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

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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 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 a pending subject. 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. After this transient period and under the influence of a gradient of Activin/nodal or BMP signalling a decision between differentiating into neuroectoderm and contributing to Primitive Streak fates is made. Whilst cells emerge from pluripotency with an intrinsic competence for the neuroectoderm fate, the competence for Primitive Streak occurs over a discrete temporal window. Surprisingly Wnt signalling aids both fates in a context dependent manner.

Introduction

Embryonic Stem (ES) cells are clonal populations derived from early mammalian blastocysts which 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; Nichols & Smith, 2011; Smith, 2001). 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 (Borowiak et al, 2009; Borowiak & Melton, 2009; Gadue et al, 2005; Lupo et al, 2013; Nostro et al, 2008; Spence et al, 2011) 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 (Chen et al, 2013; Gadue et al, 2006). Therefore, gaining insights into the mechanisms of differentiation in culture will have an impact in our ability to harness the potential of these cells.

Differentiation from pluripotency represents a good model system to understand the general process of fate decision making (Gadue et al, 2005; Murry & Keller, 2008). In the embryo, the blastocyst differentiates into the epiblast where, within a single cell layered epithelium and under the influence of spatially organized 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 and the anterior nervous system, and 2) a group of cells in the proximal posterior region that will contribute to a dynamic structure, the Primitive Streak (PS), and act as a seed for the endoderm and the mesoderm (Arkell & Tam, 2012; Arnold & Robertson, 2009; Rossant & Tam, 2009). The molecular components enabling and implementing the specification of these cell types are well known (Pfister et al, 2007) and the process can be mimicked in culture (Gadue et al, 2006; Kubo et al, 2004), albeit in a disorganized manner and often with low yields of cells of a particular fate. Notwithstanding this, the details of the interactions between signalling and transcriptional networks mediating this decision remain poorly understood.

A widely held view of the differentiation process entertains that, as cells exit pluripotency, they choose between NECT and PS from a naïve state under the influence of their local signalling environment that enforces a rearrangement of the pluripotency network (Loh & 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 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 & 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 or PS. Our observations support suggestions that ES cells have an intrinsic competence towards the NECT fate (Smukler et al, 2006; Tropepe et al, 2001; Wataya 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 Nanog. We find no evidence to support the widespread notion that Wnt/β-Catenin signalling suppresses NECT (Aubert et al, 2002b; Bhargava et al, 2013; Lupo et al, 2013; Patani et al, 2009; Smith et al, 2008; Watanabe et al, 2005) 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.

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 (Abranches et al, 2009; Engberg et al, 2010; Fehling et al, 2003; Hansson et al, 2009; Ying et al, 2003b) (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; Kalmar 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). Treating cells to a short pulse of AC (1 day) after increasing durations of N2B27 exposure revealed that during this window of time (two days N2B27) cells are most sensitive to PS-inducing stimuli (Fig. 1E). In addition, as this N2B27 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 differentiation, we made use of the ability of BMP signalling to inhibit this event (Bertacchi et al, 2013; Di-Gregorio et al, 2007; Finley et al, 1999; Surmacz et al, 2012; Zhang et al, 2010). Treatment of ES cells with 1ng/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 (Malaguti et al, 2013; Zhang et al, 2010). Consistent with this we observe that when TNGA cells are exposed to 1ng/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. While commitment to NECT, as reflected in Sox1::GFP expression, does not appear to require specific inputs (Watanabe et al, 2005; Wataya et al, 2008; Ying et al, 2002) and has been suggested to be latent in self renewal conditions (Smukler et al, 2006; Tropepe et al, 2001), 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 (neutral) differentiation is the population, as a whole, competent to respond to AC (Fig. 2D,E).

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, 2002b; Bhargava et al, 2013; Lupo et al, 2013; Patani et al, 2009; Smith et al, 2008; Watanabe et al, 2005).To test the impact of these signals and their interactions on the NECT/PS decision during the exit from pluripotency, we treated ES cells 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 the GSK3 inhibitor CHIR99021 (Chi), either for 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 the Tankyrase inhibitor 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 very much on the period and timing of exposure. Even though β-Catenin is necessary for T::GFP expression, treatment with Chi for two days before the exposure to PS-promoting signals (AC), reduces, rather than enhances the number of T::GFP expressing cells (Fig. 2D). Furthermore, application of XAV939, enhances the response. The effects of Activin signalling followed a similar pattern: exposure to Activin (Act) during the first two days of differentiation inhibited Sox1::GFP expression and to lesser extent, T::GFP expression (Fig. 2D). On the other hand, inhibition of Activin signalling during this period with SB431542 (SB43), led to an increase in Sox1::GFP and to a slight but detectable reduction of T::GFP expression by exposure to AC (compare 65% with SB43 v 90% with N2B27; Fig. 2D). In all cases the effects were less pronounced the shorter the exposure of the cells to the signal modulators (Fig. 2 Supplementary). Tests of the interplay between the two pathways on Sox1::GFP expression (Fig. 2 Supplementary) indicate a predominant role of an inhibitory function of the signals, with the effects of Activin and Chi being dominant over the loss of function of either pathway. Interestingly, 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 (Fig. 2 Supplementary).

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 Wnt/β-Catenin signalling has been shown to promote pluripotency (Doble et al, 2007; Faunes et al, 2013b; Kelly et al, 2011; Lyashenko et al, 2011; Wray et al, 2011; Yi et al, 2011), 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, functional tests (colony-forming assays; data not shown), flow cytometric analysis and live imaging of ES cells (Figs. 2 and 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 cells pluripotent whereas inhibition promotes their differentiation (Figs. 2 and 3A,B,D). This effect on pluripotency is illustrated through flow cytometry analysis (Fig. 3A,B) and live-imaging (Fig. 3C,D): Wnt/β-Catenin signalling (Chi) maintains the expression profile of both the Nanog::GFP (TNGA cells) and Rex1::GFP reporters whereas its inhibition (XAV939) results in both their downregulation (Fig. 3A,B,C).

The notion that the impact of β-Catenin on differentiation reflects its activity in promoting pluripotency are confirmed by its effects on the PS differentiation. Although Wnt/β-Catenin signalling is required for PS, 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 block 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,B,C), 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) and Fig. 3) and therefore must reflect an active suppression of this fate; the effects of SB43 on Sox1::GFP confirm this possibility (Fig. 2A,C). On the other hand, the effect of SB43 on T::GFP expression (Fig. 2D) is surprising and might reflect a delayed differentiation associated with the requirement for Activin/Nodal in the

maintenance of the EpiSC state (Galvin-Burgess et al, 2013; Vallier et al, 2009). This possibility is supported by our observation of a small delay in the decay of Nanog expression during differentiation and the gene expression profile of these cells (Fig 3A,C,E and Fig.4).

Altogether these observations show that, as cells exit pluripotency, they integrate several signals and emphasize 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 a 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 BMP and β-Catenin will enhance this state, while Activin/Nodal and FGF/ERK signalling will contribute to loss of Nanog expression and thereby promote differentiation in these cells (Galvin et al, 2010; Galvin-Burgess et al, 2013; Kunath et al, 2007; Stavridis et al, 2010; Stavridis et al, 2007). 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, 2013b; Trott & Martinez Arias, 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 fates. Our experiments also support the notion that NECT might not need specific signals and that the effect of all the signals is to induce PS (Fig. 2 and Fig. 2 supplementary data).

The impact of signals on gene expression

To better characterize the impact of the signalling pathways on the exit from pluripotency, we monitored the expression of genes associated with pluripotency (E-Cadherin, Nanog, Rex1, Klf4), exit from pluripotency (Tcf15), 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 different fates are due to its effects on pluripotency: Chi maintains the levels of all pluripotency markers with little effect on differentiation, whereas XAV939 promotes differentiation, with a bias towards NECT (Fig. 3E). 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 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.

A fate restriction/commitment point at the exit from pluripotency

Our results support the contention that BMP delays differentiation (Malaguti et al, 2013; Zhang et al, 2010) 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 (Fig. 2 supplementary data). 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; Hansson et al, 2009; Irion et al, 2010; Nostro et al, 2008). 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 and Fig 5 supplementary data). 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 (Bernemann et al, 2011; Sterneckert et al, 2010; 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). To understand the molecular basis of ES cell differentiation, we monitored the expression of a collection of genes associated with pluripotency and lineage restriction during the exit from pluripotency, as well as early markers of signalling activity. 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) and at the level of single cells (Fig. 4B,C,D). 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 characterized 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 in the expression of direct targets, e.g. Dkk1 and Axin2, which rise during day 3 (Fig. 4A).

Analysis of gene expression in single cells indicates that the driver of the process is the decrease in genes associated with pluripotency with the top elements of the pluripotency network (Essrb, Oct4, Dax1, Klf4, Sox2, Nanog, and Rex1) amidst the top 15 genes driving the change (Fig. 4B,C,D). 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, 2013a). As differentiation progresses, single cells start to favour the high expression of genes associated with the NECT (Fig 4C,D).

These results suggest that decisions in ES cells are taken at the level of individual cells and that they occur against the backdrop of a primary neural fate, which might be latent in the pluripotent state. They also support the notion for the progressive emergence of a competence to respond to PS promoting signals.

Signals, commitment and differentiation at the decision point

After three days in N2B27, cells are unable to return to the naïve state 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 (Fig. 5). As expected, exposure to Activin or BMP promotes T::GFP expression and a PS fate (Fig. 5A and Fig. 5 Supplementary data) 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 Chi promotes both Sox1::GFP and T::GFP expression in a context dependent manner: it both enhances the effects of Activin on the PS fate and the effect of SB43 on the NECT (Fig. 5C).

Altogether these results indicate that the competence of cells to respond to AC and acquire a PS fate arises after 2 days of undirected differentiation (Fig. 1E), whereas the competence for NECT is present from the initial stages of differentiation but declines probably due to exposure of the cells to paracrine Activin/Nodal signalling. Inhibition of both Activin with SB43 and Wnt/β-Catenin signalling with XAV939 enhance both the proportion and rate of cells exiting the pluripotent state (Fig. 3A,B,E) and also lead to an enhanced population of Sox1::GFP. The increase in proportion of the population of NECT cells could be attributed to an enhanced rate of exit from pluripotency, which would confer an advantage to the NECT fate, or to an enhanced commitment to NECT fate. To test this, cells were exposed to SB43 or XAV939 for the initial 2 or 3 days of differentiation before switching to either N2B27, Activin or BMP for the remainder of the 5 day assay (Fig. 5D). Whereas populations of cells initially exposed to XAV939 resembled those cultured in N2B27 (with respect to the proportion of Sox1::GFP-positive cells), cells initially grown in SB43 showed an enhanced commitment to the NECT fate and the longer the exposure the greater the enhancement (Fig. 5D).

These results suggest that whereas Wnt/β-Catenin signalling regulates the dynamics of the exit from pluripotency, it has little effect on the fate of the cells during the first two days of differentiation. On the other hand, Nodal/Activin controls both the rate of differentiation and the fate of the cells. At day 3 of differentiation in culture, cells become committed to either of the fates under the influence of the signalling environment. Activin and BMP signalling promote PS and inhibit NECT whereas Wnt/β-Catenin signalling appears to play a role of enhancement of both fates in the context of the decision

Discussion

We have analysed the role of signalling in fate assignment during the early stages of differentiation of mouse ES cell populations in culture. While there are many studies of ES cell differentiation into particular cell types, there is no integrated analysis of the kind that we have performed here. Our observations reveal the importance of the state of the cell when responding to signals, particularly to those that promote both self-renewal and differentiation like Wnt and BMP. Both signals have been suggested to suppress neural differentiation (Aubert et al, 2002a; Bertacchi et al, 2013; Finley et al, 1999; Lupo et al, 2013; Smith et al, 2008; Surmacz et al, 2012; Watanabe et al, 2005; Watanabe & Sasai, 2005; Zhang et al, 2010), however we find that at the exit from pluripotency, their effects on differentiation are mediated through their effects on pluripotency. Our results support the conclusions that BMP delays the exit from pluripotency (Malaguti et al, 2013; Zhang et al, 2010) whilst simultaneously priming the PS fate (Engberg et al, 2010; Hansson et al, 2009; Malaguti et al, 2013). In the case of Wnt/β-Catenin, we find that the observed suppression of neural fates is an indirect consequence of its effects on pluripotency (Faunes et al, 2013b; Munoz Descalzo et al, 2013). High levels of β-Catenin maintain cells pluripotent but this effect is not stable and, over time, cells exit pluripotency and when they do, in the presence of high levels of β-Catenin they adopt the PS fate. We find that in cells committed to differentiation on which BMP suppresses the neural fate, Wnt/β-Catenin signalling promotes both NECT and PS fates 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 & 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. 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 as well as of the neuromuscular precursor. 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 Fig. 7G in (Thomson et al, 2011) but is ignored by the authors. The lack of lineage specificity in the effects of Wnt/β-Catenin signalling contrast 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 & Hayward, 2006; Munoz Descalzo & 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 and the co-expression of genes associated with PS and NECT (Trott & Martinez Arias, 2013; Zhang et al, 2010). A prevalent view of this 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 which can be overridden for a short period of time by pro-PS signals. The notion of a “primary” or “default” NECT fate has been suggested before (Smukler et al, 2006; Tropepe et al, 2001) and is supported by the observation that it will develop in the absence any external signals (Watanabe et al, 2005; Wataya et al, 2008; Ying et al, 2002) as well as by our observation of the prevalence of NECT gene expression in self renewal and, particularly, during the early stages of differentiation. 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 neutral 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 (Engberg et al, 2010; Nordin et al, 2008) (and here). 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 (Trott & Martinez Arias, 2013) (and here). However, the large dilution effects of the culture are likely to prevent significant differentiation of PS derivatives in this condition 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 (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, 2012).
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 (Karwacki-Neisius et al, 2013; Munoz Descalzo et al, 2013; Munoz Descalzo et al, 2012; Radzisheuskaya et al, 2013).
3. The elements of the pluripotency network prime particular fates, with Oct4 priming all differentiation but then, together with Nanog, promoting PS and Sox2 promoting NECT (Loh & Lim, 2011; Thomson et al, 2011).
4. Differentiation is associated with Wnt/β-Catenin signalling (Faunes et al, 2013b; Trott & Martinez Arias, 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 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; Martinez Arias & Brickman, 2011; Munoz Descalzo et al, 2013). In this condition, 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 (Faunes et al, 2013a; Malaguti et al, 2013; Wray et al, 2011; Ying et al, 2003a; Ying et al, 2008) 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 (Faunes et al, 2013b; Kunath et al, 2007; Stavridis et al, 2007). 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 fate. 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 race of two mutually excluding fates with two different triggering thresholds. 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 dynamical model that accounts for our observations (Fig. 6C). In the model the two competing cellular fates adopt different dynamic strategies and shows that there is a lag period of about two 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 organized 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 & 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 (Arkell & Tam, 2012; Beddington & Robertson, 1998; Beddington & Robertson, 1999).

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 1μM PD0325901, 3μM Chi and LIF (insert Chambres paper). Cell medium was changed daily and cells passaged every other day. For differentiation experiments, cells were plated at a concentration of $4x10^3$ cells/cm^2 in the indicated differentiation medium (day 0). Differentiation medium consisted of an N2B27 base medium supplemented with combinations of Activin (100 ng/ml), CHIR99021 (Chi; 3 μM), XAV939 (1μM; (Huang et al, 2009)) SB431542 ((Inman et al, 2002); 10 μM), BMP4 (1ng/ml) and Dorsomorphin-H1 (0.5μM). Differentiation medium was completely replaced daily to reduce the influence of increased concentrations of secreted factors.

Flow cytometry and cell sorting

Cells were analyzed for GFP fluorescence using an LSR Fortessa (BD Bioscience) using a 488 laser and emission measured using 530/30 filter, Dapi exclusion was used to determine live cells and measured using 405 laser and emission at 450/50. Data was analyzed using Flowjo software. TNGA cells were sorted according to their GFP fluorescence using a MoFlo sorter (Beckman Coulter) using the same laser and filter sets described above. Cells were collected in SL media, counted and re-plated into the media indicated in the described experiments.

Immunofluorescence & Confocal Microscopy

Immunofluorescence and image analysis were carried out as described previously (Munoz Descalzo et al, 2012; Turner et al, 2013)in 8-well (Ibidi), plastic tissue-culture dishes. Samples were washed in BBS+$CaCl_2$ (50mM BES Sodium Salt, 280mM NaCl, 1.5mM Na_2HPO_4, 1mM $CaCl_2$ adjusted to pH 6.96 with 1M HCl) and fixed for 15 minutes in 4% paraformaldehyde. Samples were washed and permeablised with BBT (BBS+$CaCl_2$ supplemented with 0.5% BSA and 0.5% TritonX-100) before overnight antibody staining, following which, the samples were washed with BBT and incubated for 2h with the desired fluorescently-conjugated secondary antibody. Prior to imaging, samples were washed with BBS+$CaCl_2$ and covered in mounting medium (80% spectrophotometric grade glycerol, 4% w/v n-proply-gallatein in BBS+$CaCl_2$). 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 imaged using and LSM700 on a Zeiss Axiovert 200M with a 40x 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)

Wide-field Epifluorescence Microscopy

For live imaging, cells were imaged by wide-field microscopy in a humidified CO_2 incubator (37°C, 5% CO_2) every 10 min for the required duration using a 20x LD Plan-Neofluar 0.4 NA Ph2 objective with correction collar adjusted for imaging through plastic. All medium was 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 100U/mL LIF and 10ng/mL BMP4 (Department of Biochemistry, University of Cambridge). Subsequently, cells were trypsinised and reseeded in N2B27 alone at a density of 10 x 10^3 cells/cm^2 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 (5μl) was mixed with 9μl Specific Target Amplification mix (STA mix – 7.5μl TaqMan Pre-Amp mastermix (Invitrogen), 1.5μl 10X STA Primer Mix, 0.075μl 0.5M EDTA, pH 8.0). STA Primer Mix is composed of 48 or 96 primer pairs (see supplementary information) diluted in DNA suspension buffer (Teknova) at a concentration of 500nM per primer. Individual amplicons were further amplified using the following thermal cycling protocol: 10 min at 95°C, 20 cycles of 5s 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 manufacturers 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 ~$5x10^5$ trypsinised and pelleted mES cells using the RNeasy Mini kit (Qiagen, 74104) according to the manufacturers instructions, and resuspended in 30µL distilled water. RNA was reverse transcribed as follows. RNA samples (1µg in 38µL nuclease free water) were combined with 2µL 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: 1.5µl dNTPs (Life technologies, 18427-013), 12 µl 5X First Strand buffer (Life technologies, 18080-400), 3µl 0.1M DTT (Life technologies, D-1532), 1.5µl RNaseOUT (Life technologies, 10777-019) and 2µl 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 1µL RNaseH (Life technologies, 18021071) was added and samples were incubated at 37°C for 20 minutes and then at 80°C for 20 minutes. Samples were diluted 1 in 100 before being analysed for gene expression using the Fluidigm platform (from Specific Target Amplification step using the same primers as for single cell qPCR).

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 λ_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: λ_N for NECT and λ_M for PS. In addition to these cellular types and the corresponding transitions, we also consider a signal (*s*) that builds up as cells loose 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 Fig. 6C.

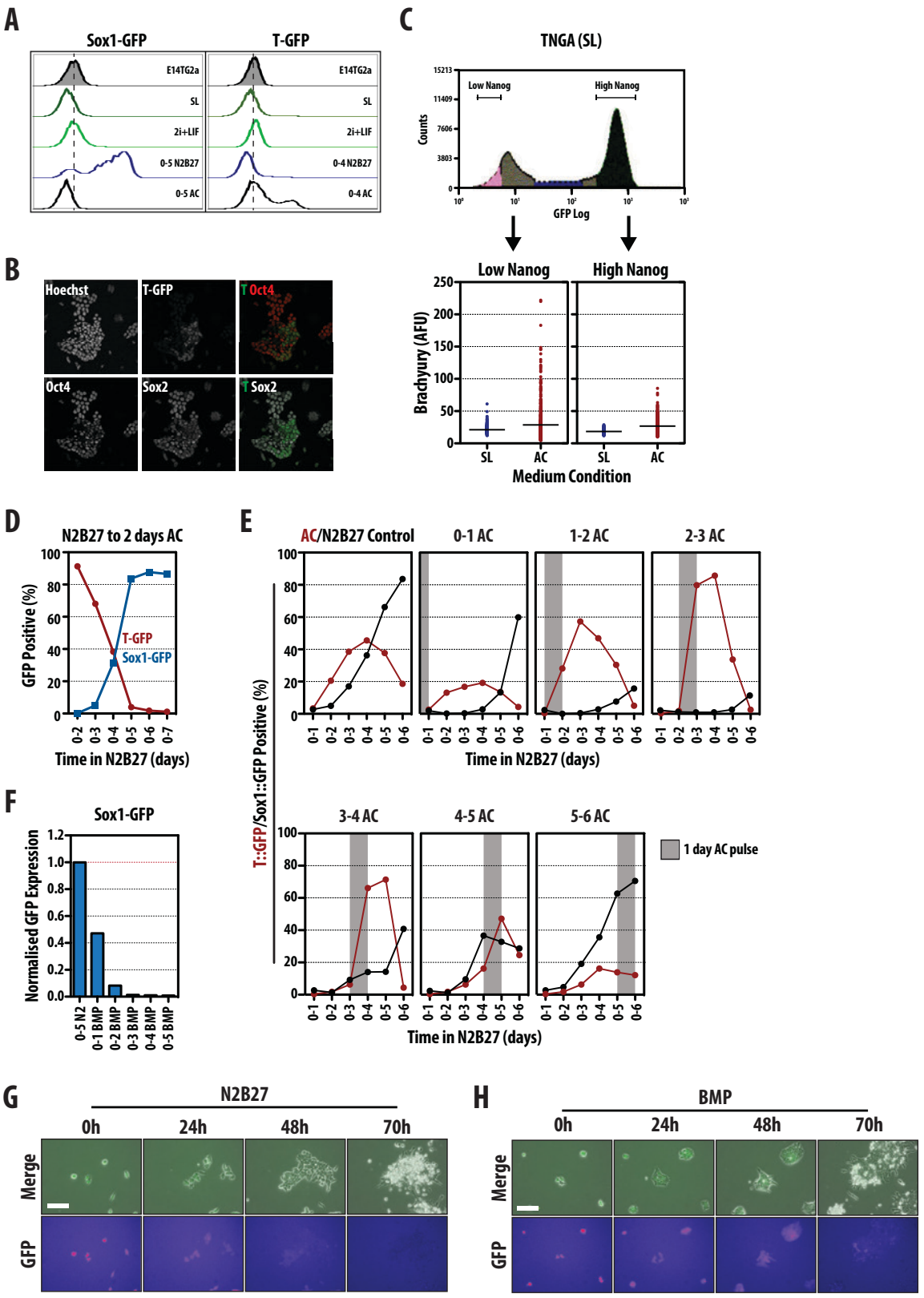

Figure 1: The exit from pluripotency determines the ability of mESCs to differentiate. (A) Sox1::GFP (left) or T::GFP (right) mESCs exposed to serum and LIF (SL), 2i and LIF (2i+L), N2B27 or Activin and Chi (AC) for the indicated durations and GFP expression analysed by flow cytometry. Hashed vertical line bisecting the population profile plots indicates the peak maximum of the negative control (B) T::GFP mESCs differentiated in AC for 2 days (0-2), immunostained for Hoechst, Oct4 and Sox2 and imaged by confocal microscopy. Scale bar indicates 100µm. (C) TNGA mESCs cultured in SL and FACS sorted into low- (indicated in pink) and high- (indicated in dark blue) expressing populations (top) were re-plated in AC conditions for 2 days, immunostained for Brachyury and analysed by confocal microscopy (bottom). TNGA

cells cultured in SL conditions served as a negative control for Brachyury immunostaining. (D) T::GFP (red) and Sox1::GFP (blue) mESCs were differentiated for 2 days in AC conditions after exposure to N2B27 for the durations indicated on the abscissa. GFP expression was measured by flow cytometry. (E) T::GFP (Red) or Sox1::GFP (black) mESCs plated and treated with N2B27 for 6 days with single, 1 day pulses of AC on days indicated above graphs. For the control, T::GFP or Sox1::GFP cells were incubated with 6 days of either AC or N2B27 respectively. Grey bar indicates period of AC pulsing. (F) Sox1::GFP mESCs treated with 1ng/ml BMP4 for durations indicated on the abscissa or 5 days (0-5) in N2B27. GFP expression measured by flow cytometry and fluorescence values displayed are normalised to the N2B27 control. (G,H) Live-cell imaging of TNGA cells in N2B27 alone (G) or supplemented with 1ng/ml BMP4 (H). Scale-bar represents 100µm.

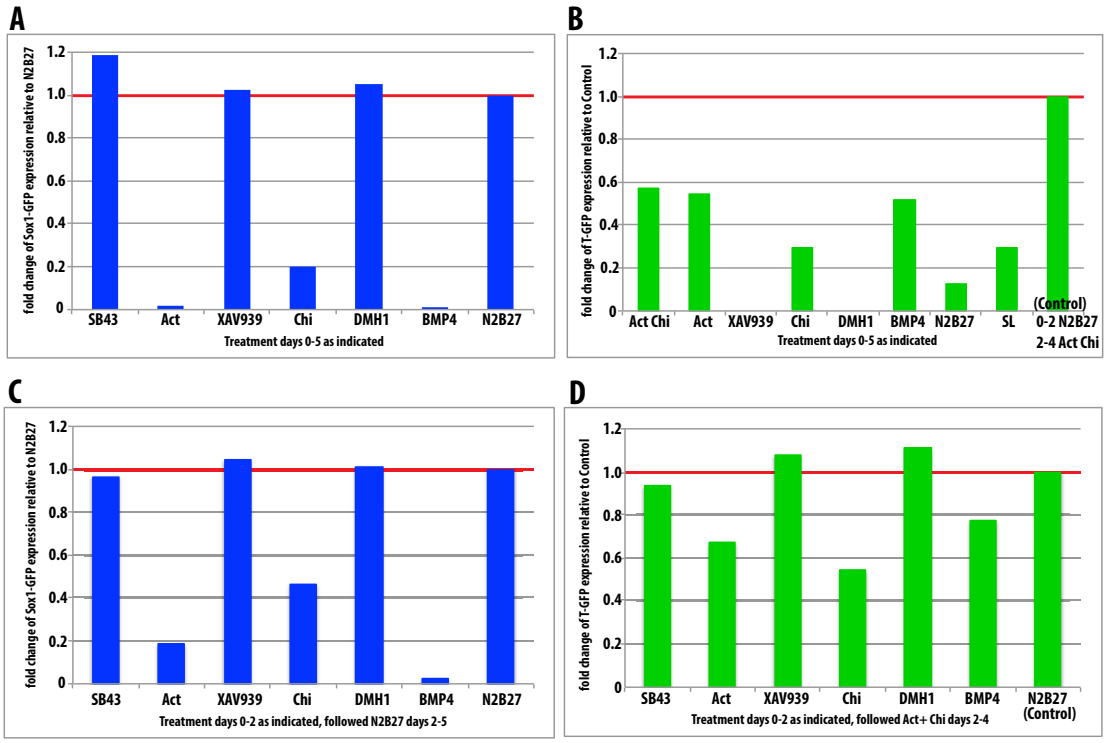

Figure 2: In the initial stages of differentiation factors predominantly contribute to or inhibit the exit from pluripotency. (A, B) Proportion of GFP positive cells in Sox1::GFP (A) or T::GFP (B) cells exposed to the indicated factors for 5 days. C, D) Proportion of GFP positive cells in Sox1::GFP (C) or T::GFP (D) cells exposed to the indicated factors for 2 days prior to switching media to N2B27 for 3 days (A, Sox1::GFP) or AC conditions for 2 days (B, T::GFP). All data presented here is normalised to 5 days in N2B27 (Sox1::GFP) or 2 days N2B27 followed by 2 days AC conditions (T::GFP).

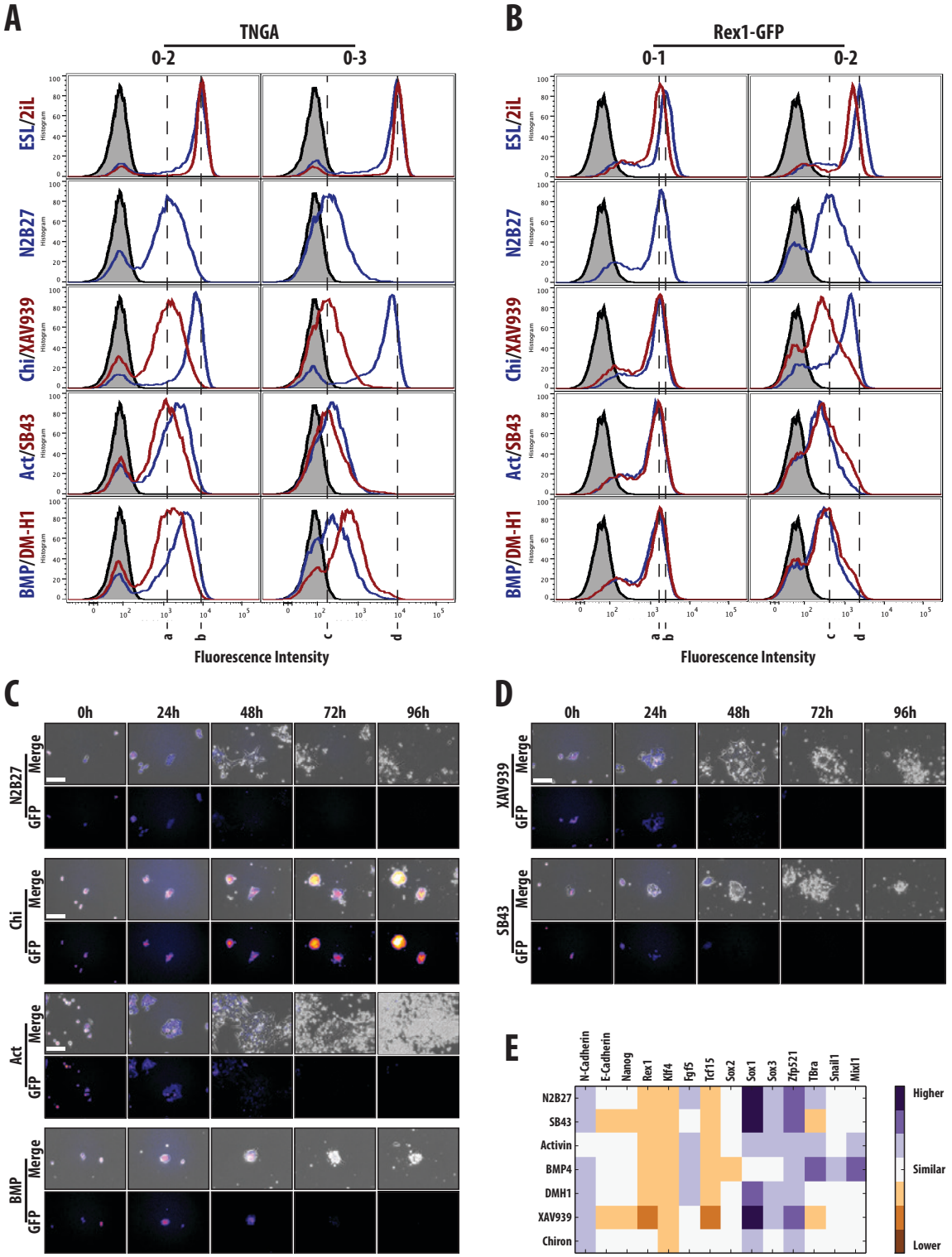

Figure 3: A fate restriction point at the exit from pluripotency. (A,B) Population profiles of cells analyzed by flow cytometry from either (A) Nanog::GFP (TNGA) or (B) Rex1::GFP mESCs after 2 (0-2) or 3 (0-3) days in the medium indicated on the ordinate (SL, 2iL, N2B27, Chi, XAV939, Activin (Act), SB43, BMP, DM-H1). Hashed vertical lines correspond to the peak maximum of the fluorescence from cells in either N2B27 (a,c) or SL (b,d) conditions at each time-point. (C) Live-cell imaging of TNGA mESCs differentiated for 96h in N2B27, Chi, Act or BMP. (D) Live-cell imaging of TNGA mESCs differentiated for 96h in either SB43 or XAV939. Scale-bar in all live-cell imaging experiments indicates 100µm. (E) 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 (abscissa). Data normalised to the house-keeping gene Ppia (see supplemental data for Fig. 3) and displayed as values higher, similar or lower than the SL control.

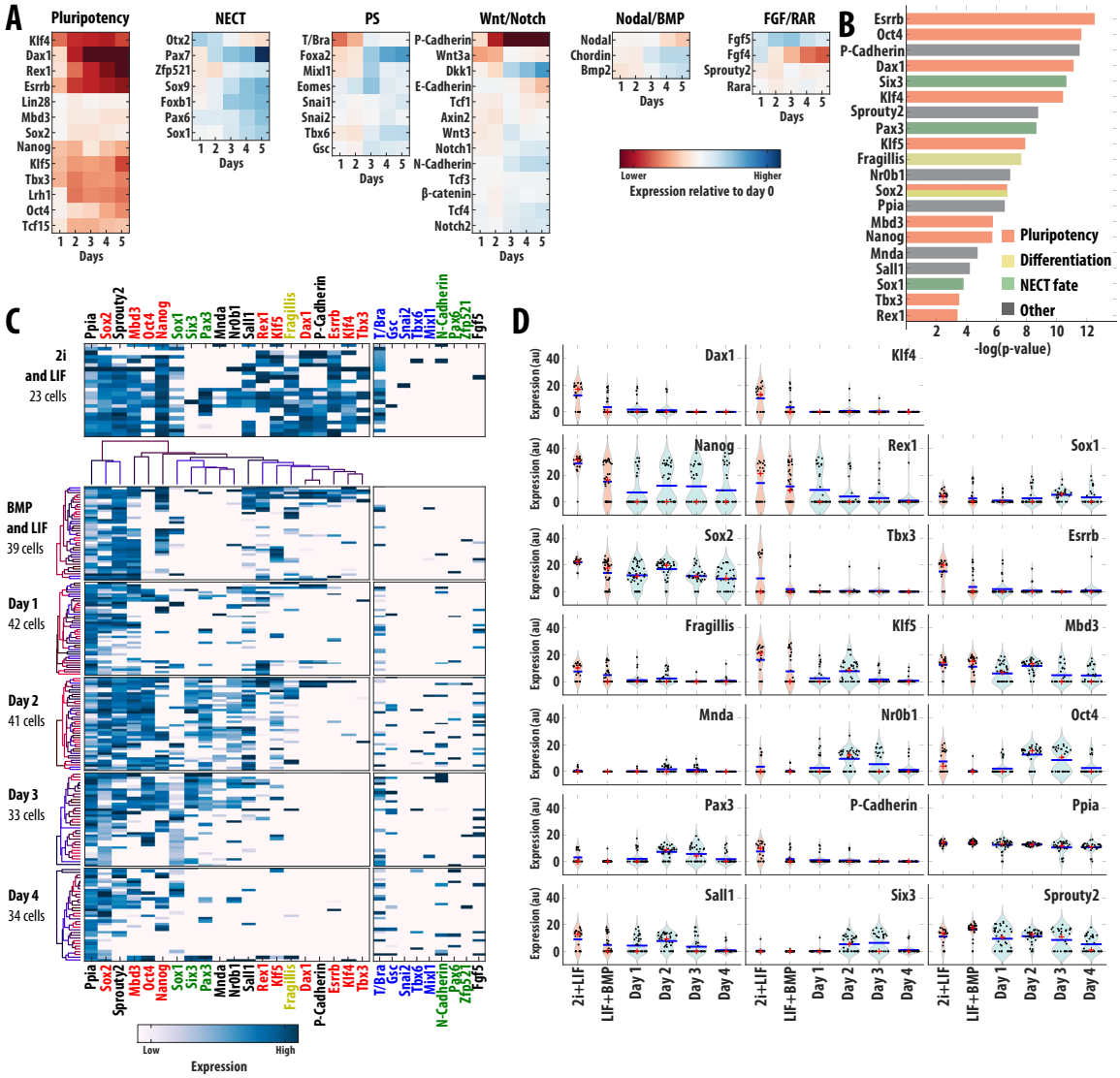

Figure 4: Dynamics of gene expression during the exit from pluripotency. Mouse ES cells cultured in LIF and BMP were transferred to N2B27 and allowed to differentiate for 5 days. (A) Average (bulk) expression levels of several genes associated with pluripotency, NECT and PS fate restriction as well as targets for Wnt/Notch, Nodal/BMP and FGF/RAR signalling were measured daily using fluidigm quantitative RT-PCR. Color-coded values indicate the log-fold change values with respect to the initial LIF+BMP condition of Gapdh-normalised expression levels measured by RT-PCR. (B-D) Expression levels of over 90 genes were analysed using fluidigm techology and single-cell RT-PCR over the 5-day differentiation experiment. Panel (B) shows the 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). (C) Heat maps of single-cell 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 is also shown on the panel on top. (D) Violin plots of the data in (C) showing kernel density estimates of the mRNA distribution (clear blue) together with single cell expression levels (black dots), the population average (blue line) and median (red dot).

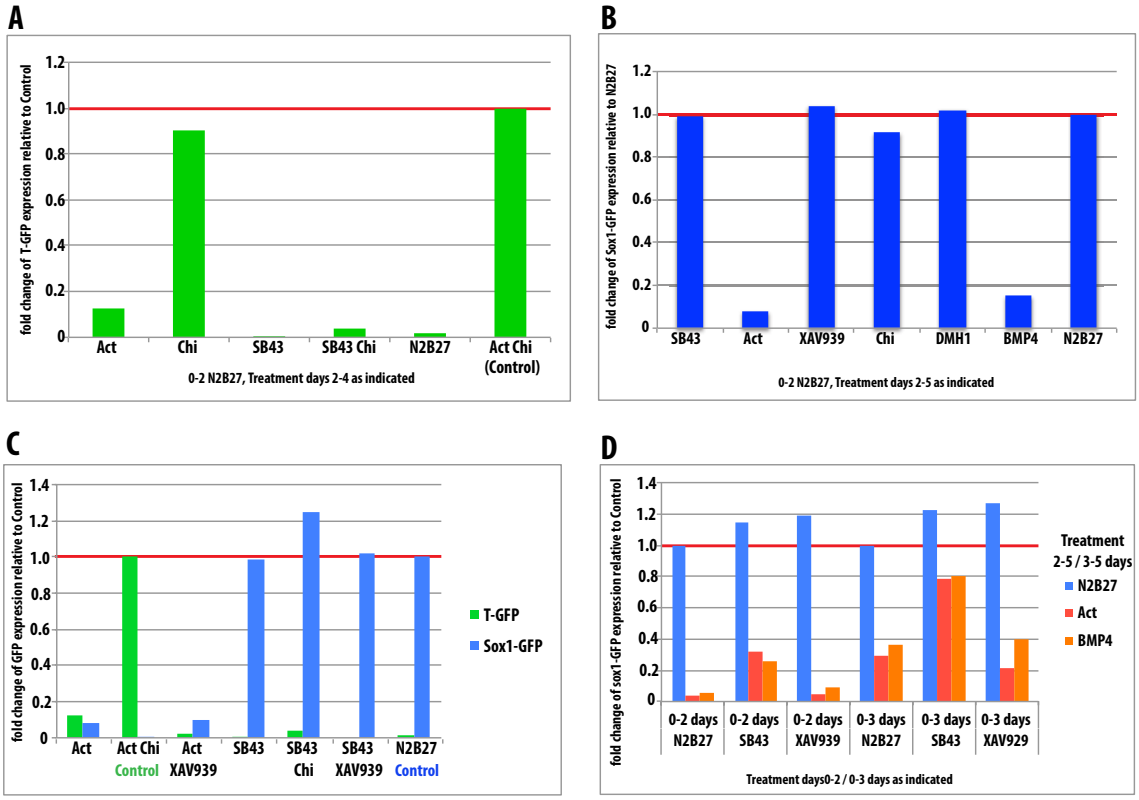

Figure 5: Activin activity but not Wnt/β-Catenin activity controls fate decisions between PS and NECT. (A-C) T::GFP (A) and Sox1::GFP (B, C) cells were grown in N2B27 for 2 days prior to transferring to the indicated conditions for 2days (T::GFP) or 3 days(Sox1-GFP). GFP expression was assessed by flow cytometry and data normalized to control conditions (0-2 days N2B27, 2-4 days AC for T::GFP, 0-5 days N2B27 for Sox1::GFP) and presented in bar charts. (D) Sox1::GFP cells were plated in either N2B27, SB43 or XAV939 for the first 2 or 3 days of culture and then switched to N2B27, ACT or BMP4 for the remaining period. GFP expression was assessed by flow cytometry on day 5. Data is displayed as a heat map relative to control conditions (0-5 N2B27).

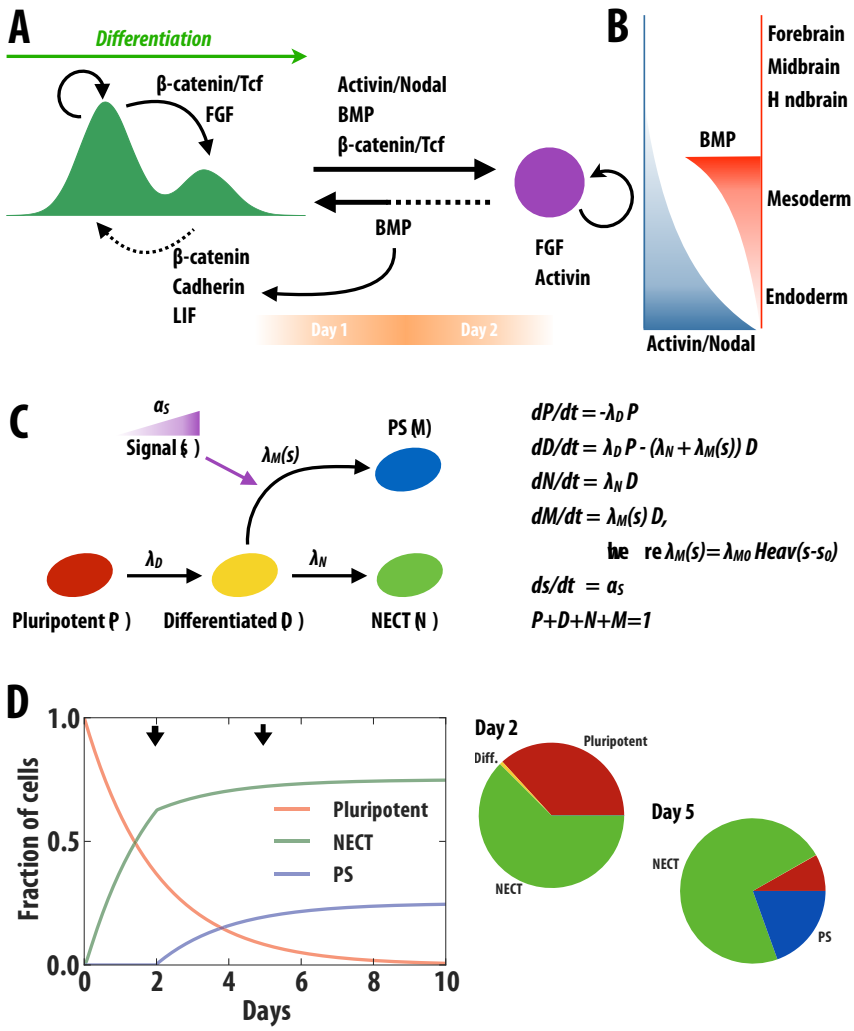

Figure 6: Fate choices at the exit from pluripotency. (A-B) Summary of interactions between signalling pathways and cell states during the exit from pluripotency and the commitment to different cell fates (for details see main text). Embryonic Stem cells self renew in a metastable state governed by the ratio of Nanog and Oct4 (indicated in the levels of green) and exhibit a bimodal distribution of the kind shown. The ratios are implemented by a combination of β-Catenin/E-Cadherin and inhibition of ERK signalling. When differentiation conditions are implemented in the culture ERK and Wnt/β-Catenin/Tcf signalling promote differentiation with levels of Nodal/Activin and BMP determining the fates of the cells. After two days of differentiation, cells enter in an Epiblast like state –maintained by FGF and Activin- where their fate is determined by the relative levels of BMP and Nodal/Activin as indicated. Wnt/β-Catenin signalling is required for all fates. (C) A simple mathematical of cell population dynamics describing for the transitions from different cellular states: from pluripotent (*P*) to differentiating (*D*), and from the latter to either NECT (*N*) or PS (*M*); and the signalling effects in the transition rates accounts for the observed dynamics of cell fate adoption (D). The model assumes that in the initial stages of differentiation cells are more prone to adopt a NECT fate than a PS fate ($\lambda_N > \lambda_M(s)$, see materials and methods). As cells differentiate and the global levels of signal (e.g., Nodal/Activin) raise, the PS fate becomes dominant within the remaining differentiating cells.

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