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FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment

Tilo Kunath[1,*], Marc K. Saba-El-Leil[2], Marwa Almousailleakh[1], Jason Wray[3], Sylvain Meloche[2] and Austin Smith[3,*]

Pluripotent embryonic stem (ES) cells must select between alternative fates of self-replication and lineage commitment during continuous proliferation. Here, we delineate the role of autocrine production of fibroblast growth factor 4 (Fgf4) and associated activation of the Erk1/2 (Mapk3/1) signalling cascade. Fgf4 is the major stimulus activating Erk in mouse ES cells. Interference with FGF or Erk activity using chemical inhibitors or genetic ablations does not impede propagation of undifferentiated ES cells. Instead, such manipulations restrict the ability of ES cells to commit to differentiation. ES cells lacking Fgf4 or treated with FGF receptor inhibitors resist neural and mesodermal induction, and are refractory to BMP-induced non-neural differentiation. Lineage commitment potential of *Fgf4*-null cells is restored by provision of FGF protein. Thus, FGF enables rather than antagonises the differentiation activity of BMP. The key downstream role of Erk signalling is revealed by examination of *Erk2*-null ES cells, which fail to undergo either neural or mesodermal differentiation in adherent culture, and retain expression of pluripotency markers Oct4, Nanog and Rex1. These findings establish that Fgf4 stimulation of Erk1/2 is an autoinductive stimulus for naïve ES cells to exit the self-renewal programme. We propose that the Erk cascade directs transition to a state that is responsive to inductive cues for germ layer segregation. Consideration of Erk signalling as a primary trigger that potentiates lineage commitment provides a context for reconciling disparate views on the contribution of FGF and BMP pathways during germ layer specification in vertebrate embryos.

KEY WORDS: Pluripotency, Mitogen activated protein kinase, Neural induction, Epiblast, Mesoderm induction, Mouse

INTRODUCTION

Embryonic stem (ES) cells are immortal cell lines derived from the epiblast of mammalian blastocysts (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). ES cells have the ability to differentiate into multiple cell types representative of the three definitive germ layers of the embryo, a property defined as pluripotency. Through a process of self-renewal, ES cells maintain this potency while expanding in culture (Smith, 2001b). These properties make ES cells a unique system in which to study developmental decisions and differentiation (Kouskoff et al., 2005; Nishikawa et al., 1998; Niwa et al., 2005; Smith, 2001a; Tada et al., 2005), and also a promising tool for biotechnological and biomedical applications (Keller, 2005).

Although the requirements for maintaining mouse ES cells in a self-renewing pluripotent state are increasingly being defined (Chambers and Smith, 2004; Ivanova et al., 2006), the process by which ES cells initially enter into lineage commitment remains obscure. Fibroblast growth factors (FGFs) and downstream activation of the Ras-Erk signalling cascade are critical stimuli for proliferation and differentiation in many cell types (Roux and Blenis, 2004; Thisse and Thisse, 2005). Fgf4 is produced in an autocrine fashion by undifferentiated ES cells (Ma et al., 1992; Rathjen et al., 1999). However, previous studies have suggested that Fgf4 and Erk activation may be dispensable for propagation of undifferentiated mouse ES cells (Burdon et al., 2002; Burdon et al., 1999; Jirmanova et al., 2002; Qu and Feng, 1998; Wilder et al., 1997). Here we delineate the role of Fgf4 and the Ras-Erk signalling cascade in the decision between self-renewal and commitment.

MATERIALS AND METHODS

ES cell lines and culture

E14Tg2a and 46C parental mouse ES cell lines have been described previously (Ying et al., 2003b). *Fgf4*$^{+/-}$ (clone 342) and *Fgf4*$^{-/-}$ (clone FD6) ES cells were a kind gift from Angie Rizzino (Wilder et al., 1997). *Erk2*$^{+/-}$ ES cells were generated by targeting and two *Erk2*$^{-/-}$ ES cell lines (B1 and B3) were derived from blastocysts from *Erk2*$^{+/-}$ intercrosses (Saba-El-Leil et al., 2003). All ES cell lines were maintained in GMEM (Sigma, G5154) supplemented with 10% FCS (Invitrogen), 100 μM 2-mercaptoethanol (BDH, 441413), 1×MEM non-essential amino acids (Invitrogen, 1140-036), 2 mM L-glutamine, 1 mM sodium pyruvate (both from Invitrogen), and 100 units/ml LIF (made in-house) on gelatinised tissue culture flasks (Smith, 1991).

ES cell monolayer differentiation

The serum-free neural induction protocol was applied as described (Ying and Smith, 2003; Ying et al., 2003b). ES cells were plated in 6-well plates at a density of 1.5×10^5 cells/well in N2B27 medium with LIF (100 units/ml). The next day (day 0), the medium was changed to N2B27 without LIF (plus ligands/inhibitors). Medium was renewed daily thereafter. For assays at clonal density, ES cells were plated at 0.75 cells/mm^2 (720 cells/6-well or 150 cells/4-well) in N2B27 plus LIF for 2 days. The medium was then changed to N2B27 (plus ligands/inhibitors) and cells fed every other day. Human recombinant FGF4 (R&D Systems, 235-F4), FGF2 (R&D Systems, 233-FB) and FGF5 (Sigma, F4537) were used at 5 ng/ml, 5 ng/ml and 10 ng/ml, respectively, in the presence of 1 μg/ml heparin (Sigma, H3149). Human recombinant BMP4 (R&D Systems, 314-BP) was used at

[1]Centre Development in Stem Cell Biology, Institute for Stem Cell Research, School of Biological Sciences, University of Edinburgh, Edinburgh, UK. [2]Institut de Recherche en Immunologie et en Cancérologie, and Department of Pharmacology, Université de Montréal, Montréal, Québec, Canada. [3]Wellcome Trust Centre for Stem Cell Research, and Department of Biochemistry, University of Cambridge, Cambridge, UK.

*Authors for correspondence (e-mails: tilo.kunath@ed.ac.uk; ags39@cscr.cam.ac.uk)

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10 ng/ml. PD173074 (Sigma, P2499) was used at 100 ng/ml (Mohammadi et al., 1998), PD184352 (gift from Philip Cohen) at 25 μM (Davies et al., 2000) and SU5402 (Calbiochem, 572630) at 5 μM (Mohammadi et al., 1997). Mesoderm induction was performed as described (Nishikawa et al., 1998) on collagen IV plates (BD Biosciences, 354428).

FACS analysis

ES cells were collected with Cell Dissociation Buffer (Gibco, 13151-014), washed with PBS+1% FBS, incubated with anti-Pdgfrα antibody at 1:100 (Clone APA5; Chemicon, CBL 1366) and labelled with a secondary antibody (anti-rat IgG-PE), before analysis on a CyAN FACS machine.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde (room temperature, 10 minutes), washed three times with PBS, then incubated for 1 hour in blocking buffer (PBS, 2% goat serum, 0.1% Triton X-100). Primary antibodies were diluted in blocking buffer and applied for at least 1 hour at room temperature or overnight at 4ºC, followed by three washes in PBS. Goat secondary antibodies conjugated to Alexa fluorophores (Molecular Probes) were diluted 1:1000 in blocking buffer and applied for 1 hour at room temperature. The cells were washed twice in PBS and a third time in PBS containing DAPI (10 μg/ml) before obtaining pictures on an Olympus inverted fluorescence microscope. Confocal images were obtained with a Leica TCSNT confocal microscope and associated software (Leica Microsystems). Primary antibodies used were anti-phospho-p44/42 MAPK at 1:200 (20G11; Cell Signaling Technology, 4376), anti-p44/42 MAPK at 1:200 (Cell Signaling Technology, 9102), anti-phospho-histone H3 at 1:20,000 (HTA28; Sigma, H9908), anti-fibrillarin at 1:1000 (Abcam, ab4566), anti-Oct4 (also known as Pou5f1 – Mouse Genome Informatics) at 1:200 (C-10; Santa Cruz Biotechnology, sc-5279), anti-Sox2 at 1:200 (Chemicon, AB5603), anti-TuJ1 (also known as Tubb3) at 1:1000 (Covance, MMS-435P), anti-nestin at 1:10 (Rat-401, DSHB), anti-E-cadherin (also known as cadherin 1) at 1:200 (ECCD2, 2 mg/ml, gift from Masatoshi Takeichi, Kobe, Japan) and anti-keratin 14 at 1:10,000 (MK14, Covance). A phospho-peptide, but not an unphosphorylated control peptide (Abcam, ab5255), blocked all activated phospho-Erk1/2 staining (data not shown).

Immunoblotting

Western blotting was performed as described (Saba-El-Leil et al., 2003). Briefly, whole cell lysates (100 μg) were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and blotted for anti-phospho-p44/42 MAPK at 1:1000 and anti-p44/42 MAPK at 1:1000 (both described above). The secondary antibody, anti-rabbit IgG-peroxidase (Sigma, A6154), was used at 1:3000 and peroxidase activity was visualised with the SuperSignal West Pico Kit (Pierce).

Reverse transcriptase (RT)-PCR

Total RNA was isolated using the Absolutely RNA Kit (Stratagene, 400800), and cDNA was made from 1 μg of total RNA using SuperScript II RT (Invitrogen) and oligo-dT primers. PCR primers and conditions are listed in Table 1. Real-time PCR was performed with the LightCycler 480 using the Universal Probe Library System (Roche). Quantitative PCR primers for nestin (forward, 5′-CTGCAGGCCACTGAAAAGT-3′; reverse, 5′-TTCCAGGATCTGAGCGATCT-3′) were used with UPL probe #2 (Roche, 04684982001) and primers for TATA-binding protein (TBP) (forward, 5′-GGGGAGCTGTGATGTGAAGT-3′; reverse, 5′-CCAGGAAATAAT-TCTGGCTCA-3′) were used with UPL probe #97 (Roche, 04692144001).

RESULTS AND DISCUSSION

We first examined the ability of *Fgf4* mutant ES cells (Wilder et al., 1997) to undergo differentiation in the absence of other inducers. ES cells were cultured in N2B27, a defined medium that lacks serum or leukaemia inhibitory factor (LIF) (Ying and Smith, 2003; Ying et al., 2003b). Unlike wild-type and $Fgf4^{+/-}$ ES cells, $Fgf4^{-/-}$ ES cells produced very few nestin-positive neural precursors by day 6, and only gave rise to sporadic TuJ1-positive neurons by day 10 (Fig. 1A-E). The blockade in neural lineage differentiation was evident at a very early stage, as shown by the failure of *Fgf4*-null cells to upregulate primary neural markers *Sox1* or nestin after 48 hours (Fig. 1F). Quantitative analysis of nestin mRNA expression at day 5 confirmed the immunostaining results (Fig. 1G). Neural differentiation could be fully restored by supplementing the culture medium with recombinant FGF4 or FGF2 at 5 ng/ml (Fig. 1G-I). Cell counts of nestin-positive versus Oct4-positive cells showed that differentiation of $Fgf4^{-/-}$ ES cells is restored to wild-type efficiency by Fgf4 protein (Fig. 1J). The FGF receptor (FGFR) inhibitor PD173074 (Mohammadi et al., 1998) at 100 ng/ml completely blocked FGF-mediated rescue (data not shown). These results establish that *Fgf4*-null cells retain neural differentiation capacity and that their deficiency in monoculture commitment is directly attributable to the absence of FGF4. Intriguingly, FGF5 failed to rescue neural commitment indicating that there is selectivity for FGF ligands in this action. This is noteworthy because *Fgf5* is upregulated upon LIF withdrawal (see below). In agreement with Stavridis et al. (Stavridis et al., 2007), addition of FGF4 for the first 24 hours (of a 10-day assay) was sufficient to elicit complete rescue of neural and neuronal differentiation, suggesting that it acts as a trigger to initiate commitment rather than being required continuously.

The majority of $Fgf4^{-/-}$ cells retain expression of the pluripotency marker Oct4 after LIF withdrawal (Fig. 1D,E). These Oct4-positive cells remain viable and proliferative for several days and cell numbers remain similar to those of wild-type cultures for at least 8 days. Therefore, the decreased number of neural derivatives arises primarily from a reduced ability of ES cells to enter this lineage, rather than from death of neural precursor cells in the absence of FGF stimulation. Neural differentiation can readily be induced if FGF4 is added 3 days after LIF withdrawal (data not shown), indicating that the ES cells persist in an undifferentiated state rather than selecting alternative commitment programmes. These observations corroborate and extend previous arguments that Fgf4 is an autoinductive stimulus for neural commitment in ES cells (Lowell et al., 2006;

Table 1. Primers and conditions for reverse transcriptase PCR

Gene	Forward primer	Reverse primer	Product (bp)	Annealing temp. °C	Cycles
Oct4	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACCACATCCTTCTCT	313	55	25
Nanog	ATGAAGTGCAAGCGGTGGCAGAAA	CCTGATGGAGTCACAGAGTAGTTC	440	65	26
Fgf5	ACCCTTTGAGCTTTCTACCC	CCGTCTGTGGTTTCTGTTGAGG	188	58	35
Rex1	AGGCCAGTCCAGAATACCAG	GGAACTCGCTTCCAGAACCT	233	58	30
brachyury	GTGACTGCCTACCAGAATGA	ATTGTCCGCATAGGTTGGAG	336	57	33
Id1	TCCGCCTGTTCTCAGGATCA	GTAGCAGCCGTTCATGTCGT	217	55	30
Id3	CGACATGAACCACTGCTACT	CTCCTCTTGTCCTTGGAGAT	221	55	30
β-actin (Actb)	GGCCCAGAGCAAGAGAGGTATCC	ACGCACGATTTCCCTCTCAGC	460	55	25

Primer sequences are shown 5′ to 3′.

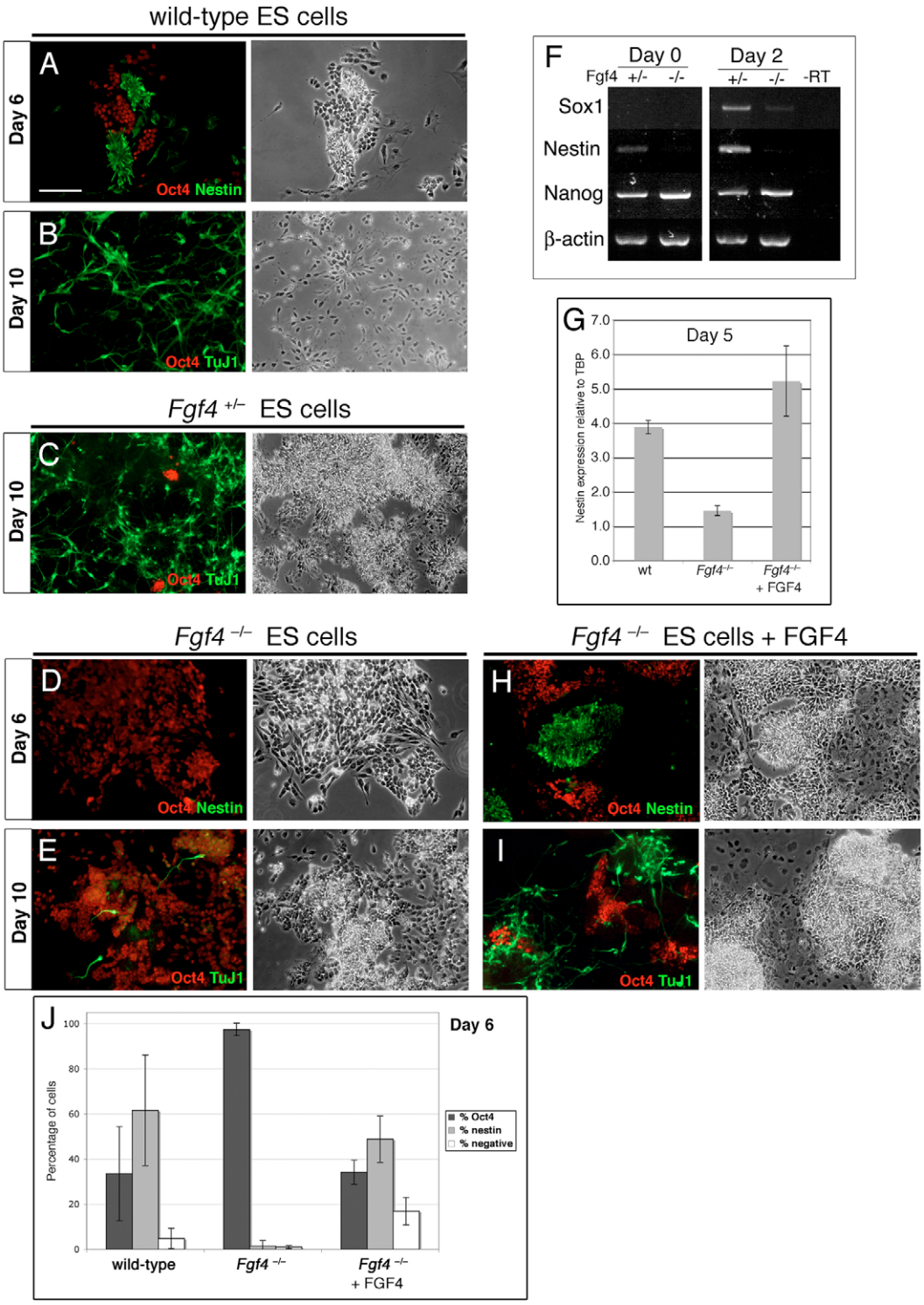

Fig. 1. *Fgf4*$^{-/-}$ ES cells are deficient in neural induction.
(**A-E**) Immunostaining (left; phase-contrast, right) for Oct4 and nestin (A,D) and Oct4 and TuJ1 (B,C,E) in wild-type (A,B), *Fgf4*$^{+/-}$ (C) and *Fgf4*$^{-/-}$ mouse ES cells on day 6 (A,D) and day 10 (B,C,E) of the monolayer neural differentiation protocol. (**F**) RT-PCR for neural markers *Sox1* and nestin in *Fgf4*$^{+/-}$ and *Fgf4*$^{-/-}$ ES cells in self-renewing conditions on day 2 of the neural induction protocol. *β-actin* was used as a loading control.
(**G**) Quantitative RT-PCR for nestin on day 5 of the neural differentiation assay. TBP, TATA-binding protein.
(**H**,**I**) Immunostaining for Oct4 and nestin (H) and Oct4 and TuJ1 (I) of *Fgf4*$^{-/-}$ ES cells cultured in FGF4 (5 ng/ml) for the first 24 hours of a 6 day (H) and 10 day (I) neural monolayer assay. Scale bar: 100 μm.
(**J**) Cell counts of Oct4-positive, nestin-positive, and double-negative cells for wild-type, *Fgf4*$^{-/-}$ ES cells, and *Fgf4*$^{-/-}$ ES cells treated with FGF4 after 6 days culture in N2B27 (n=3 for each sample).

Ying et al., 2003b) and are consistent with evidence for a requirement for FGF signalling for neural induction in vertebrate embryos (Streit et al., 2000; Wilson et al., 2000; Stavridis et al., 2007).

Addition of BMP to ES cells in N2B27 without LIF suppresses neural differentiation and causes the entire population to form sheets of large, flat cells (Fig. 2A,B) (Ying et al., 2003b). The identity of these BMP4-induced cells is not known. They are negative for neural markers Sox1 and nestin, but positive for E-cadherin, and therefore highly unlikely to be mesodermal. A small subset expressed keratin 14, suggesting they might be immature ectodermal derivatives (see Fig. S1 in the supplementary material). One mechanism proposed for the neuralising action of FGF/Erk signalling is the inhibition of BMP signal transduction through phosphorylation of Smad1 (Kretzschmar et al., 1997; Kuroda et al., 2005; Pera et al., 2003). We therefore examined the BMP responsiveness of *Fgf4*$^{-/-}$ ES cells. These cells completely resisted differentiation in response to BMP4 and remained Oct4-

positive and undifferentiated (Fig. 2D). The rare neuronal differentiation observed in N2B27 alone was eliminated, suggesting that BMP signalling was operative (Fig. 2C,D). This was confirmed by upregulation of *Id1* and *Id3* expression (Ying et al., 2003a) following a 45-minute BMP4 stimulation of *Fgf4*$^{-/-}$ ES cells (Fig. 2J). Strikingly, when FGF4 was added, the differentiation response to BMP was regained (Fig. 2E). Independent confirmation of these findings was obtained by applying the FGFR inhibitor PD173074 to wild-type ES cells. This prevented differentiation consequent to LIF withdrawal (Fig. 2F,G) and to BMP4 treatment (Fig. 2H,I). These findings establish that an intact FGF signalling pathway is essential for the switch of BMP signalling from supporting self-renewal (Ying et al., 2003a) to driving non-neural differentiation.

To examine whether the requirement for FGF stimulation is restricted to ectodermal lineages, we investigated mesoderm differentiation. After 5 days on collagen IV-coated plates, wild-type ES cells exhibited the expected induction of the paraxial mesoderm marker Pdgfrα (Nishikawa et al., 1998). However, in the presence of PD173074, this induction was completely

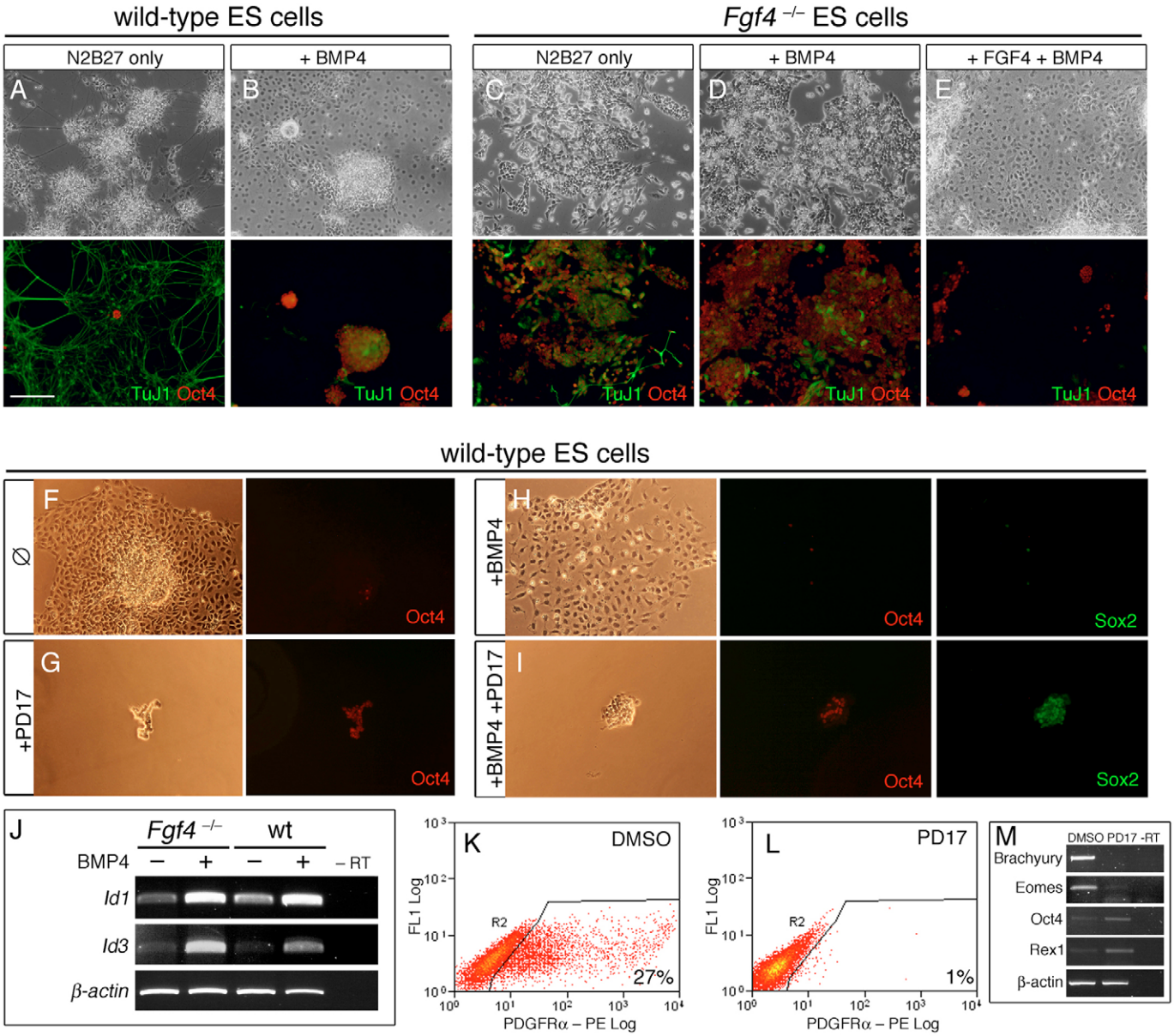

Fig. 2. *Fgf4*$^{-/-}$ and wild-type ES cells require FGFR signalling for multilineage commitment. (**A-E**) Immunostaining (below; phase-contrast, above) of Oct4 and TuJ1 in wild-type (E14Tg2a) mouse ES cells on day 7 of monolayer culture in N2B27 alone (A) or N2B27 with Bmp4 (B) and on *Fgf4*$^{-/-}$ ES cells in N2B27 alone (C), with BMP4 (D), or with BMP4 and FGF4 (E). Note that the green fluorescence in C and D is not specific and both immunopositivity and neuronal morphology are required to identify cells as neurons (Svendsen et al., 2001). (**F**,**G**) Immunostaining (right; phase-contrast, left) for Oct4 in a colony of E14Tg2a ES cells in the absence of LIF (F) or absence of LIF and presence of PD173074 (G). (**H**,**I**) Immunostaining for Oct4 and Sox2 in E14Tg2a ES cells cultured in BMP4 (H) or BMP4 and PD173074 (I). Scale bar: 100 μm. (**J**) RT-PCR for *Id1* and *Id3* after a 45-minute BMP4 stimulation. *β-actin* was used as a loading control. (**K**,**L**) FACS analysis for Pdgfrα expression of wild-type ES cells after 5 days on collagen IV plates in the absence (K) or presence (L) of the FGFR inhibitor PD173074. (**M**) RT-PCR analysis of wild-type cells after 4 days on collagen IV plates in the presence or absence of PD173074.

abrogated (Fig. 2K,L). Gene expression analysis at day 4 showed that expression of brachyury and eomesodermin (*Eomes*) was prevented by PD173074, and downregulation of the pluripotency markers *Oct4* and *Rex1* (also known as *Zfp42* – Mouse Genome Informatics) did not occur (Fig. 2M). This suggests that FGF signalling in ES cells might initiate commitment of ES cells to multiple lineages.

Absence of Fgf4 is not sufficient to prevent ES cell differentiation completely, however. Following multicellular aggregation, *Fgf4*$^{-/-}$ ES cells show some induction of germ layer markers (not shown), and they can form complex teratomas albeit at a lower frequency than *Fgf4*$^{+/-}$ ES cells (Wilder et al., 1997). This could be due to upregulation of other FGFs, notably Fgf8, upon aggregation (Wang et al., 2006) and to FGFs or other stimuli provided by the host after grafting.

To assess the mechanism of FGF action, we investigated activation of the mitogen-activated protein kinases Erk1/2 (Erk1 is also known as Mapk3 and p44 MAPK, and Erk2 is also known as Mapk1 and p42 MAPK – Mouse Genome Informatics). Culture in the FGFR inhibitor SU5402 decreased, but did not entirely eliminate, activated phospho-Erk1/2 (pErk1/2) immunostaining, compared with ES cells cultured in PD184352, a potent antagonist of the Erk activating enzymes Mek1/2 (Map2k1/k2 – Mouse Genome Informatics) (Davies et al., 2000) (Fig. 3A-D). We therefore examined the specific role of Fgf4 in Erk1/2 activation in ES cells. To eliminate autocrine stimulation we again took advantage of *Fgf4*-null ES cells (Wilder et al., 1997). Immunoblotting revealed a massive reduction in steady-state Erk1/2 phosphorylation in *Fgf4*$^{-/-}$ ES cells, compared with heterozygous cells in serum-free medium (Fig. 3E). Presence of the self-renewal cytokine LIF, which activates Erk in addition to Stat3 (Burdon et al., 2002; Burdon et al., 1999), only partially restored pErk levels in the null cells and did not further augment pErk in the heterozygous cells. Consistent with these observations, acute (15 minute) stimulation with FGF4 resulted in a massive increase in Erk1/2 phosphorylation, whereas LIF and serum stimulation gave a more moderate increase (not shown). These data, and those in the accompanying manuscript (Stavridis et al., 2007), establish that Fgf4 is a potent activator of the pErk pathway in undifferentiated ES cells.

We examined the distribution of active Erk1/2 in wild-type ES cells. Immunofluorescence staining for pErk1/2 was both nuclear and cytoplasmic (see Fig. S2A,B in the supplementary material). Occasional cells showed an intense immunofluorescence signal over the entire cell. Co-localisation with the mitotic marker phospho-histone H3 (Goto et al., 1999) identified these as mitotic cells (see Fig. S2C in the supplementary material), as also reported for pErk immunostaining in the egg cylinder embryo (Corson et al., 2003). In cells outside of M phase, diffuse cytoplasmic staining was evident, along with punctate nuclear bodies in most cells. This subnuclear localisation coincided with the nucleolar marker fibrillarin (see Fig. S2D in the supplementary material), and is consistent with the role of Erk1/2 in RNA polymerase I activation and rRNA synthesis (Zhao et al., 2003). We conclude that the Erk pathway is continuously activated in undifferentiated ES cells predominantly by signalling through FGFRs, and is potentially functional in both nucleus and cytoplasm.

Since Erk is strongly activated by FGF signalling in ES cells (Fig. 3E), we examined whether this pathway may have a crucial role in lineage commitment. Erk1 and Erk2 are thought to have equivalent biochemical activity, but Erk2 is present at higher levels in ES cells (Fig. 3E). *Erk2*$^{-/-}$ embryos form a blastocyst, implant and produce epiblast, but they fail to make mesoderm (Yao et al., 2003) and die owing to severe trophoblast defects (Saba-El-Leil et al., 2003). ES cell lines were derived from blastocysts homozygous for the null *Erk2* allele. They are viable and proliferate with similar kinetics to normal ES cells. Although, morphologically, they appear more flattened than wild-type ES cells, they express the full range of pluripotency markers. These cells exhibited massively reduced pErk1/2 by immunofluorescence (not shown) and immunoblotting analyses (Fig. 4A). In the defined neural induction protocol, two

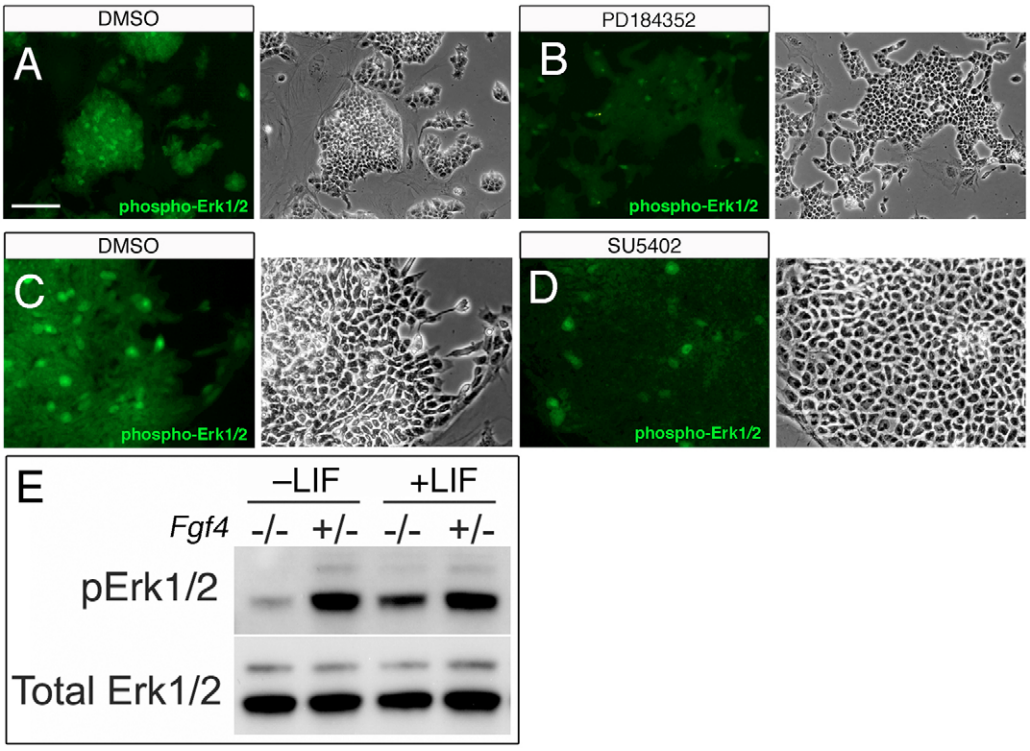

Fig. 3. Erk1/2 activity in ES cells is FGF dependent. (**A-D**) Immunostaining (left; phase-contrast, right) for phospho-Erk1/2 (pErk1/2) in mouse ES cells cultured in serum plus LIF for 14 hours in vehicle control (DMSO, A,C), inhibitor of MEK activation PD184352 (B), or FGFR inhibitor SU5402 (D). Each pair of images was taken at the same exposure time. Scale bar: 100 μm for A,B; 50 μm for C,D. (**E**) Immunoblot of *Fgf4*$^{-/-}$ and *Fgf4*$^{+/-}$ ES cell lysates cultured in serum-free N2B27 medium with or without LIF for 24 hours.

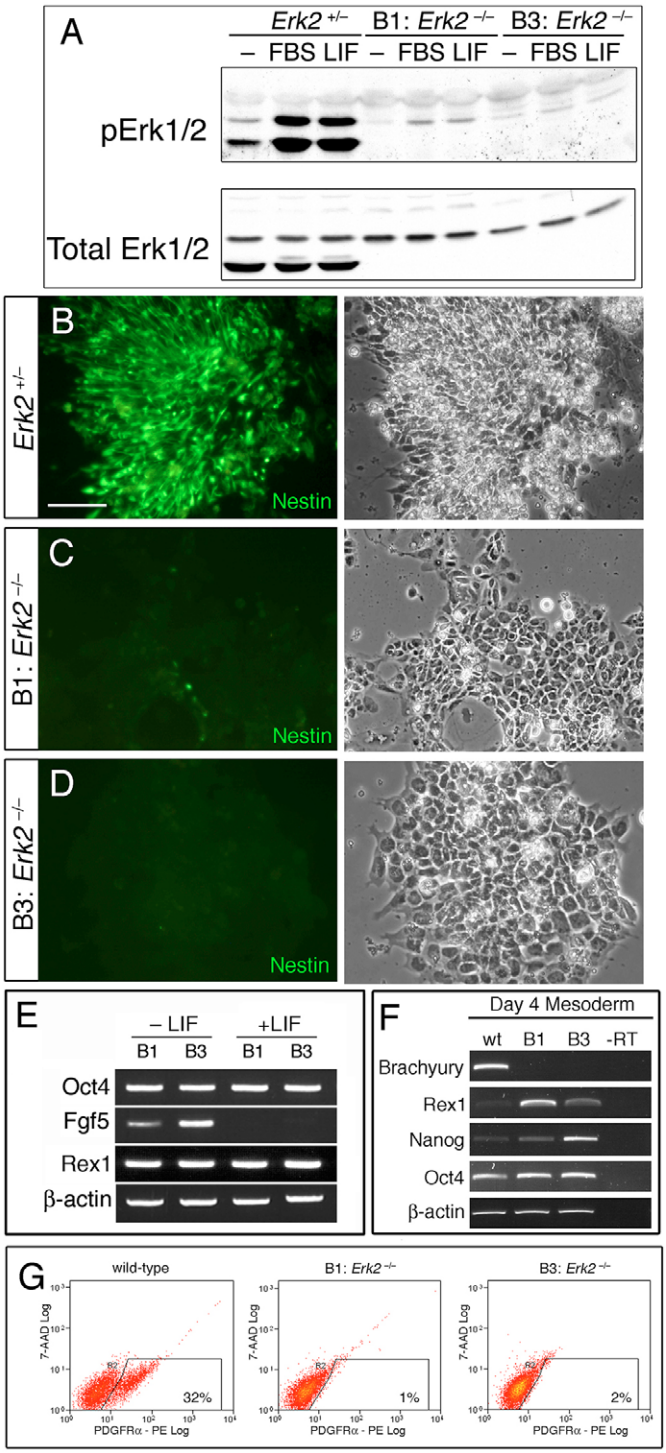

Fig. 4. *Erk2*$^{-/-}$ ES cells are severely deficient in neural and mesodermal commitment. (**A**) Immunoblotting for phospho-Erk1/2 (pErk1/2) and total Erk1/2 from serum-starved *Erk2*$^{+/-}$ and *Erk2*$^{-/-}$ mouse ES cells that were stimulated with foetal bovine serum (FBS), LIF, or unstimulated (–). (**B-D**) Immunostaining (left; phase-contrast, right) for nestin in *Erk2*$^{+/-}$ cells (A), and two independent *Erk2*$^{-/-}$ ES cell clones (B,C) fixed on day 6 of neural monolayer culture. Scale bar: 50 μm. (**E**) RT-PCR for *Oct4*, *Fgf5*, *Rex1* and *β-actin* (as loading control) on RNA isolated from two *Erk2*$^{-/-}$ ES cell lines (B1, B3) that were maintained in N2B27 medium without LIF. Parallel cultures were exposed to LIF for 2 days (+LIF) before isolation of RNA. (**F**) RT-PCR analysis of wild-type and two *Erk2*$^{-/-}$ (B1, B3) ES cell lines on day 4 of mesoderm monolayer differentiation. (**G**) FACS analysis for Pdgfrα expression of wild-type and *Erk2*$^{-/-}$ ES cells on day 5 of mesoderm monolayer differentiation.

Erk2$^{-/-}$ ES cell clones (B1, B3) showed scant evidence of differentiation (Fig. 4B-D). In fact, both clones could be passaged in the absence of LIF in serum-free N2B27 medium and continued to express the ES cell markers *Oct4* and *Nanog*. Without LIF they exhibited upregulation of *Fgf5* (Fig. 4E), which is normally expressed at low levels in ES cell cultures. *Fgf5* is widely employed as an early marker of ES cell differentiation and is suggested to mark formation of a population corresponding to the egg-cylinder-stage epiblast (Haub and Goldfarb, 1991; Rathjen et al., 1999; Shen and Leder, 1992). However, we did not observe any corresponding change in expression of *Rex1*, which is downregulated in post-implantation epiblast (Rogers et al., 1991) and is reported to show reciprocal expression with *Fgf5* during ES cell differentiation (Rathjen et al., 1999). Furthermore, when LIF was added back to the cultures, *Fgf5* expression was lost within 2 days, with no change in cell proliferation or evident cell death (Fig. 4E). Therefore, expression of *Fgf5* appears to be reversible and directly or indirectly regulated by the LIF pathway.

In aggregate culture, *Erk2*$^{-/-}$ cells formed highly compacted clumps with few signs of differentiation. However, this could be due in part to death of differentiated cells with reduced pErk. Therefore, we utilised a monolayer mesoderm differentiation protocol (Nishikawa et al., 1998) to assess the direct consequences of *Erk2* deletion on commitment. Both *Erk2*$^{-/-}$ clones were viable and proliferative in these conditions. However, they showed no upregulation of brachyury expression and maintained the pluripotency markers *Rex1*, *Nanog* and *Oct4* (Fig. 4E) and failed to differentiate into Pdrfrα-positive lateral mesoderm cells (Fig. 4G).

Collectively, these findings demonstrate that the FGF-Erk1/2 pathway is crucial for ES cells to differentiate into both neural and non-neural lineages. Our data do not exclude involvement of the PI3-kinase pathway in FGF-mediated differentiation (Chen et al., 2000) [but see accompanying study (Stavridis et al., 2007)]. However, a central role for the Erk pathway is consistent with previous observations on the effect of mutation in the adaptor molecule Grb2 on differentiation in response to LIF withdrawal in the presence of serum (Cheng et al., 1998; Hamazaki et al., 2004), the suppression of neural differentiation by MEK inhibitors (Lowell et al., 2006; Ying et al., 2003b; Stavridis et al., 2007), and the requirement for Erk2 for mesoderm formation in the embryo (Yao et al., 2003). We show that FGF-Erk does not act by blocking BMP signal transduction in ES cells but is necessary to redirect the effect of BMP signalling. Only after FGF-Erk stimulation does BMP act to divert ES cells exiting self-renewal away from a neural fate. These ES cell data are consistent with the evidence from chick and *Xenopus* embryo studies that the anti-neural action of BMP is secondary to FGF action on naïve epiblast (Stern, 2005). The perspective of phased progression of pluripotent cells towards lineage specification allows ready reconciliation of the default model of neural induction in vertebrate embryos (Wilson and Hemmati-Brivanlou, 1995) with an initiating FGF signal.

The key finding in this study is that without FGF-Erk1/2 input, progression of ES cells to either neural or mesodermal lineage commitment is arrested and substantive alterations in expression of

key pluripotency markers Oct4, Nanog and Rex1 are not observed. Based on these observations, we propose that unrestrained activity of the Ras-Erk1/2 cascade is the primary stimulus for naïve ES cells to exit self-renewal and acquire competence for germ layer segregation. In self-renewing ES cell cultures, provision of LIF acts via Stat3 and intervenes downstream of pErk to override the autoinductive capacity of Fgf4. In the absence of LIF, we suggest that the FGF-Erk pathway primes cells to enter a transitional stage, analogous to egg cylinder epiblast. Cells in this competent state will proceed to neural fate in response to ongoing FGF and Notch stimulation (Lowell et al., 2006), but are highly susceptible to redirection by other inductive cues such as TGFβ superfamily members.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/16/2895/DC1

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