erk1/2 is activated by FGFRs and drives neural specification

In order to determine which pathways are activated in response to endogenous FGF during the period of neural specification, we employed the specific FGF receptor inhibitor PD173074 (Mohammadi et al., 1998). 46C cells were grown under differentiation conditions in the presence of PD173074 and analysed daily by flow cytometry for Sox1-EGFP, as well as by western blotting. At a concentration sufficient to inhibit the emergence of *Sox1*-expressing cells (Fig. 1A), PD173074 prevented the activation of Erk1/2 with no inhibitory effect on the phosphorylation of PKB or PLCγ1 (Fig. 1B). This result indicates that Erk1/2 is downstream of FGF signalling in this context and that this transduction cascade is likely to be responsible for initiating neural specification in murine ES cells. To test this hypothesis, we interfered specifically with Erk activity. In the presence of the specific MEK (Mdk) inhibitor PD184352 (Davies et al., 2000), Erk1/2 phosphorylation is lost in a dose-dependent manner (Fig. 1B and see Fig. S1 in the supplementary material) concurrently with a reduction in the proportion of Sox1-EGFP cells assayed by flow cytometry (Fig. 1A, data not shown) (see also Lowell et al., 2006; Kunath et al., 2007). A similar effect was observed when Erk1/2 activation was inhibited by overexpression of the Erk1/2 phosphatase MKP3 (also known as Dusp6 – Mouse Genome Informatics; see Fig. S1 in the supplementary material) (Eblaghie et al., 2003).

Previous work has revealed that Erk1/2 can act to inhibit BMP signalling (Kretzschmar et al., 1997; Kretzschmar et al., 1999; Pera et al., 2003) and that FGF signals lead to *Bmp4* and *Bmp7* repression in the embryo (Wilson et al., 2000). However, the negative effect of FGFR inhibition on neural specification of ES cells cannot be rescued by addition of the BMP inhibitors noggin and chordin (Ying et al., 2003), suggesting that it is not exerted by a derepression of this anti-neurogenic pathway. Experiments in the early chick embryo also suggest that FGF acts independently of BMP activity in neural induction (see Stern, 2005b). To determine whether Erk1/2 signalling acts by interfering with BMP signalling during neural specification of ES cells, we assessed whether the addition of noggin could prevent the PD184352-mediated reduction in *Sox1* expression. Inhibition of Erk1/2 did not result in increased Smad C-terminal (activating) or reduced linker (inhibitory) (Kretzschmar et al., 1997; Pera et al., 2003; Sater et al., 2003) phosphorylation under monolayer differentiation conditions (Fig. 1D). Moreover, PD184352 treatment continued to suppress neural specification when *Bmp4* signalling was exogenously blocked with noggin (Fig. 1C,D), so we conclude that FGF and Erk can act independently of attenuating BMP in this context. On the contrary, *Bmp4* induced non-neural differentiation of ES cells is also inhibited by PD184352 (M.P.S. and K.G.S., unpublished) (Kunath et al., 2007).

Because neural induction in the chick embryo is also dependent on FGF signalling (Stern, 2005b; Streit et al., 2000; Wilson et al., 2000), we hypothesised that if Erk signalling is part of a fundamental mechanism that mediates neural specification it must be required in the embryo as well as in ES cells. Next, we therefore interfered with Erk1/2 activity that is detected in the prospective neural plate at Hamburger and Hamilton (HH) stage 3-3+ (Eblaghie et al., 2003; Lunn et al., 2007) by overexpressing MKP3. Strikingly, MKP3 caused a downregulation of the preneural marker *Sox3* in transfected cells (Fig. 2A), indicating a conserved requirement for Erk1/2 signalling for neural specification.

PKB signalling is dispensable for neural induction

The finding that the activation of the other two pathways (PI3K-PKB and PLCγ1) is not FGF dependent during ES cell neural specification does not exclude the possibility that they play a role in this process. In order to investigate a possible role of PDK1 (the kinase linking PI3K activity to the activation of PKB and several other kinases) in neural specification, we examined neural differentiation in PDK1-deficient ES cells (Williams et al., 2000). These cells can not activate PKB, p90RSK (also known as Rps6ka2), SGK or p70S6 kinase (also known as Rps6kb) (Williams et al., 2000) and have lower levels of at least six PKC isoforms (Balendran et al., 2000), but they can still activate *Sox1* (Fig. 1D) and readily generated nestin-expressing neural progenitors following monolayer differentiation (see Fig. S1 in the supplementary material). Furthermore, beads soaked in the PI3K inhibitor LY294002 failed to interfere with *Sox3* expression when implanted in the chick prospective neural plate (Fig. 2B). These findings are consistent with the phenotype of mice lacking PDK1 or with inactivating mutations in key PDK1 residues that mediate PKB or S6K activation, which have forebrain defects, but still all form some neural tissue (Lawlor et al., 2002; McManus et al., 2004). Taken together, these findings demonstrate that the PI3K pathway is not necessary for neural specification.

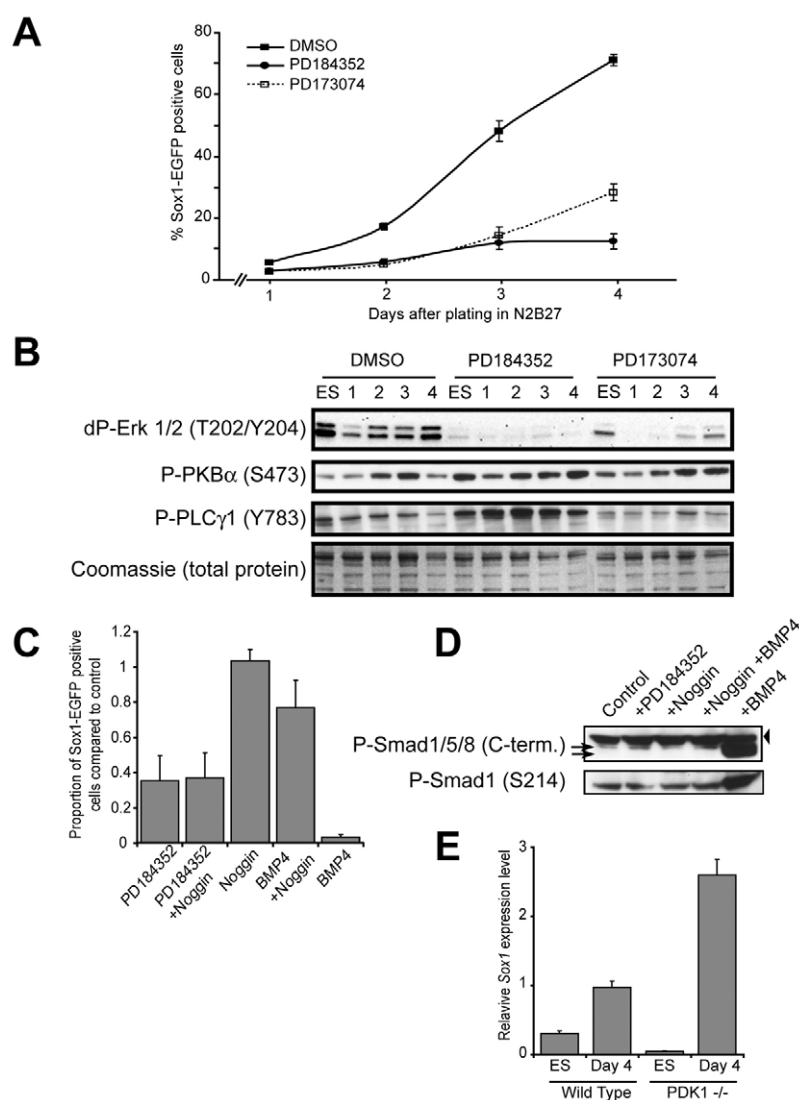


Fig. 1. Differentiation of ES cells is arrested in the absence of Erk1/2 signalling. (A) Activation of *Sox1-EGFP* expression during monolayer differentiation of 46C cells monitored daily by flow cytometry under different conditions (means of three experiments in duplicate \pm s.e.m.). (B) Western blotting for activated signal transduction cascade components during differentiation in N2B27 media (representative blots). (C) Efficiency of differentiation (relative proportion of *Sox1-EGFP*-expressing cells) at day 3 in the presence of the factors shown (normalised to the no-treatment control for each of three experiments performed in duplicate \pm s.e.m.). (D) 46C cells were differentiated for 24 hours under the conditions shown, then lysed. Western blotting for C-terminal phosphorylated Smad1, Smad5 and Smad8 (top) shows a robust response to Bmp4 but no appreciable Smad activation in N2B27 containing 3 μ M PD184352 (arrows). Arrowhead indicates a non-specific contaminating band. S214 (linker; bottom) phosphorylation of Smad1 appears to not be affected by Erk1/2 inhibition in these conditions. (E) Real-time PCR on cDNA from PDK1^{-/-} and wild-type ES cells as well as from PDK1^{-/-} and wild-type cells after 4 days of monolayer differentiation. Results are the expression levels relative to wild type at day 4 \pm s.e.m. ES, embryonic stem cell.

Erk1/2 signalling is required for ES cell differentiation beyond the primitive ectoderm state

Activation of the Erk1/2 pathway has previously been linked to ES cell differentiation (e.g. Chen et al., 2006), and treatment of ES cells with the MEK inhibitor PD098059 has been shown to maintain the expression of the pluripotency marker Oct4 (also known as Pou5f1 – Mouse Genome Informatics) under embryoid body differentiation conditions (Burdon et al., 1999). Furthermore, the morphology of 46C cells grown in N2B27+PD184352 for 3 days appeared similar to that of ES cells (Fig. 3C), so next we sought to determine whether treatment with PD184352 inhibits ES cell differentiation completely. ES cells were plated in N2B27+PD184352 for 2 days then analysed for the expression of the pluripotency determinant *Nanog* (Chambers et al., 2003; Mitsui et al., 2003) and the primitive-ectoderm marker *Fgf5*. ES cells express high levels of *Nanog* but do not express *Fgf5*; however, the latter is quickly upregulated during differentiation and is thought to be one of the earliest differentiation markers of ES cells (Rathjen et al., 1999), although its activation has not been shown to be an irreversible commitment step. Treatment with PD184352 during differentiation in N2B27

prevented the onset of *Sox1* expression but could not inhibit the transcription of *Fgf5*, as assayed by quantitative (QPCR-PCR; Fig. 3A) or immunocytochemistry (see Fig. S1 in the supplementary material) and had no significant effect on the cell cycle profile (see Fig. S2 in the supplementary material). Expression of *Nanog* (Fig. 3A) was also maintained, suggesting that, in the absence of Erk1/2 signalling, ES cells cannot progress past a primitive-ectoderm-like stage [this is also observed in *Erk2*-null ES cells (Saba-El-Leil et al., 2003; Kunath et al., 2007)]. The effect of PD184352 is readily reversible; when the inhibitor-containing medium was removed 2 days after plating and replaced with fresh media the cells resumed their differentiation and expressed *Sox1-EGFP* within the subsequent 24 hours (Fig. 3B and data not shown). Taken together, these results suggest that ES cell differentiation (beyond the upregulation of *Fgf5*) requires Erk signalling.

A short window of Erk1/2 requirement for vertebrate neural specification

Next, we sought to determine the duration of Erk1/2 signalling required for the progression of neural differentiation. The addition of PD184352 to ES cells at the time of plating robustly blocked the

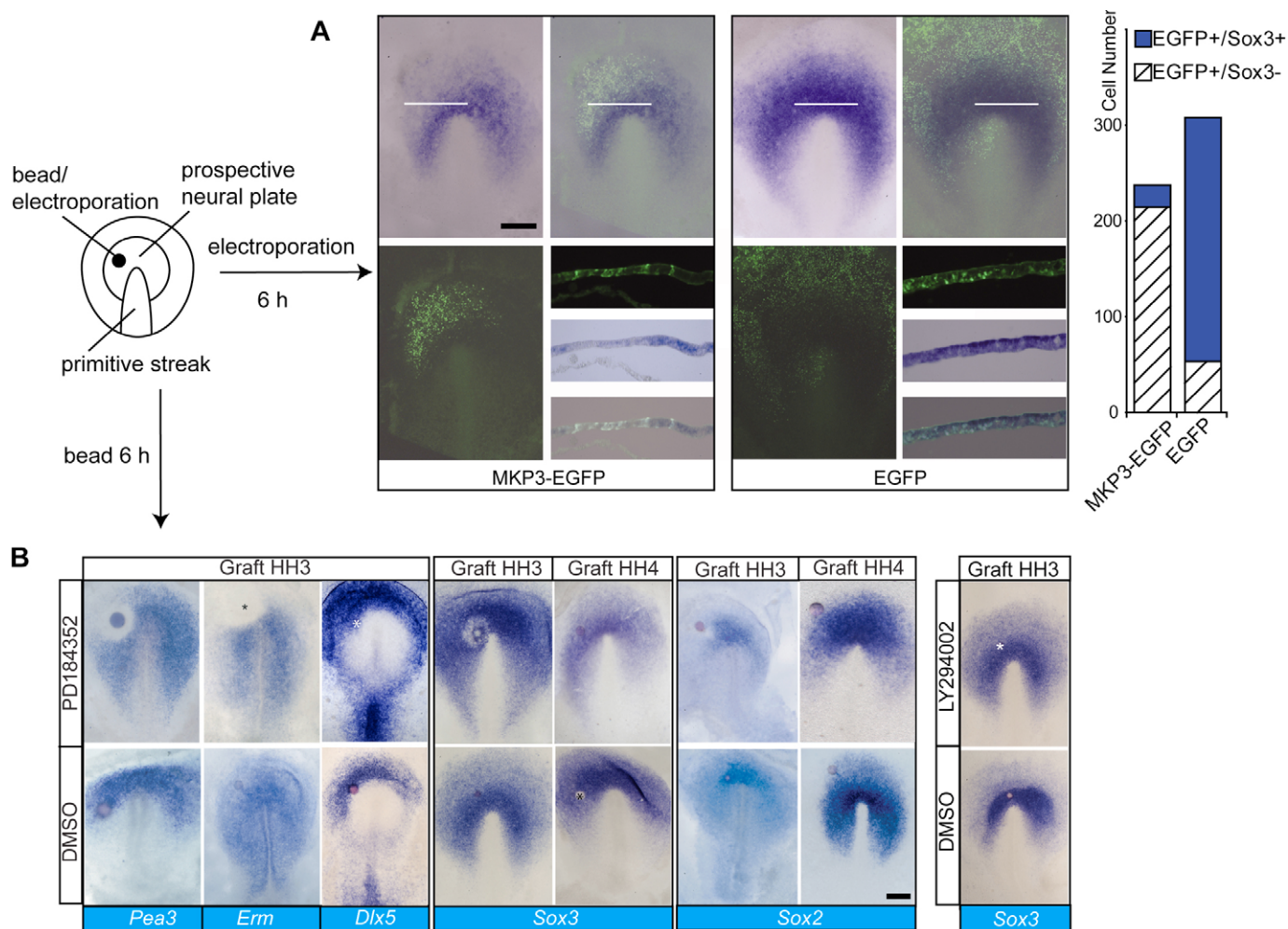


Fig. 2. Erk1/2 activity is required for the activation of neural gene expression in the chick epiblast. Diagram (top, left) shows a schematic of a stage 3 embryo and the experimental manipulations for A and B. **(A)** Hamburger and Hamilton (HH) stage 3/3+ chick embryos were electroporated with an expression plasmid on one side of the prospective neural plate and incubated for 6 hours before fixation and in situ hybridisation followed by anti-EGFP-antibody staining. Overexpression of MKP3-EGFP (left) caused a reduction in the expression of *Sox3* (6/7 embryos and 215/238 cells of three sectioned embryos), unlike overexpression of EGFP alone (four EGFP controls had no overt *Sox3* downregulation; 53/308 cells affected in the two embryos scored). White bars mark the level of sections. Graph (right) shows the number of cells expressing both EGFP and *Sox3* (blue) or only EGFP (hatched) in embryos electroporated with MKP3-EGFP or EGFP only. **(B)** Beads soaked in 10 mM PD184352 were implanted in the prospective neural plate of HH stage 3 (HH3) or HH stage 4 (HH4) embryos and left to develop for 6 hours. The expression of the *Ets* family genes *Pea3* and *Erm* was locally inhibited around the bead at stage 3 (6/6 and 6/6 embryos, respectively), whereas *Dlx5* expression was upregulated in 5/7 cases. (4/4 DMSO control embryos had normal gene expression levels.) When implanted at HH stage 3, PD184352-soaked beads caused a decrease in *Sox3* expression (12/13 embryos) and, in 4/6 embryos, caused a decrease in *Sox2* expression. Corresponding control DMSO-soaked beads ($n=4$ and 5 , respectively) all showed normal expression levels. At HH stage 4, neither PD184352- (8/8 embryos) nor DMSO-soaked control (4/4 embryos) beads showed a decrease in *Sox3* expression. PD184352-soaked beads also did not affect *Sox2* expression at this stage (4/4 embryos). Beads soaked in 10 mM LY294002 or DMSO were placed in the prospective neural plate of HH stage 3 chick embryos, which were then left to develop for 6 hours. Expression of the early neural marker *Sox3* was not affected by LY294002 treatment, whereas LY294002 did reduce phospho-PKB expression over a range of concentrations, as seen by western blotting of whole chick embryos (K. Fishwick and K.G.S., unpublished). Asterisks indicate the position of the bead in cases where it was lost during processing. Scale bars: 200 μ m.

normal emergence of *Sox1*-EGFP-expressing cells after 3 days of culture, whereas application of the inhibitor at 17 or 24 hours after plating resulted in a substantial proportion of *Sox1*-EGFP-positive cells at the same time point (Fig. 3C and data not shown). This suggests that differentiation of at least some of the ES cells commences during this time and that a relatively short exposure to Erk signalling is sufficient for neural specification.

In the chicken embryo, implantation of PD184352-soaked beads into the prospective neural plate at HH stage 3 resulted in the downregulation of the Erk1/2-target *Ets* genes *Erm* (also

known as *Etv5*) and *Pea3* (also known as *Etv4*) and of the preneural marker *Sox3* (similar to the results observed when MKP3 was overexpressed; Fig. 2), whereas a border marker, *Dlx5*, was locally upregulated. Exposure to PD184352 at this stage also prevented the onset of *Sox2* expression (Fig. 2B). This suggests that Erk1/2 signalling is required for the maintenance of an early preneural state and for the progression to a committed neural state, marked by the continued expression of *Sox3* and the onset of *Sox2* expression. However, when implanted at HH stage 4 (late primitive streak stage), PD184352-soaked beads no longer

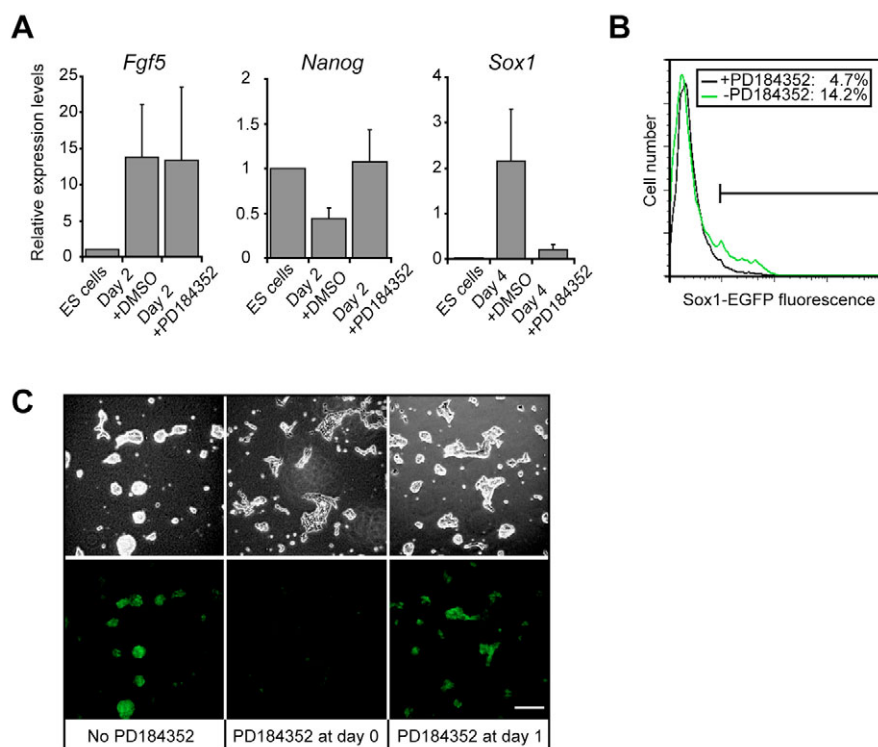


Fig. 3. Erk1/2 inhibition reversibly blocks ES cell differentiation at the primitive-ectoderm stage. (A) Real-time RT-PCR for *Fgf5* and *Nanog* was performed at day 2 of differentiation. Expression of *Sox1* was determined for the same conditions at day 4. Results are from duplicate determinations of three experiments \pm s.e.m. relative to the expression level in ES cells (*Fgf5*, *Nanog*) or day 4 of differentiation (*Sox1*). (B) Flow cytometry profile of 46C ES cells plated in N2B27 plus 3 μ M PD184352 for 2 days before media was changed to either N2B27 (–PD184352; green line) or N2B27 containing fresh inhibitor (+PD184352; black line) for 24 hours. (C) Images of 46C cells at day 3 of differentiation under the conditions shown. Scale bar: 200 μ m.

inhibited *Sox3* or *Sox2* expression (Fig. 2B, right), identifying an early and discrete requirement for Erk signalling in neural specification. This falls within the period during which the chick epiblast is competent to respond to endogenous neural-inducing signals (Storey et al., 1992; Streit et al., 1997) and prompted us to characterise further the apparently analogous period of Erk activity required for neural specification in ES cells.

To refine the window of Erk1/2 requirement during ES cell differentiation, we decided to allow the cells to progress to the epiblast-like *Fgf5*-expressing state in the presence of PD184352 and then determine which period of Erk1/2 activity is required to commit these cells to their differentiation programme. To this end we plated 46C ES cells in N2B27+PD184352 and allowed them to differentiate for 2 days, by which time cells expressed *Fgf5* and

Nanog but had negligible levels of Sox1-EGFP (Fig. 3A). The media was then removed and, after two washes, replaced with media with or without inhibitor for varying lengths of time (see Fig. 4B,C). At the end of this period, cells were washed again and either lysed for western blotting analysis or incubated in fresh media plus PD184352 for 15 hours before flow cytometry analysis for Sox1-EGFP (Fig. 4A). Western blotting analysis shows that phosphorylated Erk1/2 first appeared around 5 minutes after PD184352 withdrawal, with levels peaking around 30 minutes after its removal and remaining elevated for a further 3 hours before dropping to background levels by 9 hours (Fig. 4B), indicating that the inhibitor was washed off the cells efficiently. Flow cytometry analysis showed that a period of 9 hours in the absence of PD184352 resulted in a significantly higher proportion of Sox1-EGFP-positive cells compared with inhibitor-

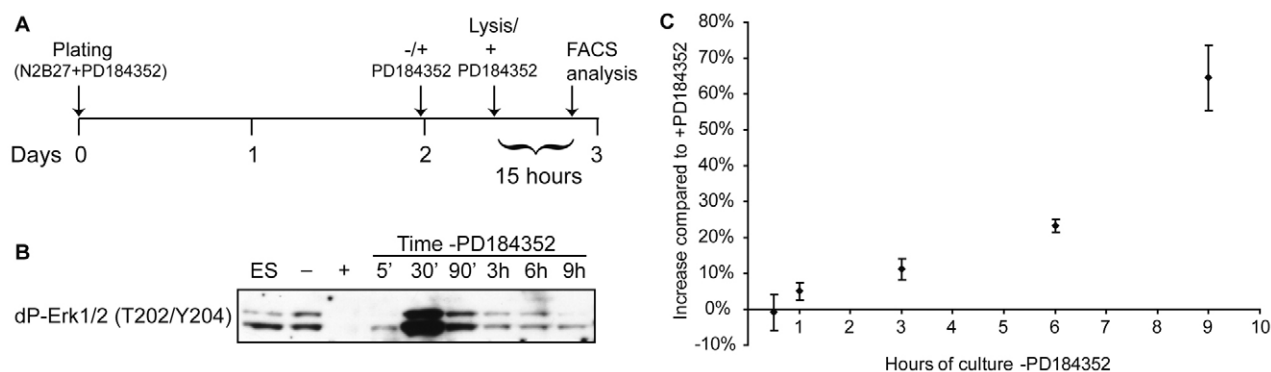


Fig. 4. A brief exposure to Erk1/2 signalling is sufficient to trigger neural specification. (A) Schematic of experiment. ES cells were differentiated for 2 days in N2B27+PD184352 before media was changed to N2B27 \pm PD184352 for the specified time. Following this, the cells were lysed and analysed for activation of Erk1/2 (B) or media containing PD184352 was replaced and cells were cultured for 15 hours before FACS analysis (C). (B) Erk1/2 activation following inhibitor withdrawal at day 2. ES, ES cell lysate; –/+, cells differentiated for 2 days in constant absence/presence of PD184352. (C) Effect of different time periods of Erk1/2 activity on neural specification. y-axis marks the percentage increase of Sox1-EGFP-expressing cells following culture in the absence of PD184352 over culture for the same time in the presence of PD184352. Results are averages \pm s.e.m. from three experiments performed in triplicate.

treated controls (Fig. 4C). This effect decreased with shorter periods of incubation in the absence of inhibitor, but a statistically significant increase in the proportion of Sox1-EGFP-positive cells compared to the N2B27+PD184352 condition was maintained until the 1 hour time-point. Taken together, these results indicate that Erk1/2 activity sustained over a period of approximately 1 hour is sufficient to drive neural specification in ES cell-derived primitive-ectoderm cells.

In summary, our experiments define a conserved period during cell fate determination in mouse ES cells and in the chick embryo when Erk1/2, but not PKB, activity is required for neural specification. Additionally, we show that, in ES cells, this requirement is independent of BMP signal transduction and determine the minimum duration of sustained Erk1/2 activity required to control the transition to a committed neural fate.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/16/2889/DC1>

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