An Epiblast Stem Cell derived multipotent progenitor population for axial extension

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Abstract

The Caudal Lateral Epiblast (CLE) of the mammalian embryo harbours a stem cell/progenitor zone of bipotent progenitors that contribute to both the elongating spinal cord and the paraxial mesoderm of the embryo. These progenitors, called the Neural Mesodermal Progenitors (NMPs), are characterized by the coexpression of Sox2 and T/Bra. A number of in vitro studies have been able to produce NMP-like cells from Pluripotent Stem Cells (PSCs). However, in contrast with embryonic NMPs these in vitro derived ones do not self renew. Here we use different protocols for NMP-like cells and find that, in addition to NMPs, all produce cells with the potential to differentiate into Lateral Plate and Intermediate Mesoderm precursors. We show that Epiblast Stem Cells are a better starting source to produce NMPs and show that a specific differentiation protocol yields cells with the potential to self renew in vitro and to make large contributions to axial elongation in both neural and mesodermal tissue. These cells are derived from a population that resembles the Caudal Epiblast (CE) of the embryo at the time of the appearance of the node and we show that a balance between Nodal and BMP signalling might be important to define this population. Our study suggests that at the end of gastrulation and associated with the node, a multipotential progenitor population emerges that will give rise to the spinal cord and the postoccipital mesodermal populations.

Introduction

The spinal cord defines the postoccipital path of the vertebrate Central Nervous System (CNS) and plays a central role in the integration of sensory and motor information as well as acting as the gateway for this information to the brain. Its structure has been defined in great details and is well conserved across species, with neurons of different identities and functions. The spinal cord is generated from a set of embryonic progenitors distributed along the anteroposterior axis of the embryo. A widespread view posits that these progenitors arise during gastrulation within a neural plate induced by underlying mesoderm shortly after gastrulation and become 'posteriorized' by the activity of Wnt signalling (Niehrs, 2004; Stern, 2005). However, fate mapping and lineage tracing studies suggest that while this process may apply to the brain and the hindbrain, the spinal cord has a different origin (Henrique et al., 2015; Stern, 2005; Steventon and Martinez Arias, 2017).

Anatomically and physiologically it is possible to distinguish two different tracts in the spinal cord: the thoracic and tail regions. In both amniote and anamniotes the tail tract is generated from cells located in the Chordoneural hinge (CNH), a structure derived from the Primitive Streak that also gives rise to the more posterior somatic mesoderm (Cambray and Wilson, 2002; Cambray and Wilson, 2007; Davis and Kirschner, 2000; Gont et al., 1993; Steventon et al., 2016). The thoracic tract has different origins in different organisms: in anamniotes e.g. fish and frogs, it is inferred to arise during gastrulation from a pool of pre-existing cells within the epiblast, while in amniotes e.g. chickens and mice, it arises from the expansion of the Caudal Lateral Epiblast (CLE), a proliferative region located at the caudal end of the embryo where the Primitive Streak (PS) persists and that, in addition, acts as source for paraxial, intermediate and lateral plate mesoderm (Henrique et al., 2015; Stern, 2005; Steventon and Martinez Arias, 2017; Sweetman et al., 2008; Wilson et al., 2009). Lineage tracing studies have shown that the CLE harbours a population of bipotential progenitors for neural and mesodermal progenitors located behind the Node, at the Node Streak Border (NSB). These cells have been called Neuromesodermal progenitors (NMPs), are often characterized by simultaneous expression of T/Bra and Sox2 (Cambray and Wilson, 2007; Wymeersch et al., 2016) and are able of self renewal (Cambray and Wilson, 2002; McGrew et al., 2008; Tzouanacou et al., 2009).

Recently a number of studies have claimed the generation of NMP-like (NMP-l) cells in adherent cultures of mouse and human embryonic PSCs (Gouti et al., 2014; Lippmann et al., 2015; Turner et al., 2014). In these studies, Embryonic Stem Cells (ESCs) are coaxed into a transient T/Bra and Sox2 coexpressing state that, depending on the culture conditions, can be differentiated into either paraxial mesoderm or spinal cord progenitors and their derivatives. However, there is no evidence that these presumptive NMPs are propagated in vitro as they are in the embryo (Tsakiridis and Wilson, 2015), and while it has been shown that they can contribute to both somites and spinal cord in a xenotransplant assay (Gouti et al., 2014), these contributions are not extensive and lack the sustained integration in the NMP region of the host embryo (P. Baillie-Johnson and B. Steventon in preparation).

Coexpression of T/Bra and Sox2 might not be a unique characteristic of NMPs as it is a signature of Epiblast Stem Cells (EpiSC) populations (Kojima et al., 2014), and this does not imply that EpiSCs, a PSC population, are NMPs. While other markers have been used to identify NMPs in vitro e.g. Nkx1.2, Cdx2, Cdh1 and Oct4, these are also expressed in the epiblast and in the primitive streak during gastrulation (see Appendix supplementary information file S1), emphasizing the notion that such gene expression

signatures are not a unique feature of NMPs. In terms of the ability of T/Bra, Sox2 expressing cells to differentiate into neural and mesodermal progenitors, it has been shown that exposure of ESCs to Wnt and FGF signalling can lead to thoracic neural progenitors, perhaps bypassing the NMP state (Mazzoni et al., 2013; Nordstrom et al., 2002; Nordstrom et al., 2006). Altogether these observations raise questions about the identity of the T/Bra, Sox2 coexpressing cells derived from ESCs.

Here, we show that T/Bra, Sox2 coexpressing cells derived from ESC and EpiSCs based differentiation protocols display differences at the level of gene expression and represent different developmental stages of the transition between naïve, primed pluripotency and neuro-mesodermal fate choices. We find that, in adherent culture, all protocols generate a multipotent population in which NMPs are mixed with progenitors of the Lateral Plate and Intermediate Mesoderm (LPM and IM) as well as the allantois. Moreover, we discover that a new protocol based on EpiSCs leads to an NMP population with many of the attributes of the embryonic NMPs, in particular their ability to self renew and to make long contributions in xenotransplants assays. Our study leads us to propose a model for the emergence, amplification and differentiation of this important population in the mammalian embryo.

Results

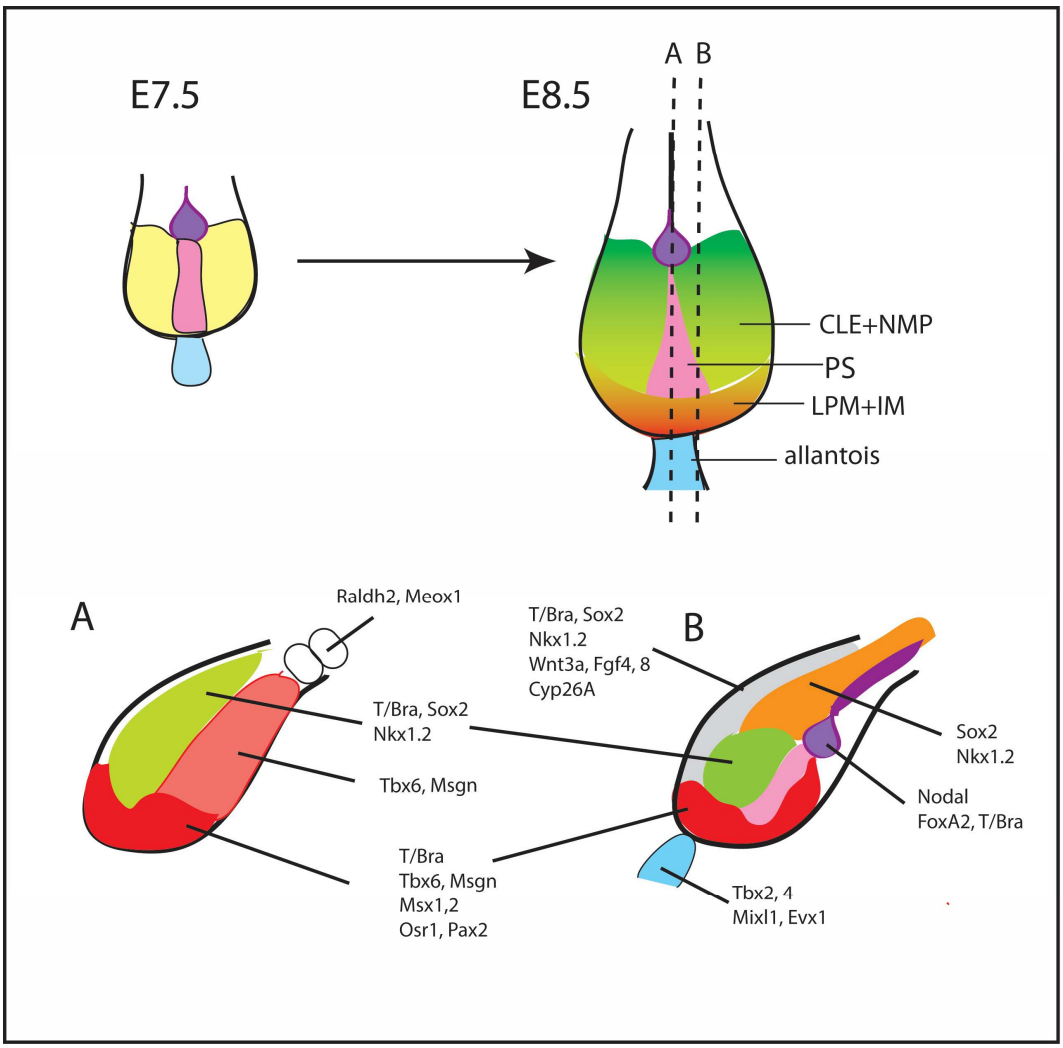
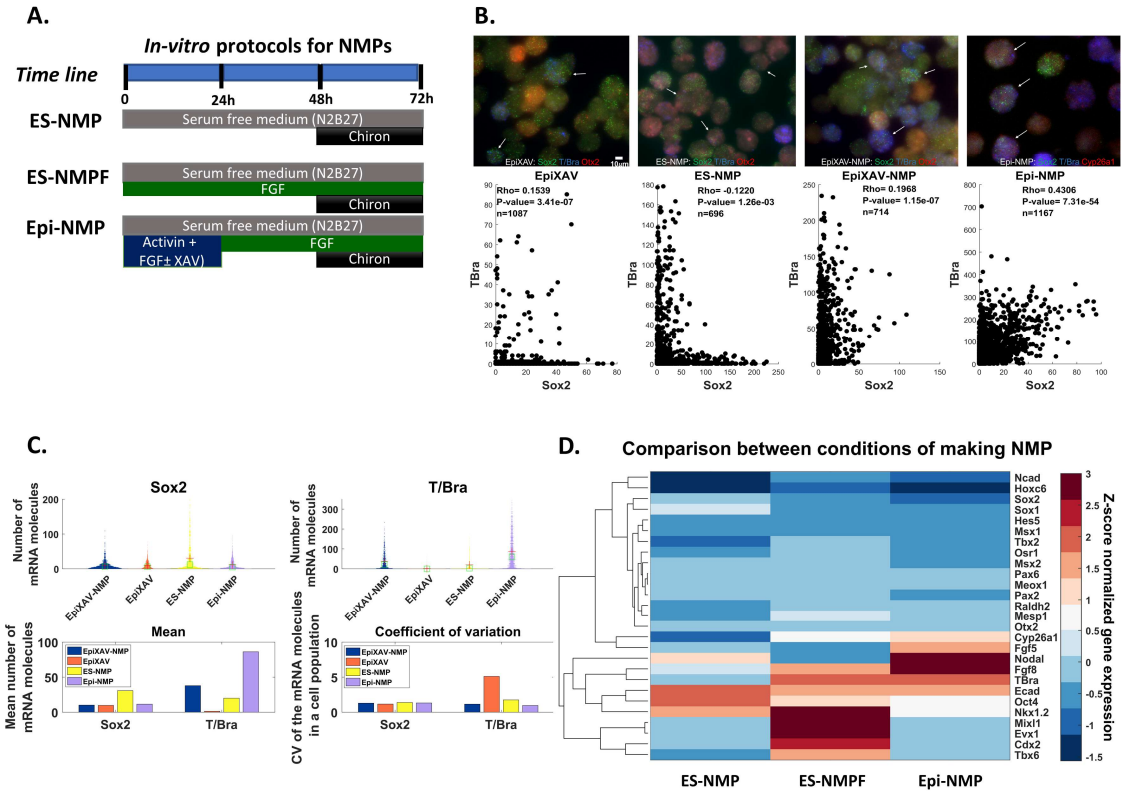


Fig. 1 - Organization and gene expression patterns in the caudal region of the mouse embryo at E8.5; top, ventral views; bottom: lateral (A) and medial (B) views. The caudal region of the embryo is derived from the posterior epiblast of E7.5 (yellow) when the primitive streak (pink) reaches the most distal region of the embryo and the node (purple) appears. This region proliferates and undergoes a number of morphogenetic events which lead to the organization visible at E8.5 and indicated in the figure. The sources for the outlines shown here can be found in Supplementary information S1.

In order to evaluate whether the NMP-I cells derived in culture correspond to the NMPs in the embryo, we need to identify genetic and functional hallmarks for the comparison. As a first step, we have used data available in the literature (see Appendix, supplementary information file S1) to create a coarse grained reference map of the CLE at E8.5 (Fig. 1), as this is the first time at which self renewing NMPs can be identified and tested functionally. These progenitors do not change until the emergence of the CNH at E9.5, and thus are a good reference for our study.

At E8.5 the caudal region of the mouse embryo, defined as a structure posterior and lateral to the node, has a clear organization across its AP (anterior – posterior) and DV (dorsal – ventral) axes in terms of gene expression and developmental potential (Steventon and Martinez Arias, 2017; Wymeersch et al., 2016) and we have used this to divide this region into a several domains, each with a specific gene expression signature (see Fig.1 and supplementary information file S1 for details). Dorsally two very clear domains can be observed: an A to P gradient of Sox2 and Nkx1.2 expression and a P to A gradient of T/Bra expression. This region also expresses Cdx1,2 and 4, Fgf4,8 Wnt3a, Cdh2, CYP26a1 and harbours the NMPs within the CLE (Cambray and Wilson, 2007; Wymeersch et al., 2016). In the most posterior region lie the progenitors of the LPM, IM and the allantois, a mesodermal derivative that will give rise to fetal blood and the umbilical cord with characteristic signatures of Msx1,2 (LPM), Pax2 and Osr2 (IM) and Evx1, Tbx2,4, Mixl1 (allantois). Ventrally, it is possible to identify the presomitic mesoderm (PSM), characterized by the expression of Mesogenin (Msgn) and Tbx6 and, posterior to it, the tail bud, that overlaps with the dorsal CLE and is characterized by expression of T/Bra, Nkx1.2, Msgn and Tbx6. On the dorsal region, anterior to the node there is the so called pre-neural tube (PNT) and further anterior and in a gradual manner, the spinal cord (SC). Anterior to the PSM, there are the somites, which express, amongst others, Meox1 and Raldh2. The CLE is identified as much by a gene expression signature as well as by what genes expresses earlier, at E7.0, that are missing at E8.5 e.g. Cdh1, Oct4, Otx2 and Fgf5 (see supplementary information file S1 for details).

EpiSCs yield a postimplantation epiblast population that resembles the CLE



<u>Fig.2</u> – Comparison between *in vitro* protocols that produce NMP like (NMP-l) cells A. Diagram of the protocols: ES-NMP {Turner, 2014 #2266} ES-NMPF {Gouti, 2014 #1936} and Epi-NMP (see Materials and Methods). **B.** images, obtained by using single molecule fluorescence in-situ hybridization (sm-FISH), of cells coexpressing Sox2 (in green) and T/Bra (in blue) mRNA in different conditions: EpiXAV, ES-NMP, EpiXAV-NMP and Epi-NMP (Otx2 or Cyp26a1 genes are represented in red, see materials and Methods). Quantification plots of the number of mRNA molecules in a cell below each of the conditions; each dot represents a cell with the x-axis and y-axis representing the number of Sox2 and T/Bra molecules respectively in a cell. The Spearman coefficient correlation between Sox2 and T/Bra and the total number of cells, noted as Rho, P-value and n respectively, can be found as an inset in each plot. **C.** Violin distribution plots of Sox2 and T/Bra expression show the distribution shape and probability density of the number of mRNA molecules in a cell at the level of the population of cells imaged in B. The red cross and the green square represent the mean and the median of the distribution of each distribution. The plots underneath the histogram display the mean and the coefficient of variation (CV) of the distribution of Sox2 and T/Bra in the different condition: EpiXAV-NMP in blue, EpiXAV in orange, ES-NMP in yellow and Epi-NMP in purple. **D.** Expression heatmap of 27 genes, obtained by RT-qPCR, in cells grown in the different protocols (A). The normalized expression of each gene across the different conditions was scaled via calculating the Z-score (see Materials and Methods and supplement Fig. S2)

There are several protocols that allow the differentiation of ESCs into NMPs, defined as cells that coexpress T/Bra and Sox2 and, in all cases, the resulting population can be further differentiated into neural and mesodermal progenitors (summarized in (Henrique et al., 2015)). However, it is not clear whether these NMP-l cells are similar to each other

and, importantly, how each relates to the NMPs in the embryo. To begin to answer these questions we compared NMPs obtained from the following three different protocols: two published that start from ESCs that we call ES-NMPs (Turner et al., 2014) and ES-NMPFs, as in this case the differentiation medium contains FGF (Gouti et al., 2014) and a third one that we call Epi-NMPs which we have developed starting from EpiSCs (see Materials and Methods and Fig. 2A). In all cases, exposure to Chiron on the third day yields cells coexpressing T/Bra and Sox2 (see Fig. 2B and Fig.S9). Measuring the number of transcripts of Sox2 and T/Bra in the different populations reveals that they differ in the degree of correlated expression of the two genes (Fig. 2B). ES-NMP exhibit a negative correlation (ρ = -0.12, P-value < 0.01) whereas Epi-NMP exhibit a very high correlation (ρ = 0.43, P-value << 0.01); as a reference, the correlation in EpiSCs treated with XAV (see Materials and Methods) is lower (ρ = 0.15, P-value << 0.01). The three EpiSCs populations (EpiXAV, EpiXAV-NMP, and Epi-NMP) exhibit low levels of Sox2 expression, which is a hallmark of posterior epiblast (Corsinotti et al., 2017) and analysis of the mean and the variability values of the expression of T/Bra highlights Epi-NMP as the population with the higher average and the lower variance in the expression of T/Bra (Fig. 2B). These results suggest that Epi-NMPs exhibit a higher number of T/Bra-Sox2 positive cells than the other ES derived NMPs and that they might be found in a transcriptional stable state (low variance of T/Bra and Sox2) in comparison to the other conditions.

To characterize the different putative NMP populations further, we investigated the expression levels of 37 genes in total throughout this work, out of which we tested 27 genes in the comparison of the three different protocols to produce NMP that we have identified as reflecting the CE at E8.5 (see Fig. S2). Both of the ES derived NMPs exhibit clear expression of Cdh1 and Oct4 and low levels of Fgf5 and Otx2, with ES-NMPF cells expressing high levels of genes associated with mesendoderm differentiation: Mixl1 (endoderm), Tbx6 (paraxial mesoderm) and extraembryonic mesoderm (Evx1) (Fig. 2D, S2 and see also (Gouti et al., 2014)). This suggests that these populations contain high numbers of differentiating cells. On the other hand, Epi-NMPs are in a very different state: in addition to T/Bra, Sox2 and Nkx1.2 these cells express significant levels of Nodal, Fgf8, Fgf5, FoxA2 and Oct4 together with CYP26a1 (see Fig. 2D and Fig. S2); this signature suggests that, on average, these cells are in a state that resembles the late epiblast (about E7.5), when the node appears. The differences between the different populations are further emphasized by examination of the protein levels of some of these markers (see Fig S9-10). NMPs derived from ESCs exhibit high levels of Sox2, Oct4 and Ecad expression with some cells expressing Otx2 and, in the case of ES-NMPF we observe a surprising combination at the level of single cells of NCad and ECad. In contrast, the Epi-NMPs exhibit lower level of Sox2 and Oct4 and a mutual exclusive expression of Ecad together with Ncad, which is a characteristic of the late Epiblast.

A.

Origin	D1	D2	D3		D4	D5	Outcome
ESCs	N2B27	N2B27	Chiron	↗	N2B27	N2B27	ES-neuro
				↘	FGF + Chiron	FGF + Chiron	ES-meso
EpiSCs	Activin + FGF	FGF	FGF + Chiron	↗	N2B27	N2B27	Epi-neuro
				↘	FGF + Chiron	FGF + Chiron	Epi-meso

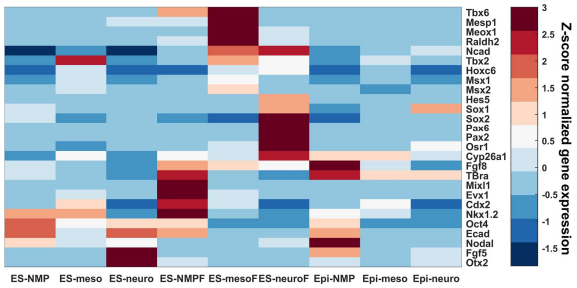
* All the different media are N2B27 based

B.

Origin	D1	D2	D3	D4	D5	Outcome
ESCs	GFF	FGF	FGF + Chiron	Retinoic Acid	Retinoic Acid	ES-neuroF
ESCs	FGF	FGF	FGF + Chiron	Chiron	Chiron	ES-mesoF

* All the different media are N2B27 based

C.



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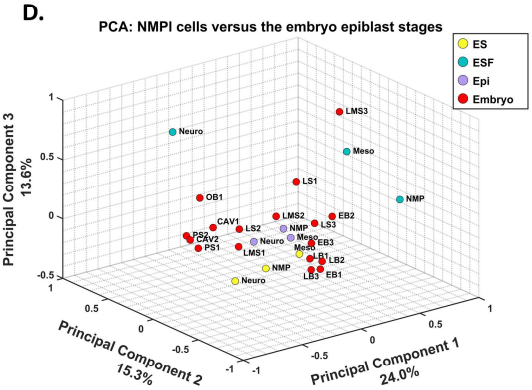

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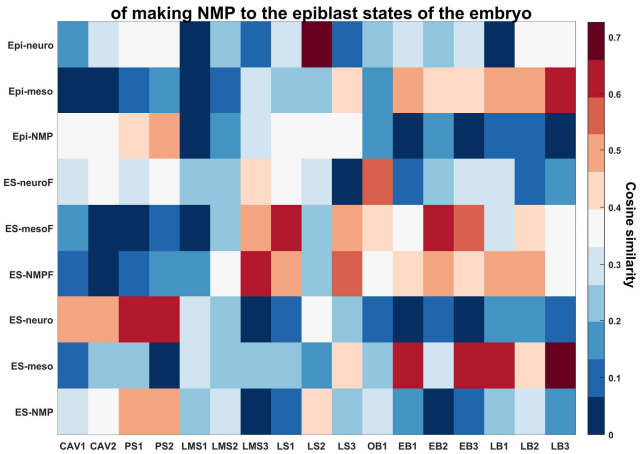

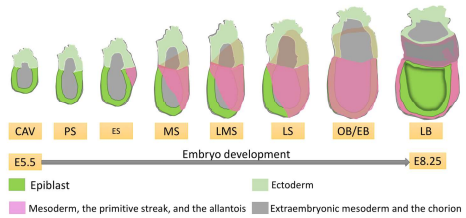

Fig 3. Differentiation NMP-l cells into neural and mesodermal precursors. A-B. Differentiation protocols followed for each of the NMP-l cells. **A.** ES cells and EpiSC were grown for 3 days accordingly to specific protocols (see Fig.2 and text), the base medium in all conditions is N2B27. After 3 days, the NMP-l cells were split into 2 flasks and cultured for 2 days in a medium that allows differentiation to either neural or mesodermal cells: N2B27 alone for neural or FGF + Chiron for mesoderm (see Materials and Methods for further details). **B.** In the case of the ES-NMPF we followed the published protocol {Gouti, 2014 #1936} and did not split/passage the cells which were grown for 5 days in the same flask in the neural or mesodermal conditions: FGF – FGF - FGF + Chiron - Retinoic acid - Retinoic acid for neural differentiation; FGF – FGF – FGF + Chiron- Chiron - Chiron for mesodermal differentiation, in all conditions the base medium is N2B27. **C.** Expression heatmap of 27 genes, obtained by RT-qPCR, in cells grown in the different conditions, as indicated (and see A). The normalized expression of each gene across the different conditions was scaled via calculating the Z-score (see Materials and Methods and supplement Fig. S2). **D.** Microarray gene expression data of the epiblast/ectoderm (excluding the

primitive streak) from different stages of the mouse embryo {Kojima, 2014 #2318} was used as an anchor to compare the different protocols to the embryo; staging is shown at the right-bottom of the figure (after Kojima et al. 2014), where green indicates the epibast/ectoderm; pink the mesoderm, the primitive streak, and the allantois and grey the extraembryonic mesoderm and the chorion: CAV, cavity; PS, pre-streak; ES, early-streak; MS, mid-streak; LMS, late mid-streak; LS, late streak; OB/EB, no bud/early bud; LB, late bud. Principal component analysis (PCA) was performed on the expression of 27 genes in C. for the in vitro protocols, their differentiation and the different stages of the epiblast mouse embryo (see text for details). **E.** The red-blue heatmap reflects the value of the pairwise cosine similarity measure that was calculated based on the expression of the 27 genes as in C. between the NMP in vitro protocols or their differentiation and the different stages of the epiblast mouse embryo. Dark blue (value of 0) indicates dissimilarity and dark red (value of 1) indicates maximal similarity (see Materials and Methods and supplementary Fig. S1A). Notice that the ES-NMPF is a more heterogeneous and differentiated population than the other two.

To test the ability of the NMP-I populations to differentiate into mesodermal and neural precursors we set them into specific differentiation conditions (see Materials and Methods and Fig. 3A-B). We named the resulting populations ES-neuro/ES-neuroF and ES-meso/ES-mesoF for those with an ES-NMP/ES-NMPF origin, and Epi-neuro and Epi-meso for those with an Epi-NMP origin. The resulting populations were tested for the expression of either 37 or 27 of our test genes (see Fig. 3A-C and Fig. S2). In all cases the cells differentiated but did that in a different manner depending on their origin (see Fig. 3C and supplement Fig. S6-8). In particular, ES-NMPF cells in contrast to the other NMP protocols, express high levels of mesendodermal affiliated genes (Tbx6, Mixl1 and Evx1) and the profile does not change much when cells are differentiated into mesoderm (ES-mesoF) confirming the mesodermal bias of this population (Fig. 2D and Fig. 3C). Surprisingly, when these cells are placed in neural differentiation conditions we observe the emergence of a mixed set of markers associated with LPM and IM (Pax2, Osr1 and neural fates (Pax6 and Sox1).

Altogether these results suggest that different protocols yield related but different NMP-1 populations which might have different functional properties.

Developmental staging of in vitro derived NMP populations

The differences between the candidate NMP populations derived in vitro suggest that they might represent different stages of the transition between the postimplantation epiblast and the CLE. To test this, we created a developmental stage reference using a microarray study of the epiblast at different embryonic stages between early postimplantation (E5.5) and pre-CLE (E8.25) (Kojima et al., 2014), and mapped the NMP populations, as well as their differentiated cells, onto it. A Principal Component Analysis (PCA) of these data using the 27 genes selected for our study (see Fig. 3D and Fig. S1A) or only using 23 genes out of the 27 genes (excluding Pax2, Msx1, Msx2 and Osr1, see Fig. S5) enabled us to recapitulate, mainly along PC1, the trajectory obtained using the complete microarray data, thus validating the use of our reduced set of genes for mapping states (see Materials and Methods, Figure 3D, Fig. S1B-C and Fig. S5A). With this as a reference, we observe that in PC1-3 space, ES-NMP and Epi-NMP mapped closely to the late mid streak (LMS) and the late streak (LS) embryonic stages, whereas the ES-NMPF and its neural and mesodermal differentiation populations are separate from these conditions and from the embryonic stages (see Fig. 3D and Fig. S1B). The ES-meso and Epi-meso project closely to each other within the embryo trajectory between the LMS and early bud (EB) stages (see supplement Fig. S1B). The ES-neuro population seems to be comparable to the early stages of the embryo epiblast

(projected closely to the cavity (CAV) and PS stages), whereas the Epi-neuro is a more progressive population which is mapped closely to LMS-LS stages.

We also used our developmental reference to explore the proximity of the in vitro derived populations to specific epiblast states in vivo by finding a gene expression metric. To do this we calculated the cosine similarity between the different cells populations and the different epiblast stages as shown in Figure 3E (see Materials and Methods and Fig. S1A). This 'similarity score' suggests that the ES-NMP and ES-NMPF correlate with different stages: the ES-NMPF population appears spread over several late stages of epiblast development and, in agreement with the expression analysis, resemble ES-mesoF, though the ES-NMP population show a similarity to earlier stages than the ES-NMPF. The Epi-NMP population appears not to show a clear similarity to any stages, maybe a minor similarity to the PS stage. ES-neuro show a clear similarity to an earlier stage (PS) and according to its gene expression profile, those cells represent an anterior neural differentiation. ES-meso exhibit high similarity to the EB and late bud (LB) stages, which ranges between E7.75-E8.25. Epi-meso, interestingly resembles only the LB stages, which correspond to E8.25, the stage where the CLE harbours the NMP.

Altogether these results show that different starting conditions and differentiation protocols lead to populations with different identities and representations. The NMP-l population derived from EpiSCs, here stated under the name of Epi-meso, is the one closer to the CLE in the embryo, where the NMPs are established. An interpretation of these results is that ES-NMP represents a heterogeneous early population, while ES-NMPF is a heterogeneous population dispersed over several stages with much differentiation. Epi-NMPs appear to be a tighter population resembling a later epiblast.

Multiple tailbud fates emerge from differentiating ESCs and EpiSCs in culture

In the course of our survey of cell type markers in the different populations, we noticed that all protocols that lead to coexpression of T/Bra and Sox2 also lead to the expression of genes not associated with NMPs e.g. Mesp1, Evx1, Gata6, BMP4, Msx1,2, Osr1, Pax2 and Tbx2 (see Fig. 3C and Fig. S2 and see (Amin et al., 2016; Gouti et al., 2014)). A survey of the literature shows that in the embryo between E7.0 and E8.5, roughly the stage of the differentiating cells, these genes are expressed in the CE and in the posterior region of the Primitive Streak in the progenitors of the allantois (Tbx2,4, Mesp1 and Evx1), the LPM (msx1,2) and the IM (Pax2, Osr1) (see supplementary information file S1). This suggests that the three populations are not restricted to harbour merely NMPs and to differentiate to SC and paraxial mesoderm progenitors, but rather that they represent multi-potential populations with different degrees of bias.

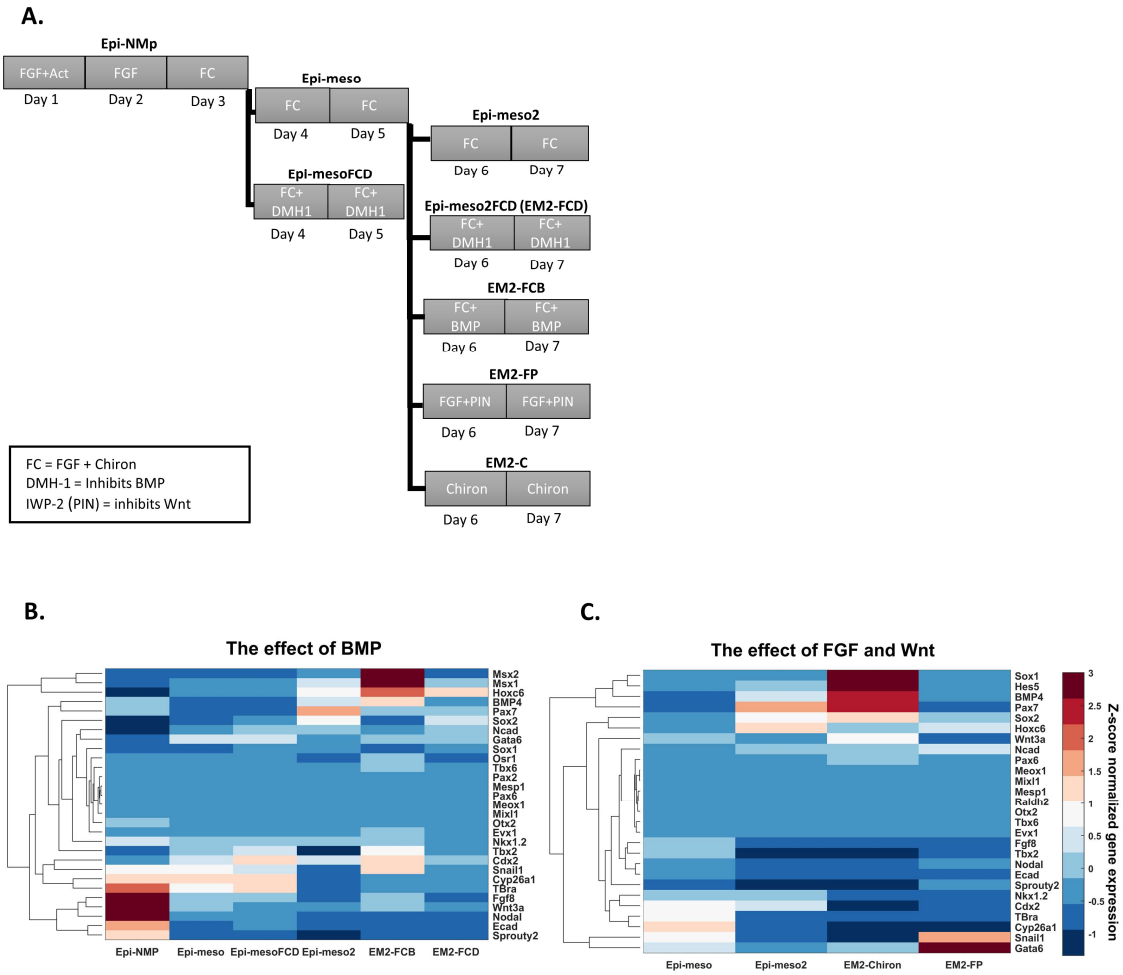

Fig 4. Effect of Wnt, FGF and BMP on mesodermal differentiation of the Epi-NMP population. **A.** Differentiation protocols for Epi-NMP into Epi-meso and Epi-meso2 with modulation of BMP signalling (DMH-1 as an inhibitor and BMP4 as an agonist), Wnt signalling (IWP-2 an inhibitor of Wnt secretion as a Wnt signalling antagonist) and without the addition of exogenous FGF (see Materials and Methods). **B -C.** Expression heatmap of the expression of a set of chosen genes to monitor the differentiation of cells grown as indicated. In all cases, the effect of differentiation was tested on the second passage. All measurements were from RT-qPCR and the normalized expression of each gene across the different conditions was scaled via calculating the Z-score (see Materials and Methods and supplement Fig. S3 and Fig. S4A). **B.** The effect of BMP: Epi-NMP and Epi-meso are compared to Epi-mesoFCD which is Epi-meso that was grown with FGF, Chiron and DMH-1 condition which inhibit BMP. Epi-meso and Epi-meso2 are compared to Epi-meso2 populations that their growing conditions either contained BMP (EM2-FCB) or DMH-1 (EM2-FCD). **C.** The effect of FGF and Wnt: Epi-meso and Epi-meso2 are compared to Epi-meso2 populations that their growing conditions either contained Chiron and no exogenous FGF (EM2-Chiron) or exogenous FGF with IWP-2 that blocks WNT (EM2-FP).

In the embryo, the differentiation of the CE is under the control of BMP signalling, that favours more posterior fates (LPM, IM and allantois progenitors) at the expense of more anterior, i.e. NMPs. To test this, we altered the levels of BMP on the Epi-meso population. Inhibition of BMP signalling at the stage of Epi-meso elevates the expression of NMP markers T/Bra, Sox2 and Cdx2 (Epi-meso versus Epi-mesoFCD in Fig. 4B and Fig. S3A). When passaging Epi-meso to make Epi-meso2 and maintain the NMP

signature in culture (see Fig. 4A), addition of BMP to this population (EM2-FCB) increases the levels of the genes on the posterior fates: BMP4, Msx1,2 and Tbx2 together with Cdx2 and Snail, compared to Epi-meso2 and EM2-FCD (Epi-meso2 with BMP signalling inhibition, see Fig. 4B and Fig. S3B).

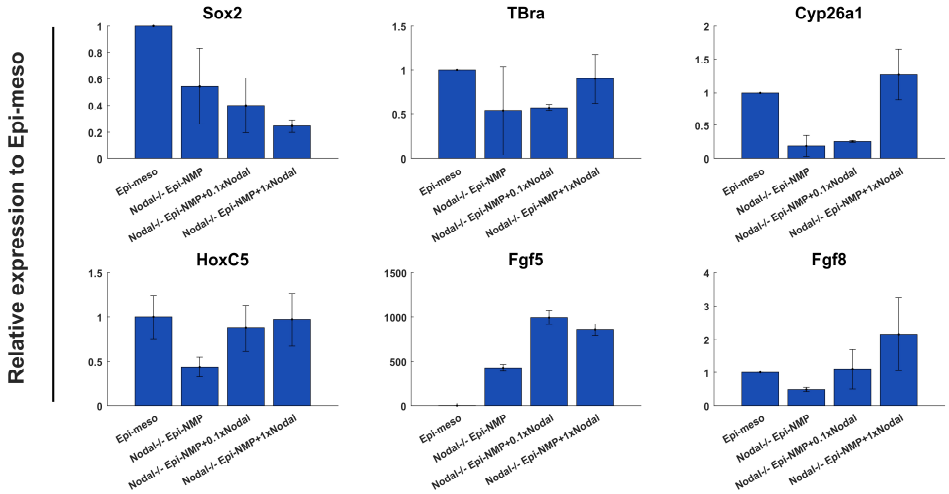

Fig 5. Comparison of expression of set of genes under the effect of Nodal signalling. Nodal null cells were cultured under the Epi-NMP protocol (Nodal -/- Epi-NMP). This population of cells was compared to the same population just with the addition of 2 doses of Nodal concentration to the growing medium of the Epi-NMPs on their 3rd day: FGF, Chiron and either 100ng/ml of Nodal (Nodal-/- Epi-NMP+0.1xNodal) or 1µg/ml of Nodal (Nodal-/- Epi-NMP+1xNodal). The bars represent the genes average expression across biological replicas obtained by RT-qPCR and the error bars indicate the standard error between those replicas. The gene expression across the different conditions was normalized to Epi-meso condition (see Materials and Methods).

In the embryo, the anterior region of the CLE is dominated by the node and we observe the expression of Nodal and FoxA2 in the Epi-NMP population (see Fig. 3C and Fig. S2A). We have observed before that suppressing Nodal signalling reduces the T/Bra, Sox2 coexpressing cells (Turner et al., 2014) and wondered whether Nodal signalling might have an effect on the NMP-I signature of this population. To do this, we cultured Epi-NMP from Nodal mutant EpiSCs (Nodal -/- Epi-NMP see Materials and Methods and Fig. 5) compared to Nodal mutant Epi-NMPs supplemented with 2 different doses of Nodal in the culturing medium on the third day: FGF, Chiron and either 100ng/ml of Nodal (Nodal-/- Epi-NMP+0.1xNodal) or 1µg/ml of Nodal (Nodal-/- Epi-NMP+1xNodal). Loss of Nodal leads to an increment of Sox2 and a decrease of Cyp26a1, Fgf5, Fgf8 and Hoxc5 expression. Addition of Nodal reduces the levels of Sox2 and increases the levels of T/Bra in these populations. These results suggest that Nodal signalling is necessary to maintain the relative levels of Sox2 and T/Bra characteristic of the NMP-I population. In agreement with this we also observe that Nodal influences the levels of Fgf8 and Cyp26a1, which are characteristic and associated with the CLE and the NMPs.

These results suggest that in all cases, differentiation of PSCs into a caudal population does not result in the specification of NMPs only, but rather of multipotent populations for all axial derivatives. The differences between the different protocols might not only result in different stages of development but also in different proportions of the different mesodermal populations.

Epi-NMPs create a population that can be propagated in vitro

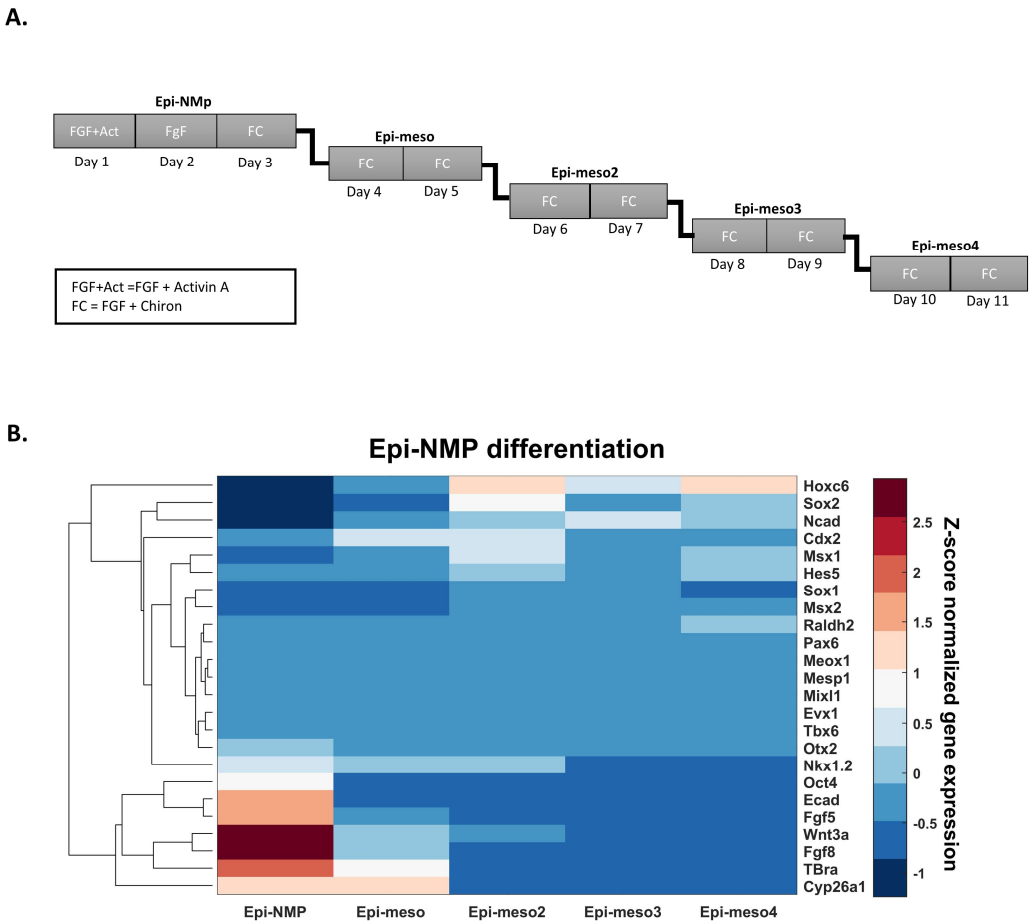

Fig 6. Differentiation of Epi-NMP. **A.** Differentiation protocol for Epi-NMP into mesodermal precursors; cells were passaged and cultured in FGF and Chiron at every passage to generate the different generation of Epi-meso cells (Epi-meso, Epi-meso2, Epi-meso3, etc. see Materials and Methods). **B.** Expression heatmap of 24 chosen genes, obtained by RT-qPCR, of the different cell populations. The normalized expression of each gene across the different conditions was scaled via calculating the Z-score (see Materials and Methods and supplement Fig. S4B).

In the embryo, the initial NMP population needs to be amplified if it is to account for the cellular mass along the length of the postoccipital region (Steventon and Martinez Arias, 2017; Wymeersch et al., 2016) and this amplification should be a criterion to identify the NMPs in vitro. Earlier studies have shown that ESC derived NMPs are not able to maintain the T/Bra, Sox2 coexpressing cells when passaged in Chiron alone or FGF and Chiron, the conditions in which they were generated (Gouti et al., 2014; Turner et al., 2014) and unpublished observations). We were surprised to observe that when Epi-NMPs are induced to differentiate into mesoderm by exposure to FGF and Chiron, they maintain T/Bra and Sox2 expression for at least two passages (see Fig. 6 and Fig S4B). We call these populations Epi-meso, Epi-meso2, Epi-meso3 (EM1, EM2, EM3…) and have explored their identity following the expression of our collection of markers.

During the passages, we observe a progressive decrease in the expression of NMP markers (T/Bra, Cyp26a1, Fgf8 and Nkx1.2) and a slow increase in the expression of

differentiation genes associated with neural fates: Ncad, Sox2, and Hes5 (see Fig. 6B and Fig. S4B). This is surprising since, while Epi-NMP cells differentiate well into neural progenitors when placed in N2B27, we expected them to differentiate into mesoderm when exposed to Wnt and FGF signalling and not to maintain a neural fate. When Epi-meso is grown in N2B27 supplemented with Chiron alone (EM2-Chiron, see Fig. 4A, 4C and Fig. S4A), we observe high expression of neural markers (Sox1, Sox2 and Hes5) together with Pax7 and BMP4. Furthermore, inhibition of Wnt in the Epi-meso2 state (EM2-FP, see Fig. 4A, 4C and Fig. S4A) reduces the expression of neural progenitor markers and elevates the expression of mesodermal ones (Gata6 and Snail1). Wnt and FGF create a signalling environment comparable with the CLE and our observations suggest that, when applied to Epi-NMPs, they induce a population that exhibit short term self renewal progenitors (EM1, EM2…) that resembles the NMPs. These observations are consistent with the report that, in the context of FGF, Wnt signalling promotes proliferation of the CLE/NMP population (Wymeersch et al., 2016) and does not affect neural specification nor Sox2 expression (Garriock et al., 2015). Furthermore, in the transition from Epi-NMP to Epi-meso, cells lose expression of epiblast markers e.g. Fgf5, Nodal, Otx2, Oct4 and Ecad (see Fig. 3C, Fig. S2A and protein expression of Oct4 in Fig. S9), hence this suggesting that Epi-meso population is a refined sate containing many features of the NMPs that are a subset of the CLE.

Epi-NMPs and the Epi-meso contribute to axial extension

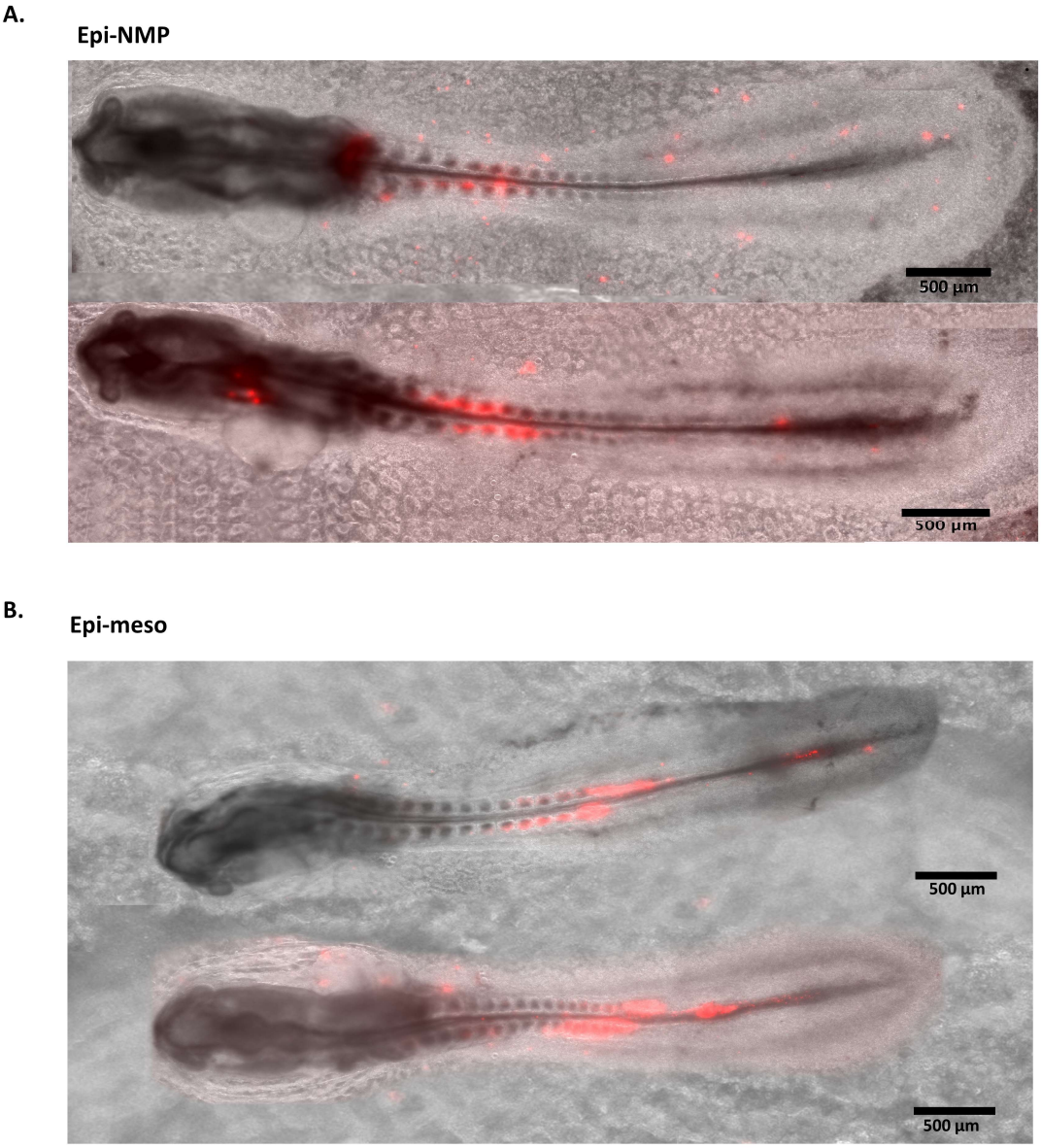

Fig 7. Epi-NMP and Epi-meso are competent to produce NMP-l contributions on transplantation in a chicken embryo. **A-B.** Labelled Epi-NMP (A.) and Epi-meso (B.) (marked in red) were transplanted into the caudal and lateral region to the node. The embryos are position that their head (A) are on the left-hand side and their tail (P) on the right-hand side.

A most important feature of a cell type is its ability to fulfil a function in a developing embryo. In the case of mouse NMPs, in addition to their ability to differentiate into neural and mesodermal progenitors, they contribute to axial elongation (Amin et al., 2016; Cambray and Wilson, 2002; Tzouanacou et al., 2009). Therefore, NMP-l populations should be tested for this ability in vivo. While the most stringent test would be the transplantation of these cells into mouse embryos (Gouti et al., 2014), chicken embryos have been shown to be reliable hosts for the functionality of neural and mesodermal progenitors (Fontaine-Perus et al., 1997; Fontaine-Perus et al., 1995; Gouti et al., 2014).

Application of this technique to ESCs and different ESC derived NMPs confirms that it is a reliable assay of developmental potential (P. Baillie-Johnson and B. Steventon in preparation) and shows that ESC derived NMPs have some ability to differentiate into neural and mesodermal precursors but do not make long contributions to axial elongation. When we use Epi-NMP and Epi-meso in this assay, we observe significant axial contributions of both populations in the chicken embryo that contrast with the limited and discontinuous of the ESC derived NMPs. Significantly, in the case of the Epi-meso we observe contributions to presomitic, somatic, preneural and neural tissues (see Fig. 7). We also observe an association of the transplanted cells with the node region of the host, a characteristic of the NMPs in the embryo and as likewise can be observed in the positive control: transplant of the chicken node in a host embryo (P. Baillie-Johnson and B. Steventon in preparation).

These results suggest that, in this assay, Epi-NMP and Epi-meso behave more like the NMPs of the embryo than the ESC based NMP-like cells.

Discussion

A large number of studies over the last few years have shown that PSCs represent excellent models for the study of cell fate decisions in development as their differentiation follows paths and temporal schedules similar to those in embryos (Keller, 2005; McCauley and Wells, 2017; Turner et al., 2016). In particular, the experimental versatility of the culture system allows an exploration of the processes in a manner that is not possible in the embryo and these studies have shown that while there are events and features of the differentiation process that occur in embryos which are not represented in culture, most if not all that happens in culture has a correlation with the embryo. However, for these studies to be significant it is important to establish the correlation between events in vitro and the events in the embryo. Here we have used ESCs and EpiSCs to study, in vitro, the emergence of a population of bipotential progenitors, NMPs, that, in the embryo, give rise to the paraxial mesoderm and spinal cord of the thoracic tract. Our results show that each of these protocols produce populations of cells with different gene expression signatures, functional ability and with two common denominators: coexpression of T/Bra and Sox2 as well as of a number of genes associated with LPM, IM and allantois i.e. all protocols produce multipotent populations. Therefore, the coexpression of T/Bra and Sox2 is not a univocal criterion to identify NMPs. We believe that the identification of these cells requires additional criteria, in particular self renewal and the ability to make long contributions to axial extension in association with the node (Gouti et al., 2015; Henrique et al., 2015; Steventon and Martinez Arias, 2017; Wilson et al., 2009). Applying these to differentiating PSCs populations we identify a specific EpiSC based protocol that yields NMPs similar to those of the embryo in terms of cellular function and gene expression. This population emerges from a late epiblast like state and can also give rise to LPM, IM and extraembryonic mesoderm in a signalling dependent manner. ESC based protocols yield similar cells that can be differentiated into mesodermal and neural progenitors but lack several features characteristic of NMPs. Furthermore, as we have shown here, these cells appear to be spread throughout several, and different, epiblast stages and might represent different populations. It has been suggested recently that differentiating cells can reach a final state through a variety of paths (Briggs et al., 2017) and this might be also the case for the ESC derived NMPs. However, these studies are based, mainly, on patterns of gene expression and whether the differentiated cells behave as bona fide postoccipital neural and mesodermal progenitors will require additional functional tests.

In the case of NMPs there are many studies in which Wnt signalling can caudalized epiblast like populations (Amin et al., 2016; Mazzoni et al., 2013; Neijts et al., 2016; Nordstrom et al., 2002; Nordstrom et al., 2006) but in these cases, which for the most part are ESC based, the NMP-l cells fail to self renew as they do in vivo.

In contrast to the ESCs based protocols, here we have shown that exposure to FGF and Chiron of pre-treated EpiSCs generates a population with a gene expression signature characteristic of a late CE, around the time of the appearance of the node. Upon further exposure to Wnt and FGF signalling, this population evolves and generates cells with many of the hallmarks of the NMPs, including limited but robust self renewal as well as ability to differentiate into neural and mesodermal progenitors in a Wnt dependent manner and long contributions to axial extension in a xenotransplant assay. For these reasons, we shall call the Epi-NMP population Epi-CE, and the Epi-meso Epi-NMP. A significant feature of the Epi-CE, that is in common with the ESC derived populations, and, also of the Epi-NMPs is the expression of markers for LPM, IM and allantois, suggesting that the NMPs are derived from a multipotent population that is likely to also exist in the embryo. We find that the fate of the Epi-CE cells is dependent on a balance between BMP and Nodal signalling and has a strict requirement for Wnt signalling in both neural and mesodermal lineages. The dependence on BMP and Nodal mirrors events in the embryo where BMP signalling promotes posterior (LPM, IM and allantois (Sharma et al., 2017)) and Nodal anterior (NMPs) fates. The importance of Nodal in the establishment of the multipotent population and perhaps also in the definition of the NMP domain is underpinned by our studies with mutants. Consistent with this, embryos mutant for FoxA2 which lack a node, exhibit deficiencies in the organization of the CLE and axial elongation (Ang and Rossant, 1994; Weinstein et al., 1994) and the same can be observed in embryos mutant for Smad2,3 (Vincent et al., 2003).

It is interesting that the protocol that we use to derive Epi-CE an Epi-NMP is based on a protocol used to derived these progenitors from human ESCs which have been shown to partially self renew (see Materials and Methods, Fig. 6 and (Lippmann et al., 2015)). EpiSCs are closer to human ESCs than mouse ESCs and therefore this observation emphasizes the possible importance of the initial state of the population for the paths and outcomes of differentiation

The transition between the Epi-CE and Epi-NMP is linked to the loss of expression of several genes that are associated with the epiblast e.g. Fgf5, Otx2 and, specially, Oct4, a POU domain transcription factor that, together with Sox2, maintains pluripotency. A similar transition can be observed in the embryo where Oct4 expression ceases at around E8.5/9.0 (Downs, 2008; Osorno et al., 2012), the time at which cells start differentiating. It is possible that the combination of Oct4 and Sox2 promotes multipotency and that only when Oct4 expression ceases then Sox2 starts playing a pro-neural role. A function for Oct4 in axial elongation can be gauged from the severe axial truncations that follow loss of Oct4 activity from E7.0/7.5 (DeVeale et al., 2013) and the extended axial elongations associate with overexpression of Oct4 (Aires et al., 2016) which may reflect an increase in the initial size of the multipotent CE pool rather than a specific alteration in the NMP population.

During the passage of the Epi-NMP population in the presence of Wnt and FGF signalling, we notice that the cells progressively lose T/Bra expression and increase Sox2. This is surprising since Wnt signalling is thought to suppress neural differentiation and promote mesodermal. However, while in the embryo this is true during the first phase of gastrulation, before E7.5, it might not be during the development of the caudal region of the embryo. When the trunk develops, there is a clear evidence that Wnt/β-

catenin signalling is required for the expansion of the neural progenitors in the spinal cord (Zechner et al., 2003) and an increase in Wnt/β-catenin signalling do not supress neural development (Garriock et al., 2015). Furthermore, the fact that Sox2 gene has a Tcf response element (Takemoto et al., 2006) and responds to Wnt signalling, suggests that the dependence of Sox2 that we observe is associated with the development of neural structures. Altogether these observations can account for our results and contrast with previous reports in which Wnt signalling suppresses neural fates (Gouti et al., 2017; Gouti et al., 2014; Turner et al., 2014). We suggest that the antagonism of Wnt signalling and Sox2 expression does not reflect an effect on neural fate but the assignation of posterior identity in the early epiblast (Corsinotti et al., 2017). This would be consistent with the observation that NMP-I cells derived from ESCs reflect early stages of development and that their differentiation bypasses the embryo path. On the other hand, there is ample evidence for a requirement for Wnt/β-catenin signalling in axial elongation (Cunningham et al., 2015; Dunty et al., 2008; Yamaguchi et al., 1999). This requirement might reflect a necessity for the proliferation of the CLE which has been hinted in some studies (Wymeersch et al., 2016).

In summary, we have identified a specific experimental protocol that, starting with EpiSCs yields a multipotent progenitor population for all postoccipital structures (Epi-CE) that can be further differentiated in vitro into NMPs (Epi-NMP) or LPM/IM depending on the signalling environment. Significantly the Epi-NMP population exhibits a limited but robust self renewing ability which, together with its gene expression signature, lead us to believe that it is an in vitro correlation of the in vivo NMPs. Our results suggest that there might not be NMP specific protocols that bypass the multipotent state observed in this work; we have found the signature of this state in the data of all published protocols.

Acknowledgements
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Materials and Methods

Cell culture

E14-Tg2A were grown in tissue-culture plastic flasks coated with either 0.1% gelatine (Sigma-Aldrich, G1890-100G) in PBS (with Calcium and Magnesium, Sigma-Aldrich, D8662) or 0.5% Plasma Fibronectin (FCOLO, 1mg/ml, Temecula) in PBS (with Calcium and Magnesium). The differentiation protocols are as the following:

<u>ES-NMP</u>

Cells were plated at a density of 4.44×10^3 cells/cm^2 in a 0.1% gelatine coated flask with a base medium of N2B27 (NDiff 227, Takara Bio) for 2 days. After 48hr N2B27 is supplemented with 3μM of CHIR99021 (Chiron 10mM, Tocris Biosciences) for additional 24hr, which are in total 72hr.

<u>ES- meso and ES-neuro</u>

ES-NMP cells were detached from the culture flask using Accutase (BioLegend 0.5Mm) and divided into 2 flasks coated with 0.5% Fibronectin at a dense of 7.5×10^3 cells/cm^2. To get ES-neuro the cells were grown for 2 days in N2B27 and for ES-meso the cells were grown in N2B27 supplemented with 20ng/ml FGF2 (R&D systems, 50μg/ml) and 3μM Chiron.

ES-NMPF, ES-neuroF, ES-mesoF(Gouti et al., 2014)

Cells were plated at a density of $5x10^3$ cells/cm^2 in a 0.1% gelatine coated CellBINDSurface dish (Corning) with a base medium of N2B27 supplemented with 10 ng/ml FGF2. After 48hr N2B27 is supplemented with 10 ng/ml FGF2 and 5μM Chiron for additional 24hr, which are in total 72hr. To induce neural SC identity (ES-neuroF) or mesodermal identity (ES-mesoF) day 3 – day 5 was followed by either N2B27 supplemented with 100nM RA (Sigma) or by N2B27 supplemented with 5μM Chiron respectively.

Epi-NMP

E14-Tg2A were grown on a 0.5% Fibronectin coated culture flask with N2B27 supplemented with 12ng/ml FGF2 and 25ng/ml Activin A (Stem Cells Institute 100μg/ml), this known as Epi-media with or without 20μM XAV939 (XAV Tocris Biosciences, 10mM) for at least 4 passages. Those cells known as EpiSCs (or EpiXAV when the β-catenin inhibitor XAV is used). Those cells can be tested to be EpiSC by seeding them in a colony assay density (67 cells/cm^2) in restricted medium (2i: N2B27 supplemented with 3μM Chiron and 1μM PD0325901 (PD03, Tocris Biosciences, 10mM)), resulting in no growth of cells, ensuring that the cells are no longer in the naïve pluripotent state and they moved on to the prime pluripotent state (data are not shown).

EpiSCs (treated with or without XAV) were plated at a $5x10^4$ cells/cm^2 density in a 0.5% Fibronectin pre-coated flask with Epi-media for the first day. Day 2 is followed by increasing the concentration of FGF2 to 20ng/ml in the base medium of N2B27 and removing Activin A or XAV if originally was used to grow the EpiSCs. On day 3, N2B27 is supplemented with 3μM Chiron which is added to the 20ng/ml FGF2. After 72hr those cells known as the Epi-NMP or EpiXAV-NMP if XAV was used in the Epi-media. This protocol is a variation of one that has been used to derive NMP-l cells from human ESCs (Lippmann et al., 2015)

Epi- meso and Epi-neuro

Epi-NMP (without XAV) cells were detached from the culture flask using Accutase and divided into 2 flasks coated with 0.5% Fibronectin at a dense of $5x10^4$ cells/cm^2. To get Epi-neuro the cells were grown for 2 days in N2B27 and for Epi-meso the cells were grown in N2B27 supplemented with 20ng/ml FGF2 and 3μM Chiron.

Epi-meso differentiation

Epi-meso (without XAV) cells were detached from the culture flask using Accutase and plated back to a 0.5% Fibronectin coated flask at a dense of $5x10^4$ cells/cm^2 for 2 days in N2B27 supplemented with 20ng/ml FGF2 and 3μM Chiron. The first passage from Epi-meso is named Epi-meso2 (EM2), the second passage is named Epi-meso3 (EM3) and so forth.

BMP, FGF and Wnt signalling

Epi-mesoFCD

Epi-NMP (without XAV) cells were detached from the culture flask using Accutase and plated back to a 0.5% Fibronectin coated flask at a dense of $5x10^4$ cells/cm^2 for 2 days in N2B27 supplemented with 20ng/ml FGF2, 3μM Chiron and 500nM dorsomorphin-H1 (DMH-1 5mM, Tocris Biosciences) which is a BMP inhibitor.

EM2-FCD

Epi-meso (without XAV) cells were detached from the culture flask using Accutase and plated back to a 0.5% Fibronectin coated flask at a dense of $5x10^4$ cells/cm^2 for 2 days in N2B27 supplemented with 20ng/ml FGF2, 3μM Chiron and 500nM DMH-1.

EM2-FCB

Epi-meso (without XAV) cells were detached from the culture flask using Accutase and plated back to a 0.5% Fibronectin coated flask at a dense of $5x10^4$ cells/cm^2 for 2 days in N2B27 supplemented with 20ng/ml FGF2, 3μM Chiron and 1ng/ml BMP4 (R&D Systems, 100μg/ml).

EM2-Chiron

Epi-meso (without XAV) cells were detached from the culture flask using Accutase and plated back to a 0.5% Fibronectin coated flask at a dense of $5x10^4$ cells/cm^2 for 2 days in N2B27 supplemented with 3μM Chiron alone.

EM2-FP

Epi-meso (without XAV) cells were detached from the culture flask using Accutase and plated back to a 0.5% Fibronectin coated flask at a dense of $5x10^4$ cells/cm^2 for 2 days in N2B27 supplemented with 20ng/ml FGF2 and 1μM IWP-2 (PIN 5mM, STEMGENT) which is a Wnt pathway inhibitor.

Nodal Null cells

Cells mutant for Nodal (Nodal-/-) were provided by J. Collignon and were derived from the 413.d mutant mouse line (Conlon et al., 1991), They were grown on a 0.5% Fibronectin coated culture flask with Epi-media: N2B27 supplemented with 12ng/ml FGF2 and 25ng/ml Activin A for at least 4 passages. The Nodal null EpiSCs were plated in $5x10^4$ cells/cm^2 density on a 0.5% Fibronectin pre-coated flask with Epi-media for the first day. Day 2 is followed by increasing the concentration of FGF2 to 20ng/ml, in the base medium of N2B27, and removing the Activin A. On day 3, N2B27 is supplemented with 3μM Chiron which is added to the 20ng/ml FGF2. After 72hr those cells known as the Nodal-/- Epi-NMP. In order to examine the role of Nodal in establishing the NMPs the Nodal mutant Epi-NMPs were supplemented with 2 different doses of Nodal in the culturing medium on the 3rd day: 20ng/ml FGF2, 3μM Chiron and either 100ng/ml of Nodal (R&D systems, sample name: Nodal-/- Epi-NMP+0.1xNodal) or 1μg/ml of Nodal (sample name: Nodal-/- Epi-NMP+1xNodal) in the base medium of N2B27.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cells using Trizol. First strand cDNA synthesis was performed with Superscript III system (Invitrogen) and the quantification of double-stranded DNA obtained with specific genes designed primers, using Quantifast SYBR Green PCR Master Mix (Qiagen) and the standard cycler program (Qiagen RotorGene Q). The qPCR was done in technical triplicates. The primers that have been used are available in Supplementary Material Table S1. All experiments were performed in biological duplicates or triplicates. Expression values were normalized against the housekeeping gene Ppia. To compare different experiments of qRT-PCR, each run of the qPCR included one common condition, in our case it was the Epi-meso. Each condition in every run was normalized to Epi-meso and averaged across biological replicas. Here are the steps to calculate the normalized gene expression values:

1. Identifying the C_t (threshold cycle) for each gene (technical triplicates) and calculating the expression values (2^{-Ct}).
2. Calculating the average and the standard deviation (std) for each gene from the triplicate expression values.

3. Dividing the average and the std of each gene in the expression value of Ppia.
4. The normalized gene expression values in condition x will be divided with the normalized gene expression values in the common condition in every qRT-PCR experiment (Epi-meso) as the following:
   F=x/y, where x denotes the expression of a gene at any condition and y denotes the expression of the same gene at Epi-meso condition. Both x and y have an error, the std that have been calculated in step 3: Dx and Dy, hence the total error is:

$$DF = \sqrt{\left(\frac{dF}{dx}Dx\right)^2 + \left(\frac{dF}{dy}Dy\right)^2} = \sqrt{\left(\frac{1}{y}Dx\right)^2 + \left(-\frac{x}{y^2}Dy\right)^2}$$

5. Averaging the biological replicas: F_1 and F_2 are biological replicas of the same gene in the same condition and their expression was normalized as the above steps. The average of the normalized expression and the error is calculated as the standard error:

$$\bar{F} = \frac{1}{N}\sum_{i=1}^{N} F_i$$

$$SE(F) = \frac{1}{\sqrt{N}(N-1)}\sqrt{\sum_{i=1}^{N}(F_i - \bar{F})^2}$$

   where N is the number of replicas (between 2 and 3).
6. Standardizing the normalized expression values of a gene to Z-score values across conditions was done according the equation below:

$$z = \frac{\bar{F} - \mu}{\sigma}$$

   Where μ and σ denote the average and the standard deviation of the normalized expression of a gene across all the conditions examined in this work respectively (the average and standard deviation of $\bar{F}$ for a specific gene across all the conditions).

Single molecule fluorescence *in-situ* hybridization (sm-FISH)

Single molecule RNA FISH was carried out as described previously (Nair et al., 2015). Cells were dissociated using Accutase, washed in PBS, fixed in 37% formaldehyde at room temperature and permeabilized and stored in 70% ethanol at 4°C. All washes and hybridizations were carried out in suspension. Wash buffers included 0.1% Triton X-100 to minimize losses to sticking on the walls. Samples were mounted between a slide and #1 cover glass, in the glucose-oxidase-based 2 × SSC anti-fade buffer. The probes for the genes (supplementary Table S2) were designed using StellarisTM website and bought via StellarisTM FISH probes (Biosearch Technologies)(Raj et al., 2008). Additional information about how the probes were designed, prepared and used can be found in (Raj et al., 2008). Cells were imaged within 24 to 48 h on a Nikon Ti-E wide field microscope, using a 60X oil-immersion objective and a cooled camera (Orca flash 4.0, Hamamatsu). The cells in the images were segmented manually and the spot-detection was done semi-automatic using a MATLAB graphic user interface (GUI) developed by Marshall J. Levesque and Arjun Raj at the University of Pennsylvania or home-made protocols written in ICY(de Chaumont et al., 2012).

Principal Component Analysis

Principal component analysis (PCA) (Nair et al., 2015) involves the assignment of data, in our case gene expression, to new coordinates named principal components or PCs. The variance of observed coordinates in each PC occurs in a decreasing order, observations (the samples) projected on PC1 have a greater variance than the same observations projected on PC2 and so on. The PCs were calculated according to the Z-scores expression values of the 27 genes expressed (see Fig. 3D and Fig. S1B-C) in the different stages of the mouse embryo epiblast/ectoderm and in the 3 in vitro protocols and their neural and mesodermal differentiation: ES-NMP, ES-NMPF and Epi-NMP.

Cosine similarity

Here we used cosine similarity as a measure of similarity between Z-scores expression values of list of genes in one condition versus other condition (i.e. Epi-NMp versus the mouse embryo epiblast stages (Kojima et al., 2014) per the same list of genes). The cosine similarity was calculated as the following:

$$similarity = \frac{\sum_{i=1}^{n} A_i B_i}{\sqrt{\sum_{i=1}^{n} A_i^2}\sqrt{\sum_{i=1}^{n} B_i^2}}$$

Where A and B represents the list of genes with their values of Z-score gene expression in two conditions and A_i and B_i are the components of these two vectors. The similarity was constrained to the positive space, where 0 indicates that the two vectors are dramatically opposite and 1 indicates maximal similarity.

Bright field images

All bright field images were taken on a Nikon Ti-E wide field microscope, using a 20X objective and a cooled camera (Orca flash 4.0, Hamamatsu).

Confocal and immunostaining

In 4-well (Ibidi), plastic tissue-culture dishes the different samples were grown. Samples were washed in BBS + $CaCl_2$ (50 mM BES Sodium Salt, 280 mM NaCl, 1.5 mM Na2HPO4, 1 mM $CaCl_2$ 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 + $CaCl_2$ supplemented with 0.5% BSA and 0.5% Triton X-100) before overnight antibody staining, following a wash with BBT and then the samples were incubated for 2hr with the desired fluorescently-conjugated secondary antibody. Prior to imaging, samples were washed with BBS + $CaCl_2$ and covered in a mounting medium (80% spectrophotometric grade glycerol, 4% w/v n-propyl-gallatein in BBS + $CaCl_2$).

The following primary antibodies were used: Brachyury N19 (goat; Santa Cruz Biotechnologies, sc17743, dilution 1:100), Oct3/4 (mouse; Santa Cruz Biotechnologies, sc5279, dilution 1:100), Sox2 (rabbit; Millipore, AB5603, dilution 1:200), Otx2 (goat, R&D AF1979 dilution 1:200), Ncad (mouse, BD Bioscience 610920 dilution 1:200) and Ecad (rat, Takara M108, dilution 1:100). Secondary antibodies (Goat-A488, Rabbit-A633, Mouse-A568, Rat-A633) were from Molecular Probes, made in donkey and used in a 1 in 500 dilution with Hoechst 33342 (H3570, dilution 1 in 1000; Invitrogen ThermoFisher). Samples were imaged using an LSM700 on a Zeiss Axiovert 200 M with a 63× 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).

Chicken Embryo Culture

Fertilised chicken eggs were stored in a humidified 10°C incubator for up to one week until required. Eggs were transferred to a humidified, rocking 37°C incubator for 24 hours prior to the preparation of embryo cultures, which was done according to a modified version of New Culture (New, 1955). Embryo cultures were incubated at 37°C prior to grafting and were fixed in 4% paraformaldehyde within 24 hours.

Graft Preparation and Transplantation: Cell cultures were prepared as described above. Adherent cell cultures were detached mechanically using a cell scraper in PBS (with calcium and magnesium) to lift intact colonies with minimal sample dissociation. The tissues were labelled by transferring them to a FBS-precoated FACS tube and were centrifuged at 170 x *g* for five minutes. The supernatant was discarded and the colonies washed by gentle resuspension in PBS (with calcium and magnesium), before the centrifugation step was repeated. The tissues were then resuspended gently in PBS (without calcium and magnesium) for labelling with DiI (Thermo Fisher Scientific Vybrant® V22885, 1% v/v) for 25 minutes in the dark, on ice. The labelled tissues were centrifuged at 170 x *g* for five minutes and the pellet was gently resuspended in PBS (with calcium and magnesium) for grafting.

Labelled tissues were grafted into the region of the chick caudal lateral epiblast as described in (P. Baillie-Johnson and B. Steventon in preparation), using an eyebrow knife tool. Embryo cultures were imaged as single time points and as time-lapses for 15-18 hours after grafting.

Embryo Microscopy and Image Analysis

Widefield images were acquired with a Zeiss AxioObserver Z1 (Carl Zeiss, UK) using a 5x objective in a humidified 37°C incubator, with the embryo cultures positioned on the lid of a six-well plate. An LED white light illumination system (Laser2000, Kettering, UK) and a Filter Set 45 filter cube (Carl Zeiss, UK) was used to visualise red fluorescence. Emitted light was recorded using a back-illuminated iXon800 Ultra EMCCD (Andor, UK) and the open source Micro-Manager software (Vale Lab, UCSF, USA). The open source FIJI ImageJ platform (Schindelin et al., 2012) and the pairwise stitching plugin (Preibisch et al., 2009) were used for image reconstruction and analysis.

Supplementary figures

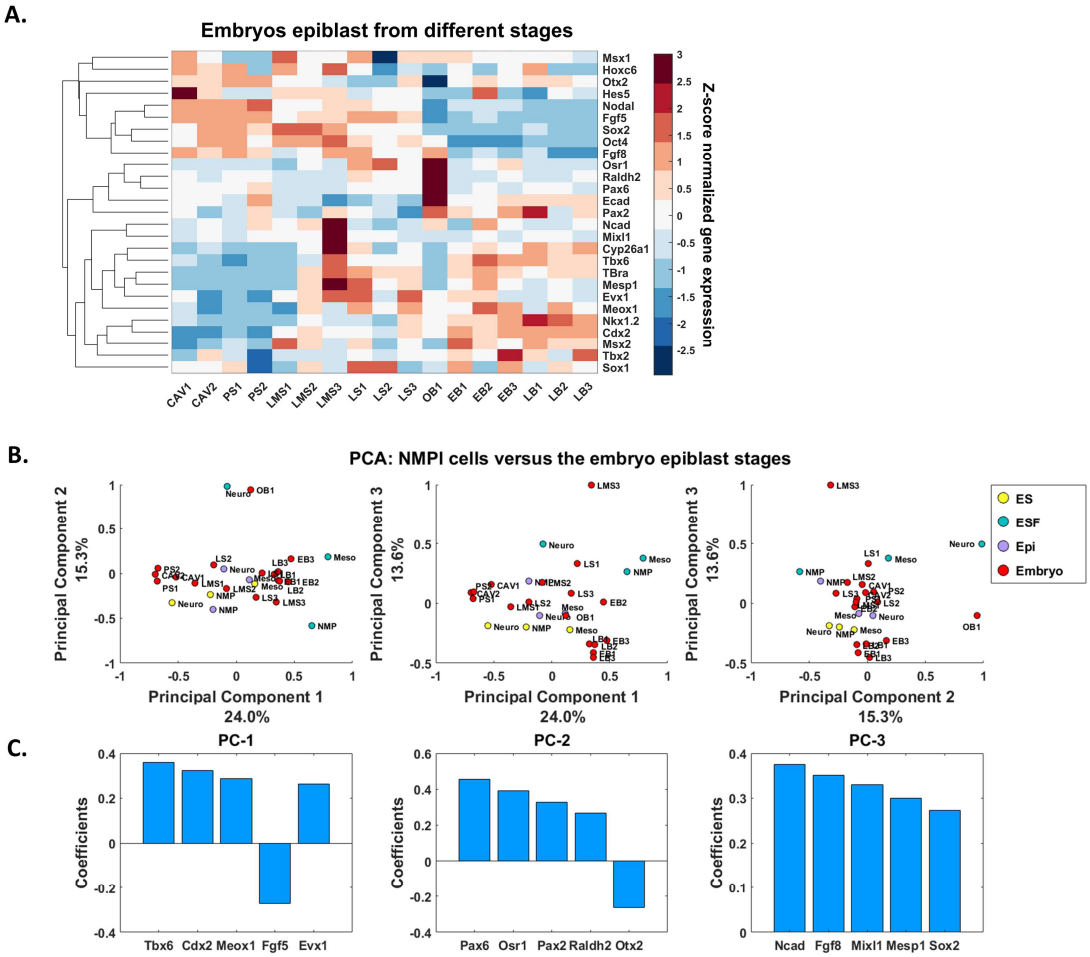

Fig S1. Comparison of expression of set of genes between the 3 in vitro protocols and the different embryo epiblast stages. **A.** Gene profile of the embryo epiblast in the different stages. Heatmap of the Z-score expression of the 27 genes (see Materials and Methods), was calculated from the microarray gene expression data of the epiblast/ectoderm (excluding the primitive streak) from different stages of the mouse embryo as published **{**Kojima, 2014 #2318**}. B - C**. PCA was performed on the expression of the 27 genes in the NMP in vitro protocols, their differentiation and the different stages of the epiblast mouse embryo as aforesaid in A. **B.** The expression data is presented by the first three components. **C.** The first five genes that most correlated with the three components.

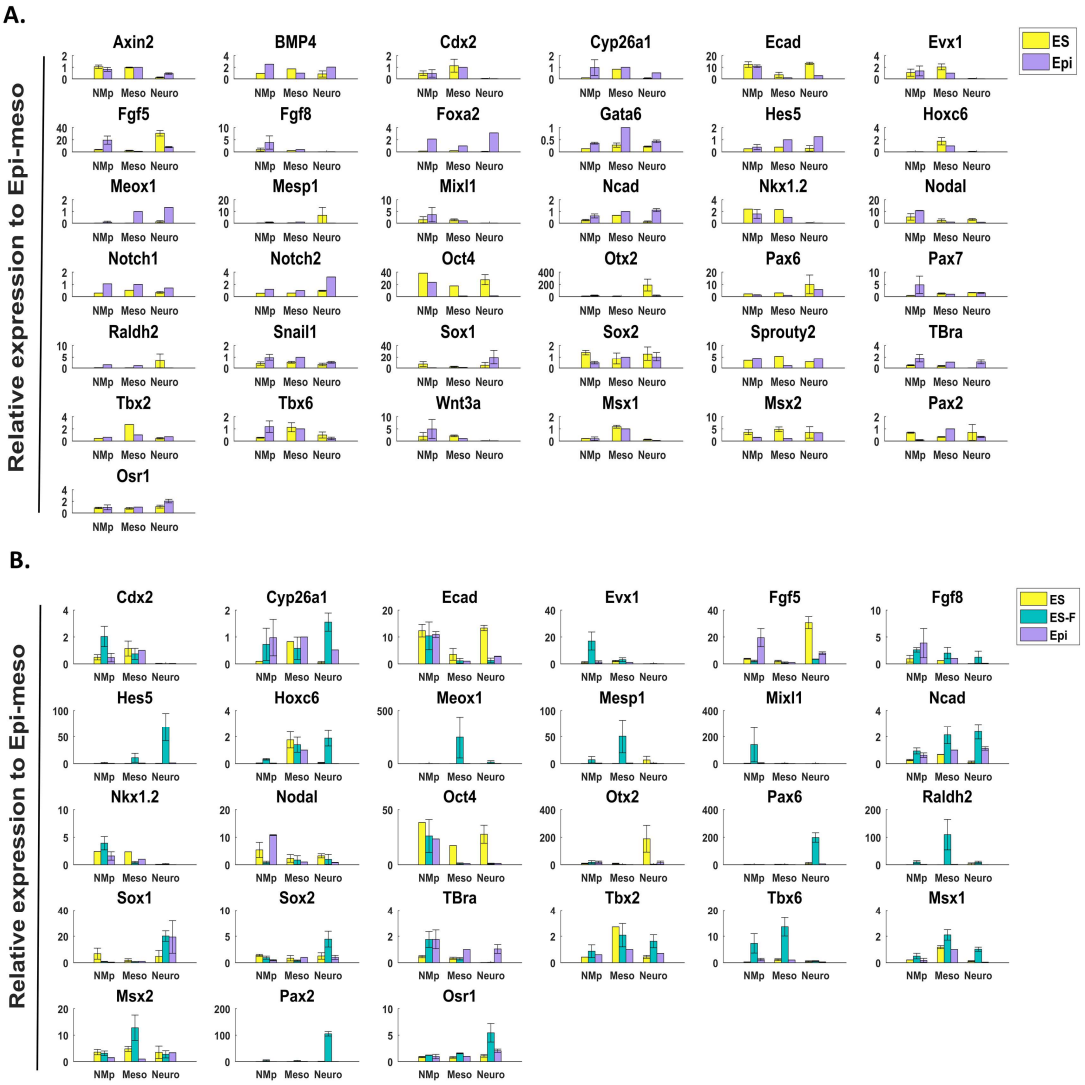

Fig S2. Comparison of expression of set of genes in the 3 in vitro protocols. **A.** Measuring the expression of 37 genes in ES-NMP and Epi-NMP. **B.** Measuring the expression of 27 genes in: ES-NMP, ES-NMPF and Epi-NMP. The bars represent the genes average expression across biological replicas obtained by RT-qPCR and the error bars indicate the standard error between those replicas. The gene expression across the different conditions was normalized to Epi-meso condition (see Materials and Methods).

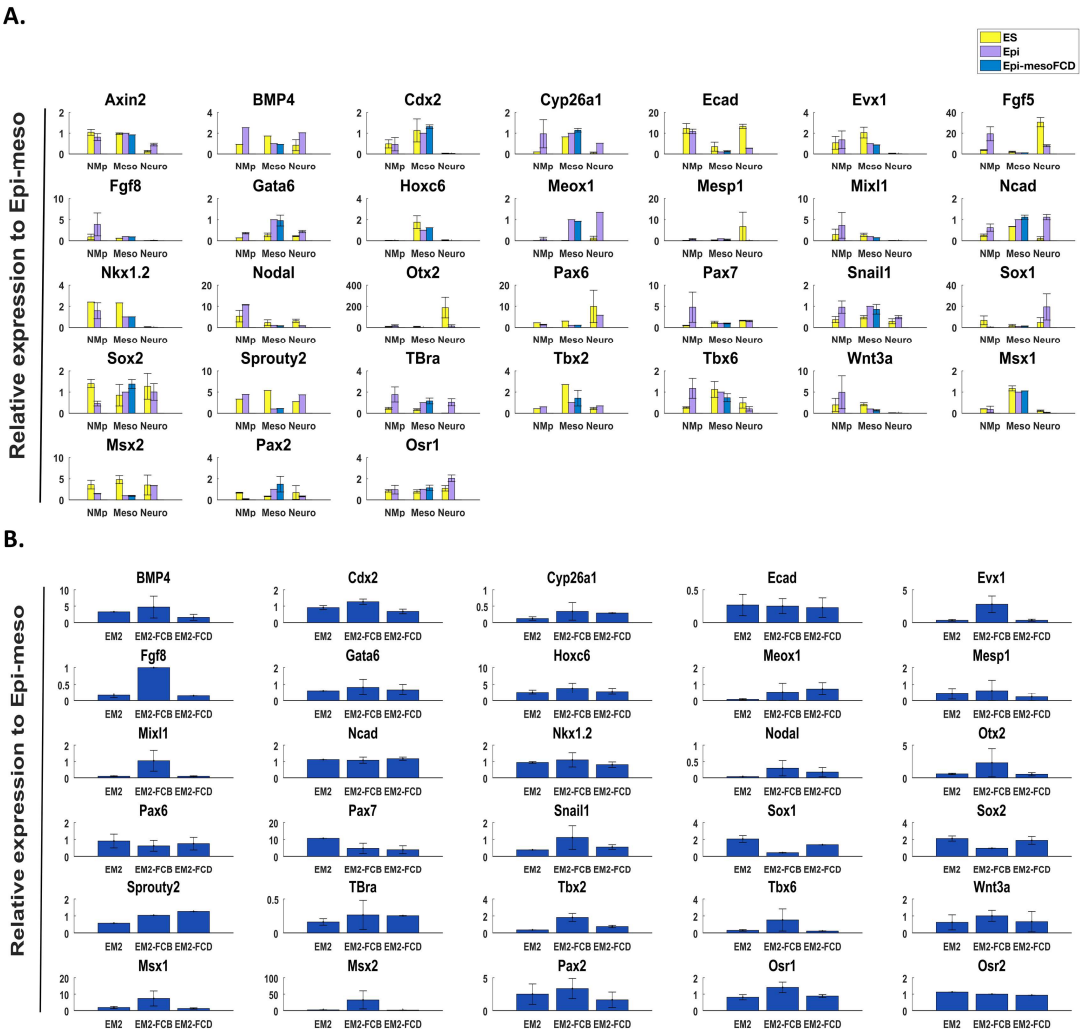

Fig S3. The influence of promoting or inhibiting BMP. A. Measuring the expression of 31 genes in ES-NMP and Epi-NMP and their differentiation to neural and mesodermal in comparison to Epi-meso condition supplement with BMP inhibitor, DMH-1. **B.** Measuring the expression of 30 genes in Epi-meso2 in comparison to Epi-meso2 condition supplement with either BMP or DMH-1 (BMP inhibitor) (see Materials and Methods). The bars represent the genes average expression across biological replicas obtained by RT-qPCR and the error bars indicate the standard error between those replicas. The gene expression across the different conditions was normalized to Epi-meso condition (see Materials and Methods).

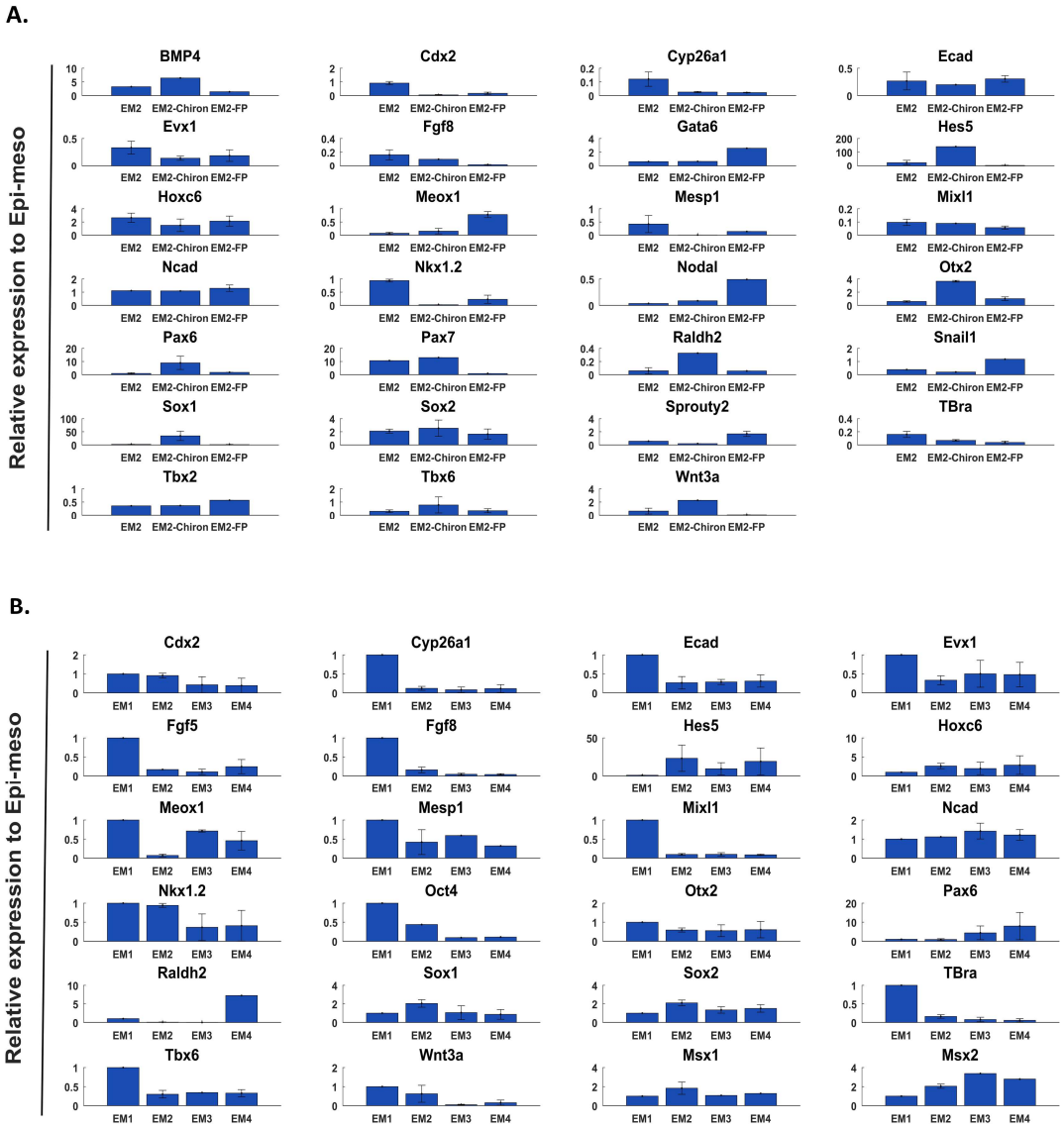

Fig S4. The influence of promoting or inhibiting Wnt and cultering Epi-meso over time. **A.** Measuring the expression of 27 genes in Epi-meso2 in comparison to Epi-meso2 condition with Chiron alone (no FGF, EM2-Chiron) or FGF with Wnt pathway inhibitor, IWP-2 (EM2-FP) (see Materials and Methods). **B.** Measuring the expression of 24 genes in Epi-meso (EM1) and its derivatives: Epi-meso2, Epi-mes3 and Epi-mes4 (EM2, EM3 and EM4). The bars represent the genes average expression across biological replicas obtained by RT-qPCR and the error bars indicate the standard error between those replicas. The gene expression across the different conditions was normalized to Epi-meso condition (see Materials and Methods).

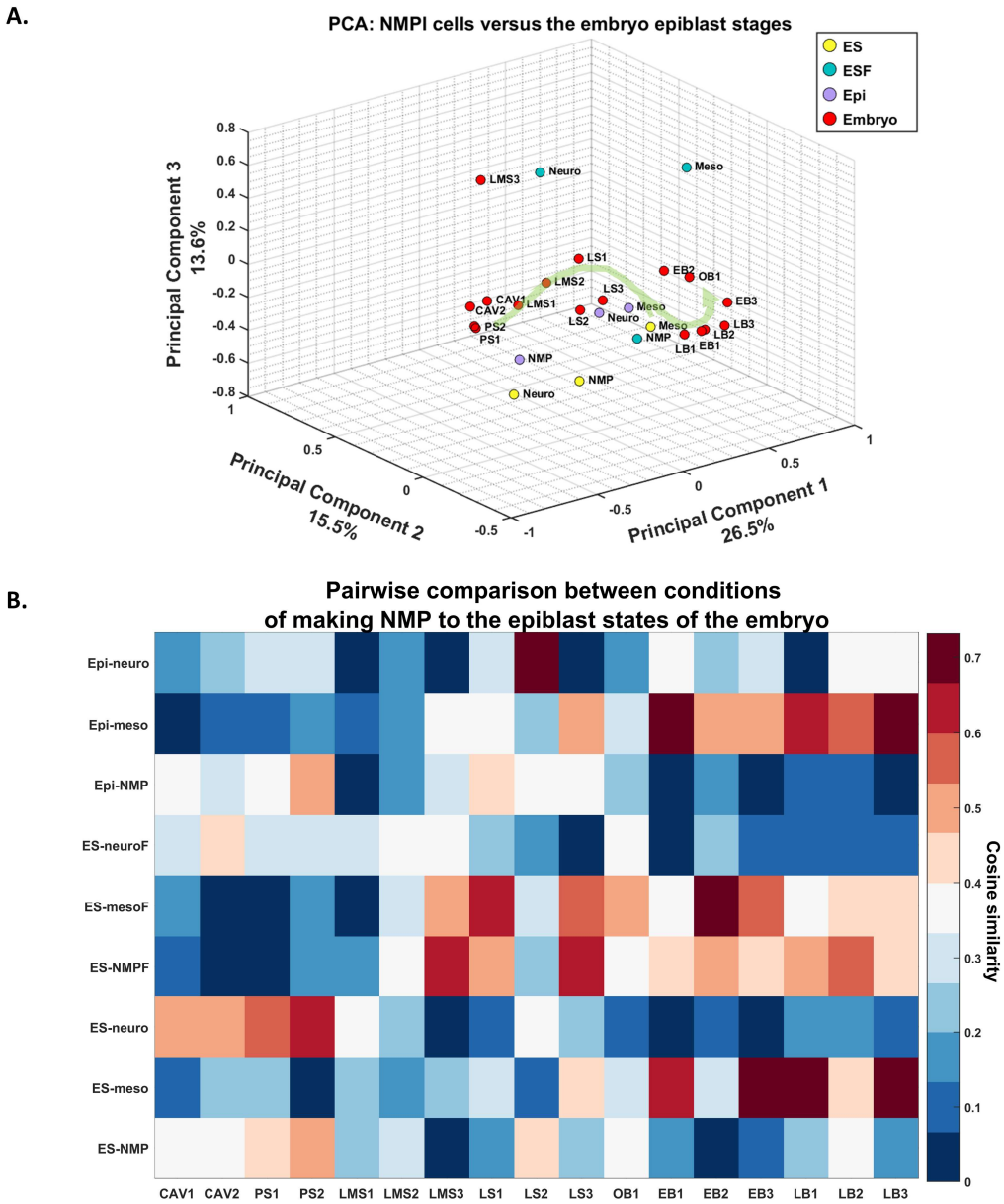

Fig. S5. Comparison of the NMP-I cells to the epiblast stages of the embryo according to 23 out of the 27 genes, excluding Pax2, Msx1, Msx2 and Osr1 (known to be expressed in the IM and LPM). A. Microarray gene expression data of the epiblast/ectoderm (excluding the primitive streak) from different stages of the mouse embryo as published in {Kojima, 2014 #2318}, was used as an anchor to compare the different protocols. Principle component analysis (PCA) was performed based on the expression of 23 genes (mentioned in Fig. 3C excluding Pax2, Msx1, Msx2 and Osr1) in the NMP in vitro protocols, their differentiation and the different stages of the epiblast mouse embryo {Kojima, 2014 #2318}. **B.** The red-blue heatmap reflects the value of the pairwise cosine similarity measure that was calculated based on the expression of the 23 genes as in A. between the NMP in vitro protocols or their differentiation and the different stages of the epiblast mouse embryo {Kojima, 2014 #2318}. Dark blue (value of 0) indicates dissimilarity and dark red (value of 1) indicates maximal similarity (see Materials and Methods).

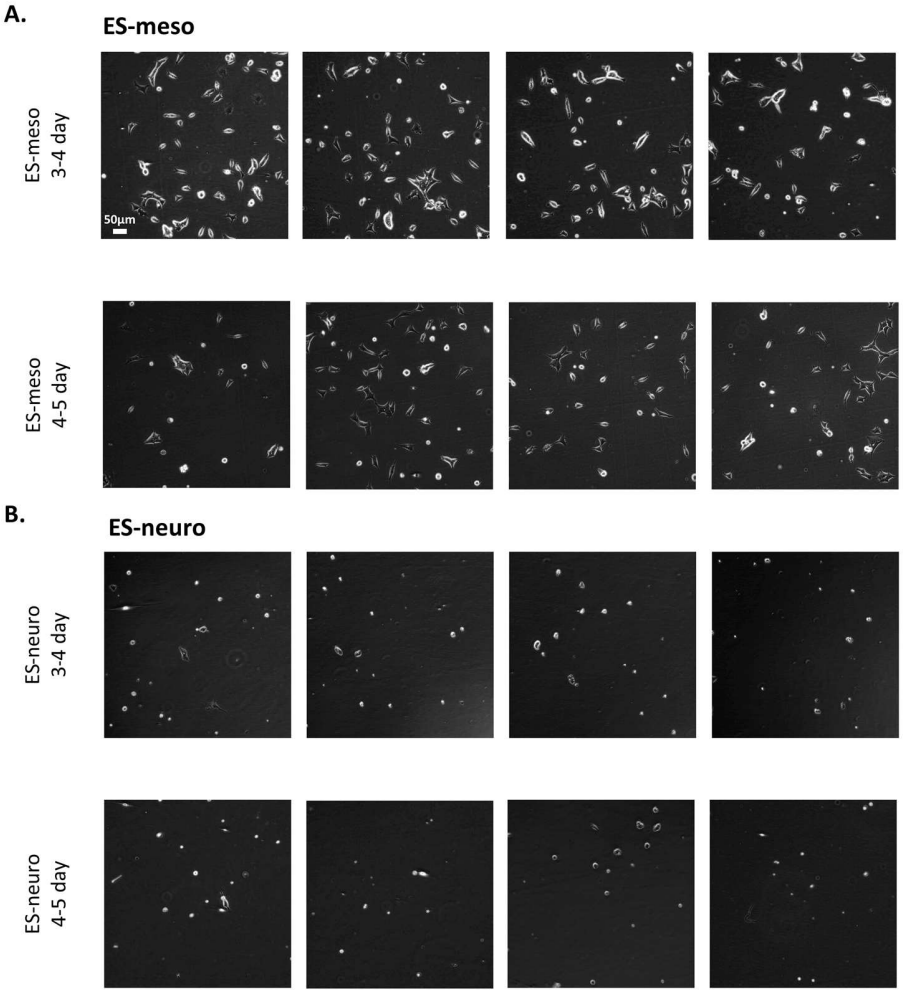

Fig S6. Brightfield images of ES-NMP differentiation. **A.** ES-meso cells were imaged between their 3rd and 4th day (1st row) and 4th and 5th day (2nd row), the cells were treated according to the ES-meso protocol (see Materials and Methods). **B.** ES-neuro cells were imaged between their 3rd and 4th day (1st row) and 4th and 5th day (2nd row), the cells were treated according to the ES-neuro protocol (see Materials and Methods). All the images were taken on a Nikon Ti-E wide field microscope, using a 20X objective and a cooled camera (Orca flash 4.0, Hamamatsu).

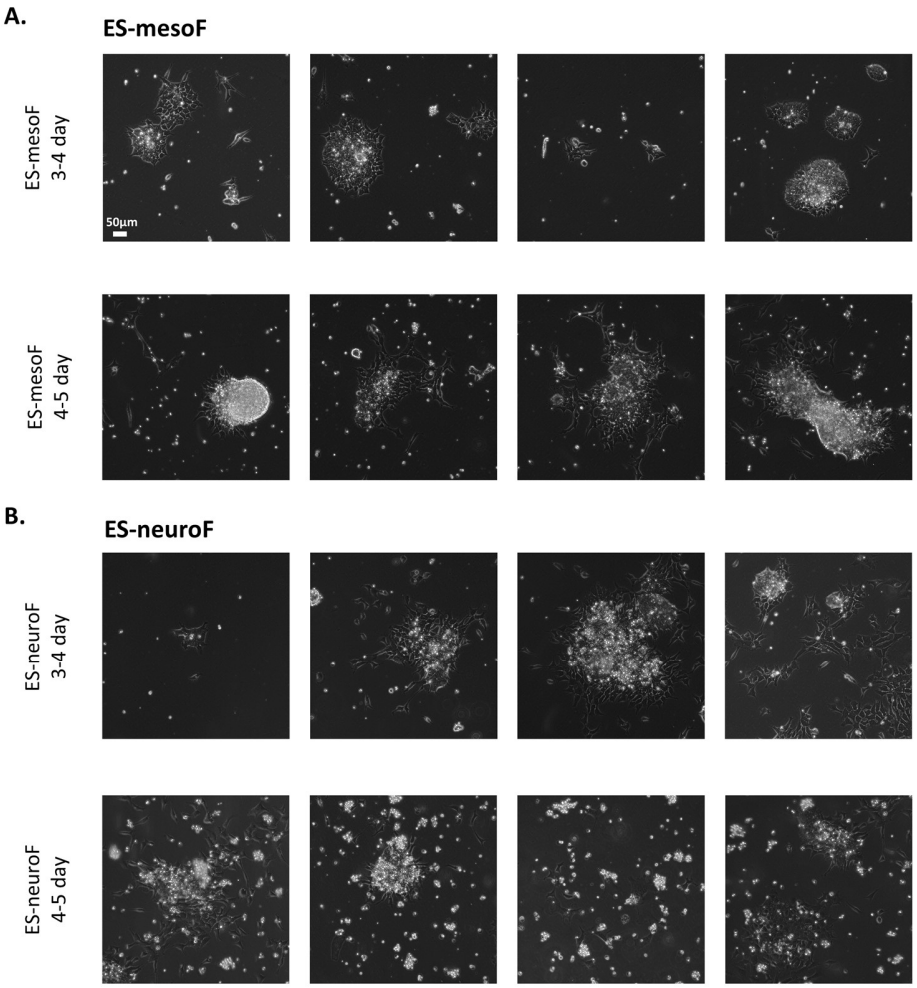

Fig S7. Brightfield images of ES-NMPF differentiation. **A.** ES-mesoF cells were imaged between their 3rd and 4th day (1st row) and 4th and 5th day (2nd row), the cells were treated according to the ES-mesoF protocol (see Materials and Methods). **B.** ES-neuroF cells were imaged between their 3rd and 4th day (1st row) and 4th and 5th day (2nd row), the cells were treated according to the ES-neuroF protocol (see Materials and Methods). All the images were taken on a Nikon Ti-E wide field microscope, using a 20X objective and a cooled camera (Orca flash 4.0, Hamamatsu).

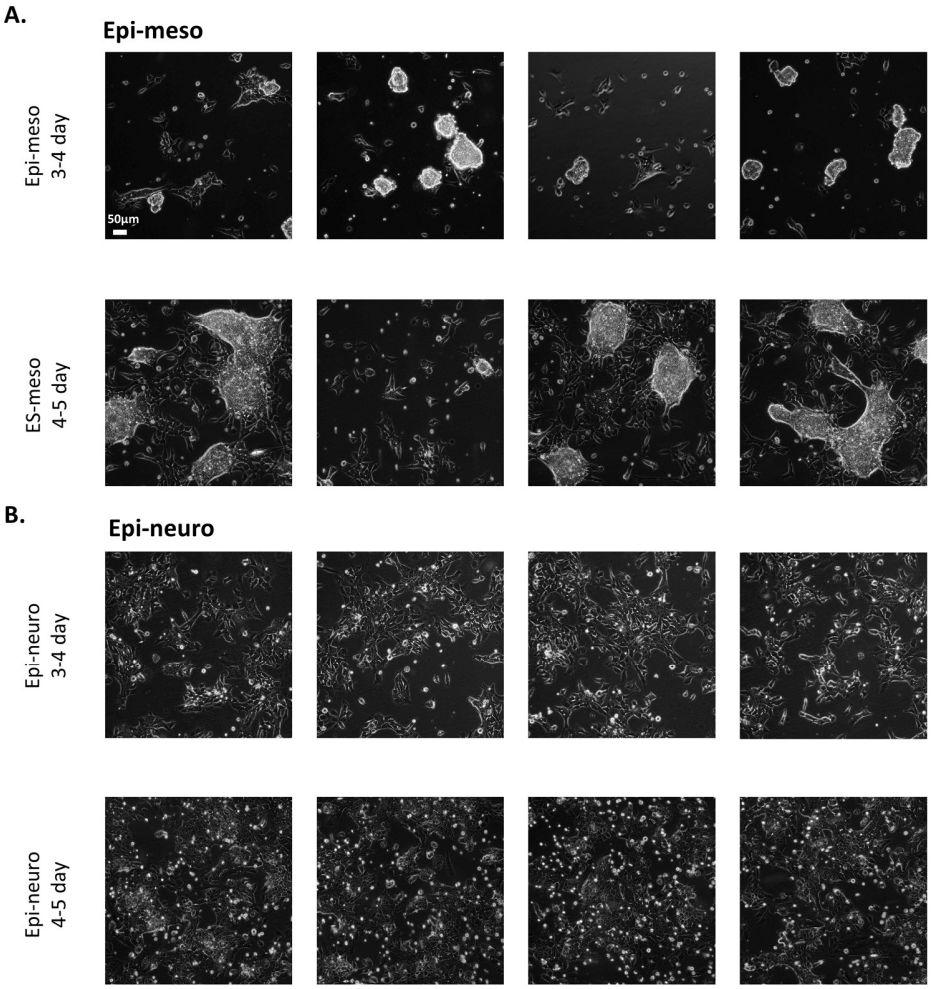

Fig S8. Brightfield images of Epi-NMP differentiation. A. Epi-meso cells were imaged between their 3rd and 4th day (1st row) and 4th and 5th day (2nd row), the cells were treated according to the Epi-meso protocol (see Materials and Methods). **B.** Epi-neuro cells were imaged between their 3rd and 4th day (1st row) and 4th and 5th day (2nd row), the cells were treated according to the Epi-neuro protocol (see Materials and Methods). All the images were taken on a Nikon Ti-E wide field microscope, using a 20X objective and a cooled camera (Orca flash 4.0, Hamamatsu).

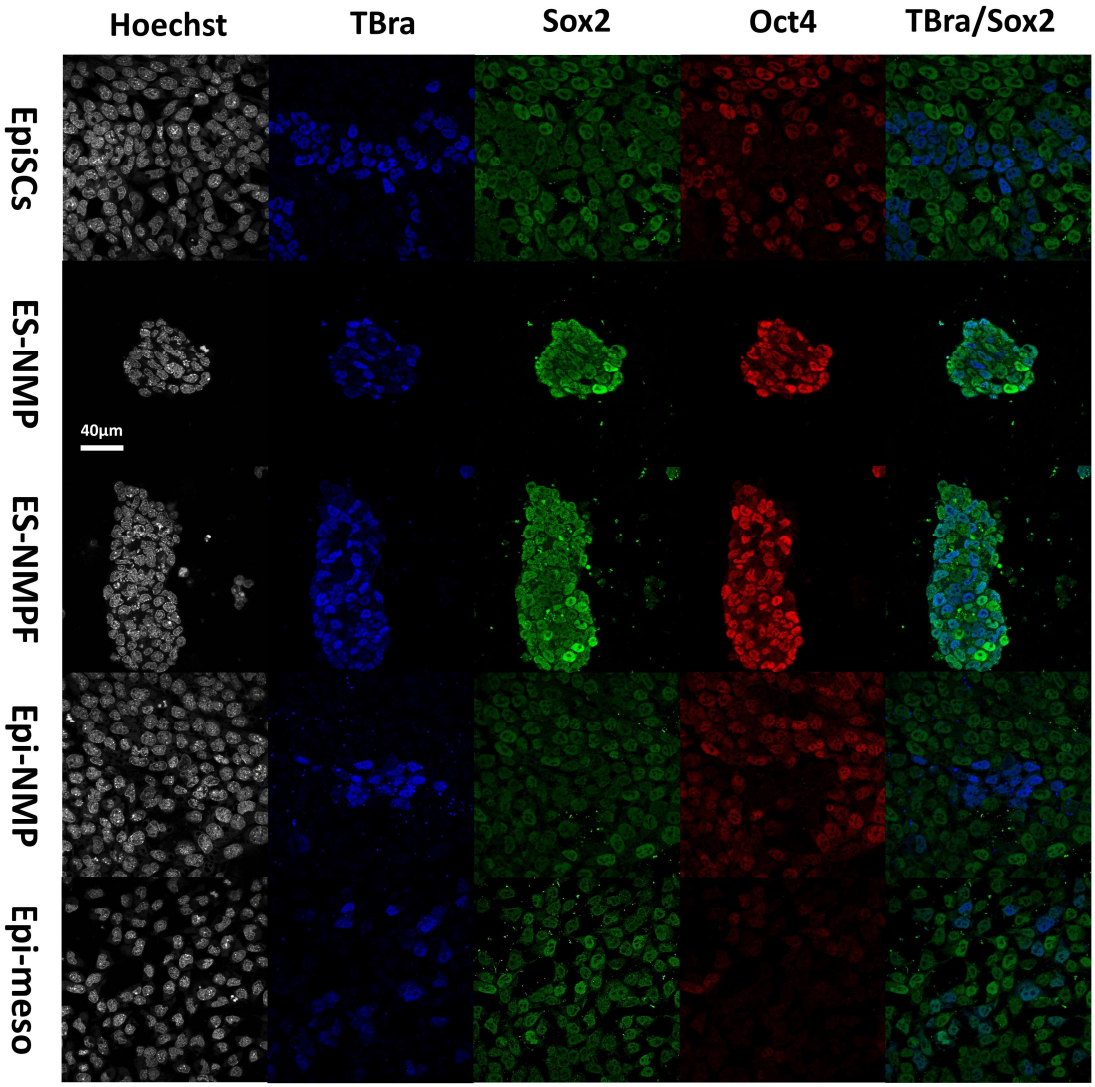

Fig. S9. Immunostaining of EpiSC, ES-NMP, ES-NMPF and Epi-NMP on their 3rd day and Epi-meso on its 2nd day. Hoechst (dapi) in grey, T/Bra in blue, Sox2 in green and Oct4 in red were imaged by a confocal microscopy. The composite image of T/Bra (blue) and Sox2 (green) is presented on the right-hand side column (TBra/Sox2). All the images were taken using a LSM700 on a Zeiss Axiovert 200 M with a 63× EC Plan-NeoFluar 1.3 NA DIC oil-immersion objective (see Materials and Methods).

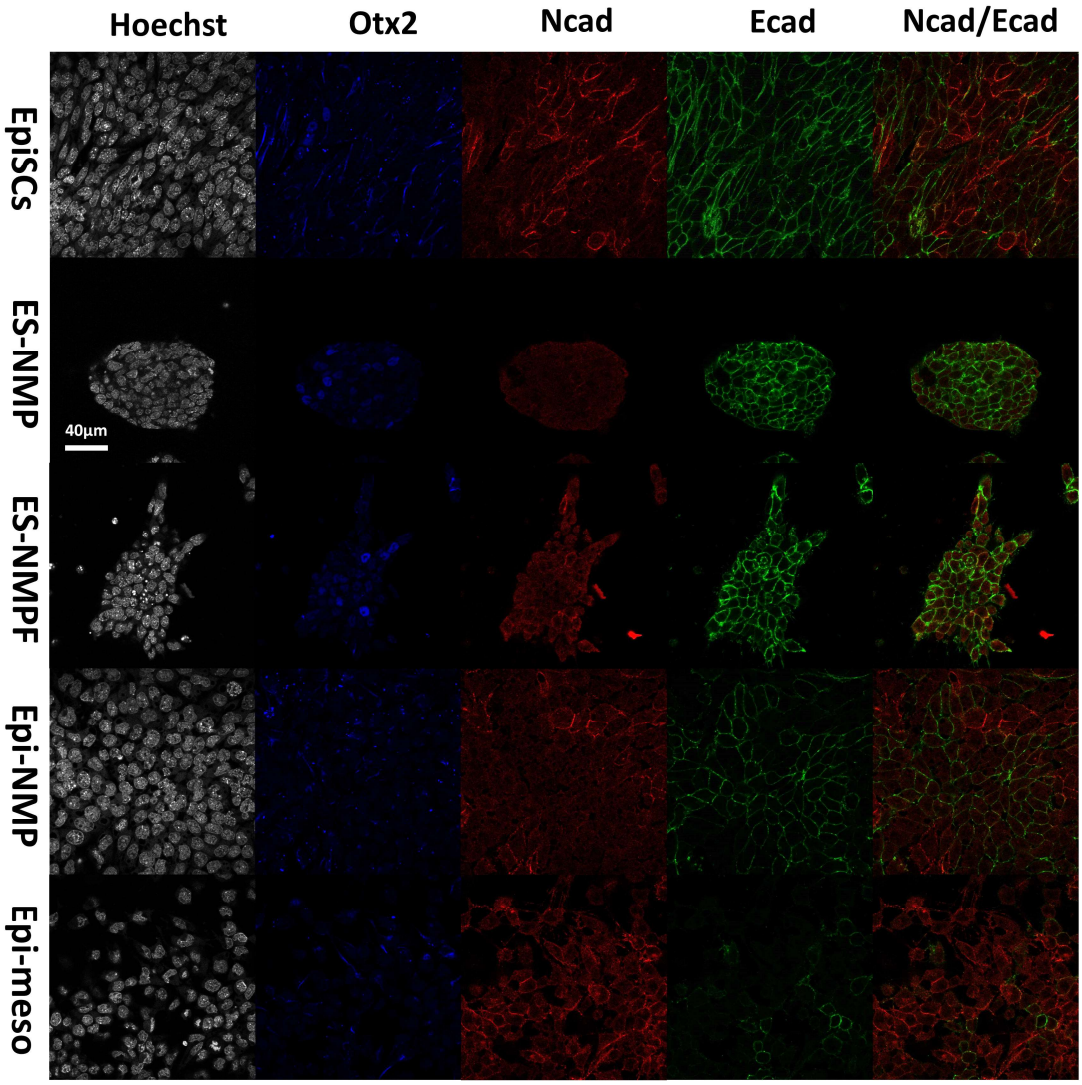

Fig. S10. Immunostaining of EpiSC, ES-NMP, ES-NMPF and Epi-NMP on their 3rd day and Epi-meso on its 2nd day. Hoechst (dapi) in grey, Otx2 in blue, Ncad in red and Ecad in green were imaged by a confocal microscopy. The composite image of Ncad (red) and Ecad (green) is presented on the right-hand side column (Ncad/Ecad). All the images were taken using a LSM700 on a Zeiss Axiovert 200 M with a 63× EC Plan-NeoFluar 1.3 NA DIC oil-immersion objective (see Materials and Methods).

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