RESEARCH ARTICLE

An interplay between extracellular signalling and the dynamics of the exit from pluripotency drives cell fate decisions in mouse ES cells

David A. Turner¹,*, Jamie Trott¹,², Penelope Hayward¹,*, Pau Rué¹ and Alfonso Martinez Arias¹,‡

ABSTRACT

Embryonic Stem cells derived from the epiblast tissue of the mammalian blastocyst retain the capability to differentiate into any adult cell type and are able to self-renew indefinitely under appropriate culture conditions. Despite the large amount of knowledge that we have accumulated to date about the regulation and control of self-renewal, efficient directed differentiation into specific tissues remains elusive. In this work, we have analysed in a systematic manner the interaction between the dynamics of loss of pluripotency and Activin/Nodal, BMP4 and Wnt signalling in fate assignment during the early stages of differentiation of mouse ES cells in culture. During the initial period of differentiation, cells exit from pluripotency and enter an Epi-like state. Following this transient stage, and under the influence of Activin/Nodal and BMP signalling, cells face a fate choice between differentiating into neuroectoderm and contributing to Primitive Streak fates. We find that Wnt signalling does not suppress neural development as previously thought and that it aids both fates in a context dependent manner. Our results suggest that as cells exit pluripotency they are endowed with a primary neuroectodermal fate and that the potency to become endomesodermal rises with time. We suggest that this situation translates into a “race for fates” in which the neuroectodermal fate has an advantage.

KEY WORDS: Mouse embryonic stem cell, Pluripotency, Cell differentiation, Wnt/β-catenin signalling, Activin/Nodal and BMP signalling, Cell fate decision making, Single cell analysis

INTRODUCTION

Embryonic Stem (ES) cells are clonal populations derived from early mammalian blastocysts that have the ability to self-renew as well as the capacity to differentiate into all embryonic cell types of an organism in culture and contribute to the normal development of an embryo into an organism, i.e. they are pluripotent (Bradley et al., 1984; Smith, 2001; Nichols and Smith, 2011). Controlled application of defined cocktails of signalling molecules has been used as a means to differentiate ES cells into specific tissues, e.g. cardiac, blood, pancreatic cells, lung, gut, neurons (Gadue et al., 2005; Nostro et al., 2008; Borowiak et al., 2009; Borowiak and Melton, 2009; Spence et al., 2011; Lupo et al., 2013) and, in some instances, into organ-like structures resembling the in vivo counterparts (Sasai et al., 2012). However, despite notable successes, these protocols remain tinkering exercises performed with limited understanding of the routes and mechanisms that control differentiation. Furthermore, perhaps for this reason, questions remain as to the similarities and differences between the events in ES cells and in the embryo (Gadue et al., 2006; Chen et al., 2013). Therefore, gaining insights into the mechanisms of differentiation in culture will have an impact in our ability to harness the potential of these cells.

The experimentally controlled differentiation of pluripotent cells represents a good model system to understand the general process of fate decision making in development (Gadue et al., 2005; Murry and Keller, 2008). In the embryo, the blastocyst differentiates into the epiblast where, after implantation, within a single cell layered epithelium and under the influence of spatially organised signalling centres, cells are assigned to one of two fates: 1) an anteriorly located neural primordium or neuroectoderm (NECT), that will give rise to most of the brain, the anterior nervous system and epidermis, and 2) a group of cells in the proximal posterior region that, for the most part, will contribute to a dynamic structure, the Primitive Streak (PS), which acts as a seed for the endoderm and the mesoderm (Arnold and Robertson, 2009; Rossant and Tam, 2009; Arkell and Tam, 2012). The spatiotemporal organisation of the molecular components enabling and implementing the specification of these cell types are well known (Pfister et al., 2007) and the sequence of events can be mimicked in culture (Kubo et al., 2004; Gadue et al., 2006), albeit in a spatially disorganised manner and often with low yields of cells of a particular fate. Notwithstanding these experiments, the details of the interactions between signalling and transcriptional networks mediating this decision remain poorly understood.

A widely held view of the differentiation of mammalian ES cells entertains that, as cells exit pluripotency, they choose between fates that resemble NECT and PS from a naïve state under the influence of their local signalling environment that enforces a rearrangement of the pluripotency network (Loh and Lim, 2011; Thomson et al., 2011). In this process, Retinoic Acid (RA) is deemed to promote NECT whereas Wnt/β-Catenin signalling might bias the decision towards the PS-like fate (Thomson et al., 2011). However, NECT can emerge in the absence of RA (Watanabe et al., 2005; Wataya et al., 2008) and Wnt/β-Catenin signalling has been reported to be required for the differentiation of neural precursors (Otero et al., 2004; Slawny
and O’Shea, 2011). Therefore these observations raise questions on the prevalent model and led us to analyse in detail the influence of external signals on the early stages of differentiation of ES cells into either NECT-like or endomesoderm fated PS-like (which also include a neuronesodermal precursor (Tasikiridis et al., 2014)); from hereon referred to simply as NECT and PS.

Our observations support suggestions that ES cells have an intrinsic competence towards the NECT fate (Tropepe et al., 2001; Smukler et al., 2006; Watabu et al., 2008) and show that, after two days of undirected (neutral) differentiation, they enter a transient period of competence to become mesendoderm. Competence towards this fate emerges progressively and requires the downregulation of the pluripotency factor Nanog. We find no evidence to support the widespread notion that Wnt/β-Catenin signalling suppresses NECT (Aubert et al., 2002; Watanabe et al., 2005; Smith et al., 2008; Patani et al., 2009; Bhargava et al., 2013; Lupo et al., 2013) and instead find that, within the period of competence, it potentiates NECT and mesendoderm in a context-dependent manner. In the presence of high levels of Activin/Nodal and BMP, Wnt/β-Catenin signalling promotes mesendoderm whereas in their absence it produces anterior neurons.

**MATERIALS AND METHODS**

**Routine cell culture and differentiation**

E14Tg2A, Bra::GFP (Fehling et al., 2003), TNGA (Chambers et al., 2007) and Sox1::GFP (Ying et al., 2003b) mESCs were cultured on gelatin (0.1%) in GMEM (Gibco, UK) supplemented with non-essential amino acids, sodium pyruvate, GlutaMAX™, β-mercaptoethanol, foetal bovine serum and LIF (SL). To obtain serum-free pluripotency conditions, cells were cultured in 2i + LIF; 2i uses an N2B27 base medium (NDiff 227, StemCells Inc., UK) supplemented with 1 mM CaCl₂ adjusted to pH 6.96 with 1 M HCl) and fixed for 15 minutes in 4% paraformaldehyde. Samples were washed and permeabilised with BBT (BBS + CaCl₂ supplemented with 0.5% BSA and 0.5% Triton X-100) before overnight antibody staining, following which the samples were washed with BBT and incubated for 2 h with the desired fluorescently-conjugated secondary antibody. Prior to imaging, samples were washed with BBS + CaCl₂ and covered in mounting medium (80% spectrophotometric grade glycero, 4% v/v n-propyl-gallatine in BBS + CaCl₂). The primary antibodies used were as follows (all at a 1 in 200 dilution): Brachyury (goat; Santa Cruz Biotechnologies, sc17743), Oct3/4 (mouse; Santa Cruz Biotechnologies, sc5279) and Sox2 (rabbit; Millipore, AB5603). Secondary antibodies were from Molecular Probes and used in a 1 in 500 dilution with Hoechst (1 in 1000; Invitrogen). Samples were imaged using an LSM700 on a Zeiss Axiovert 200 M with a 40× EC Plan-Neofluar 1.3 NA DIC oil-immersion objective. Hoechst, Alexa488, -568 and -633 were sequentially excited with a 405, 488, 555 and 639 nm diode lasers, respectively. Data capture carried out using Zen2010 v6 (Zeiss), image analysis performed using Fiji (Schindelin et al., 2012).

**Flow cytometry and cell sorting**

Cells were analysed for GFP fluorescence using an LSR Fortessa (BD Bioscience) equipped with an 80 mW Argon laser to excite GFP at 488 nm and a 200 mW Helium-Neon laser (633 nm) used to excite fluorescent proteins. Emitted light was recorded using an MRRn AxioCam (Zeiss) and data recorded with Axiovision (2010) release 4.8.2. Analysis performed using Fiji (Schindelin et al., 2012).

**Wide-field epifluorescence microscopy**

For live imaging, cells were imaged by wide-field microscopy in a humidified CO₂ incubator (37°C, 5% CO₂) every 10 min for the required duration using a 20× LD Plan-Neofluar 0.4 NA Ph2 objective with correction collar adjusted for imaging through plastic. All media were changed daily (see above). An LED, white-light system (Lumencor) was used to excite fluorescent proteins. Emitted light was recorded using an MRm AxioCam (Zeiss) and data recorded with Axiovision (2010) release 4.8.2. Analysis performed using Fiji (Schindelin et al., 2012).

**Single cell qRT-PCR**

E14Tg2A wild-type (129/Ola) mES cells were cultured in NDiff N2B27 (StemCells Inc) supplemented with 100 U/ml LIF and 10 ng/ml BMP4 (Department of Biochemistry, University of Cambridge). Subsequently, cells were trypsinised and resuspended in N2B27 alone at a density of 10×10⁶ cells/cm² to induce differentiation. FACS was used to distribute individual mES cells into the wells of a 96-well plate containing lysis buffer (see below). The transcriptomes of isolated cells were amplified according to a previously published protocol (up to step 30) (Tang et al., 2010). The resultant cDNA samples were purified using a PCR purification kit (Qiagen) and diluted 1 in 10 in PCR-grade water.

Diluted cDNA (5 µl) was mixed with 9 µl Specific Target Amplification mix (STA mix – 7.5 µl TaqMan Pre-Amp mastermix (Invitrogen), 1.5 µl 10× STA Primer Mix, 0.075 µl 0.5 M EDTA, pH 8.0). STA Primer Mix is composed of 48 or 96 primer pairs (supplementary material Figs S1–S5) diluted in DNA suspension buffer (Teknova) at a concentration of 500 nM per primer. Individual amplicons were further amplified using the following thermal cycling protocol: 10 min at 95°C, 20 cycles of 5 s at 96°C followed by 4 min at 60°C, 4 min at 60°C. Samples were exonuclease treated and the levels of individual amplicons determined using 48×48 or 96×96 Dynamic Arrays on the BioMark HD platform (Fluidigm) as per the manufacturer’s instructions (using the EvaGreen based protocol, and the STA primer mix described above). Ct values were extracted automatically using the Biomark Data Collection Software. Melt curves arising from primer-dimer amplification were identified and removed from the dataset manually by comparison with a positive control sample.

**qRT-PCR**

RNA was isolated from ~5×10⁶ trypsinised and pelleted mES cells using the RNase Mini kit (Qiagen, 74104) according to the manufacturer’s instructions, and resuspended in 30 µl distilled water. RNA was reverse transcribed as follows. RNA samples (1 µg in 38 µl nuclease free water) were combined with 2 µl Oligo-dT anchored primers (Life Technologies, 12577-011) and incubated at 80°C for 2 minutes before transferring immediately to ice for 2 minutes. PCR master mix was then added to each sample: 1.5 µl dNTPs (Life Technologies, 18080-400), 12.5 µl 5× First Strand buffer (Life Technologies, 18080-400), 3 µl 0.1 M DTT (Life Technologies, D-1532), 1.5 µl RNaseOUT (Life Technologies, 12577-011) and 2 µl Superscript III Reverse Transcriptase (Life Technologies, 18080-400). Thermal cycling was carried out as follows: 25°C 10 minutes, 50°C 30 minutes, 70°C 15 minutes. To remove RNA from the resultant cDNA samples 1 µl RNaseH (Life Technologies, 10777-019) and 2 µl RNaseOut (Life Technologies, 12577-011) and incubated at 80°C for 2 minutes before transferring immediately to ice for 2 minutes. PCR master mix was then added to each sample: 1.5 µl dNTPs (Life Technologies, 18427-013), 12.5 µl 5× First Strand buffer (Life Technologies, 18080-400), 3 µl 0.1 M DTT (Life Technologies, D-1532), 1.5 µl RNaseOUT (Life Technologies, 10777-019) and 2 µl Superscript III Reverse Transcriptase (Life Technologies, 18080-400). Thermal cycling was carried out as follows: 25°C 10 minutes, 50°C 30 minutes, 70°C 15 minutes. To remove RNA from the resultant cDNA samples 1 µl RNaseH (Life Technologies,
Mathematical modelling of cell-fate adoption dynamics

The model considers 4 different cellular types: pluripotent cells (P), differentiating cells (D), cells committed to NECT (N) and cells committed to PS (M). In the model, we consider initially all cells to be pluripotent. Cells then are allowed to differentiate at a certain rate $\lambda_D$. Once each cell abandons the pluripotent state, it rapidly and irreversibly adopts a final fate: either NECT or PS. Cells are assumed to acquire either fate at a particular rate: $\lambda_N$ for NECT and $\lambda_M$ for PS. In addition to these cellular types and the corresponding transitions, we also consider a signal (s) that builds up as cells lose pluripotency. Once the signal crosses a certain threshold, it biases the rates of fate adoption. In particular, we consider that the rate of PS conversion is negligible in the absence of signal ($\lambda_M<<\lambda_N$) and becomes the much larger ($\lambda_M>>\lambda_N$) once the signal reaches the threshold. In this simple model we disregard both cellular death and cell division, assuming that both processes are effectively balanced within all cellular fates. The model equations are described in supplementary material Fig. S5A. These define a simple linear ODE system with a piecewise constant rate $\lambda_M$ (which depends on the levels of accumulated signal). Thus a solution can be built up by gluing the analytical solutions of each range of constant $\lambda_M$ (supplementary material Fig. S5B). Two of the three model parameters, $\lambda_D$ and $\lambda_S$, have been fitted to the experimental data of N2B27 differentiation by assuming that no differentiation to PS occurs, i.e. $\lambda_M=0$ (supplementary material Fig. S5C,D). With the obtained parameter values, and assuming a switch in the dynamic regime ($\lambda_M>>\lambda_N$) in the presence of Activin and Chiron, the model reproduces to a great extent the experimental pulse-chase results (cf. Fig. 1D and supplementary material Fig. S5E).

RESULTS

The exit from pluripotency establishes competence to differentiate

We have monitored the NECT/PS fate choice in differentiating ES cell populations using a Sox1::GFP reporter line for NECT, and a Brachyury::GFP (T::GFP) line for PS. The expression of both is negligible in self renewing conditions (Serum + LIF (SL), LIF + BMP (data not shown) or 2i + LIF) but can be detected when cells differentiate (Fehling et al., 2003; Ying et al., 2003b; Abranches et al., 2009; Hansson et al., 2009; Engberg et al., 2010) (Fig. 1A). Low density growth in N2B27, which favours neural fates, elicits Sox1::GFP expression in about 70-90% of the cells, while growth in Activin and CHIR99021 (Chi, a GSK3 inhibitor that acts as an agonist of Wnt signalling; AC) activates T::GFP in about 30-50%, of cells (Fig. 1A).

The relatively low yield of cells expressing T::GFP in PS differentiation conditions was surprising. The differentiated population contains a mixture of T::GFP-positive and negative cells interspersed with some that express Nanog (data not shown), Oct4 and Sox2 (Fig. 1B), indicating that some cells did not exit pluripotency and suggesting an explanation for the low percentage of T::GFP expressing cells. In self-renewing conditions ES cells exhibit a variegated differentiation potential with low Nanog expressing cells primed for differentiation (Chambers et al., 2007; Kalmär et al., 2009). Thus, it might be that only these cells can respond to AC. To test this we used TNGA cells, a Nanog::GFP reporter line that allows the identification of low Nanog expressing cells by their levels of GFP (Chambers et al., 2007). Sorting cells with high and low Nanog::GFP expression and exposing them to AC revealed that only the low Nanog population is capable to develop T::GFP expression effectively (Fig. 1C), i.e. lowering of Nanog might be a prerequisite for PS differentiation. In agreement with this, the percentage of T::GFP cells that respond to AC increases with the time of exposure to N2B27, reaching a peak of more than 80% after two days when all cells in the population have low levels of Nanog (Fig. 1D,E). The rise in the percentage of T::GFP positive cells is, in addition, highly correlated with a decrease of Sox1::GFP positive cells (correlation coefficient of $-0.97$, Fig. 1D). Treating cells to a short pulse of AC (1 day) after increasing durations of N2B27 exposure revealed that cells are most sensitive to PS-inducing stimuli after two days (Fig. 1F); as this duration was extended, cells began to favour a NECT fate (Fig. 1E).

To test if there was a similar change in the competence for NECT over the first two days of induced differentiation, we made use of the ability of BMP signalling to inhibit this event (Finley et al., 1999; Di-Gregorio et al., 2007; Zhang et al., 2010; Surmaz et al., 2012; Bertacchi et al., 2013). Treatment of ES cells with 1 ng/ml BMP4 for different periods of time (Fig. 1F) confirmed previous reports: treatment during the first day of differentiation vastly reduced Sox1::GFP expression, whilst exposure for longer periods, and even for the second day of differentiation only, eliminated Sox1::GFP expression (Fig. 1F). However, this effect might not be related to an inhibition of the NECT fate, but rather to an enhancement of pluripotency (Zhang et al., 2010; Malaguti et al., 2013). Consistent with this we observe that when TNGA cells are exposed to 1 ng/ml BMP4 and filmed over time, they retain Nanog::GFP expression for about two days before abruptly differentiating (Fig. 1G,H); this is confirmed in large population studies using flow cytometry (Fig. 1F and see below).

Altogether these results suggest that at the exit from pluripotency, ES cells respond differently to NECT and PS differentiation signals. Our results agree with previous observations that commitment to NECT, as reflected in Sox1::GFP expression, does not appear to require specific inputs other than those produced by the cells (Ying et al., 2002; Watanabe et al., 2005; Wataya et al., 2008) and may be latent in self renewal conditions (Tropepe et al., 2001; Smukler et al., 2006). On the other hand we notice that the ability to respond to AC emerges as cells shut down the pluripotency network. In both cases, commitment to a particular fate requires a downregulation of Nanog and we observe that only after 2 days of undirected differentiation is the population, as a whole, competent to respond to AC (Fig. 1D,E,G).

A balance of Wnt, Nodal/Activin and BMP signalling controls fate decisions at the population level during the exit from pluripotency

There is evidence that, in addition to BMP, Activin/Nodal and Wnt/β-Catenin signalling can suppress NECT specification during ES cell differentiation (Aubert et al., 2002; Watanabe et al., 2005; Smith et al., 2008; Patani et al., 2009; Bhargava et al., 2013; Lupo et al., 2013). In agreement with this, treatment of mES cells induced to differentiate in N2B27 for 5 days with Activin, BMP4, or the GSK3 inhibitor CHIR99021 (Chi) to induce Wnt/β-Catenin signalling, results in inhibition of Sox1::GFP expression (Fig. 2A). Whereas inhibition of Activin activity with SB431542 (SB43), BMP activity with DMH1 or β-Catenin activity with the Tankyrase inhibitor XAV939 resulted in enhanced Sox1::GFP expression (Fig. 2A). To test the impact of each of these signals and their interactions on the NECT/PS decision during the exit from pluripotency, we treated ES cells with...
with agonists and antagonists of each pathway for the first two days of differentiation, then transferred them to either N2B27 (3 days) or AC (2 days) and recorded Sox1::GFP or T::GFP expression (Fig. 2).

Activation of Wnt/β-Catenin signalling with Chi, either for the first two days or for the period of differentiation, resulted in a suppression of Sox1::GFP expression (Fig. 2A,C), whereas inhibition of β-Catenin activity with XAV939, results in some apoptosis, but the remaining cells exhibit a robust activation of Sox1::GFP (Fig. 2A,C). The effects of Wnt/β-Catenin signalling on T::GFP expression depend on the period and timing of exposure. Even though β-Catenin is necessary for T::GFP expression, treatment with Chi for the first two days before the exposure to PS-promoting signals (AC), reduces, rather than enhances the number of T::GFP expressing cells (Fig. 2D).

Furthermore, exposure to XAV939 during the exit from pluripotency enhances the response to AC. The effects of Activin signalling followed a similar pattern: exposure to Activin during the first two days of differentiation inhibited Sox1::GFP expression (Fig. 2C) and, to a lesser extent, T::GFP expression (Fig. 2D). On the other hand, inhibition of Activin signalling during this period with SB43, led to an increase in Sox1::GFP and to a slight but detectable reduction of T::GFP expression by exposure to AC (Fig. 2D). In all cases the effects were less pronounced the shorter the exposure of the cells to the signal modulators (supplementary material Fig. S1). Tests of the interplay between the two pathways on Sox1::GFP expression (supplementary material Fig. S1) indicate a predominant role of
an inhibitory function of these signals, with the effects of Activin and Chi being dominant over the loss of function of either pathway. We noticed that, in contrast to the effects of BMP, one day exposure to either Activin or Chi was not sufficient to suppress Sox1::GFP expression (supplementary material Fig. S1). Altogether these results suggest that, in agreement with published reports, both Wnt/β-Catenin and Activin/Nodal signalling can suppress NECT fate during the first two days of differentiation. However, as β-Catenin signalling has been shown to promote pluripotency (Doble et al., 2007; Kelly et al., 2011; Lyashenko et al., 2011; Wray et al., 2011; Yi et al., 2011; Faunes et al., 2013), there is a possibility that, as in the case of BMP, its effects on Sox1::GFP expression reflect a function in the maintenance of pluripotency rather than on neural differentiation. Consistent with this flow cytometric analysis and live imaging of ES cells (Fig. 2, Fig. 3A–D) show that the effects of Wnt/β-Catenin signalling on the early stages of differentiation are a consequence of its effects on the stability of pluripotency: activation of β-Catenin maintains Nanog::GFP (TNGA cells) and REX1::GFP reporters, whilst inhibition with XAV939 promotes their downregulation (Fig. 2, Fig. 3A–D).

The notion that the impact of β-Catenin on the early stages of differentiation reflects its activity in promoting pluripotency is confirmed by its effects on PS differentiation. Although Wnt/β-Catenin signalling is required for PS fate, treatment of ES cells with Chi before exposing them to AC decreases rather than increases, the proportion of cells that develop as PS (Fig. 2D). This surprising observation can only be understood in terms of the effect that β-Catenin has in promoting pluripotency, which will thus reduce the possibility to commit to any differentiation route.

Whereas exposure to Activin or SB43 during the exit from pluripotency does not alter the dynamics of the Nanog::GFP or Rex1::GFP reporters with respect to the N2B27 control (Fig. 3A–D), exposure to Activin suppresses Sox1::GFP and, to a lesser degree, T::GFP expression (Fig. 2C,D). The effect on NECT cannot be explained in terms of effects on pluripotency since Activin/Nodal promotes exit from pluripotency (Galvin-Burgess et al., 2013) (Fig. 3) and therefore must reflect an active suppression of the NECT fate; the effects of SB43 on Sox1::GFP confirm this possibility (Fig. 2A,C). On the other hand, the reduction of T::GFP expression upon exposure to Activin (Fig. 2D) is surprising and might reflect a delayed differentiation associated with the requirement for Activin/Nodal in the maintenance of the EpiSC state (Vallier et al., 2009; Galvin-Burgess et al., 2013) i.e. the exposure to Activin induces a slow transition through an Epiblast-like state and a delay in cell fate decisions through a pause in an Epi-like state.

The impact of signals on gene expression
To better characterise the impact of the signalling pathways on the exit from pluripotency and the initial stages of differentiation, we monitored the expression of genes associated with pluripotency (E-Cadherin, Nanog, Rex1, Klf4), the exit from pluripotency (Tcf15), the epiblast (Fgf5), neural (N-Cadherin, Sox1, Sox2, Sox3 and Zfp521) or PS differentiation (T/Bra, Snail, Mixl1) under different conditions. Cells were kept for 2 days in the different conditions and cultured for a further day in N2B27 before their state being assessed (Fig. 3E). The results provide some insights into the action of the different signals. For example they confirm that the effects of β-Catenin on the commitment to
Fig. 3. See next page for legend.
different fates are due to its effects on pluripotency: Chi, which increases β-Catenin, maintains the levels of pluripotency markers with little effect on differentiation, whereas XAV939, which reduces β-Catenin, promotes differentiation, with a bias towards NECT (Fig. 3E) in the absence of additional signals. The effect of BMP is more subtle and gene expression confirms that it delays, rather than suppresses, differentiation; in this experiment cells seem to be in an Epi-like state but the high levels of expression of T/Bra, Snail and Mixl1 in these cells suggest that they have a strong bias towards PS (see also Malaguti et al., 2013). The profile also confirms the effects of Nodal/Activin on NECT differentiation but also provides some support for their effect in delaying differentiation through a maintenance of an Epi-like state as reflected in the high levels of Fgf5 and the mixture of neural and PS gene expression within the population.

Altogether these observations show that, as cells exit pluripotency, they integrate several signals in a time dependent manner. They also emphasise the need to take into account the dynamics of the pluripotency network in cell populations when considering the impact of different signals in the process of differentiation. Cells will respond to individual signals depending on the state they are in. In an ES culture maintained in Serum and LIF, there is a mixture of cells with varied differentiation potential. Cells with high levels of Nanog are pluripotent and both BMP and β-Catenin signalling will enhance this state, while Activin/Nodal and FGF/ERK signalling will contribute to loss of Nanog expression and thereby promote differentiation (Kunath et al., 2007; Stavridis et al., 2007; Galvin et al., 2010; Stavridis et al., 2010; Galvin-Burgress et al., 2013). On the other hand, the low Nanog expressing population is an heterogeneous mixture of cells in different states: some primed to return to the high Nanog state, some in an epiblast (or Epi Stem Cell)-like state and some differentiating or even already differentiated. This population with low levels of Nanog exhibits higher levels of Wnt/β-Catenin transcriptional activity (Faunes et al., 2013) and its response to signals will depend on the particular state of the cell. For those primed to differentiate, the combined activity of Wnt, Activin, BMP and FGF will favour differentiation and, specifically, differentiation into PS states. Our experiments also support the notion that NECT might not need specific external signals whose effect is, for the most part, to induce a PS state (Fig. 2, Fig. 3E; supplementary material Fig. S1).

A fate restriction/commitment point at the exit from pluripotency
Our results support the contention that BMP delays differentiation (Zhang et al., 2010; Malaguti et al., 2013) and show that this effect is dominant over pro-differentiation signals as BMP suppresses the effects of SB43 and XAV939 on the exit from pluripotency (supplementary material Fig. S1). However, BMP does not abolish differentiation. Monitoring persistent exposure of ES cells to BMP reveals an abrupt loss of Nanog expression at day 3 (Fig. 3A,C), and the emergence of T::GFP expression (Fig. 2B). Furthermore, BMP boosts the effects of Activin and Chi on the expression of T::GFP, in particular in the context of differentiation into PS derivatives (Gouon-Evans et al., 2006; Nostro et al., 2008; Hansson et al., 2009; Irion et al., 2010). This effect is more obvious when BMP is applied after two days of differentiation and results in a total suppression of Sox1::GFP expression without reverting the cells to a pluripotent state (Fig. 5; supplementary material Fig. S4). Taken together these observations highlight a change in the responsiveness of the differentiating population of ES cells after 2/3 days of differentiation in culture. The most significant change is the emergence of a competence to respond to PS-promoting signals, which emerges at this time and lasts for two days (Fig. 1D,E).

A number of studies show that at day 3 of differentiation, ES cells go through a state similar to that of Epiblast stem cells, which can be mapped onto the post-implantation epiblast E5.0–E6.0 (Zhang et al., 2010). It has been further suggested that at this moment cells are primed for both NECT and PS and that they choose between the two fates (Sterneckert et al., 2010; Bernemann et al., 2011; Thomson et al., 2011). This is supported by our observation that at this point cells lose the ability to return to the naïve state (not shown) and can be seen most clearly with live-cell imaging and the FACS profiles where we observe morphological and dynamical changes in the cells around this time (Fig. 3A–D).

Gene expression during exit from pluripotency
To understand the molecular basis of fate choice during mouse ES cell differentiation, we monitored the expression of a collection of genes associated with pluripotency and specific lineages as well as elements of various signalling pathways in ES cells as they exit pluripotency. In a first experiment, cells in LIF and BMP were allowed to differentiate in N2B27 for five days and gene expression was monitored both in the population (Fig. 4A–C) and at the level of single cells (Fig. 4D,E). Initial and strong changes in expression are largely restricted to pluripotency-related genes but lineage affiliated gene expression emerges as cells begin to differentiate (Fig. 4A); in addition, we can see that at days 2 and 3, the number of genes expressed is maximal (data not shown). A survey of specific genes confirms that the exit from pluripotency is associated with a downregulation of genes associated with the pluripotency network (Nanog, Oct4, Sox2, Klf4, Esrrb) and that at about day 3 the cells go through an Epi-like state as characterised by the expression of Fgf5 and a transient rise in Nanog expression (Fig. 4A). In terms of differentiation, whilst genes associated with NECT (e.g. Sox1, Sox2, Sox3, Pax6, Pax7, Otx2) are expressed as the cells begin to differentiate and continue to be expressed during the differentiation period, genes associated with PS (e.g. Foxa2, Gsc, T/Bra, Eomes, Mixl1, Snail, Tbx6, Wnt3) emerge around day 3 (Fig. 4A). This study also confirms the rise of Wnt/β-Catenin transcriptional activity described before (Faunes et al., 2013) in the expression of direct targets, e.g. Dkk1 and Axin2, which rise during day 3 (Fig. 4A).

Principal Component Analysis (PCA) of the expression profiles of 34 genes during the exit from pluripotency in
Fig. 4. Dynamics of gene expression during the exit from pluripotency. Mouse ES cells cultured in LIF and BMP were transferred to N2B27 and allowed to differentiate for 5 days. (A) Average (bulk) expression levels of several genes associated with pluripotency, NECT and PS fate restriction, as well as targets for Wnt/Notch, Nodal/BMP and FGF/RAR signalling, were measured daily using fluidigm quantitative RT-PCR. Colour-coded values indicate the log-fold change values with respect to the initial LIF + BMP condition expression levels measured by RT-PCR and normalised to a housekeeping gene. (B) Principal Component Analysis of the bulk expression profiles of 34 genes for 2i, LIF and BMP and the 5 days of differentiation in N2B27 normalised to Gapdh. Each single point indicates a technical repeat. (C) Loadings plot of the PCA indicating the genes that contribute the most to the first two components. (D,E) Expression levels of the 29 most relevant genes of 90 analysed using fluidigm technology and single-cell RT-PCR over the 5-day differentiation experiment (see main text and supplementary material Fig. S3 for details). (D) Heat maps of single-cell log2-expression levels normalised to Gapdh (each row represents an individual cell) for the most significant genes. Genes (in columns) were clustered according to the pairwise similarities in their expression levels. Additional genes of interest are also displayed in the right-most panels. Data for 2i and LIF conditions are also shown on the panel on top. (E) Violin plots of the data in panel D showing kernel density estimates of the mRNA distribution (pale red and blue areas) together with single cell expression levels (black dots), the population average (blue line) and median (red dot).
undirected differentiation conditions (Fig. 4B,C; supplementary material Fig. S3A–F) provides additional insights into the process. The first component (component of maximum variance, PC1 in Fig. 4B) clearly delineates a differentiation axis, with all differentiation stages clustered in chronological order from left to right. This first component accounts for more than 60% of the total variance (supplementary material Fig. S3B) and is mainly defined by genes of the pluripotency network, such as Rex1, Dax1, Klf4 and Tbx3 (Fig. 4C; supplementary material Fig. S3C), with a contribution of others, like Fgf5, associated with the transient Epi-like state. The second component, PC2, which can be interpreted as the main axis of change in gene expression once the effect of loss of pluripotency genes (PC1) has been removed, amounts to about 15% of the total variance. Interestingly, this component highlights an inflection point on day 3 of differentiation (Fig. 4B; this discontinuity is even more evident when 2i conditions are not considered for the analysis, see supplementary material Fig. S3F). Genes strongly contributing PC2 include some that are highly expressed during the first two days: Otx2 and Foxd3; as well as genes that are very lowly expressed in 2i, all of which appear to be lineage affiliated (Fig. 4C; supplementary material Fig. S3C).

The PCA analysis highlights that the loss of pluripotency gene expression is a major driver of the differentiation process. This is confirmed by the analysis of gene expression in single cells with the core elements of the pluripotency network (Essrb, Oct4, Dax1, Klf4, Sox2, Nanog, and Rex1), which are amidst the top 15 genes driving the change (Fig. 4D,E; supplementary material Fig. S3G). An interesting observation in this list is Mbd3, a core member of the NurD complex, whose expression decreases during development and which our analysis suggests it is a driver of the process. Another interesting one is P-Cadherin, which has been highlighted before (Faunes et al., 2013). As differentiation progresses, single cells start to favour the high expression of genes associated with the NECT (Fig. 4D; supplementary material Fig. S3G).

Signals and a decision point for commitment and differentiation
After three days in N2B27, cells are unable to return to the naive state (supplementary material Fig. S2C) and we presume that they will differentiate according to their local signalling environment. We have explored this by exposing cultures to different signals and signal combinations from day 2 of differentiation (Fig. 5). As expected, exposure to Activin or BMP promotes T::GFP expression and a PS state (Fig. 5A) while suppression of Activin signalling (with SB43) or BMP (with DMH1) promotes Sox1::GFP expression and the NECT fate (Fig. 5B). Surprisingly, we find that exposure to Chi from this time promotes both Sox1::GFP and T::GFP expression in a context dependent manner: it both enhances the effects of Activin on the PS state and the effect of SB43 on the NECT (Fig. 5C). Altogether these results can be interpreted in the context of a competence for NECT being present from the initial stages of differentiation.
Both fates in the context of the decision. BMP and BMP signalling promote PS and inhibit NECT whereas Wnt/β-Catenin maintains cells pluripotent but this activity is not stable. Over time, cells exit pluripotency and when they do, in the presence of high levels of β-Catenin they adopt the PS state. With their committed to differentiation on which BMP suppresses the neural fate, Wnt/β-Catenin signalling promotes both NECT and PS states in a signalling context dependent manner. While its effect on PS is in agreement with well-established observations (Gadue et al., 2006; Nostro et al., 2008; Turner et al., 2013), the effect on NECT development is, at first sight, surprising. However, there is evidence that Wnt/β-Catenin signalling promotes the differentiation of neural precursors (Otero et al., 2004; Slawny and O’Shea, 2011) and some of our observations are likely to reflect this activity.

Our results suggest that the effects of β-Catenin depend on the signalling context it is embedded in. For example, in the presence of high levels of β-Catenin, it is the levels of Activin that determine the fate of the cells: high levels, as provided experimentally, promote endoderm, whereas lower levels promote the development of mesoderm. It is also possible that even within the anterior neural fate, which develops in the absence of Activin/Nodal signalling, the levels of β-Catenin might determine fates. In this context, it is of interest that an effect of β-Catenin (Chi) on NECT differentiation can be observed in figure 7G of Thomson et al. but is ignored by the authors (Thomson et al., 2011). The lack of lineage specificity in the effects of Wnt/β-Catenin signalling contrasts with those of Nodal/Activin and BMP, which promote PS and suppress NECT and are consistent with a proposed role of β-Catenin affecting the probability with which a cell adopts a fate rather than the implementation of such fate (Martinez Arias and Hayward, 2006; Muñoz-Descalzo and Martinez Arias, 2012).

Our results support the notion that after two days of differentiation, ES cells enter a state in which they commit to particular fates (Thomson et al., 2011). This state is closely related to that of cells in the E5.5 epiblast and is reflected in the expression of Fgf5, the lowering of E-Cadherin, a transient rise in Nanog and the co-expression of genes associated with PS and NECT (Zhang et al., 2010; Faunes et al., 2013). A prevalent view of this state of commitment is that cells make choices from a naïve state (Thomson et al., 2011); however, our results are consistent with a different view in which ES cells have a primary NECT fate that can be overridden for a short period of time by pro-PS signals. The notion of a “primary” or “default” NECT fate in ES cells has been suggested before (Tropepe et al., 2001; Smukler et al., 2006) and is supported by the observation that in the absence any external signals ES cells will adopt a neural fate (Ying et al., 2002; Watanabe et al., 2005; Wataya et al., 2008), as well as by our observation of the prevalence of NECT gene expression in self-renewal and, particularly, during the early stages of differentiation. On the other hand, the competence to become PS emerges over time with a peak at days 3 and 4, and is associated with a decrease in Nanog expression, the loss of Oct4 expression and the emergence of β-Catenin transcriptional activity. These events can be observed in neural differentiation conditions e.g. N2B27, where in the course of the time of the experiment, the levels of Activin/Nodal, BMP and Wnt can be seen to rise as reflected in the expression of PS genes e.g. T/Bra, Mix1, Eomes (Nordin et al., 2008; Engberg et al., 2010; this paper). These signals are likely to be diluted in the culture but can be effective in small patches and we can observe multiple fates arising over four or five days differentiation in N2B27 (Faunes et al., 2013; this paper). However, the large dilution effects of the culture are likely to prevent significant long term differentiation of PS derivatives in these conditions and long term culture in N2B27 selects against PS cells. Conversely, addition of agonists of Activin/Nodal, BMP and Wnt selects for PS. Our results show that there is a window of opportunity to become PS and that if the cells do not adopt the PS fate in this period, they will become NECT.

Taken together our results lead us to surmise that the fate choice that cells face in culture is of the kind “IF NOT X then Y”
i.e. “IF NOT PS then NECT”. Many of the pro-NECT effects of suppressing Wnt/β-Catenin or BMP signalling could be interpreted in this light: an early exit from pluripotency might give an advantage to commit to NECT before a build up of PS promoting signals. Consistent with this, filming of the exit from pluripotency and fate assignment shows that the cells that lower Nanog early have a higher probability to become neural (A.M.A. lab, unpublished).

**Fate choices at the exit from pluripotency: a framework**

In the context of published work, our results lead us to suggest a sequence of events and causal connections for the manner in which signals guide fate decisions at the exit from pluripotency in culture (Fig. 6A). To do this we integrate our results with a number of published facts:

1. A pluripotent population in standard self renewing conditions (Serum and LIF, BMP and LIF) is a dynamic mixture of three subpopulations: cells in the ground state, Epi stem cells and cells in transition between these two states; it also includes some differentiated cells (Chambers et al., 2007; Kalmar et al., 2009; Luo et al., 2013).

2. The state of a cell in an ES cell population is determined by the levels of Nanog and β-Catenin, which, in turn, determine the levels of free Oct4. Differentiation is determined by the Oct4:Nanog ratio: a high ratio it will promote differentiation (Muñoz Descalzo et al., 2012; Karwacki-Neisius et al., 2013; Muñoz Descalzo et al., 2013; Radziszewska et al., 2013).

3. The elements of the pluripotency network prime particular fates, with Oct4 priming all differentiation but then, together with low levels of Nanog, promoting PS and Sox2 promoting NECT (Loh and Lim, 2011; Thomson et al., 2011).

4. Differentiation is associated with Wnt/β-Catenin signalling (Faunes et al., 2013).

5. In N2B27 differentiation is driven by local signalling interactions between cells, which can be overridden by external signals in experimental conditions.

In self renewal conditions, a metastable balance of the activity of signal transduction networks maintains a ratio of Oct4:Nanog that fosters the activity of the pluripotency network and suppresses a high frequency of differentiation (Kalmar et al., 2009; Martínez Arias and Brickman, 2011; Muñoz Descalzo et al., 2013). In this situation, BMP and Wnt/β-Catenin signalling in the context of low FGF/ERK signalling and high levels of E-Cadherin, play a pivotal role in this balance (Ying et al., 2003a; Ying et al., 2008; Wray et al., 2011; Faunes et al., 2013; Malaguti et al., 2013) and maintain the pluripotency network in a homeostatic steadiness. Triggering differentiation artificially and neutrally e.g. by placing the cells in N2B27, leads to a shift in the balance of signals and the Oct4:Nanog ratio leading to an activation of FGF/ERK and β-Catenin transcriptional activities (Kunath et al., 2007; Stavridis et al., 2007; Faunes et al., 2013). This launches differentiation in the context of an intrinsic/primary NECT programme, which firms up as the pluripotency network disassembles. During this period expression of BMP, Nodal and Wnt rises and we surmise that in N2B27 the levels of these signals increase in the medium and condition cells to the PS state. However, it is clear that these signals are not sufficient to promote PS differentiation and that cells only respond effectively to them when they have disassembled the pluripotency network. At the moment we do not understand what this means in molecular terms i.e. what the molecular events are that link the disassembly of the pluripotency network and the commitment to particular fate, but our observations point out this correlation.

One can view the process as a competition or race between two programmes that lead to mutually excluding fates (NECT/PS). The later can be regarded as implementing two different strategies of fate adoption that compete to become dominant. The PS programme can suppress the NECT programme but not the other way around. However, the suppression can only take place while the cells are deciding and not passed their commitment time. Furthermore, the competence to PS is not
built into the system initially but arises during differentiation. With this in mind we have created a simple mathematical model that accounts for our observations (Fig. 6C,D; supplementary material Fig. S5); and see Materials and Methods. In the model the two competing cellular fates adopt different dynamic strategies and shows that there is a lag period of a few days in which the appropriate signalling conditions for PS, namely Nodal/Activin, have to build up population-wise in order to allow cells to become PS. Once this second fate is made available to cells, it becomes the predominant fate (i.e. cells are more prone to become PS than NECT). However, the delay introduced by the signalling mechanism has already allowed the first cells leaving pluripotency to irreversibly commit to the NECT fate. Hence, the interplay between the dynamics of pluripotency exit and the signalling mechanisms allow for a balance between the two opposing fates (Fig. 6C,D).

This situation that we have described in culture is reminiscent of, and provides insights into, the early differentiation in the embryo. The main difference is that in the embryo signalling centres are spatially organised, which determines the pattern. It is likely that here there is also a “primary” neural fate (Camus et al., 2006; Di-Gregorio et al., 2007; Levine and Brivanlou, 2007) and that PS-promoting signals override this fate. Anterior NECT fates are maintained by the antagonism of PS promoting signals by the secretion of anti-Wnt, anti-Nodal and anti-BMP from the anterior visceral endoderm (Beddington and Robertson, 1998; Beddington and Robertson, 1999; Arkell and Tam, 2012).

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References

Author contributions
Conceived and designed the experiments: J.T., D.A.T., P.H. and A.M.A. Performed the experiments: D.A.T., J.T. and P.H. Analysed the data: J.T., D.A.T., P.H. and A.M.A. Wrote the paper: D.A.T., P.H. and A.M.A. Funding
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Competing interests
The authors have no competing interests to declare.

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References


Fig. S1. Effects of signalling during the exit from pluripotency (supplementary data for Fig. 2). (A) Sox1::GFP cells were cultured in the indicated factors days 0–2, followed by days 2–5 in N2B27. (B,C) Sox1::GFP (B) or T::GFP (C) cells were maintained in N2B27 for 5 days (Sox1::GFP) or 2 days in N2B27, followed by 2 days in AC (T::GFP) and exposed to pulses of the treatment as indicated. GFP expression was analysed by flow cytometry and results were normalised to 5 days in N2B27 (Sox1::GFP) or 2 days N2B27 followed by 2 days AC (T::GFP). Note that the effect of the factors and treatments have remarkably similar effects on GFP expression in both Sox1::GFP cells and T::GFP cells.
Fig. S2. See next page for legend.
Fig. S2. A fate restriction point at the exit from pluripotency (supplementary data for Fig. 3). (A) Sox1::GFP cells were grown in the indicated medium (ordinate) for 2 days and a further day in N2B27 prior to RNA extraction and RT-qPCR analysis for the indicated genes (graph titles). Data normalised to the house-keeping gene Ppia. (B) Live-cell imaging of TNGA Nanog::GFP cells treated with a 2-day pulse of either SB43 or XAV939 before wash-off and medium replacement with N2B27. (C) E14Tg2A mES cells maintained in S + L were plated into N2B27 or 2i as indicated for either 48 or 72 hours (2 or 3 days). Then 600 cells from each population were plated into 2i medium and cultured for a further 5 days; only cells that are pluripotent will form colonies. Cells initially plated in 2i for 2 days form 220 colonies, which increases to 257 colonies after 3 days in 2i. Whereas, cells initially plated into N2B27 for 2 days form 111 colonies, this declines to 27 colonies after 3 days in N2B27. Scale bars: 100 μm.

Fig. S3. Analysis of gene expression during the exit from pluripotency (supplementary data for Fig. 4). (A–F) Principal Component Analysis of bulk gene expression. We performed PCA of the gene expression profiles of 34 genes in 2i, BMP and LIF, and the 5-days differentiation in N2B27. The first four components are shown (A). Each dot corresponds to a technical repeat of a particular experimental condition (colour coded and labelled in the left panel). PC1 and PC2 cluster data according to each condition (A) and account for more than 75% of the total variance (B). (C) The levels of expression (coded in colour and size) of each condition for several genes of interest. (D,E) The results of the PCA are robust to data normalisation procedures. We obtained similar results doing PCA with data normalised with the min–max method (D) or the Z method (E). (F) The results of the PCA are held when we do not consider the 2i conditions. (G) Single-cell gene expression analysis: top-most significant genes for differences in the distributions of expression levels of 4 days as well as the initial LIF and BMP condition (Kruskal–Wallis one-way test p-values corrected with Bonferroni for multiple testing).
Fig. S4. Activin activity but not Wnt/β-Catenin activity controls fate decisions between PS and NECT (supplementary data for Fig. 5).

Sox1::GFP cells were cultured in N2B27, SB43, XAV939 or SB43 + XAV939 for 0–2 days and, as indicated, days 2–5. GFP expression was analysed by flow cytometry, normalised to 5 days in N2B27 and is presented in the form of a heat map.
Fig. S5. A simple population model for a cell fate decision race (supplementary data for Fig. 6). (A) Model equations: the model consists of 4 different cellular types: pluripotent cells (P), differentiating cells (D), cells committed to NECT (N) and cells committed to PS (M). We consider initially all cells to be pluripotent. Cells then spontaneously lose pluripotency and start differentiating with a certain rate $l_D$. Once each cell abandons the pluripotent state, rapidly and irreversibly adopts a final fate: either NECT or PS. Cells are assumed to acquire either fate at a particular rate: $l_N$ for NECT and $l_M$ for PS. In addition to these cellular types and the corresponding transitions, we also consider a signal ($s$) that builds up and once crosses a threshold, biases the rates of fate adoption. In particular, we consider that the rate of PS conversion is negligible in the absence of signal and becomes the largest rate once the signal concentration has reached the threshold, which we arbitrarily take to $s_0=1$. We initially consider all cells to be pluripotent ($P=1$, $D=N=M=0$). The model is linear but non-smooth, with piecewise constant $l_M(s)$ depending on the signal level. The analytic solution of each region of constant $l_M$ is given in panel B. (C,D) Two of the three parameters ($l_D$ and $l_N$) can be fitted to the experimental data on Sox1::GFP in N2B27 by assuming no cells adopt the PS fate ($l_M=0$). (E) The model reproduces to a certain extent the 2-day Activin and Chiron pulse-chase experiment with the parameter values found in panel C and assuming a PS-fate adoption rate much higher than the NECT-fate adoption rate during the pulse (i.e. $l_M>>l_N$).