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NANOG at the exit of pluripotency:
new roles in the gastrulating mouse embryo

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SUMMARY

SUMMARY

Developmental biology studies the processes by which a cell divides and differentiates to generate a full functional organism.

Up to the onset of gastrulation, cells from the embryo remain pluripotent, but precisely at this stage they differentiate towards mesoderm, endoderm, ectoderm and primordial germ cells. Pluripotency factors maintain uncommitted cells of the blastocyst and embryonic stem cells in culture in the pluripotent state. However, little is known about the role played by these factors during later development, despite their being expressed in the postimplantation epiblast. At this stage, progenitors of the first hematopoietic cells in the mouse arise in the early mesodermal progenitors in the posterior-proximal region of the epiblast, but the mechanisms that specify primitive blood cells are still largely unknown. At the same time, the embryo polarizes and asymmetry arises with the formation of the anterior-posterior axis.

Using a dual transgene system for controlled expression at postimplantation stages, we found that NANOG, a pluripotency factor crucial for pluripotency maintenance, blocks primitive hematopoiesis in the gastrulating embryo, resulting in a loss of red blood cells and downregulation of erythropoietic genes. Accordingly, *Nanog* deficient embryonic stem cells are prone to erythropoietic differentiation. Moreover, *Nanog* expression in adults prevents the maturation of erythroid cells. By analysis of available data for NANOG binding during stem cell differentiation and CRISPR/Cas9 genome editing, we found that *Tal1* is a direct target of NANOG.

We also found that *Nanog* represses anteriorization of the epiblast. By analyzing *Nanog*^{KO} ES cell RNAseq during naïve to primed transition and single cell RNAseq of gastrulating mouse embryos, we discovered that *Nanog* directly downregulates *Pou3f1*, that codes for a transcription factor important for anteriorization and neural development. Our results show that *Nanog* exerts crucial functions at the exit of pluripotency, and that it regulates primitive hematopoiesis and anteriorization of the embryo by directly repressing critical lineage specifiers.

RESUMEN

La biología del desarrollo estudia los procesos por los cuales una célula se divide y se diferencia hasta generar un organismo completo y funcional.

Hasta el momento de la gastrulación, las células del embrión permanecen pluripotentes, pero a partir de este estadio embrionario se diferencian a mesodermo, endodermo, ectodermo y células del primordio germinal. Los factores de pluripotencia mantienen indiferenciadas las células madre en cultivo y del epiblasto embrionario, sin embargo, poco se sabe de su función más tardía, pese a seguir expresados más allá del epiblasto pluripotente y de la gastrulación. En este estadio los progenitores de las primeras células hematopoyéticas aparecen a partir de los progenitores tempranos del mesodermo y al mismo tiempo, el embrión se polariza dando lugar a la formación del eje anteroposterior.

NANOG es un factor de pluripotencia crucial para el mantenimiento de ésta en el blastocisto y células madre. Usando un modelo de ratón doble transgénico mediante el cual podemos controlar la expresión de *Nanog* en estadios de postimplantación, hemos descubierto que *Nanog* bloquea la hematopoyesis primitiva y genes específicos de anteriorización del embrión durante la gastrulación. Como consecuencia, los embriones que expresan *Nanog* de manera ubicua son anémicos y tienen defectos craneofaciales. Además, la expresión ectópica de *Nanog* en médula ósea de adulto tiene como consecuencia el bloqueo madurativo de los eritrocitos. Mediante el análisis de datos previos de sitios de unión al genoma de NANOG en células madre, junto con edición del genoma mediante CRISPR/Cas9, hemos demostrado que la expresión de factores críticos en la especificación de estos linajes, *Tal1* y *Pou3f1*, se regulan negativamente de manera directa por NANOG.

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LIST OF ACRONYMS

AGM	aorta-gonad-mesonephros región
A-P axis	anterior-posterior axis
AVE	anterior visceral endoderm
BFU-E	burst forming unit erythroid (CFU)
CFU	colony forming unit
CFU-E	erythroid progenitor cells (CFU)
CFU-G	Granulocyte (CFU)
CFU-GEMM	granulocyte, erythroid, macrophage, and megakaryocyte progenitor (CFU)
CFU-GM	granulocyte-macrophage progenitor (CFU)
CFU-M	Monocyte (CFU)
CFU-MEP	megakaryocyte-erythroid progenitors (CFU)
CFU-Mk	Megakaryocyte (CFU)
CMP	common myeloid progenitors
dox	doxycycline
DVE	distal visceral endoderm
EMT	epithelial to mesenchymal transition
EPC	ectoplacental cone
EpiLC	epiblast like cell
Ery-P	primitive erythroid colonies
ES cell	embryonic stem cell
ExE	extraembryonic ectoderm
GMP	Granulocyte-Monocyte progenitors
GRN	gene regulatory network
HSC	hematopoietic stem cells
ICM	inner cell mass
Kb	Kilobases

LSK	Lin- Sca1+cKit+, hematopoietic stem cells enriched population
MEP	megakaryocyte-erythroid progenitors
Nanog^{-/-}	<i>Nanog</i> knockout ES cells
Nanog^{tg}	TetON transgenic model for Nanog ectopic expression
NSC	neural stem cells
PE	primitive endoderm
PGC	primordial germ cells
RBC	red blood cells
sgRNA	guide RNA
T	<i>Brachyury</i>
TE	trophectoderm
tetO	doxycycline responsive promoter
TF	Transcription factor
TGC	trophoblast giant cells
tracrRNA	Trans-activating crRNA
VE	visceral endoderm

A todas las mujeres, que luchan cada día
por lo que a los hombres nos viene dado.

En especial a tres luchadoras:

A Carmina,

a Dori

y a Cris.

“Study hard what interest you the most
in the most undisciplined,
irreverent and original manner possible”

Richard Feynman

INTRODUCTION

INTRODUCTION

Developmental biology has been studying the embryonic growth and differentiation of different species of plants and animals from the egg to the individual for the past century. From the observation that cancer cells and embryo cells differentiate and grow differently by Calkins in 1908 or the mixed culture of embryonic tissue and tumor of Rous in 1911, the discovery of the organizer by Mangold and Spemann in 1927, going through the realization that induction of the epiblast was done by the endoderm in chicken by Waddington in 1933, or the discovery that preimplantation embryos could be cultured outside the uterus of the mother by Runner in 1947 up to the generation of embryonic stem cells out of blastocyst outgrowth by Gardner and Beddington in 1988. All these discoveries have in common that all are important breakthroughs in development and have pushed forward the understanding of life as we know it (Calkins, 1908; Gardner and Beddington, 1988; Mangold and Spemann, 1927; Rous, 1911; Runner, 1947; Waddington, 1933). This requires the dynamic interplay of many cellular processes coordinated in space and time. Scientific research in the developmental area pursues the elucidation of the general principles and mechanisms underlying the cellular organization in developing organisms. The breakthroughs in developmental biology have reshaped our society, for instance through the introduction of new technologies such as the in vitro fertilization. Developmental sciences have as well yielded huge technological advances in science, such as chimeras and transgenic mouse line or embryonic stem (ES) cell generation (Gardner, 1968; Martin, 1981a). However, scientific advances in developmental biology have also contributed to other fields such as cancer (Nieto, 2013), evolution (Conway Morris, 2000), neurosciences (England et al., 2006), immunology (Martín-Gayo et al., 2017), genetics (Duboule and Morata, 1994) or adult stem cell biology (Koo and Huch, 2016).

1. From one to many

One of the most important scientific questions of our time that developmental biology has been trying to address for the past century is: how can just one cell give rise to a whole organism? However, we are still far from having an answer (Fig1).

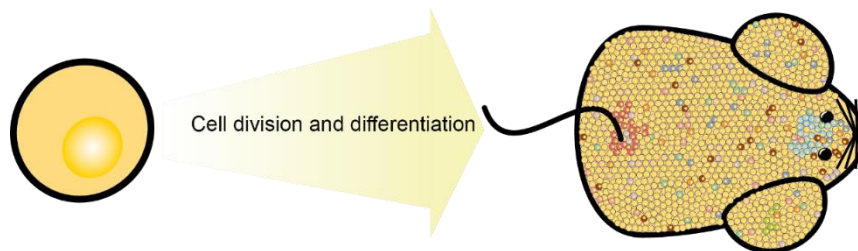


Figure 1. From one cell to a whole grown and differentiated organism: On the left one cell (zygote) that will give rise to a whole organism composed by many different types of cells (indicated with cells of various colours) on the right

Conrad Hal Waddington was one of the first to elaborate a theory on how a cell acquires a specific fate through differentiation, providing a paradigm that developmental biologists still use to visualize the differentiation potential of a cell. He imagined competence (competence being the ability of a cell to respond to inducing –differentiation- signals) as a cell in a state of instability. Induction would push the cell towards a specific developmental pathway from this point of instability. If the cell does not receive the inducing signal, it would self-differentiate according to its own tendencies. He represented this theory with the epigenetic landscape (Baedke, 2013; Fig 2).

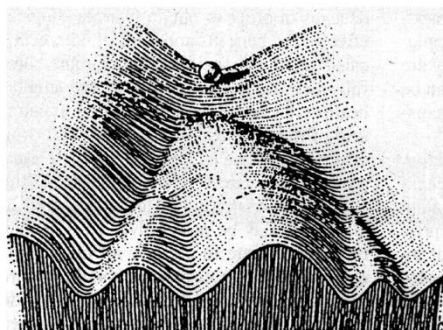


Figure 2. Epigenetic landscape of a differentiating cell as envisioned by Conrad Hal Waddington: Picture was first published in the book *Organisers and genes* (Waddington, 1940)

2. Gastrulation

The time in which cells from the embryo have to undergo important differentiation processes in animals is the gastrulation stage. As Lewis Wolpert put it, "It is not birth, marriage or death, but gastrulation which is truly the most important time of your life". In this stage, undifferentiated cells give rise to the different germs layers that will generate all different tissues in the organism.

Currently there are animal models well suited for the study of gastrulation mainly because at this stage they can be observed without much interference or handling, such as the African clawed toad *Xenopus laevis*, or the zebrafish *Danio rerio* (Heisenberg et al., 2000; Winklbauer et al., 2001), where gastrulation process can be observed in the microscope. However, one of the animal models that have been used extensively for studying the gastrulation stage has been the mouse. This is mainly because of its evolutionary resemblance to humans, both being mammals, its breeding capabilities, and its small size.

2.1 The road to gastrulation

For a mouse embryo, it takes 6 days since fertilization to reach the onset of gastrulation. To arrive to this stage from the fertilized egg, an equilibrium between pluripotency -that forces the cells to grow undifferentiated- and differentiation - that drives the generation of new tissues- must exist (Fig 3).

Once the egg is fertilized it undergoes a series of divisions until the stage of 8 to 16 cells, when the so called morula is compacted (Pratt et al., 1982). From the two cell stage (when maternal to zygotic transition occurs) through the next two division rounds, each blastomere retains full competence to develop into any cell, embryonic or extraembryonic. This ability of the cell to give rise to any tissue is called totipotency (Boroviak and Nichols, 2014; Morgani and Brickman, 2014). The first morphological differences appear during compaction, in which cells increase their cellular adhesions. Subsequent divisions -from 16 to 32 cells- result in polarized cells in the surface of the embryo and apolar cells in the inner region. At this stage, a large fluid-filled cavity is formed within the embryo called blastocoel. Blastocoel expansion reshapes the embryo and finishes with the

formation of the blastocyst (Chisholm et al., 1985; Motosugi, 2005). At the early blastocyst stage, we already observe two different populations: the trophectoderm (TE) that are epithelial cells on the outer layer of the embryo, which surround the second population, a group of cells called inner cell mass (ICM) that are sitting in contact with the TE. The ICM will give rise to the embryo proper and the yolk sac, while the TE will differentiate to form the majority of extraembryonic tissues (mainly the placenta). The ICM at this stage conserves the ability to give rise to any cell type in the embryo except for the placenta, therefore becoming pluripotent, instead of totipotent.

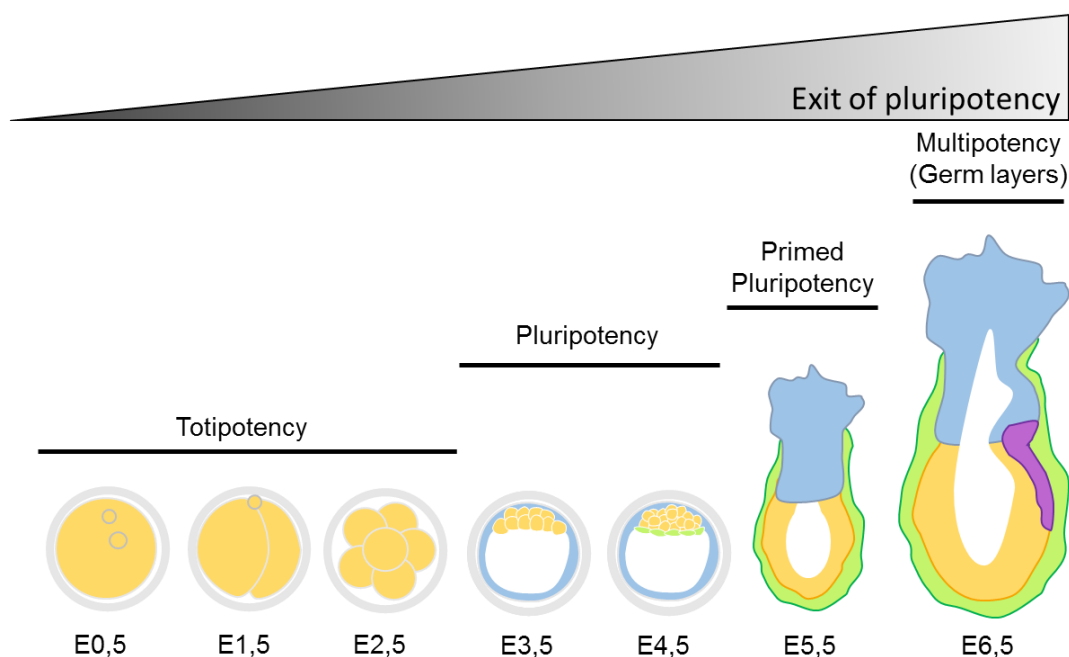


Figure 3. Stages in early mouse embryonic development: From egg fertilization to gastrulation. In orange the embryo, trophoctoderm and derivatives in blue, primitive endoderm and derivatives in green, mesoderm in purple.

One day later, at embryonic day 4.5 (E4.5), the primitive endoderm (PE) that eventually will give rise to the visceral endoderm (VE), an extraembryonic group of cells that are involved in patterning and protection of the embryo, differentiates from the ICM. Between the PE and the TE a group of cells called epiblast still retains pluripotent capabilities and will give rise to all of the tissues of the embryo (Gardner, 1982).

After hatching from the zona pellucida –a glycoprotein layer surrounding the plasma membrane of oocytes (Mainland, 1932)– the blastocyst implants in the

uterus of the mother through the mural TE, which are the trophoblast cells opposite to the ICM pole. Implantation requires a complex set of interactions between the embryo and the uterine epithelium, involving different proteases, adhesion molecules and signaling events (Aplin and Ruane, 2017).

The first definitive cell type that differentiates from the TE upon implantation are the trophoblast giant cells (TGC). The TGCs cover the embryo with the exception of the polar TE, which are the trophoblast cells in direct contact with the epiblast. The polar TE gives rise to two different cone shaped structures: the extraembryonic ectoderm (ExE), in direct contact with the epiblast, and the ectoplacental cone (EPC), that is located proximal to the ExE and that mediates early interactions with the maternal endometrium.

At the same time, the epiblast acquires a cylinder shape with cells arranged in a pseudo-stratified epithelium, and is covered by the VE. The ExE establishes a complex crosstalk with the epiblast that will be necessary for anterior-posterior axis establishment, germ cell formation and gastrulation of the epiblast (Beck et al., 2002a; Feldman et al., 1995; Yoshimizu et al., 2001).

2.2 The onset of gastrulation

Formation of the primitive streak is the morphological cue for gastrulation. Proper location of the primitive streak demands the previous establishment of the anterior-posterior (A-P) axis in the epiblast. The A-P axis arises from the opposite signals from the ExE (BMP4 and NODAL activation) and the VE, first distal (DVE) and later anterior (AVE) with the nodal agonist *Cer1* and *Lefty1*. For Nodal activation, its dephosphorylation is fundamental. Furin and Pcsk6 (protein convertases), both secreted in the ExE, dephosphorylate Nodal in the epiblast (Beck et al., 2002b). These signaling events, together with *Wnt3* that triggers the expression of *Brachyury* (T) and *Fgf8* make gastrulation possible (Fig4).

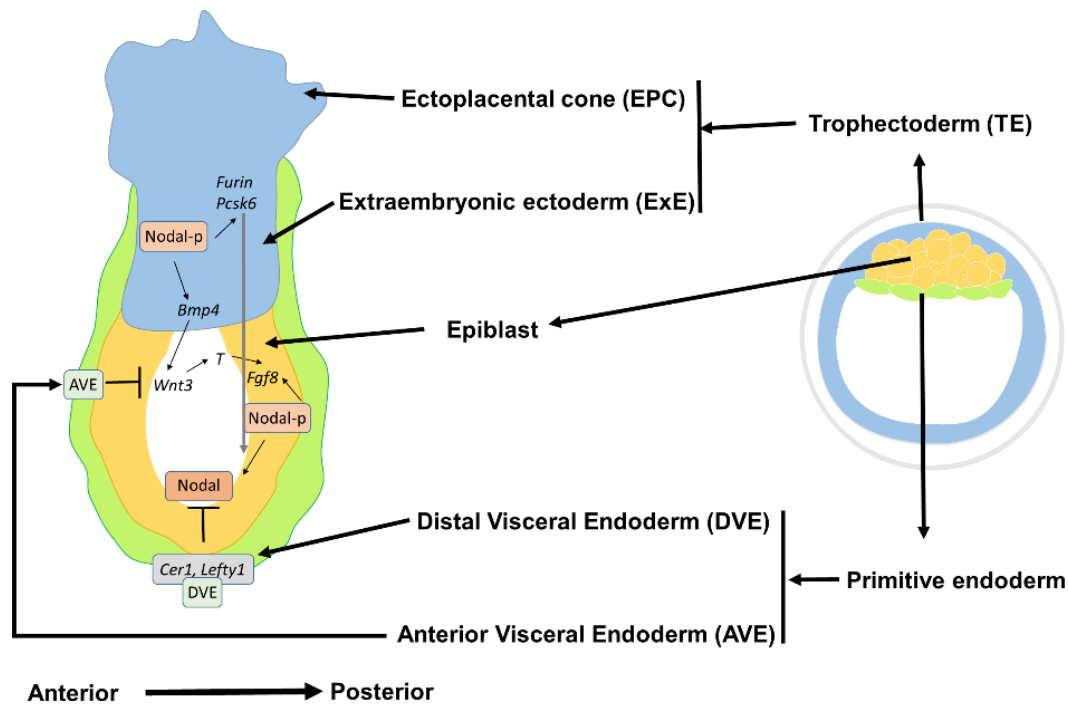


Figure 4. Signaling and gene expression in A-P axis formation: On the left a representation of an E5.5 embryo with schematic signaling for A-P axis formation; arrows indicate activation, blunt arrows indicate repression. On the right, an E4.5 blastocyst from which the different structures commented in the figure develop.

During gastrulation, cells that were attached to each other in the epiblast by adhesion molecules such as E-Cadherin lose said adhesions and separate from the epiblast undergoing an epithelial to mesenchymal transition (EMT) to migrate through the primitive streak. *Snail* genes code for key factors that regulate this primary EMT by downregulating *E-Cadherin* and promoting motility (Nieto et al., 1994; Thiery et al., 2009). The previous location of these cells in the epiblast predict which tissues they will give rise to (Padrón-Barthe et al., 2014; Tam and Beddington, 1987; Tam and Zhou, 1996). From proximal to distal, precursor mesoderm cells ingress the primitive streak in an orderly fashion. First blood, then allantois, cardiac, lateral plate and presomitic mesoderm. Finally the last cells that delaminate from the epiblast will differentiate into definitive endoderm (Scialdone et al., 2016; Fig 5). An important role here is played by the node, that acts to organize the body plan inducing the formation of posterior structures by producing Nodal. It is essential for mesoderm and endoderm differentiation (Tam and Beddington, 1987). Next to the node, at the node-streak border is where the neuromesodermal progenitors appear (Henrique et al., 2015).

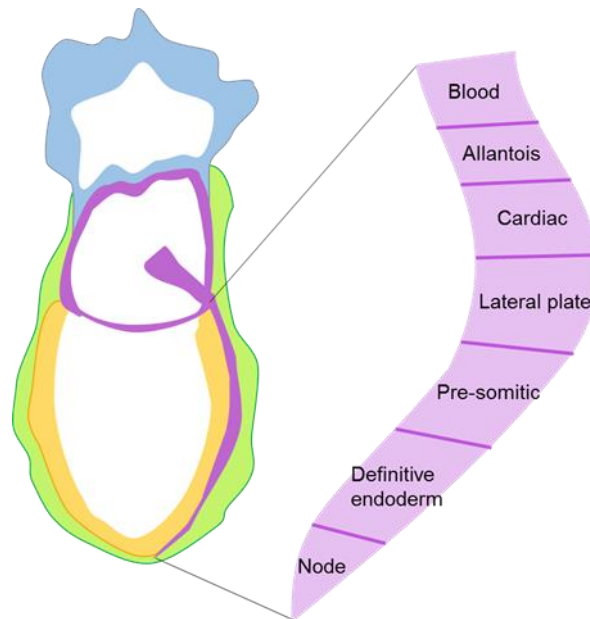


Figure 5. Mesoderm lineages distribution: Along the axis of the primitive streak in order from proximal to distal lineage specification

Up to the onset of gastrulation epiblast cells conserve the ability to give rise to any germ layer, although epigenetic and transcriptomic changes make them more prone to differentiate. Thus cells at this stage are considered to be in a state of primed pluripotency (Nichols and Smith, 2009a). Therefore, one of the most important processes that takes place during the first stages of embryo development is the exit from pluripotency, what allows these cells to differentiate down Waddington's epigenetic landscape.

3. The genetic control of pluripotency and its dismantling

Pluripotency is a steady state in which cells can self-renew and remain undifferentiated, retaining the capacity to give rise to any germ layer. This cell state is maintained thanks to an intricate network of genes that are tightly regulated by three main TFs: NANOG, OCT4 and SOX2.

NANOG is a constituent of the core set of transcription factors, together with OCT4 and SOX2, involved in establishing and maintaining embryonic pluripotency, both in the blastocyst and in embryonic stem (ES) cells in culture (Chambers and Tomlinson, 2009). ES cells were first obtained from blastocyst and cultured in teratocarcinoma stem cell conditioned medium (Martin, 1981b)

and described as pluripotent cells able to form teratocarcinomas when injected into mice. Since then, ES cells have been used for chimeras generation, differentiation assays and xenograft implants. The instrumentalization of this ES cells relies on the understanding of the pluripotency gene network. This gene regulatory network (GRN) regulates pluripotency by repressing genes involved in differentiation and activating other genes important for pluripotency (Osorno et al., 2012a; Thomson et al., 2011) (Fig 6). Most importantly, it also leads the process of exiting pluripotency by responding to extrinsic and intrinsic signals and changing the regulatory regions and partners they bind to (Kalkan and Smith, 2014; Mohammed et al., 2017a; Pfeuty et al., 2018).

3.1 The exit of pluripotency

Although multiple studies have addressed embryonic stem (ES) cell differentiation (Mendjan et al., 2014; Radziskeuskaya et al., 2013; Thomson et al., 2011), the exit of pluripotency *in vivo* is still not well understood (Tam and Behringer, 1997). Most of the work has been done in the blastocyst, given that at this stage it is the first time a lineage choice occurs (Chazaud et al., 2006; Rossant and Tam, 2009; Yagi et al., 2007), and also because of the versatility and easy handling of preimplantation embryos.

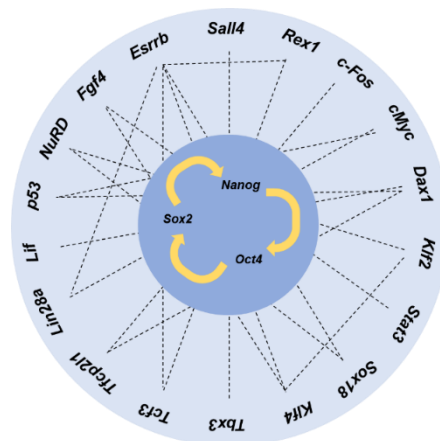


Figure 6. The pluripotency Gene Regulatory Network: Multiple genes regulate the activation and the maintenance of pluripotency *in vivo* and *in vitro*, and at its core SOX2, NANOG and OCT4 are in charge of regulating the process at a transcriptional level. This is a representation of the interconnections between genes instrumental for pluripotency instruction that at the same time are regulated by the core GRN (modified from (Parfitt and Shen, 2014)).

As we have discussed earlier, the dynamics of the core pluripotency GRN *in vivo* vary at different developmental stages of the early embryo, and these variations are actually the ones that configure the disposition of the exit of pluripotency.

Rewiring of pluripotency GNR is fundamental for the exit of pluripotency (Aksoy et al., 2013). In ES cells, SOX2 and OCT4 bind together to most genomic regions, which are also frequently bound by NANOG (Chen et al., 2008; Marson et al., 2008). Changes in levels of expression of these TFs can induce a change of partners, what can also be due to novel factors being expressed or to changes in the chromatin accessibility of key loci (Navarro et al., 2012; Niwa, 2007). This is how TFs that previously worked together to maintain pluripotency are able to adjust their function to allow differentiation when changes in the cellular environment occur (Iwafuchi-Doi et al., 2011a). This has been proven to happen both *in vitro* and *in vivo* (Avilion et al., 2003). A good example of how this network is rewired at the exit of pluripotency is the selective influence of SOX2 in either OCT4 (*Pou5f1*) or OCT6 (*Pou3f1*). These TFs interact differently in ES cells and in differentiated multipotent neural stem cells (NSC): In ES cells SOX2 binds to OCT4 in the Sox/Oct motives, but in neural stem cells OCT6 and SOX2 bind to those motives on their own (Mistri et al., 2015), without interacting with each other.

3.2 Exit of pluripotency *in vivo*

The early blastocyst is a good example of how these dynamics are important for differentiation *in vivo*. In the inner cell mass (ICM) of the blastocyst the cells are in a pluripotent stage in which *Nanog* levels fluctuate from low *Nanog* to high *Nanog* expressing cells (Abranches et al., 2013). The cells that express low levels of *Nanog* are the ones that are able to differentiate and give rise to the primitive endoderm (Chazaud et al., 2006). The late blastocyst stage contains the primitive endoderm and the epiblast. In the epiblast, all of the cells hold highly expressed levels of *Nanog*, *Sox2* and *Oct4* and hence do not differentiate towards any lineage. How these epiblast cells escape pluripotency and the role of the core pluripotency factors in this process is largely unknown.

Therefore, in order to explore the exit of pluripotency towards different germ layers and tissues we need to study later stages of postimplantation development, when gastrulation takes place (Tam and Behringer, 1997).

3.3 Pluripotency factors after implantation

Despite the key role of OCT4, SOX2 and NANOG in the preimplantation embryo and in ES cells to maintain cells in the pluripotent state, it is a fact that all three factors are expressed at later, postimplantation stages of mouse development, which suggest roles not directly related to pluripotency (Osorno et al., 2012b)

At E6.5, *Sox2* is expressed in the anterior region of the epiblast and will remain expressed in the anterior neural region up to E9.5, when it gets restricted to specific neural structures. At E6.5 *Nanog* gets reexpressed in the posterior epiblast. In the proximal region is a key factor for the induction of PGCs, where it gets restricted at E8.0 while it disappears from the rest of the epiblast (Chambers et al., 2007a; Hart et al., 2004a; Zhang et al., 2018). On the other hand, *Oct4* is expressed throughout the whole epiblast and continuously expressed up to E8.5, when it starts restricting to the posterior part of the embryo (Yeom et al., 1996) (Fig 7).

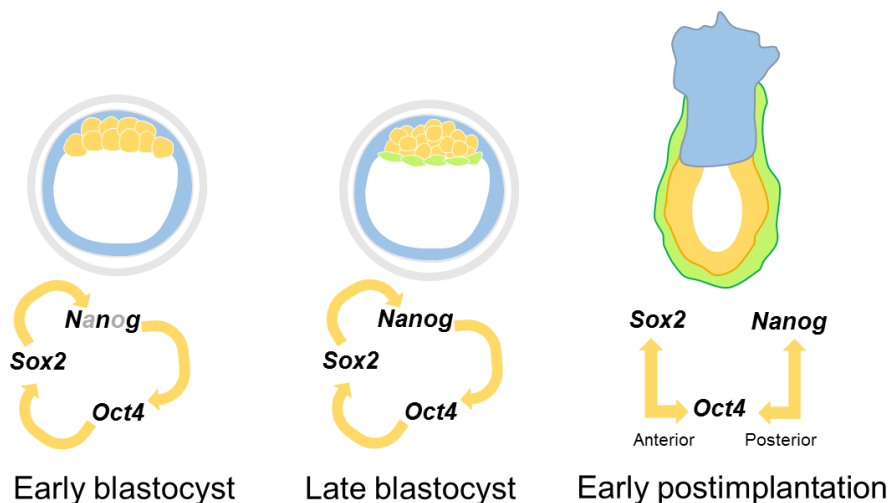


Figure 7: Pluripotency GRN during development: At the early blastocyst stage the levels of *Nanog* change from cell to cell of the ICM (*Nanog* in gray and black). In the late blastocyst all three transcription factors are highly expressed in the epiblast. After implantation *Oct4* is expressed in the whole epiblast while *Sox2* is restricted to the anterior and *Nanog* to the posterior epiblast.

To study the role of the different components of the pluripotency GRN the three core TFs have been knocked out (KO) of the embryo. In all three cases, the deletion is lethal at blastocyst stage (Masui et al., 2007; Mitsui et al., 2003a; Nichols et al., 1998). This precludes the analysis of possible postimplantation

phenotypes. In order to address this issue, conditional KO lines have been generated. In the case of *Oct4*, mutants show proliferation defects at gastrulation (DeVeale et al., 2013). *Sox2* conditional mutant embryos result in neural defects, with mild effects in brain development probably due to other *Sox* genes, such as *Sox1* and *Sox3*, that might compensate for *Sox2* absence (Bylund et al., 2003; Ferri et al., 2013; Graham et al., 2003)

However, no conditional mouse line of *Nanog* deletion in the epiblast has been published yet. Interestingly, *Nanog*-deficient ES cells are still able to maintain pluripotency, although they are prone to differentiate (Chambers et al., 2007a). *Nanog* expression is turned off during implantation only to be re-expressed again at E6.0 in the posterior part of the embryo, where gastrulation will start later. This pattern gradually disappears and at E8.0 the expression of *Nanog* is restricted to the primordial germ cells (PGCs), where *Nanog* plays an important role in their differentiation and migration (Chambers et al., 2007a; Yamaguchi et al., 2009; Zhang et al., 2018) (Fig 8). Besides its function in the germline, there is little evidence for the role of *Nanog* in the postimplantation epiblast or in the gastrulating embryo.

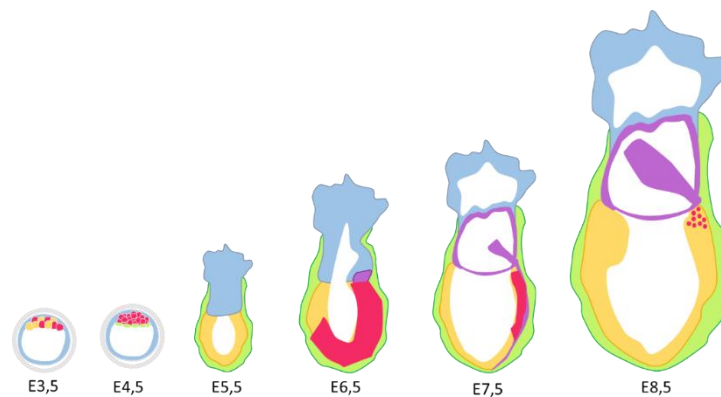


Figure 8: *Nanog* pattern of expression during development: *Nanog* expression is shown in red. Embryonic stages are indicated below embryos. *Nanog* is expressed at E3.5 in the ICM in a salt and pepper manner, and at E4.5 it is expressed in the whole epiblast. Expression turns off at E5.5, to be re-expressed at E6.5 throughout the posterior region of the epiblast. At E7.5 it is gradually downregulated until E8.0 when it is only expressed in the PGCs, where the expression is maintained until E12.5-E13.5.

Since there is no conditional KO for *Nanog* in the epiblast, we decided to address the question of the role of *Nanog* during the gastrulation stage by using a TetON transgenic model (*Nanog*^{t9}). This system has been successfully used to uncover a previously unsuspected role for *Nanog* in adult epithelia (Piazzolla et al., 2014). With this model in hand, we could express *Nanog* at will in defined time windows during postimplantation stages of mouse development. When we first extended *Nanog* expression from gastrulation up to E9.5 we observed two specific phenotypes: a complete lack of blood in the embryos, and consistent anterior neural defects (Results Fig 11). During this thesis work, we aimed to understand both processes and how they relate to the function of *Nanog* in the gastrulating embryo.

4. Emergence of hematopoiesis in the mouse embryo

Hematopoiesis first arise when diffusion of oxygen is insufficient for the maintenance of the growing embryo. Cells from the posterior epiblast are able to give rise to red blood cells during a very short period of time. The precursors of the first erythroid cells are already present at the initial stages of gastrulation, in the nascent mesoderm at the posterior end of the embryo (Huber et al., 2004; Lawson et al., 1991). Moreover, detailed fate mapping suggests that these cells are specified in the epiblast before gastrulation (Kinder et al., 1999; Padrón-Barthe et al., 2014). In fact, there are allegedly two different precursors in the posterior epiblast: those that firstly ingress the primitive streak and that will give rise to red blood cells (RBC) and those that follow this first wave and give rise to endothelial cells as well as RBC, megakaryocytes and macrophages (Padrón-Barthe et al., 2014; Scialdone et al., 2016). Hematopoietic precursors are specified after the determination of the early mesoderm from the epiblast, which is driven by the sequential action of the transcription factors encoded by *Brachyury* and *Mesp1* and finally in the expression of FLK1 (encoded by *Kdr*), which marks most mesodermal cells at gastrulation (Pfister et al., 2007; Scialdone et al., 2016). Subsequently, primitive hematopoiesis progenitors start expressing a battery of lineage-specific transcription factor genes such as *Tal1*,

Gata1, and *Klf1* as they migrate to the extraembryonic region and generate the blood islands of the yolk sac (Baron et al., 2012; Doré and Crispino, 2011).

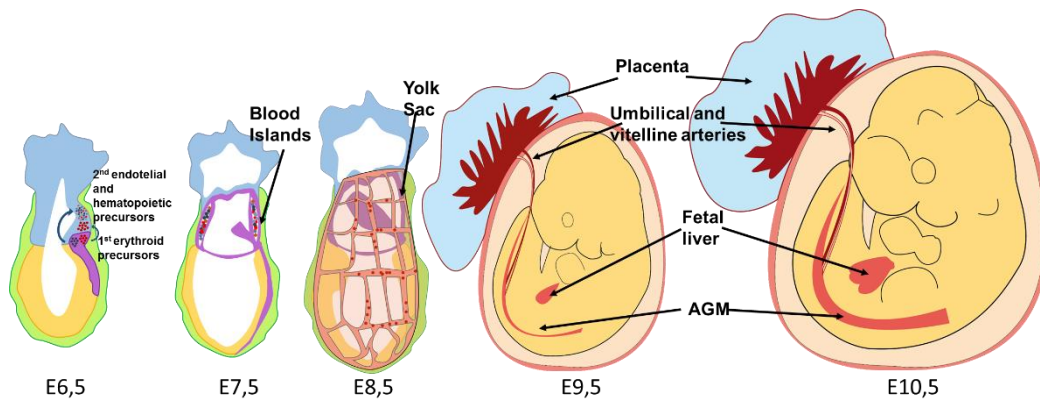


Figure 9: Hematopoietic development: Embryo in orange, ExE and placenta in blue, VE in green, hematopoietic sites in red. Arrows indicate anatomic sites of hematopoiesis during development.

The first erythroid cells are immature RBC with unique characteristics and are found only in the early embryo (Palis et al., 1999). They are much larger than definitive erythrocytes and express embryonic globins, and upon maturation they lose the nucleus as occurs with adult erythrocytes (Tober et al., 2008). Macrophages and megakaryocytes progenitors are produced as well at the first stages of primitive hematopoiesis (Tober et al., 2007) but they are indistinguishable from their definitive counterpart. At E8.0 erythro-myeloid progenitors arise and are able to produce mature erythrocytes and most myeloid lineages. At E9.5, B and T cells appear both in the yolk sac and the aorta-gonad-mesonephros region (AGM; Yoshimoto et al., 2011). It is at this stage that resident macrophages are generated (Ginhoux and Guilliams, 2016). Since the first burst of erythroid cells occurs at E7.5, at E9.5 still most of the circulating RBC population derives from the primitive, first wave of hematopoiesis (Lux et al., 2008; Fig 9).

It is not until E10.5 that the first long-term hematopoietic stem cells (HSC) are generated (Medvinsky and Dzierzak, 1996). HSCs are produced in the major arteries of the embryo: AGM, and vitelline and umbilical arteries. Later they are also found in the placenta and yolk sac (de Bruijn et al., 2000; Gekas et al., 2005). HSCs migrate to the fetal liver where their numbers increase massively and by

E16.5 they colonize the bone marrow, where they will engraft and stay during all the adult life.

Since *Nanog* is highly expressed at E6.5 in the posterior region of the epiblast, which is when and where the first RBCs come from, we hypothesize that *Nanog* might be blocking the RBC lineage at E6.5, and that property of *Nanog* is echoed in the anemic phenotype of the E9.5 embryos.

5. Anterior patterning and neural fate

As we have described above, anterior-posterior axis determination of the mouse embryo relies on gastrulation, signaling from extraembryonic tissues, and morphological changes. However, studies in gastruloids have shown that ExE and AVE are not strictly necessary for triggering gastrulation in culture (Turner et al., 2017). Furthermore, growth of human and mouse ES cells on micro-patterned cultures have demonstrated that no shape cue is necessary for gastrulation or exteriorization (Deglincerti et al., 2016; Hemmati-Brivanlou and Melton, 1997; Morgani et al., 2018; Muñoz-Sanjuán and Brivanlou, 2002). Therefore, this evidence suggests that epiblast-intrinsic properties are responsible for driving anterior-posterior fates and neural specification, and not dependent exclusively on signaling processes driven by extraembryonic tissues.

Nevertheless, the precise timing of the regulation at a transcriptional level of anterior neural formation *in vivo* is still not fully understood. Transcription factors (TF) such as SOX2 have been identified as neural fate-promoting factors. Actually, it has been demonstrated that in chicken SOX3 repress *Snail* genes and vice versa for defining anterior and posterior territories. This is conserved in mice –which mechanism involves Sox2- and in human cancer cells (Acloque et al., 2011). The earliest TF expressed in postimplantation epiblast involved in anteriorization is *Pou3f1*, it is known to activate Sox2 in the epiblast and later in anterior neural differentiation (Zhu et al., 2014).

This, together with our observation that *Nanog* can interfere with the development of the anterior neural region, suggests that rewiring of the pluripotency gene regulatory network could also be at play during this process at gastrulation.

OBJECTIVES

OBJECTIVES

The general objective of this thesis is to elucidate the role of *Nanog* at the exit of pluripotency.

The specific objectives addressed during this thesis work are:

1. To study the role of *Nanog* in hematopoietic differentiation by:

- 1.1 Investigating the effect of continued expression of *Nanog* in the embryo on hematopoietic differentiation at gastrulation.
- 1.2 Analyzing the hematopoietic differentiation potential of embryonic stem cells in the absence of *Nanog*.
- 1.3 Assessing the effect of *Nanog* gain-of-function in the adult bone marrow.
- 1.4 Identifying hematopoietic genes that could be direct targets of NANOG at gastrulation.

2. To study the role of *Nanog* in regionalization of the gastrulating embryo by:

- 1.1 Exploring the role of *Nanog* at the exit of pluripotency by analyzing the transition of naïve to primed embryonic stem cells.
- 1.2 Identifying putative targets of NANOG at this transition by integration of different transcriptomic analysis.
- 1.3 Analyzing genomic loci of selected target genes to look for NANOG responsive regulatory elements.

OBJETIVOS

El objetivo general de esta tesis es esclarecer el papel de *Nanog* en la salida de la pluripotencia.

Los objetivos específicos desarrollados durante la tesis han sido:

1. Estudiar el rol de *Nanog* en la diferenciación hematopoiética:

- 1.1 Investigando el efecto de la expresión continua de *Nanog* en la diferenciación hematopoiética del embrión durante la gastrulación.
- 1.2 Analizando el potencial de diferenciación hematopoiética de células madre embrionarias en ausencia de *Nanog*.
- 1.3 Evaluando el efecto de la ganancia de función de *Nanog* en la médula ósea adulta.
- 1.4 Identificando genes hematopoiéticos que pudieran ser dianas directas de NANOG durante la gastrulación.

2. Estudiar el papel de *Nanog* en la regionalización del embrión durante gastrulación:

- 1.1 Explorando el papel de *Nanog* en la salida de la pluripotencia mediante el análisis transcripcional de la transición de células madre embrionarias naïve a diferenciadas.
- 1.2 Identificando posibles dianas de NANOG en esta transición mediante la integración de diferentes análisis transcriptómicos.
- 1.3 Analizando diferentes *loci* del genoma para buscar posibles elementos reguladores que respondan a NANOG.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Animal model

We obtained the *Nanog/rtTA* mouse line (*R26-M2rtTA;Col1a1-tetO-Nanog*) (Piazzolla et al., 2014) from Manuel Serrano (CNIO, Madrid) and Konrad Hochedlinger (Harvard Stem Cell Institute). This is a double transgenic line that carries the *M2-rtTA* gene inserted at the *Rosa26* locus and a cassette containing *Nanog* cDNA under the control of a doxycycline-responsive promoter (tetO) inserted downstream of the *Col1a1* locus (Fig 10). Mice were genotyped by PCR of tail-tip DNA as previously described (Hochedlinger et al. 2005; Piazzolla et al. 2014). Mice were housed and maintained in the animal facility at the Centro Nacional de Investigaciones Cardiovasculares (CNIC, Madrid, Spain) in accordance with national and European Legislation. Procedures were approved by the CNIC Animal Welfare Ethics Committee and by the Area of Animal Protection of the Regional Government of Madrid (ref. PROEX 196/14).

Double-homozygote transgenic males were mated with CD1 females, which were then treated with doxycycline (dox) to induce the *Nanog* cassette by replacing normal drinking water with a 7.5% sucrose solution containing dox (1 mg/ml), with replacement with fresh solution after 2 days. For transgene induction in embryos to be harvested at E7.5, a single 100 μ l intraperitoneal injection of 25 μ g/ μ l doxycycline was administered to pregnant females at E5.5, followed by dox administration in drinking water as above.

2. Transient transgenic analysis and transgenic line generation

For the generation of transgenic embryos, 7 weeks old F1 (C57Bl/6x CBA) females were superovulated to obtain fertilized oocytes as described (Nagy et al., 2014). Females were injected with 5 units of pregnant mare's serum gonadotropin (PMSG, Foligon 5000) and 5 units of chorionic gonadotropin (Sigma) two days later, followed by embryo collection the next day. After 1 hour incubation, viable zygotes were microinjected into the pronucleus with commercially available Cas9 protein (30ng/ μ l; PNABio) and guide RNA (sgRNA; 25ng/ μ l; Sigma). All those components were previously hybridized to generate ribonucleoprotein

complexes. First we incubated 100ng/ul of Trans-activating crRNA (tracrRNA) and sgRNA 5 minutes at 95°C and then 10 minutes at room temperature (RT). After that, we incubated them with the Cas9 for 15 minutes at RT, and stored at 4°C. Injection buffer contains Tris 50nM pH7.4, EDTA 1nM, H₂O embryo tested and is filtered through a 0.22um filter.

sgRNA were designed with an online tool (<http://crispr.mit.edu/>). Details of the sequences are shown in Table 1 and Figure 34 B.

T7 adaptor TAATACGACTCACTATA always at 5' of sequence

POU+9Kb-g1: 5' TGGAAGGTGGGTGGCTCGGTGAAGTTTTAGAGCTAGAAATAGC 3'

POU+9kb g2: 5' GGACCCTATATGGCTGAGGGGCGTTTTAGAGCTAGAAATAGC 3'

POU-11kb-g1: 5' GGGCTCTCAGAAGGGTCGTTGGGTTTTAGAGCTAGAAATAGC 3'

POU-11kb-g2: 5' GGGCGGGCTCCCTTGGATGTTGGTTTTAGAGCTAGAAATAGC 3'

POU-9kb-g1: 5' GGCGAGAGGATATTGCCGTTTCGTTTTAGAGCTAGAAATAGC 3'

POU-9kb-g2: 5' GGCACTAGGAGGGGGGCTATTGTTTTAGAGCTAGAAATAGC 3'

Homology for trac GCTATTTCTAGCTCTAAAAC at 3' of sequence

Primer Trac-Rev:

5' AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTT 3'

Table 1. List of sequences necessary to obtain the sgRNA+tracrRNA: T/ sequence was add to all the sgRNAs.

Primers for genotyping the deletions are detailed in table 2:

"Pou3f1 del-9kb Fw": 5'CTAGTATGCCCGGGTTTGTG 3'

"Pou3f1 del-9kb Rv": 5' TCAATGGTCCTTCTGGCTCT 3'

"Pou3f1 del-11kb Fw": 5' TCTCTCTCTCCCCCTGATGA 3'

"Pou3f1 del-11kb Rv": 5' AAGCCTCCACGACAGAAGAA 3'

"Pou3f1 del +9kb Fw": 5' AACCAGCCTCAGACATTTGG 3'

"Pou3f1 del +9kb Rv": 5' TCCAGCACTTGGACTCACAC 3'

Table 2. List of primers used for identifying the deletions.

The product of the PCR was run on an agarose gel, the specific band was excised and DNA was extracted with the DNA extraction kit (Qiagen). Purified DNA was transcribed for RNA collection/isolation.

After injection, embryos were cultured in M16 (Sigma) covered with mineral oil (Nid Oil, EVB) up to the two cell stage. Living embryos were then transferred into a pseudopregnant CD1 female, previously crossed with a vasectomized male.

E6.5 embryos were recovered for transient experiments. For the generation of a stable line we let the embryos be born. +9Kb Pou3f1 deletion line is maintained in an outbred background (CD1). Control mice for the line experiments are embryos from F1 males crossed with CD1 females.

3. Culturing embryos at the onset of gastrulation

We recovered the embryos at E6.25 and cultured them for 8 hours up to primitive streak (PS) stage (Downs and Davies, 1993). Culture medium has been already described (Glanville-Jones et al., 2013), it contains half rat serum and half a N2B27 based medium. Embryos were cultured in pools, as close contact among them was shown to improve growth. As rolling culture did not represent any improvement in the embryo morphology we culture them in four-well plates at 37°C and 5% CO₂.

4. Chimera generation

Chimeras were generated by the transgenic unit at CNIC. Control and *Nanog*^{-/-} ESC were injected/delivered in 8 cell stage embryos as previously described (Nagy et al., 2014). After transferring the embryos into pseudopregnant females, embryos were collected at E10.5.

5. RT-qPCR assays

RNA was isolated from ESCs, using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA from individual E6.5-7.5 embryos, sorted E9.5 or sorted bone marrow populations was isolated using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) and reverse transcribed using the Quantitect Kit (Qiagen). cDNA was used for quantitative-PCR (qPCR) with Power SYBR® Green (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression of each gene was normalized to the expression of the housekeeping genes *Actin* and *Ywhaz* (Table 3) .

	Forward Primer	Reverse Primer
<i>Nanog</i>	CTTACAAGGGTCTGCTACTGAGATGC	TGCTTCCTGGCAAGGACCTT
<i>Brachyury</i>	GTCTAGCCTCGGAGTGCCT	CCATTGCTCACAGACCAGAG
<i>Eomes</i>	TTCACCTTCTAGAGACACAG	GAGTTAACCTGTCAATTTCTG
<i>Kdr</i>	TTTGGCAAATACAACCCTTCAGA	GCAGAAGATACTGTCACCACC
<i>Runx1</i>	GCAGGCAACGATGAAAACACTAC	GCAACTTGTGGCGGATTTGTA
<i>Tal1</i>	CACTAGGCAGTGGGTTCTTTG	GGTGTGAGGACCATCAGAAATCT
<i>Gata1</i>	CTGCATCGCTCCCTGTACAC	GCAGGCTTCCATGAAACTTGG
<i>Klf1</i>	AGACTGTCTTACCCTCCATCA	GGTCCTCCGATTTCCAGACTCA
<i>Hbb-bh1</i>	GAAACCCCGGATTAGAGCC	GAGCAAAGGTCTCCTTGAGGT
<i>Stil</i>	GACACAATTCAGGACTGGTAGAC	GGATGATCCACTTTCTGTTCA
<i>Ywhaz</i>	CGTTGTAGGAGCCCGTAGGTCAT	TCTGGTTGCGAAGCATTGGG
<i>Actin b</i>	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC

Table 3. Primer sequences for RTqPCRs

6. Flow cytometry

E9.5 and E10.5 embryos were disaggregated with 0.25% collagenase type I (Stemcell Technologies) at 37°C for 30 min, and the cells were washed with PBS containing 2%FBS (Gibco) and filtered through a 70 µm mesh. The single cell suspension was then incubated for 30 min at 4°C with the following antibodies: antiCD71-FITC (BD Biosciences, 1:100), antiTer119-APC (BD Biosciences, 1:100), antiKit-PEcy7 (BD Biosciences, 1:100), and antiCD41-PE (BD Biosciences, 1:100). Samples were analyzed with the BD LSRFortessa flow cytometer.

Bone marrow of adult mice was obtained from femurs and tibias crushed in a mortar and filtered through a 70 µm mesh to obtain single-cell suspensions. For hematopoietic cell maturation assays, a small fraction of the bone marrow was separated and the rest was depleted of red blood cells by lysis in FACSLysing solution (BD Biosciences). Antibodies used for blood maturation assay were antiCD71-FITC (BD Biosciences, 1:100) and antiTer119-APC (BD Biosciences, 1:100). Antibodies for BM precursor sorting were Biotinylated lineage cocktail (BD Biosciences, 1:50), antiCD34 (RAM34)-FITC (BD Biosciences, 1:100 30min at RT), antiKit-PEcy7 (BD Biosciences, 1:100), antiCD16/32-BV605 (BD Biosciences, 1:100), and antiSca1-PerCP-Cy5.5 (BD Biosciences, 1:100).

6.1 Transplant assays

C57Bl6 CD45.1+ mice of 15 weeks old were treated with cefovecin to avoid infections and the next day irradiated in an irradiator with 5.5 grays and 3 hours later with another 5.5 grays. The same day were injected with 1.500.000 bone marrow cells CD45.2+ from *Nanog^{tg}* mice. Bone marrow was depleted of erythrocytes with sterile FACSLysing solution (BD Biosciences). All the process was performed in sterile conditions.

6.2 Methocult and cytopsin

1000 cells of sorted populations and total BM were plated in methyl cellulose (Methocult GF M3434, Stemcell technologies). After counting and assessing the different types of colonies, to verify the type of colonies, we recover them from the plate and cytopsin them to observe cell morphology, centrifuging the samples directly into microscope slides. Staining was done with hematoxylin and eosin.

7. Cell culture

ESCs were maintained in serum-free conditions with Knock out serum replacement (Thermofisher), LIF (produced in-house), and 2i (CHIR-99021, Selleckchem; and PD0325901, Axon) and over mouse embryonic fibroblast (MEFs). The ESCs used were BT12 (NanogKO) and E14Tg2a (its parental wt cell line), kindly provided by Ian Chambers and Austin Smith (Chambers et al., 2007). Gain of function ES cells (NOES) were derived in the lab from *Nanog^{tg}* mice and karyotyping of the obtained lines was performed by the pluripotent cell technology unit at CNIC (Nagy et al., 2014).

7.1 Hematopoietic differentiation

ESC were differentiated toward hematopoiesis according to modified protocols (Irion et al., 2010, Lesinski et al., 2012, Sroczynska et al., 2009). For embryoid body formation, 5000 ESCs were plated in StemPro34 medium supplemented with nutrient supplement (Gibco) and 2 mM l-glutamine (l-Gln), penicillin/streptomycin (Gibco), 50 µg/ml ascorbic acid, 200 µg/ml iron saturated transferrin, 4 ng/ml recombinant human BMP4, and 4×10^{-4} monothioglycerol. After 2.5 days, the cultures were supplemented with 5 ng/ml recombinant human

fibroblast growth factor 2 (rhFGF2; basic fibroblast growth factor [bFGF]), 5 ng/ml recombinant human activin A, 5 ng/ml recombinant human VEGF (rhVEGF), 20 ng/ml recombinant murine thrombopoietin (TPO), and 100 ng/ml recombinant murine stem cell factor (rmSCF). Cytokines were obtained from R&D Systems Inc. or Peprotech. EBs were dissociated at day 5, 6 and 7 by treatment with 0.05% trypsin-EDTA at 37°C for 2–5 minutes.

Dissociated EBs at day 5 and 6 were plated in Methocult SF M3436 methylcellulose medium for quantification of primitive erythroid progenitor cells (BFU-E). Dissociated EBs at day 5, 6, and 7 were plated in Methocult GF M3434 methylcellulose medium for quantification of erythroid progenitor cells (CFU-E), granulocyte-macrophage progenitor cells (CFU-GM, CFU-G, CFU-M), and multi-potential granulocyte, erythroid, macrophage, and megakaryocyte progenitor cells (CFU-GEMM). Cells were plated in triplicate on ultra-low attachment surface plates (Corning) at 50,000 cells per plate. Plates were incubated in high humidity chambers for 12 days at 37°C and 5% CO₂. Whole plates were counted. For qPCR, EBs were directly lysed in extraction buffer and frozen at -80°C.

7.2 Anterior neural differentiation

NOES cells were differentiated to forebrain lineage as described (Gouti et al., 2014, 2017) in monolayer using corning p24 plates with cell bind surface and with 0.1% gelatin (Sigma) added 30 min before passing. Cells were grown in N2B27 media supplemented with 10 ng/ml bFgf (R&D) for 3 days (d1–d3) and then were transferred to supplemented N2B27.

7.3 Naïve to primed pluripotency differentiation

NanogKO and control ES cells were maintained in serum-free conditions with Knock out serum replacement (KOSR, Thermofisher), LIF (produced in-house), and 2i (CHIR-99021, Selleckchem; and PD0325901, Axon) for two passes. When cells were growing at the same rate, they were resuspended and plated in adherent culture plates coated with gelatin 0.1% at a density of 7×10^4 cells/cm² (Heo et al., 2005) on the same naïve media. 12h after plating, media was removed and cells were washed with PBS x2. Differentiating media was added: DMEM (Invitrogen) with 20%FBS (Hyclone), 2mM glutamine (Biosource), 100U/ml penicillin/streptomycin (Biosource) and β mercaptoethanol (Sigma) 1:1000. Cells

were recover at 0h, 12h and 24h, considering 0 as 12h after the cells have been replated in naïve conditions. RNA was extracted by adding RLT buffer (Qiagen) directly into the well.

8. *In situ* hybridization

Embryos were collected in cold PBS, transferred to 4% PFA, and fixed overnight at 4°C. After washing, embryos were dehydrated through increasing concentrations of PBS-diluted methanol (25%, 50%, 75%, and 2X 100%). *In situ* hybridization in whole mount embryos was performed as described (Acloque et al., 2008, Ariza-McNaughton & Krumlauf, 2002). For embryos up to 8.5 we used nested wells to facilitate the process.

Signal was developed with anti-dioxigenin-AP (Roche) and BM-Purple (Roche). Images were acquired with a Leica MZ-12 dissecting microscope. Probes were obtained by PCR of cDNA with the following primers (Table 4):

	PrimerF	PrimerR
<i>Gata1</i>	ATTTAGGTGACACTATAGAA GATGGAATCCAGACGAGGAA	GTAATACGACTCACTATAGGG CACTCAGGAACTGAGGCACA
<i>Klf1</i>	ATTTAGGTGACACTATAGAA CCACAGTACCAAGGCCACTT	GTAATACGACTCACTATAGGG ATCTCACCCCAGTCCATCTG
<i>Hbb-bh1</i>	ATTTAGGTGACACTATAGAA ACTCTGGGAAGGCTCCTGAT	GTAATACGACTCACTATAGGG TTGTGCTCTCAATGCAGTCC
<i>Redrum</i>	ATTTAGGTGACACTATAGAA GTTGCACTGCAGGTTCTTCA	GTAATACGACTCACTATAGGG TTCCCGCTATGTGAGTTTCC
<i>Sox2</i>	ATTTAGGTGACACTATAGAA ATGGGCTCTGTGGTCAAGTC	GTAATACGACTCACTATAGGG TGGACATTTGATTGCCATGT
<i>Pou3f1</i>	ATTTAGGTGACACTATAGAA CCTGGGGTCTTCTAACTCC	GTAATACGACTCACTATAGGG TTCGGTTTAGTCGGGCATAC

Table 4. Primer sequences for probe preparation for ISH.

9. RNAseq analysis

9.1 *Nanog*^{KO} ES cell RNAseq

RNA from *Nanog*^{KO} ES cells and their parental line was extracted as indicated before. Next generation sequencing single read (Illumina HiSeq 2500) and library

preparation (New England Biolabs Nest Ultra RNA library prep Kit) was performed in the genomic unit at CNIC.

Sequencing reads were inspected by means of a pipeline that used FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) to assess read quality, and Cutadapt v1.3 (Martin, 2011) to trim sequencing reads, eliminating Illumina adaptor remains, and to discard reads that were shorter than 30 bp. For libraries amplified with the NuGen Ovation Single Cell kit, the first 8 nucleotides of each read were also eliminated with fastx_trimmer (<http://hannonlab.cshl.edu/fastx>). The resulting reads were mapped against the mouse transcriptome (GRCm38 assembly, Ensembl release 76) and quantified using RSEM v1.2.20 (Li and Dewey, 2011). Raw expression counts were then processed with an analysis pipeline that used Bioconductor packages EdgeR (Robinson et al., 2010) for normalization (using TMM method) and differential expression testing, and ComBat (Johnson et al., 2007) for batch correction. Only genes expressed at a minimal level of 1 count per million, in at least 3 samples, were considered for differential expression analysis. Changes in gene expression were considered significant if their Benjamini and Hochberg adjusted p-value (FDR) was lower than 0.05.

9.2 Analysis of gene set in *Nanog*^{KO} versus control

To obtain the set of genes that are upregulated in *Nanog*^{KO} or in control along time, we look into genes that had no differences at 0h between wt and *Nanog*^{KO}, and that had increase expression for either *Nanog*^{KO} or wt at 24h. For that we used this conditions to filter them in R program.

For increased expression in KO ES cells

```
# No great difference at 0h between WT and KO, CPM based
abs(data$ave_E14_0h - data$ave_BT12_0h) <
0.6*pmax(data$ave_E14_0h,data$ave_BT12_0h)
# Increased expression with time for the KO
& data$BT12_0h_vs_BT12_12h_logFC < 0
& data$BT12_12h_vs_BT12_24h_logFC < 0
& data$BT12_0h_vs_BT12_24h_logFC < -log2(3)
# Significant negative difference at 24h between WT and KO, CPM based
& -1 * (data$ave_E14_24h - data$ave_BT12_24h) > 3 *
abs(data$ave_E14_0h - data$ave_BT12_0h)
& data$E14_24h_vs_BT12_24h_logFC < 0
& data$E14_24h_vs_BT12_24h_adj.P.Val < 0.05
```

For increased expression in control ES cells

```
# No great difference at 0h between WT and KO, CPM based
  abs(data$ave_E14_0h - data$ave_BT12_0h) <
0.6 * pmax(data$ave_E14_0h, data$ave_BT12_0h)
# Increased expression with time for the WT
& data$E14_0h_vs_E14_12h_logFC < 0
& data$E14_12h_vs_E14_24h_logFC < 0
& data$E14_0h_vs_E14_24h_logFC < -log2(3)
# Significant positive difference at 24h between WT and KO, CPM based
& (data$ave_E14_24h - data$ave_BT12_24h) > 3 * abs(data$ave_E14_0h -
data$ave_BT12_0h)
& data$E14_24h_vs_BT12_24h_logFC > 0
& data$E14_24h_vs_BT12_24h_adj.P.Val < 0.05
```

Both set of genes are filtered for adjusted Pvalue in the comparison between Control 24h Vs *Nanog*^{KO} 24h

9.3 Analysis of scRNAseq of E6.5

Two different data sets from different experiments (Mohammed et al., 2017a; Scialdone et al., 2016) were normalized by quantiles and batch corrected. To plot the cells in a tSNE genes with variance=0 were eliminated.

For correlation of genes with *Nanog* we used the slope of the line and how it adjusts to the points. For mutual exclusion in nascent mesoderm of the flk1+ cells (Scialdone et al., 2016) we use a hypergeometric test. In both cases for plotting we used ggPlot package from R, where we also performed statistics.

9.4 Intersection analysis

The intersection analysis of the genes coming from different RNAseq datasets was performed in the web tool from Bioinformatics and Evolutionary Genomics (<http://bioinformatics.psb.ugent.be/webtools/Venn/>)

RESULTS

RESULTS

1. Embryonic defects induced by *Nanog* in the E9.5 mouse

1.1. A mouse model for controlled *Nanog* gain-of-function

Nanog loss of function leads to embryonic lethality at preimplantation stages (Mitsui et al., 2003a), therefore preventing the analysis of its putative role at later stages of development when it is re-expressed at the posterior part of the gastrulating mouse embryo (Hart et al., 2004b). To overcome this obstacle, we used an inducible TetON transgenic model (*Nanog*^{tg}) in which *Nanog* expression is induced by the administration of doxycycline (dox) (Piazzolla et al., 2014). It consist in a double transgenic line comprising:

1. M2-rTA transactivator under the *Rosa26* promoter, and therefore expressed throughout the whole embryo (not extraembryonic tissues). In the presence of doxycycline is able to bind a Tet-operator (Tet-OP)
2. TetOP that upon binding of the M2-rTA activates the expression of *Nanog* cDNA. It is inserted in the *Col1a1* locus.

Consequently, with this double transgenic line we can regulate *Nanog* expression temporarily by the administration of doxycycline (dox) (Fig 10).

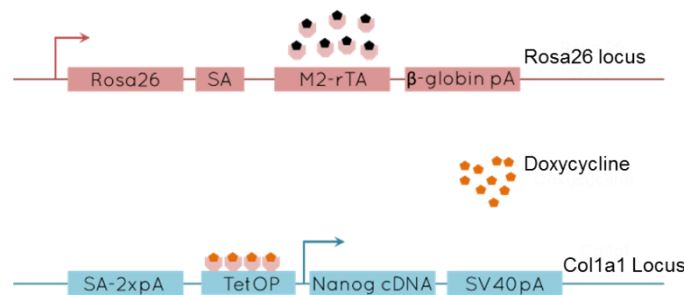


Figure 10. *Nanog*^{tg} TetON system: Schematic representation of both *loci* necessary for the expression of *Nanog*. In pink the *Rosa26* locus, that expresses the transactivator. In blue the tetracycline operator, if bound by the transactivator together with the doxycycline (in orange) it is able to express *Nanog* cDNA.

1.2 *Nanog* induces loss of blood and neural defects in the embryo

We induced *Nanog* from E6.5 in order to prolong its expression beyond E7.5, when it is normally turned off (Hart et al., 2004b), and examined the embryos at E9.5. Since we were ectopically expressing a pluripotency factor we could have found a general reversion of the embryonic tissues towards a pluripotent stage. However, based on previous works where overexpressing *Sox2* or *Oct4* leads to

a directed differentiation towards specific tissues (Niwa et al., 2000; Wang et al., 2012), we were also expecting more limited effects.

Actually, when we recovered the *Nanog*^{tg} embryos at E9.5 after treating them with doxycycline we observed a much delimited and specific phenotype: Embryos had anterior neural defects and a complete lack of blood (Fig 11). Since apparently *Nanog* was not affecting any other lineage or tissue, we wondered what could be the reason for these phenotypes and how could that relate with the physiological role of *Nanog* given its pattern of expression during gastrulation.

On one hand, red blood cells (RBC) are the first mesodermal cells to migrate through the primitive streak and they are already primed in the epiblast at the time *Nanog* is still expressed. Also, these primitive erythroid cells are conforming the majority of circulating blood cells in the E9.5 embryo.

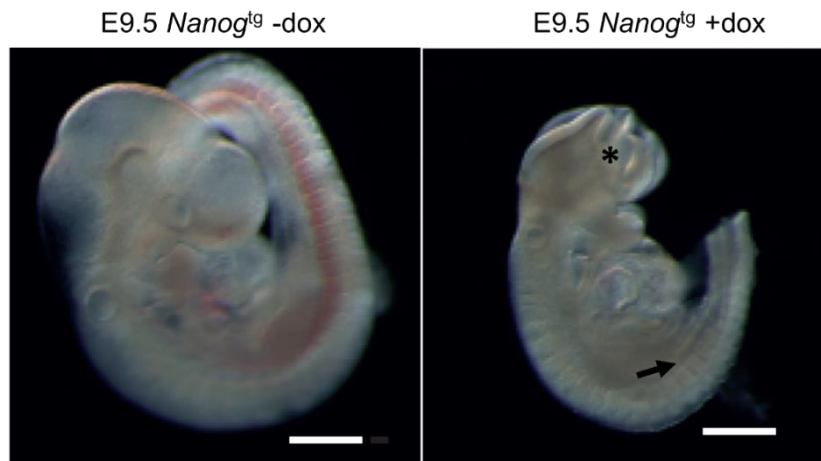


Figure 11. Phenotype of *Nanog*^{tg} embryos at E9.5 after treatment with doxycycline for three days: Representatives embryo without (-dox) and with (+dox) treatment. Asterisk marks craniofacial defects and arrow points out the lack of blood. Scale bar, 750um.

On the other hand, since *Nanog* is one of the earliest genes to show restricted expression in the posterior epiblast, it could have a role not only promoting gastrulation (Hoffman et al., 2013a; Tarafdar et al., 2013) but also repressing anterior fate.

These observations suggested that *Nanog* could be playing very specific roles at the onset of gastrulation, by maintaining hematopoietic and anterior neural determination on hold. Exploring the putative mechanism by which *Nanog* exerts these actions has been the aim of this work.

2. A role for *Nanog* in primitive hematopoiesis

2.1. *Nanog* blocks erythropoiesis in developing mouse embryos

To confirm the observation of the lack of blood we carried out whole mount in situ hybridization for *Hbb-bh1* (Wilkinson et al., 1987), encoding the *beta-like embryonic hemoglobin*, and for *Redrum*, an erythroid-specific long non-coding RNA (Alvarez-Dominguez et al., 2014; Paralkar et al., 2014). At E9.5, *Hbb-bh1* labels primitive red blood cells that are distributed throughout the yolk sac. Expression of *Nanog* up to this stage results in a nearly complete block of *Hbb-bh1* expression (Fig 12). *Redrum* expression is observed in the developing aorta-gonad-mesonephros (AGM) region and in the tail bud of the embryo. Again, induction of *Nanog* leads to loss of *Redrum* expression in the AGM, but interestingly not in the tail bud (Fig 12).

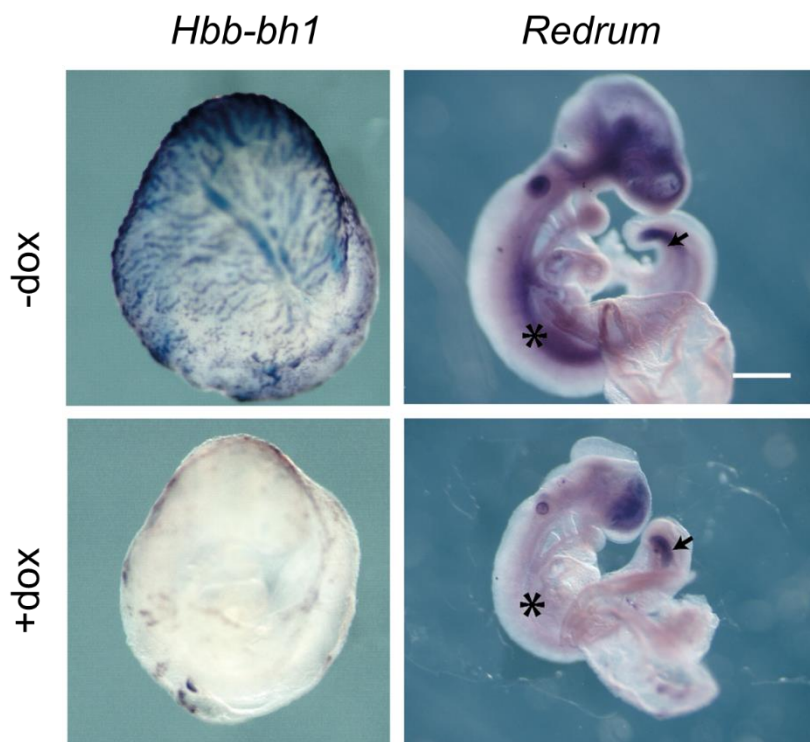


Figure 12. *In situ* hybridization of *Hbb-bh1* and *Redrum*: Effect in E9.5 embryos of sustained *Nanog* on the expression of *Hbb-bh1* (left) and *Redrum* (right). Upper panels, controls not treated with doxycycline; lower panels, 3 days doxycycline treated embryos. The asterisk indicates expression of *Redrum* in the AGM, and the arrow in the posterior region. Scale bar, 750um.

2.2. *Nanog* does not affect developing vessels

In the early gastrulating embryo, cells from the epiblast that will give rise to RBC migrate through the primitive streak to the extraembryonic region, where they can give rise to either vessels or hematopoietic cells (Padrón-Barthe et al., 2014). Therefore we checked if the apparent lack of blood was accompanied by vasculature defects. Immunostaining for Endomucin, a marker for endothelial cells expressed in embryonic endothelial cells, revealed no substantial differences at E9.5 between dox-treated and untreated *Nanog*^{tg} embryos, as is observed in the correct patterning of intersomitic vessels (Fig 13).

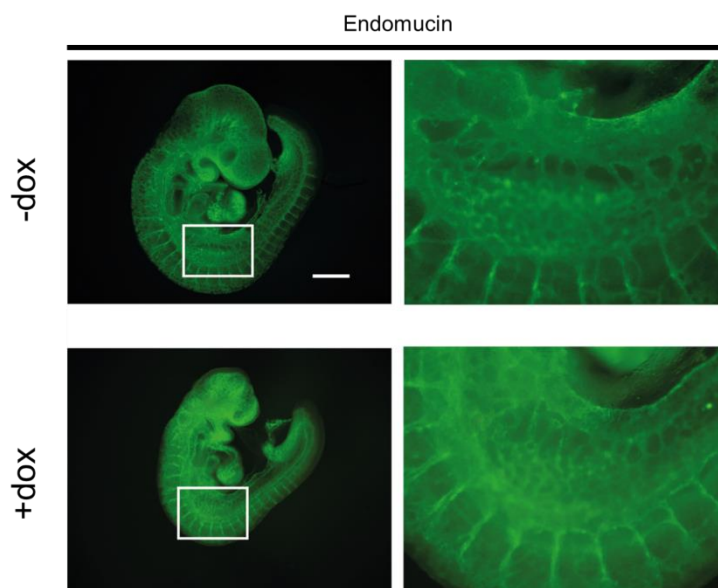


Figure 13. Endomucin staining of vessels of E9.5 *Nanog*^{tg} embryos: with (+dox) and without (-dox) treatment. Right panels show close-up view of the boxed areas on left panels. White box is zoomed in in the panel next to it. Scale bar 750um.

Prolonged *Nanog* expression in the embryo causes a deficit in primitive red blood cells that is accompanied by lack of expression of erythroid-specific genes, but does not affect early vascular development.

This anaemic phenotype was present in all stages of development. When we treated *Nanog*^{tg} embryos with dox from E10.5 to E14.5 we recovered embryos with a strong anaemic phenotype (Fig 14).

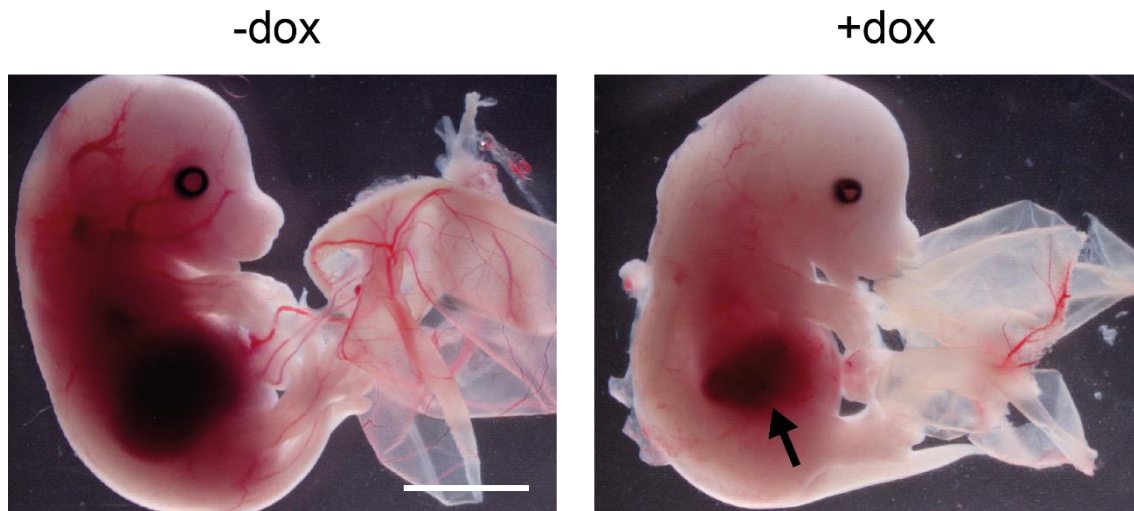


Figure 14. Freshly dissected E14.5 *Nanog*^{tg} embryos: with (+dox) and without (-dox) treatment. The arrow indicates the fetal liver, the main hematopoietic organ at this developmental stage. Scale bar 5.5mm.

2.3. Flow cytometry analysis reveals a specific effect of *Nanog* on erythroid differentiation

To characterize the effect of *Nanog* induction on hematopoiesis, we separated progenitors and red blood cells by flow cytometry of dispersed yolk sacs of E9.5 embryos using c-Kit (a marker of early uncommitted progenitors), CD41 (hematopoietic progenitors), CD71 (red blood cell precursors), Ter119 (matured red blood cells) (Baron et al., 2012; Tober et al., 2007). We found no differences in total cell number of uncommitted (c-Kit⁺) or committed hematopoietic progenitors (c-Kit⁺ CD41⁺) nor in megakaryocyte precursors (c-Kit⁻ CD41⁺) (Fig 15A,B).

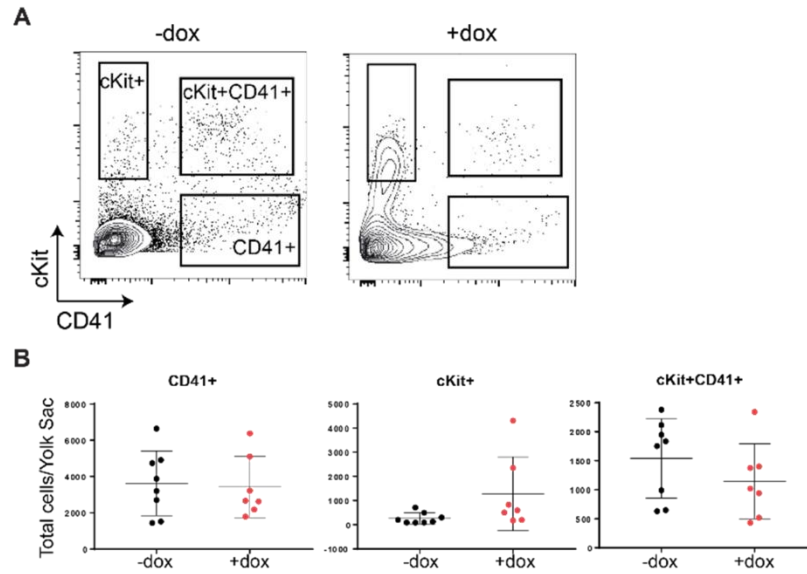


Figure 15. FACS analysis of E9.5 hematopoietic population: (A) Representative FACS images showing the distribution of cKit+CD41-, cKit+CD41+ ckit-CD41+ fractions in the E9.5 embryo. (B) Graphic representation of the cKit+CD41-, cKit+CD41+ ckit-CD41+ FACS dot plots. Each assay contained a pool of 10 E9.5 embryos per condition: 8 untreated (-dox) and 7 treated (+dox). All statistics were performed using Student's t test.

We further characterized the erythroid phenotype by analyzing cell maturation by flow cytometry using CD71 and Ter119 (Borges et al., 2012). We observed an increase in the proportion of immature types (S0 and S1) together with a reduction in more mature erythroid cells (S2), suggesting a block in the differentiation of red blood cells (Fig 16A,B). Together, these results show that *Nanog* expression in the embryo leads to a specific deficiency of the erythroid lineage.

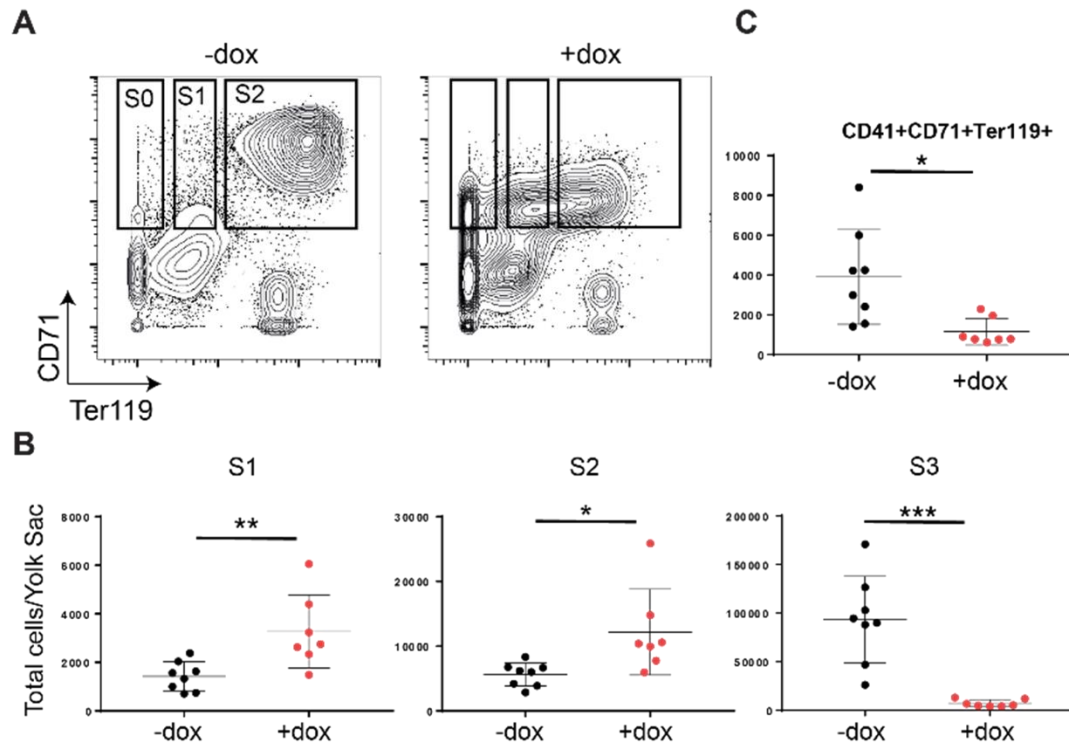


Figure 16. FACS analysis of E9.5 erythroid population: (A) Representative FACS images showing the distribution of the CD71+Ter119+ population in E9.5 single *Nanog*^{tg} embryos with and without doxycycline. S0: CD71+Ter119-, S1:CD71+Ter119low and S2:CD71+Ter119+ represent different state of progression of blood maturation. (B) Graphic representation of the Ter119 CD71 CD41+ FACS dot plots in untreated (-dox) and treated (+dox) embryos. n=7 ***P < 0.00005, **P< 0.0005, *P<0.005. (C) Graphic representation of the Ter119 CD71+ FACS dot plots. n=7 *P < 0.005. All statistics were performed using Student's t test.

2.4. *Nanog* downregulates the expression of key erythroid determination genes in the embryo

We next investigated how the extension of *Nanog* expression to E9.5 influences hematopoietic gene expression. For this, we isolated progenitor and mature populations by flow cytometry as described above (Fig 15A 16A), and conducted RT-qPCR to examine the expression of core lineage determinants of hematopoietic fate: *Runx1*, *Tal1*, *Gata1*, and *Klf1* (Chen et al., 2009; Kuvardina et al., 2015; Palis et al., 1999; Yokomizo et al., 2008). Despite consistent gain of *Nanog* expression in all populations, we only found significant changes in gene expression in mature erythrocytes (S2: CD71+Ter119+ population) where we observe significant downregulation of *Tal1* and an upregulation of *Runx1*, and in early hematopoietic progenitors (ckit+CD41-) in which we find *Tal1* and *Runx1*

downregulated, (Fig17). Expression of *Gata1* is not affected in megakaryocyte precursors (CD41+), as is also the case for *Runx1* (Fig 17). Taken together, these results suggest that *Nanog* causes a blockade in hematopoietic progenitors, preventing their differentiation towards erythroblast cells.

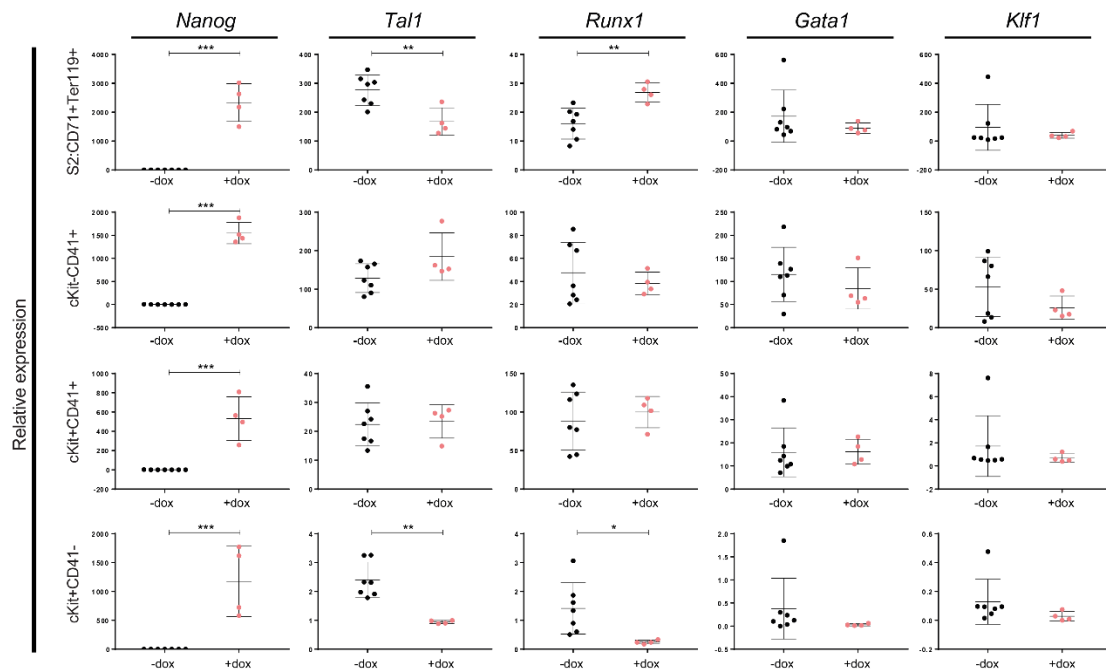


Figure 17. RT-qPCR of sorted hematopoietic populations: Differences in the expression levels of hematopoietic genes and *Nanog* between *Nanog*^{T9} with and without doxycycline of E9.5 embryos. -dox n=7, +dox n=4 ***P<0.0005, **P<0.005, *P<0.05. All statistics were performed with Student's t test.

To examine if red blood cell changes occur at earlier stages, we induced *Nanog* expression from E5.5 to E7.5, a time window spanning initiation of primitive hematopoiesis. Whole mount *in situ* hybridization showed decreased expression of *Gata1* and *Klf1* in the extraembryonic region, corresponding to the blood island domain (Fig 18).

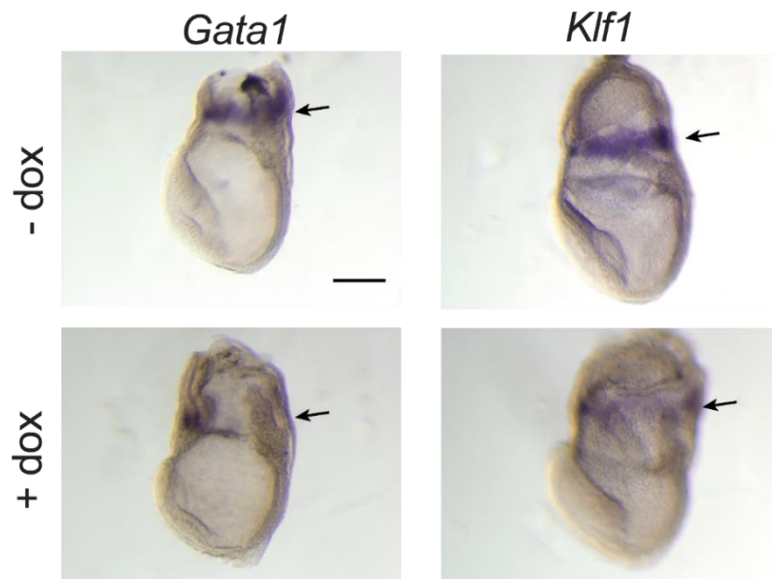


Figure 18. Whole mount in situ hybridization for *Gata1* and *Klf1*: E7.5 *Nanog^{tg}* embryos without (-dox) and with (+dox) treatment with doxycycline. Arrows indicate the location of blood islands in the extraembryonic yolk sac. Scale bar, 750 μ m.

RT-qPCR of individual dox-treated or control E7.5 *Nanog^{tg}* embryos showed decreased expression of the core erythropoietic genes *Tal1*, *Gata1*, and *Klf1* but no change in *Runx1* (Fig 19). A possible explanation for our observations would be that *Nanog* expression causes a general blockade of mesodermal specification, with the downregulation of early hematopoiesis genes being merely a secondary effect of this. We therefore tested the expression of lineage determinants expressed at gastrulation (*Brachyury* and *Eomes*) and the early mesodermal gene *Kdr* (Palis et al., 1999; Shalaby et al., 1995). Exogenous *Nanog* induced the expression of both *Brachyury* and *Eomes*, in line with published data (Teo et al., 2011), but did not alter *Kdr* expression (Fig 19).

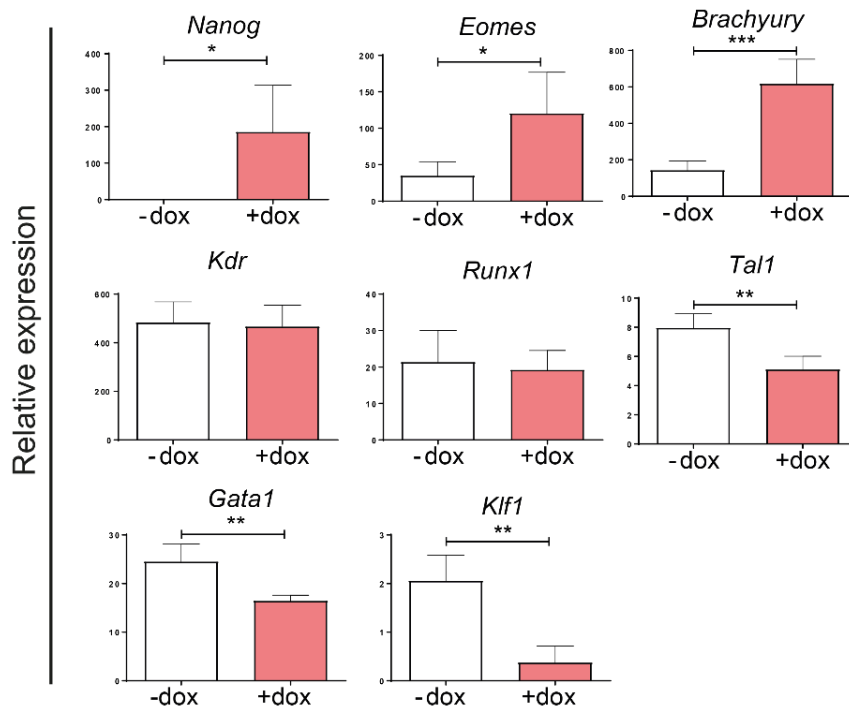


Figure 19 RT-qPCR of individual dox-treated or control E7.5 *Nanog*^{tg} : Relative expression of *Nanog*, mesodermal (*Eomes*, *Brachyury*, *Kdr*) and hematopoietic (*Runx1*, *Tal1*, *Gata1*, *Klf1*) genes in single control (-dox) or treated (+dox) E7.5 embryos (n=4 per condition). Scale bar, 750 μ m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$; All statistics were performed with Student t test.

Together, these results suggest that *Nanog* blocks erythroid fate and is able to specifically downregulate the early expression of erythropoietic genes during the initial determination of primitive haematopoiesis, after mesodermal specification in the epiblast.

3. Is the effect of *Nanog* on erythroid lineage cell-autonomous?

The results presented so far suggest that *Nanog* blocks specifically erythroid progenitors during primitive hematopoiesis. To test if this is the case, we aimed to rescue the observed genotype by generating chimeric embryos by injection of wild type ES cells constitutively expressing GFP (Díaz-Díaz et al., 2017) into *Nanog*^{tg} blastocysts.

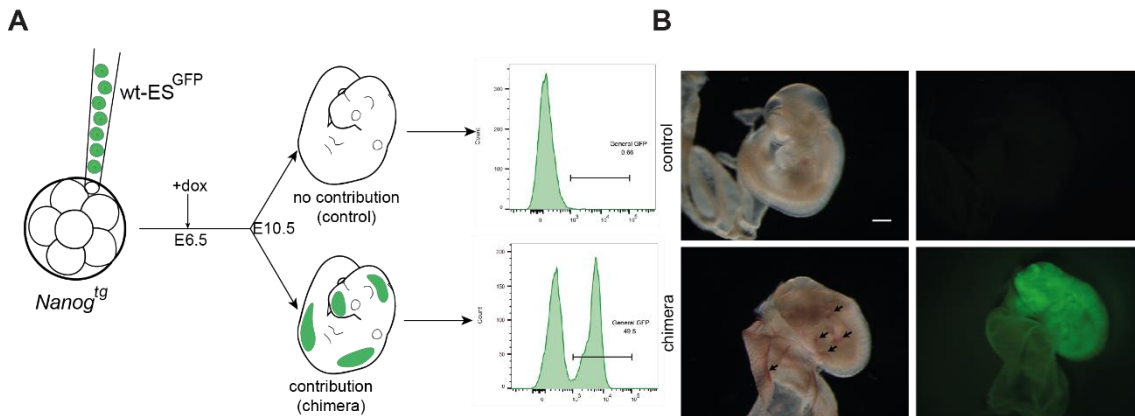


Figure 20. Wild type ES cells rescue erythroid maturation in chimeric embryos. (A) Experimental design for chimera generation and contribution of GFP cells to chimeric embryo (left hand side panels). (B) Freshly dissected dox-treated *Nanog*^{tg} E10.5 embryos without (control) and with (chimera) contribution of wt-ES^{GFP} cells (left, brightfield; right, GFP). Arrows mark the presence of blood in chimeric embryos that is absent from controls. Scale bar, 750 μ m.

The resulting embryos were treated *in utero* with dox at E6.5 and examined for GFP fluorescence at E10.5. Those showing no overall contribution (no GFP+ cells) were used as controls, whereas embryos containing GFP+ cells were considered chimeras (Figure 20 A, B). Erythroid cells were evaluated in individual embryos by flow cytometry analysis of the S2 population (CD71+ Ter119+), as described earlier (Figure 16).

Chimeras with high contribution of wild type ES cells had circulating blood in both the embryo and the yolk sac, despite dox treatment, contrasting with embryos with no contribution (Fig 20).

Chimeras showed a recovery of erythroid cells, with high contribution from GFP+ wild type ES-derived cells (Fig 21A). Quantification of erythroid populations in chimeras showed an increased content of CD71+ Ter119+ cells (Fig 21B); this increase did not occur when the experiment was repeated without dox treatment (Fig 21C). The number of GFP- cells (derived from *Nanog* expressing cells) in dox-treated chimeras did not differ from that in controls (with no contribution of GFP+ cells), demonstrating that the recovery of the erythroid populations in chimeras was entirely due to the wild type ES cells (Fig 21D). These results indicate that the effect of *Nanog* on erythroid progenitors is primarily cell autonomous, and not secondary to *Nanog*-induced changes in other cell types.

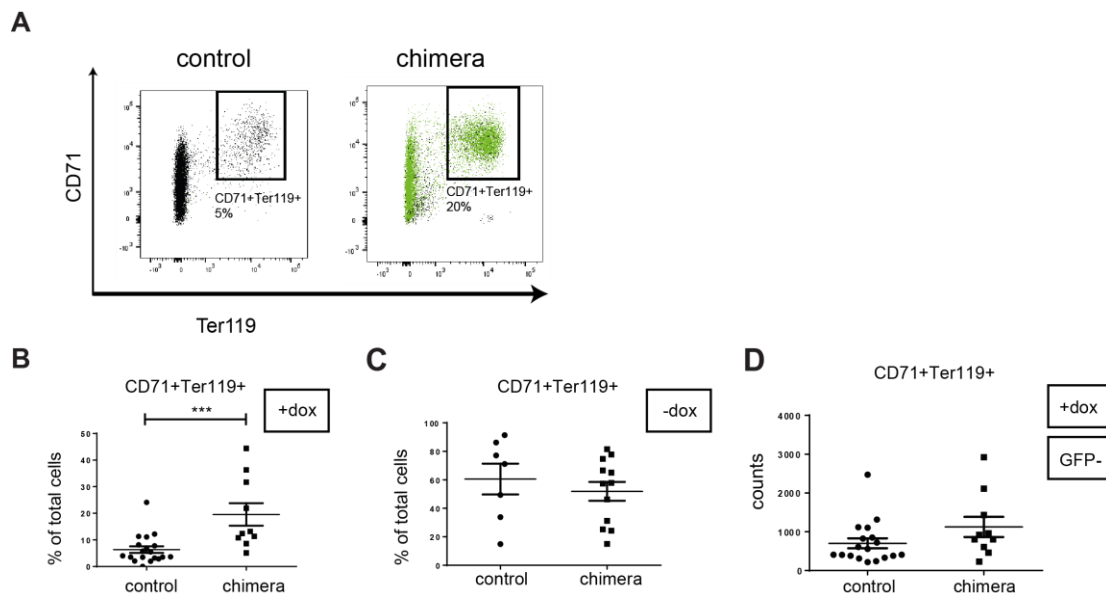


Figure 21. Wild type ES cells rescue erythroid maturation in chimeric embryos: (A) Representative FACS plots showing of red blood cell maturation as determined by CD71/Ter119 staining in single dox-treated E10.5 control (left) and chimeric (right) embryos. (B-D). Quantification of the CD71+ Ter119+ population in single dox-treated embryos (B; control, n=18; chimera, n=10), untreated embryos (C; control, n=7; chimera, n=12), and in GFP- cells (not derived from wild type ES cells) from dox-treated embryos (D; control, n=18; chimera, n=10). ***P < 0.0005. All statistics were performed with Student's t test.

4. Loss of *Nanog* enhances hematopoietic differentiation of ES cells

4.1. Colony forming units assay reveals increased potential for erythroid differentiation in *Nanog*^{-/-} ES cells

To investigate the effect of the absence of *Nanog* on the erythroid lineage, we tested the potential of ES cells with homozygous *Nanog* loss of function (Chambers et al., 2007b) to differentiate into blood cells in culture (Irion et al., 2010). *Nanog*^{-/-} and wild type control ES cells of the parental strain (E14Tg2a) were used to generate embryoid bodies (EB). EBs were allowed to differentiate for up to 7 days in hematopoietic differentiation media. After disaggregation and culture different colony forming units (CFU) were scored between days 5 and 7 (D5-D7; Fig 22). Despite a trend for a decrease in the number of common myeloid progenitors (CFU-GEMM), *Nanog*^{-/-} EBs generated significantly more primitive erythroid colonies (Ery-P) than controls, as well as a significantly higher number of mature erythroid colonies (BFU-E; burst forming unit erythroid) in the presence of cytokines driving a broader hematopoietic differentiation. Interestingly, there was no between-genotype difference in granulocyte-monocyte (CFU-GM) progenitors, but monocyte (CFU-M) or granulocyte (CFU-G) progenitors were produced more abundantly from wild type than from *Nanog*^{-/-} EBs (Fig 22). This last observation is possibly the result of the decrease in common myeloid progenitors together with the significant increase of erythroid progenitors. *Nanog*^{-/-} ES cells thus have an increased potential for specific differentiation to red blood cells.

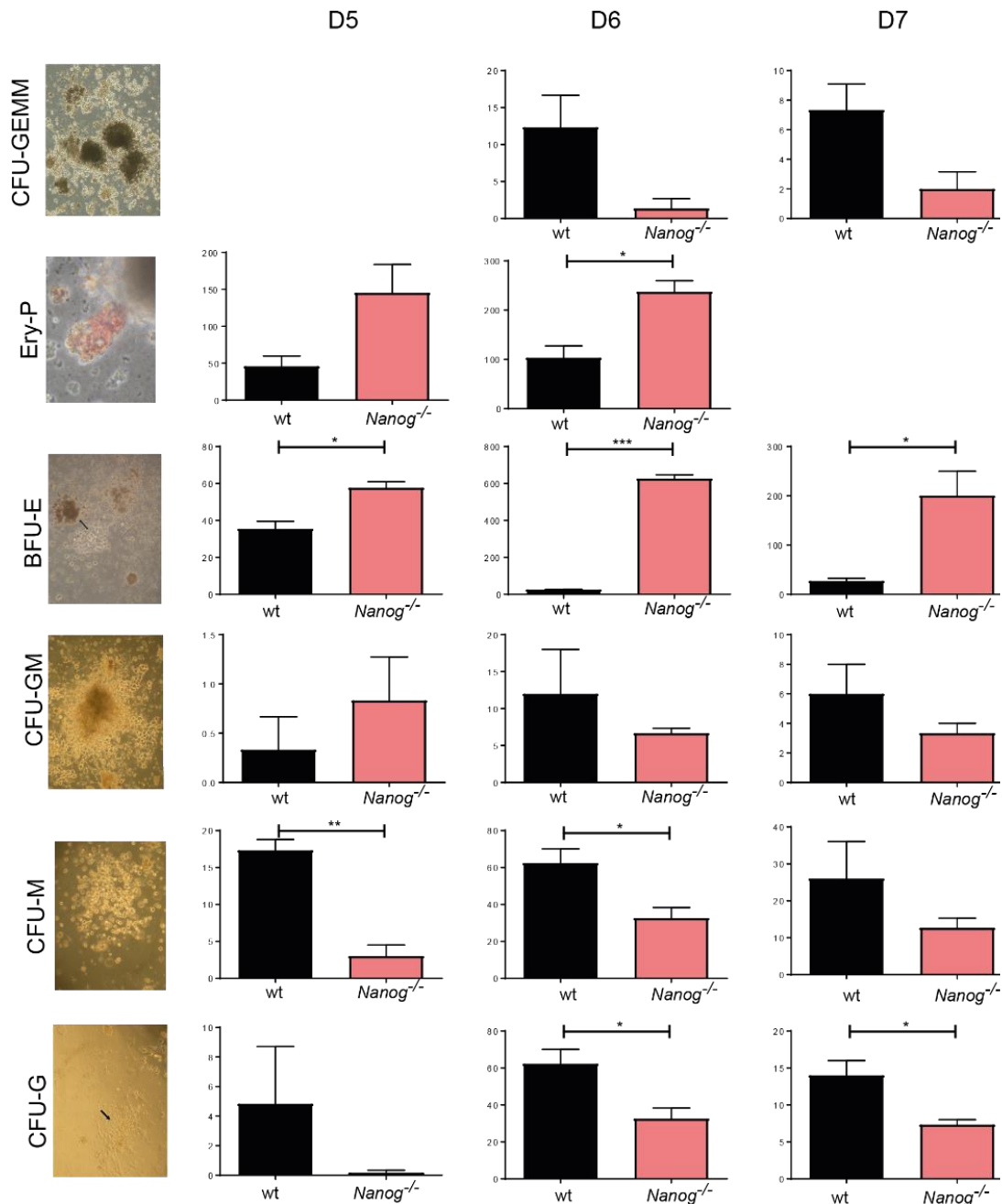


Figure 22. *Nanog*-knockout ES cells show increased potential to generate erythroid precursors: Quantification of colony-forming units generated by wild type (wt) and knockout (*Nanog*^{-/-}) ES cells after culture of EBs for 5 (D5), 6 (D6), or 7 (D7) days and plating disaggregated cells in different hemogenic-promoting conditions (n=3, each with 3 technical replicates). Panels on the left show representative images of mouse hematopoietic colonies obtained after 12 days of culture in specific media. CFU-GEMM, progenitors giving rise to granulocytes, erythrocytes, monocytes, and megakaryocytes; BFU-E, burst forming units-erythroid; Ery-P, colony forming primitive erythroid; CFU-GM, granulocyte-monocyte precursors; CFU-M, monocyte precursors; CFU-G, granulocyte precursors. No CFU-GEMM are detected at D5 and no BFU-E at D7. **P* < 0.05, ***P* < 0.005, ****P* < 0.00005. All statistics were performed with Student's t test.

4.2. Hematopoietic genes are expressed regardless of the delay in *Brachyury* expression during differentiation of *Nanog*^{-/-} ES cells

To investigate how the absence of *Nanog* affects the gene networks involved in erythroid specification, we monitored control and *Nanog*^{-/-} ES-derived EBs for the expression of selected markers over 10 days of differentiation. *Brachyury* expression was examined as a marker of initial mesoderm specification, a necessary first step for the establishment of hematopoietic lineages (Fehling et al., 2003). *Brachyury* expression markedly increased at day 3 in wild type cells, as previously described (Robertson et al., 2000), but in *Nanog*^{-/-} EBs this expression peak was delayed until day 5 (Fig 23).

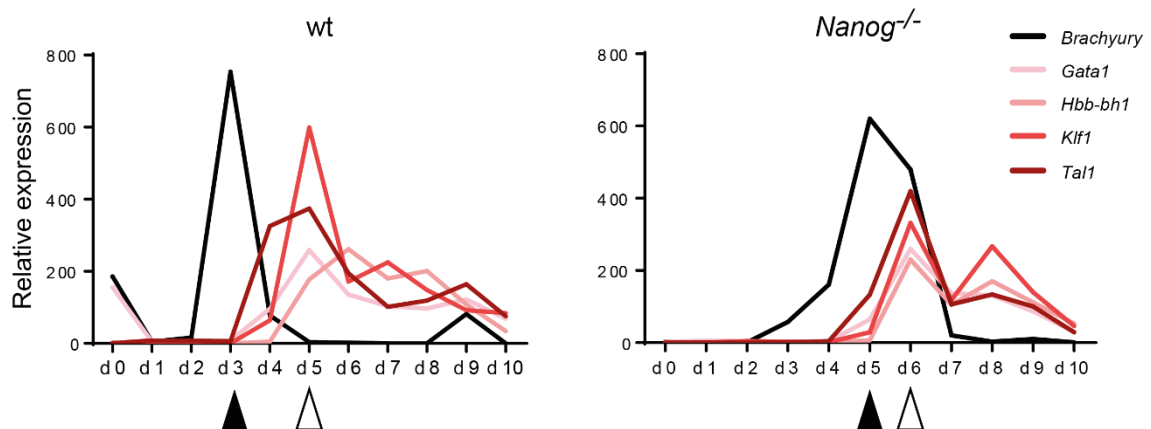


Figure 23. Relative expression of hematopoietic genes and *Brachyury*: RT-qPCR determination of the relative expression of *Brachyury* and selected hematopoietic genes in control (wt, left) and knockout (*Nanog*^{-/-}, right) ES cells (n=3) during 10 days of EB differentiation in hematopoietic-cytokine-enriched medium. Black arrowheads indicate the peak of *Brachyury* expression and white arrowheads the time of maximum hematopoietic-gene expression.

Nanog is thus likely required for the correct temporal activation of *Brachyury* during mesoderm specification. We next checked the expression of genes encoding the erythroid specific factors *Tal1*, *Gata1*, and *Klf1* and the embryonic hemoglobin gene *Hbb-bh1*. In wild type EBs, erythroid gene expression peaks around day 5, 2 days after *Brachyury* activation. In *Nanog*^{-/-} EBs, erythroid gene expression peaked a day later, at day 6. However, this is only 1 day after the onset of *Brachyury* expression, contrasting the 2-day delay in wild type EBs (Fig 23). Given the requirement of *Brachyury* expression for hematopoietic differentiation (Fehling et al., 2003), we aligned the expression dynamics of wild type and *Nanog*^{-/-} cells to the day of *Brachyury* induction (Fig 24A).

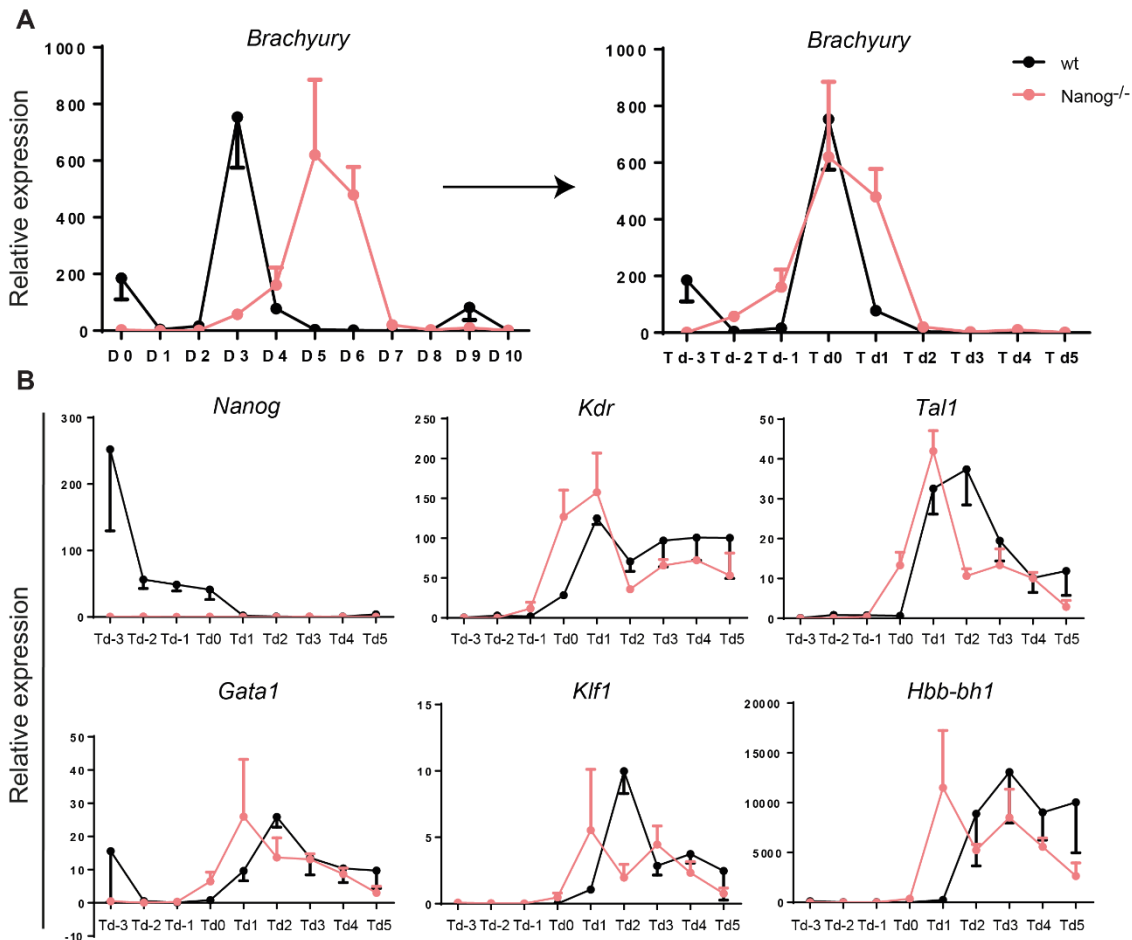


Figure 24. Expression profiles of hematopoietic genes during differentiation aligned at the peak of *Brachyury* expression: (A) Timing of *Brachyury* expression before (right) and after (left) the alignment of its peak of expression that occurs at day 3 (D3) of differentiation in wild type ES cells (wt, black) and at day 5 (D5) in *Nanog*^{-/-} cells (red). (B) Timing of expression of *Nanog* and selected hematopoietic genes when wt and *Nanog*^{-/-} cells after alignment. The time point of maximum *Brachyury* expression is labelled as T d0.

To validate this approach, we examined the expression of *Kdr*, a pan-mesodermal gene that acts downstream of *Brachyury*; relative to the timing of *Brachyury* induction, dynamics of *Kdr* expression coincided in wild type and *Nanog*^{-/-} EBs. In contrast, *Brachyury*-referenced erythroid gene activation occurred earlier in *Nanog*^{-/-} EBs than in wild type controls (Fig 24B). Thus, although mesoderm induction is delayed in *Nanog*^{-/-} EBs, once it occurs the *Nanog*^{-/-} mesodermal cells show an elevated potential for erythroid differentiation.

5. Effects of *Nanog* gain-of-function on hematopoiesis in the adult mouse

5.1 Blockade of adult erythrocyte maturation by *Nanog*

Nanog has been analyzed mainly in early developmental stages and in pluripotent stem cells. However, some reports have described its expression and role in adult tissues and cells (Kohler et al., 2011; Piazzolla et al., 2014; Tanaka et al., 2007). In light of our findings during embryonic hematopoiesis, we therefore explored the effects of *Nanog* during erythroid differentiation in the adult.

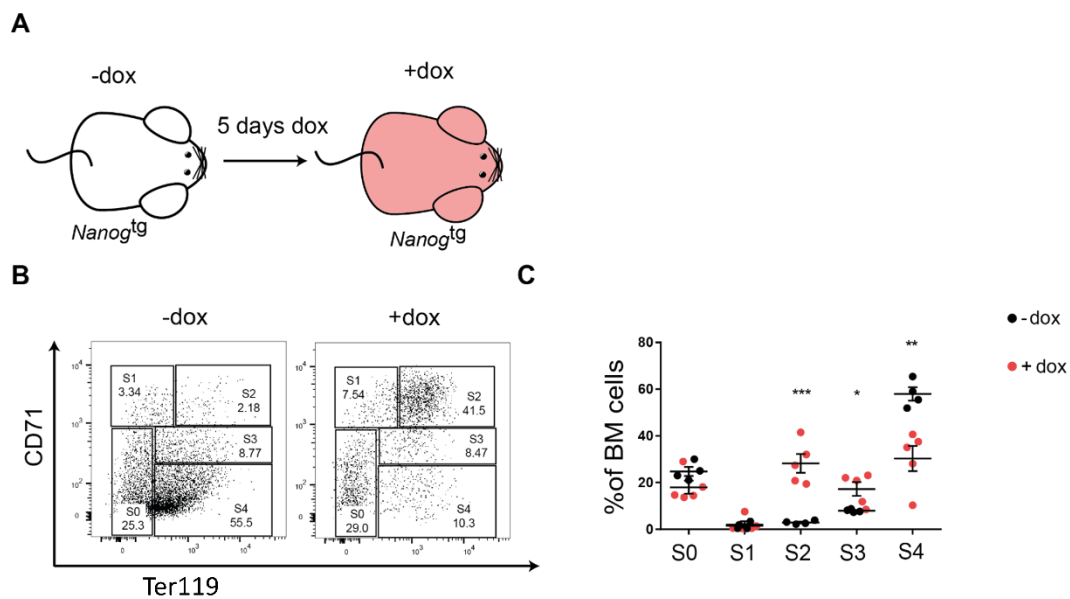


Figure 25. Induced *Nanog* expression blocks erythroid maturation in adult mice: (A) Experimental design for the treatment of adult *Nanog*^{tg} mice. (B) Representative FACS plots showing the distribution of different populations distinguished by CD71/Ter119 staining in whole bone marrow from untreated (-dox) or treated (+dox) adult mice. S0 (double negative cell), S1 (proerythroblast), S2 (basophilic erythroblast), S3 (polychromatic erythroblast), and S4 (orthochromatic erythroblast) are different stages of blood maturation. (C) Quantification of the S1-S4 erythroid populations (-dox, n=4; +dox, n=5) **P* < 0.05, ***P* < 0.005, ****P* < 0.0005. All statistics were performed with Student's t test..

Nanog expression was systemically induced in adult *Nanog*^{tg} mice by 5-day treatment with dox in drinking water, and the mice were then sacrificed and bone marrow extracted (dox+; Fig 25A). As controls, we used untreated mice of the same genotype (dox-). Analysis of erythrocyte maturation with CD71 and Ter119 (Socolovsky et al., 2001; Zhang et al., 2003) revealed an increase in immature populations (basophilic and polychromatic erythroblasts; S2 and S3, respectively) together with a decrease in the number of more differentiated erythrocytes

(orthochromatic erythroblasts, S4; Fig 25 B,C). This result suggested a block in the differentiation of erythrocyte precursors.

5.2. Differentiation of megakaryocyte-erythroid progenitors is blocked by *Nanog*

After observing the effect on the last stages of differentiation of erythroid cells, we wondered if this phenotype could be also seen in earlier progenitors and whether defects will affect only erythroid lineage or if progenitors that give rise to other cell types would be affected as well.

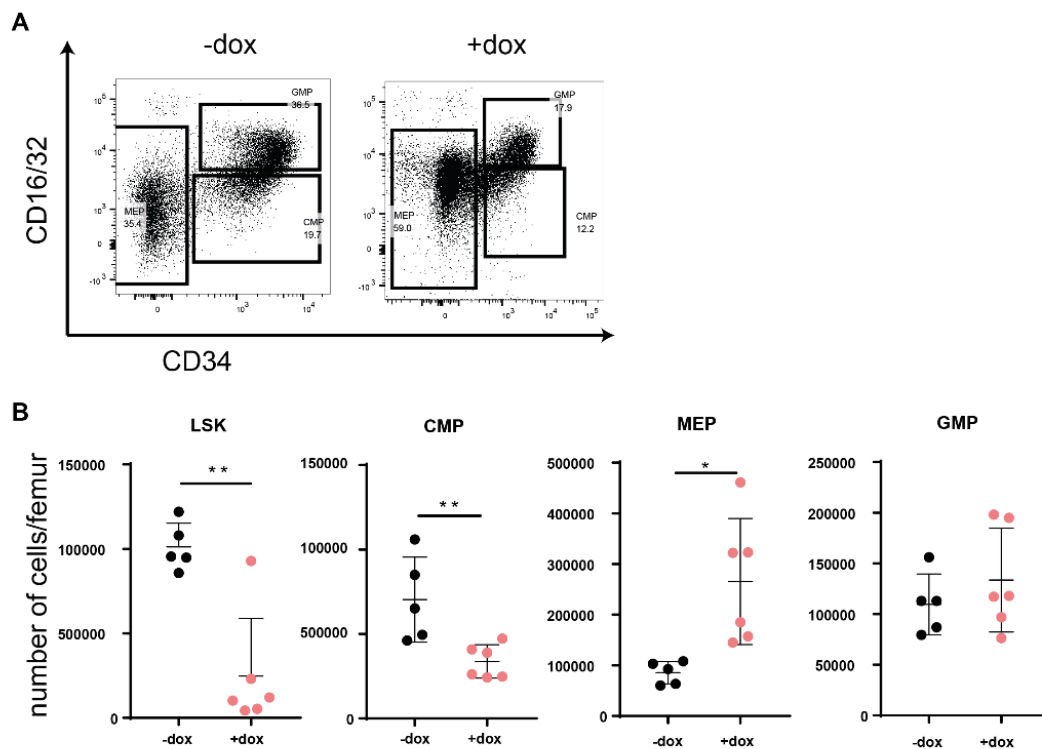


Figure 26. Induced *Nanog* expression increases MEPs in adult mice: (A) Representative FACS plots showing the distribution of CD16/32 and CD34 hematopoietic precursors sorted from the cKit+Sca1-LIN⁻ bone marrow of untreated (-dox) or treated (+dox) adult *Nanog*^{tg} mice. (B) Quantification of precursor populations based on CD16/32 and CD34 sorting, as total number of cells per individual femur (-dox, n=5; +dox, n=6). **P* < 0.05, ***P* < 0.005; Student's t-test.

Thus we quantified bone marrow progenitors by flow cytometry using lineage cocktail, ckit, Sca1, CD34 and CD16/32 (Fig 26A, Challen et al., 2009). Induced *Nanog* expression triggered a decrease in absolute cell numbers of hematopoietic stem cells (lineage-Sca1+cKit+; LSK) and common myeloid progenitors (CMP), but no changes in granulocyte-macrophage progenitors (GMP). Interestingly, this was accompanied by a significant increase in megakaryocyte-erythroid progenitors (MEP; Fig 26B). Therefore, we could see the effect of *Nanog* at different stages of erythrocyte development but not in any other cell type progenitor. The decrease in LSK and CMP could be attributed to the increase in production of MEPs that would consequently exhaust the earliest progenitors.

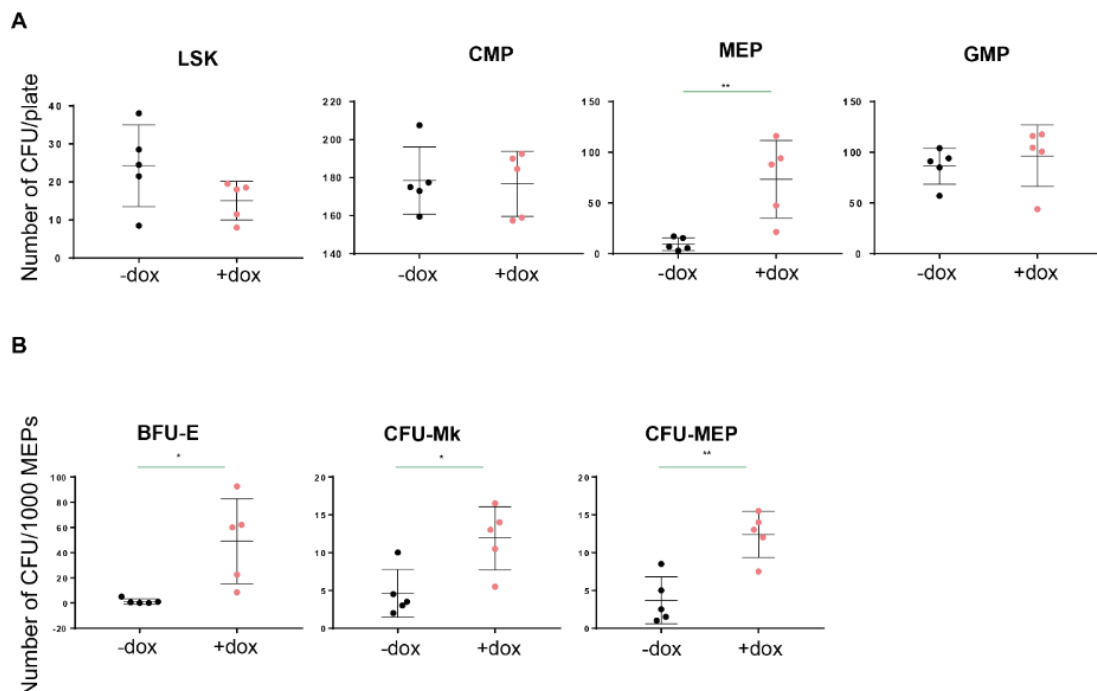


Figure 27. Induced *Nanog* expression blocks transiently the differentiation of MEPs: (A) Quantification of total number of colony forming units (CFU) from 1000 cells of different sorted precursor populations. Only megakaryocyte-erythroid progenitors (MEP) show an increase in the progenitors (-dox, n=5; +dox, n=5). ** $P < 0.005$; Student's t-test. (B) Quantification of burst forming units-erythroid (BFU-E), colony forming units-megakaryocyte (CFU-Mk), and colony forming units of a mixed erythroid/megakaryocyte phenotype (CFU-MEP) present in 1000 cells of sorted MEPs per plate from untreated (-dox; n=5) and treated (+dox, n=5) individuals. Two plates were seeded with 1000 cells each as technical replicates. * $P < 0.05$, ** $P < 0.005$; Student's t-test.

One question remained unanswered: whether the MEP population conserved their differentiation capabilities but was blocked by *Nanog* expression, or if on the contrary *Nanog* compromised the functionality of these progenitors, making them unable to further differentiate.

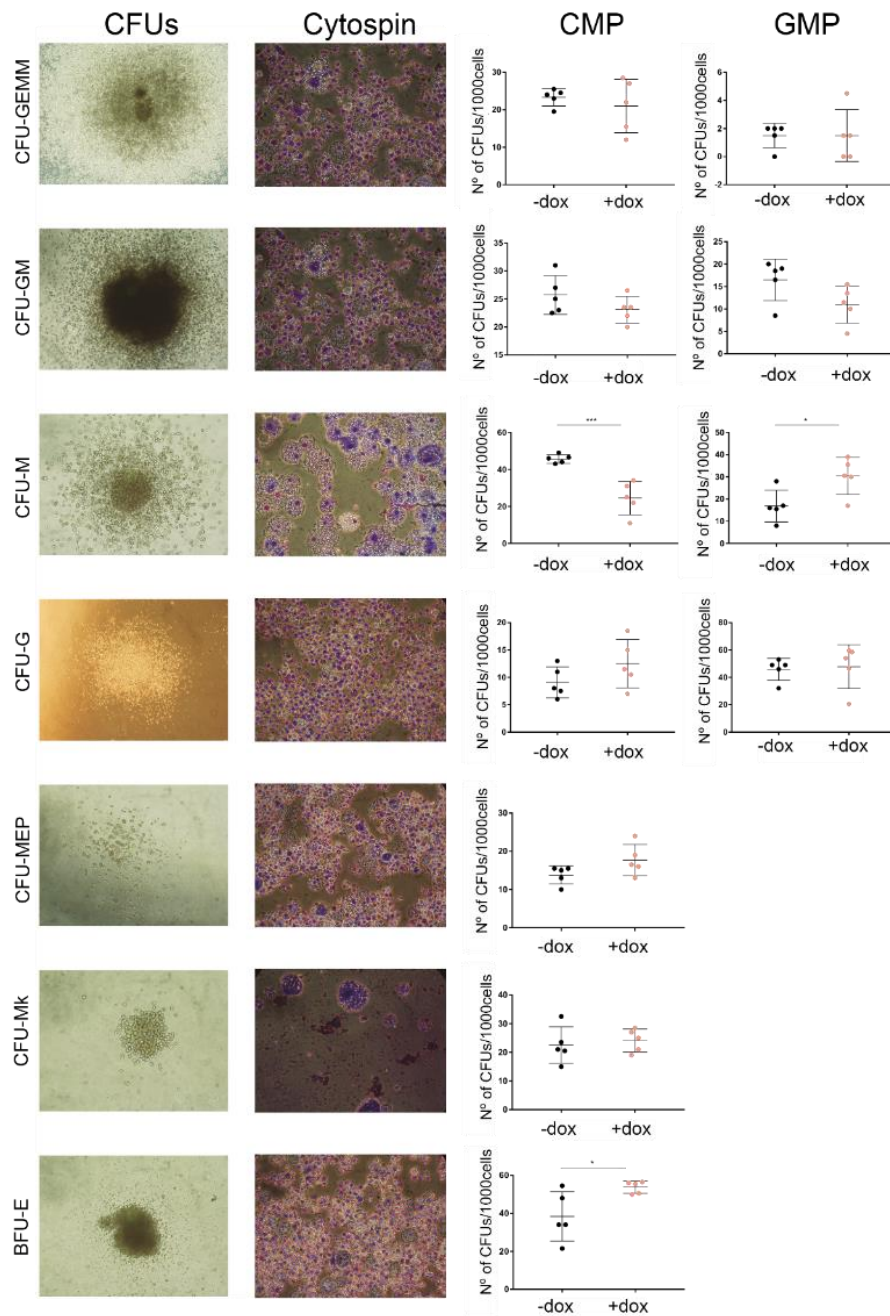


Figure 28. CFUs images, respective cytopsinings and total counts of CMPs and GMPs: **(CFUs)** Representative images of mouse hematopoietic colonies obtained from adult bone marrow. CFU-GEMM, progenitors giving rise to granulocytes, erythrocytes, monocytes, and megakaryocytes; CFU-GM, granulocyte-monocyte precursors; CFU-M, monocyte precursors; CFU-G, granulocyte precursors, CFU-MEP, mixed erythroid/megakaryocyte precursors; CFU-Mk, megakaryocyte precursors; BFU-E, burst forming units-erythroid. **(Cytospin)** Representative images of selected spun CFUs **(CMP)** Quantification of different colony-forming units generated by 1000 cells of common myeloid progenitors (CMP) per plate isolated from untreated (-dox, n=5) and treated (+dox, n=5) *Nanog^{tg}* adult mice. Two plates were seeded with 1000 cells each as technical replicates. * $P < 0.05$, *** $P < 0.0005$; Student's t-test. **(GMP)** Quantification of different colony-forming units generated by 1000 cells of granulocyte-monocyte progenitors (GMP) per plate isolated from untreated (-dox, n=5) and treated (+dox, n=5) *Nanog^{tg}* adult mice. Two plates were seeded with 1000 cells each as technical replicates. As expected, no CFU-MEP, CFU-Mk or BFU-E were produced by GMPs. * $P < 0.05$, Student's t-test.

In order to investigate this issue we tested the differentiation capability of sorted populations from the bone marrow (LSK, CMPs, GMPs and MEPs) of dox treated and untreated mice by quantifying the colony forming unit (CFU) potential of each population in the absence of doxycycline (and therefore releasing the blockade of *Nanog* on the differentiation potential). Our hypothesis was that if *Nanog* was able to block the differentiation capacities, upon *Nanog* removal we would observe normal differentiation of CFUs; on the other hand, if *Nanog* is compromising the functionality of those cells irreversibly, we should observe a deficit in the production of CFUs.

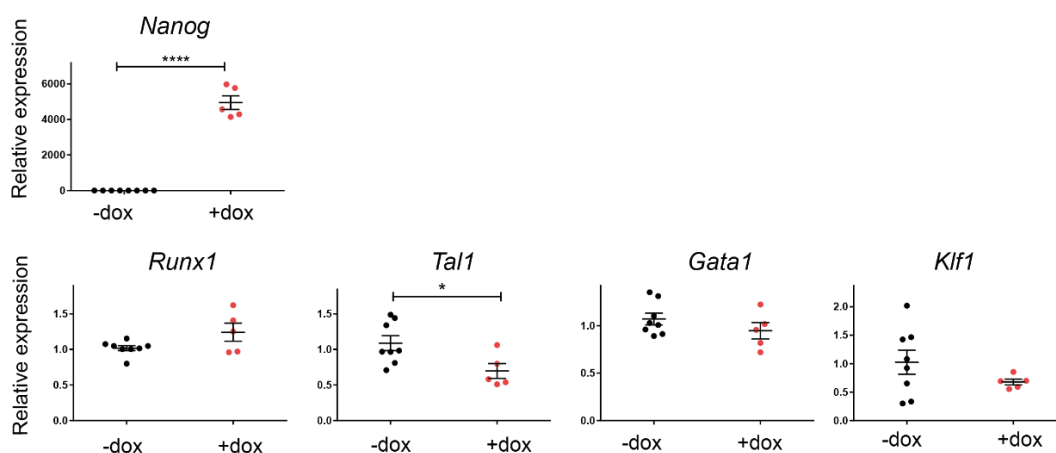


Figure 29. Induced *Nanog* expression downregulates specific hematopoietic genes in MEPs: RT-qPCR quantification of the relative expression of hematopoietic genes in megakaryocyte-erythroid progenitors (MEP) (-dox, n=8; +dox, n=5). * $P < 0.05$, **** $P < 0.00005$; Student's t-test.

We observed that only MEPs from dox-treated mice generate significantly more CFUs than controls (Fig 27A). This was also the case when BFU-E (burst forming unit-erythroid), CFU-Mk (colony forming unit-megakaryocyte) or colony forming units of a mixed erythroid/megakaryocyte phenotype (CFU-MEP) were measured (Fig 27B). CMPs and GMPs from treated and untreated mice showed similar differentiation potential, with the exception of BFU-E from CMPs, or monocyte progenitors (CFU-M) that were increased by *Nanog* in GMPs but decreased if derived from CMPs (Fig 28). Analysis of the expression of key erythroid genes by RT-qPCR in sorted MEPs revealed a significant reduction of *Tal1* in dox-treated mice (Fig 29). Together, these results indicate that *Nanog* is not altering the functions of the MEPs, but is specifically blocking transiently the

differentiation of these precursors. This leads to defective differentiation of these populations and therefore to an accumulation of their progenitors together with an exhaustion of the CMPs that do not present any impairment in functionality by CFU assay.

5.4. *Nanog*-expressing MEPs are outcompeted by the host in bone marrow transplants

To extend these observations, we next carried out transplantation of bone marrow from *Nanog*^{tg} mice to wild type recipients irradiated with 11 grays (5.5 +5.5). After 3 months of engraftment and recovery, more than 95% of peripheral blood cells were derived from the transplant (n=7; Fig 30). We treated the mice for 4 months with dox to induce *Nanog* expression only in hematopoietic cells, and found that at that point the host cells had been partially able to recolonize the bone marrow and contribute to circulating cells (ranging from 20% to 80%; Fig 30A).

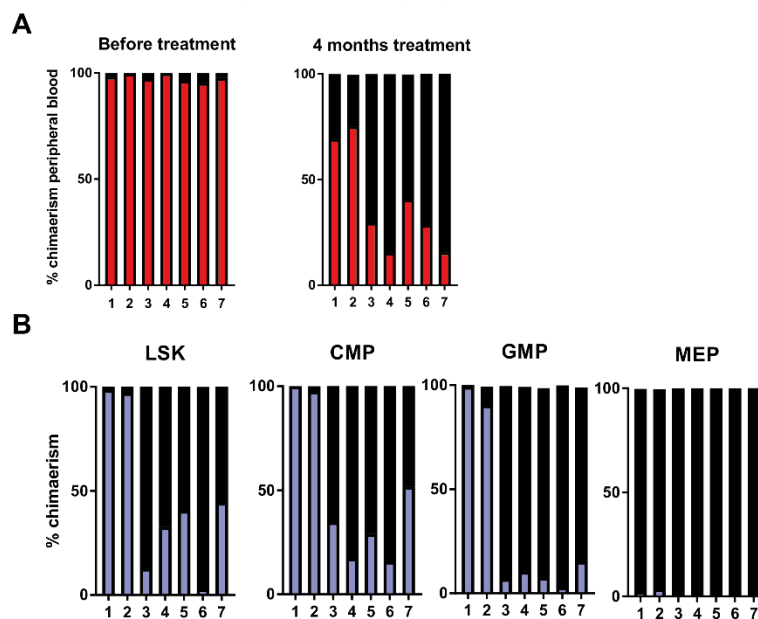


Figure 30. *Nanog*^{tg} transplanted bone marrow cells do not contribute to MEPs in a competitive transplant: (A) Contribution of *Nanog*^{tg} transplanted bone marrow cells to peripheral blood before (left) and after (right) dox treatment. Percentage of host derived cells (CD45.1+) are shown in black, and of donor derived cells (CD45.1/CD45.2 double +) in red. Individual mice are indicated on the x-axis (n=7). (B) Contribution of *Nanog*^{tg} transplanted cells to LSK, CMP, GMP and MEP populations purified from bone marrow. Percentage of host derived cells (CD45.1+) are shown in black, and of donor derived cells (CD45.1/CD45.2 double +) in blue. Individual mice are indicated on the x-axis (n=7).

We then purified bone marrow from the transplanted mice and analyzed chimaerism in different progenitor populations. While LSK, CMPs or GMPs show

variable degrees of contribution of wild type cells and *Nanog* expressing cells, MEPs are almost exclusively derived from the host (Fig 30B). These results indicate that the expression of *Nanog* in MEPs causes them to be outcompeted by wild type cells during bone marrow reconstitution, possibly due to their decreased ability to differentiate and generate mature erythroid cells.

6. *Tal1* is a target of *Nanog* at gastrulation

6.1. *Nanog* expression is mutually exclusive with that of *Tal1* in the nascent mesoderm

Nanog-mediated downregulation of erythroid-specification genes in both the embryo and the adult strongly suggests that some of these genes are likely transcriptional targets of NANOG. If so, we would expect to find mutually exclusive expression of *Nanog* and these genes at the time of initial hematopoietic specification in the gastrulating embryo. We therefore analyzed single-cell expression data from E7.0 nascent mesoderm (Scialdone et al., 2016), when *Nanog* is still expressed in the posterior-proximal region of the embryo (Hart et al., 2004), and examined the number of cells expressing both *Nanog* and markers of mesoderm (*Brachyury*, *Cdx2*) and hematopoiesis (*Tal1*, *Runx1*, *Gata1*, *Klf1*) (Fig 31A).

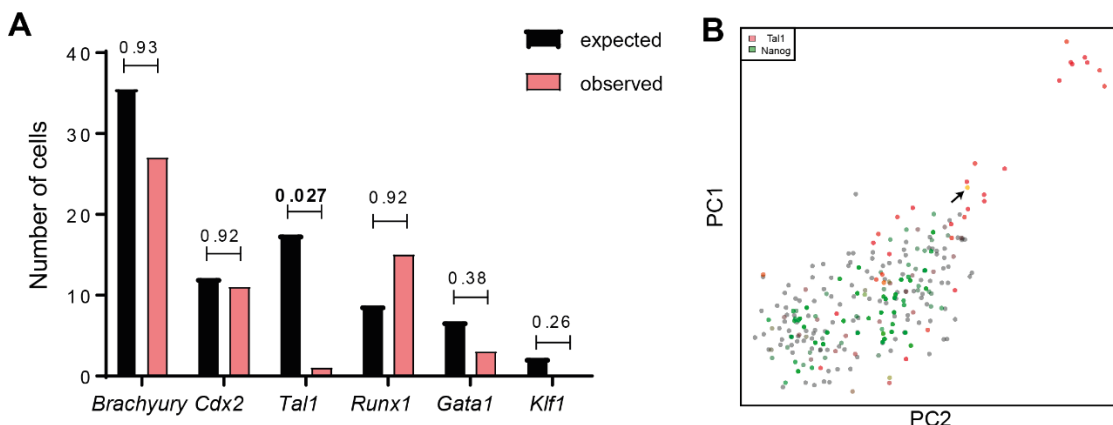


Figure 31. *Tal1* and *Nanog* are mutually exclusive in nascent mesoderm cells: (A) Expected (in black) and observed (in red) number of mesodermal (*Flk1*⁺) cells of the E7.0 mouse embryo expressing *Nanog* and selected genes expression, on single cell RNA-seq data (Scialdone et al., 2016). Statistical significance was calculated with a hypergeometric test. (B) Principal component analysis showing the distribution of *Flk1*⁺ E7.0 mesoderm cells expressing *Nanog* (green) or *Tal1* (red). The single cell expressing both genes is shown in yellow and indicated by an arrow.

For all of these genes we found the expected proportion of co-expressing cells with *Nanog* with the exception of *Tal1* (Fig 31A, B), this proportion is directly correlated with the number of cells expressing said genes. We confirmed that *Nanog* can downregulate *Tal1* at early stages by culturing *Nanog^{tg}* embryos with or without dox from E6.5 to E6.75 ex-utero, which did not alter normal development (Fig 32A). *Tal1* failed to be upregulated in dox-treated embryos, whereas other hematopoietic genes such as *Klf1* were unaffected (Fig 32B). We further confirmed that *Nanog* downregulates *Tal1* by whole mount in situ of E7.0 embryos treated with dox in utero (Fig 32C).

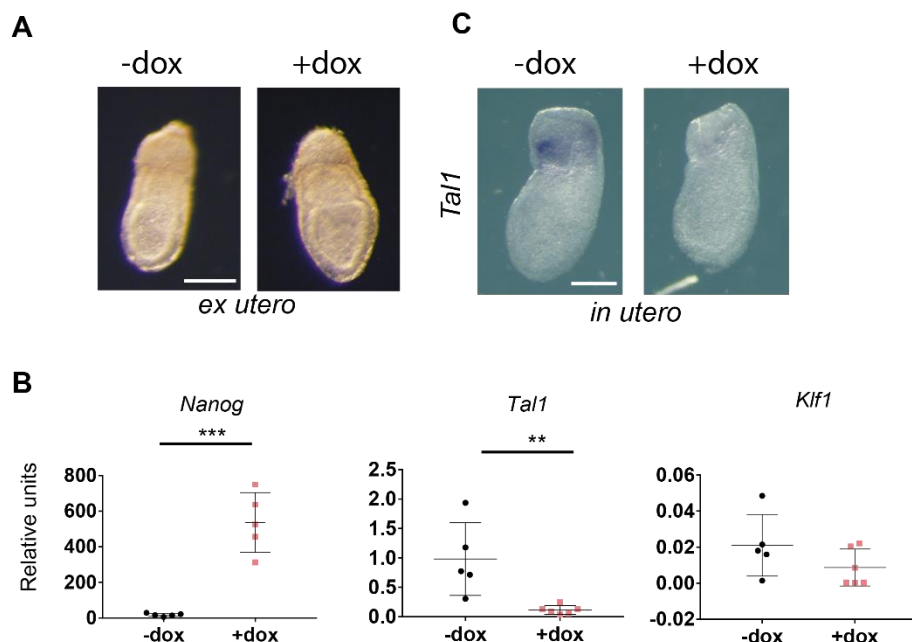


Figure 32. *Nanog* downregulates *Tal1* expression at the onset of gastrulation: (A) E6.5 *Nanog^{tg}* embryos after 8 hours ex-utero culture in the presence (+dox) or absence (-dox) of doxycycline. Scale bar, 100 μ m. (B) RT-qPCR quantification of the relative expression of *Nanog*, *Tal1*, and *Klf1* in individual untreated embryos (-dox) or treated embryos (+dox) (n=5). ** $P < 0.005$, *** $P < 0.0005$; Student's t-test. (C) Whole mount in situ hybridization of *Tal1* in E7.5 untreated (-dox) or in utero treated (+dox) *Nanog^{tg}* embryos.

This evidence strongly suggests that *Tal1* is likely a target of NANOG during early gastrulation at the onset of hematopoietic determination.

6.2. A distal NANOG-binding element represses *Tal1* expression in the embryo

To investigate the possible direct regulation of *Tal1* by NANOG, we analyzed published ChIP-seq data for NANOG binding in ES and epiblast-like cells (EpiLCs), which correspond to the E6.0 epiblast in the mouse embryo (Murakami et al., 2016). This study describes a broad resetting of NANOG-occupied genomic regions in the transition from ES cells to EpiLCs, resembling the developmental progress from the naïve inner cell mass of the blastocyst to the primed epiblast at gastrulation (Hayashi et al., 2011; Morgani et al., 2017). We examined a number of genomic loci, detecting binding at the *Nanog* locus itself in both ES cells and EpiLCs (Fig 33A) and in *Cdx2* only in ES cells (Fig 33B).

Neither cell type showed evidence of NANOG-bound regions surrounding *Runx1* (Fig 33C) or *Klf1* (Fig 33D). Interestingly, EpiLCs, but not ES cells, showed NANOG binding 22 kilobases upstream of *Tal1*, in an intron of the neighboring *Stil* gene (Fig 33E). We also detected NANOG binding downstream of *Gata1* (Fig 33F). However, these regions could be functionally related to the neighboring *Eras* and *Hdac6* genes, which are associated with pluripotency and early stem-cell differentiation (Chen et al., 2013, Takahashi et al., 2003).

Analysis of the *Tal1/Stil* NANOG-bound region in EpiLCs (Fig 34A) revealed *bona-fide* consensus binding sites (Fig 34B). To investigate the function of this region, we deleted it by CRISPR/Cas9 mediated genome editing (Ran et al., 2013) and examined the transcriptional consequences in early development. Gene expression was analyzed by RT-qPCR in individual edited E6.5 embryos.

As controls, we used embryos of the same batch showing no evidence of deletion of the *Tal1/Stil* NANOG bound region (Fig 34C). *Tal1* expression was significantly increased in deleted embryos, whereas other genes such as *Klf1*, *Gfi1b* or *Runx1* were unaffected (Fig 34D). Deletion of this genomic region did not alter *Stil* expression, despite the location of the site within this gene (Fig 34A,D). These assays provide strong evidence that this specific genomic region acts as a cis-regulatory element in the *Nanog*-mediated repression of *Tal1* in the early mouse embryo.

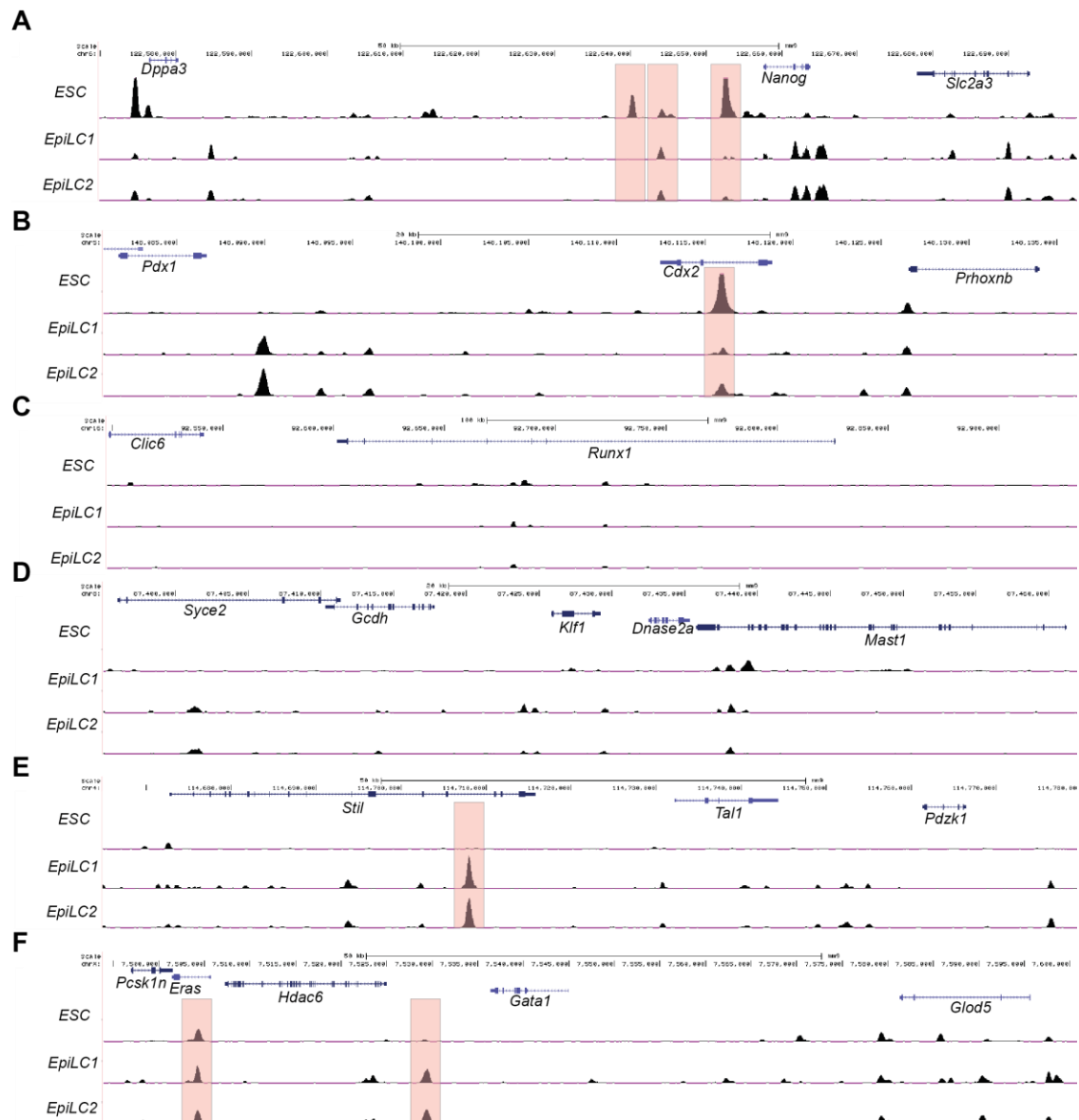


Figure 33. Distribution of NANOG bound regions at selected loci in ES and Epi-like cells (EpiLC): (A-F) UCSC browser views (mm9) of different genomic regions showing NANOG bound regions as determined by ChIP-seq in ES cells (ESC) and two replicates of epiblast-like cells (*EpiLC1*, *EpiLC2*). Selected peaks are highlighted by boxing in red. (A), *Nanog*: chr6:122,569,855-122,699,399. (B), *Cdx2*: chr5:148,080,850-148,136,544. (C), *Runx1*: chr16:92,497,828-92,940,224. (D), *Klf1*: chr8:87,395,041-87,462,459. (E), *Tal1*: chr4:114,664,868-114,780,341. (F), *Gata1*: chrX:7,493,906-7,601,563. ChIP-seq data was obtained from Murakami et al. 2016 (GEO accession number GSE71933).

Regulation of the earliest stage of RBC formation is still not well understood. Beside information extracted from lineage tracing experiments and early hematopoiesis genes deletion, not much is known about this process *in vivo* (Padrón-Barthe et al., 2014; Porcher et al., 1996; Shivdasani et al., 1995; Tam and Beddington, 1987).

Here we have described a new role for *Nanog* blocking erythroid differentiation. This effect is probably due to *Nanog* repressing *Tal1* in the early gastrulating embryo, a transcription factor crucial for the mesoderm-to-RBC induction. Furthermore, this repressive role of *Nanog* upon *Tal1* can apparently take place throughout development up to the adult bone marrow.

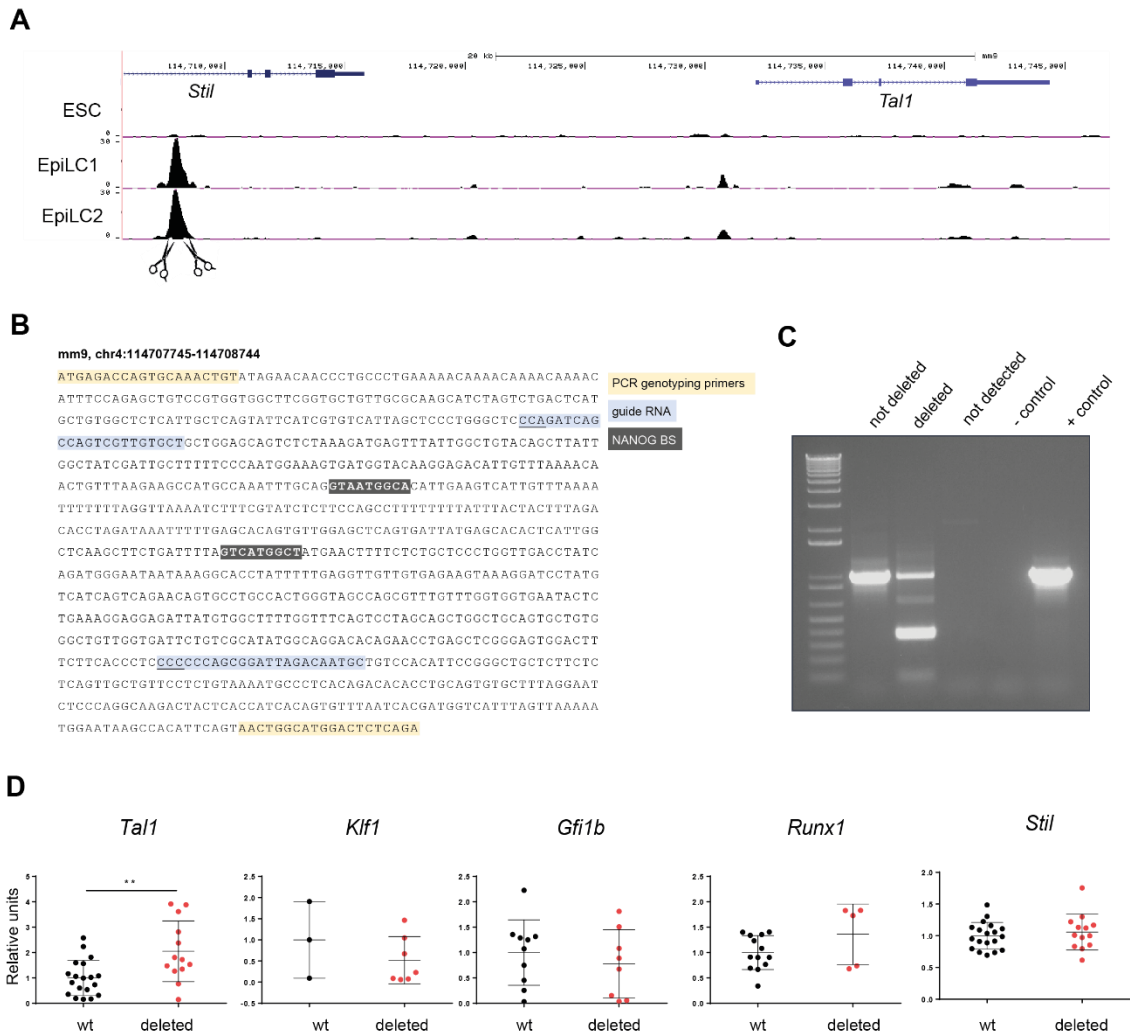


Figure 34. CRISPR/Cas9 deletion of the *Tal1* NANOG binding peak: (A) UCSC browser view of the *Tal1*/*Stil1* region (mm9; chr4:114,705,753-114,756,741), indicating the presence of the NANOG binding peak, determined by ChIP-seq, in EpiLCs (2 replicates are shown) but not in ES cells (Murakami et al., 2016); the binding peak was deleted by CRISPR/Cas9 genome editing (scissors). (B) DNA sequence of the genomic region located at -22 kb from *Tal1* bound by NANOG in EpiLC. PCR genotyping primers are highlighted in yellow, guide-RNAs in blue (PAM sequence is underlined), and two consensus NANOG binding motifs in dark grey and white bold lettering. (C) Representative gel of PCR-genotyping of individual E6.5 embryos showing not deleted, deleted, not detected, negative control (no DNA) and positive control (wild type embryo). (D) RT-qPCR determination of relative expression of *Tal1* (19 wt, 13 deleted), *Klf1* (3 wt, 7 deleted), *Gfi1b* (10 wt, 8 deleted), *Runx1* (13 wt, 5 deleted) and *Stil1* (19 wt, 13 deleted) in wild type and CRISPR-deleted embryos. * $P < 0.05$, ** $P < 0.005$, Student's t-test.

7. Regulation of anterior fates in the epiblast by *Nanog*

As we have previously shown, *Nanog* expression beyond gastrulation produces two marked and distinct phenotypes: lack of red blood cells and cranio-neural defects. This later phenotype could be due to the anteriorization of the expression of *Nanog*. At E6.5, *Nanog* is only expressed in the posterior region of the embryonic epiblast. It is at this stage that anterior and posterior fates are determined. Hence, ectopic expression of *Nanog* in the anterior epiblast could be inducing early defects that result in the phenotype we observe at E9.5.

Specification of anterior and posterior territories occurs right before gastrulation and represent the earliest event in differentiation process of pluripotent cells. At this stage, what were pluripotent cells enter a primed state from which they are more prone to differentiate. Thus we decided to study this process in vitro with *Nanog*^{KO} ES cells at the exit of pluripotency.

7.1 Lack of *Nanog* upregulates anterior neural genes at the exit of naïve pluripotency

In order to explore the role of *Nanog* and identify putative targets at the transition from pluripotency to lineage specification, we did an RNAseq of ES cells in differentiation media for 24h. We used *Nanog*^{KO} ES cells and the parental ES cell line from which they were derived as wildtype controls (Chambers et al., 2007a). Cells were cultured with 2i/LIF/KOSR for two passes and subsequently changed to serum to induce exit from pluripotency (Heo et al., 2005; Martin Gonzalez et al., 2016). To be able to follow the earliest events taking place in this process, we took samples at zero, 12, and 24 hours after adding serum to the culture (Fig 35A). We were interested in the first stages of differentiation because *Nanog* rapidly disappears from differentiating cells, and it is in these first stages in which genes of anterior or posterior character start to be expressed.

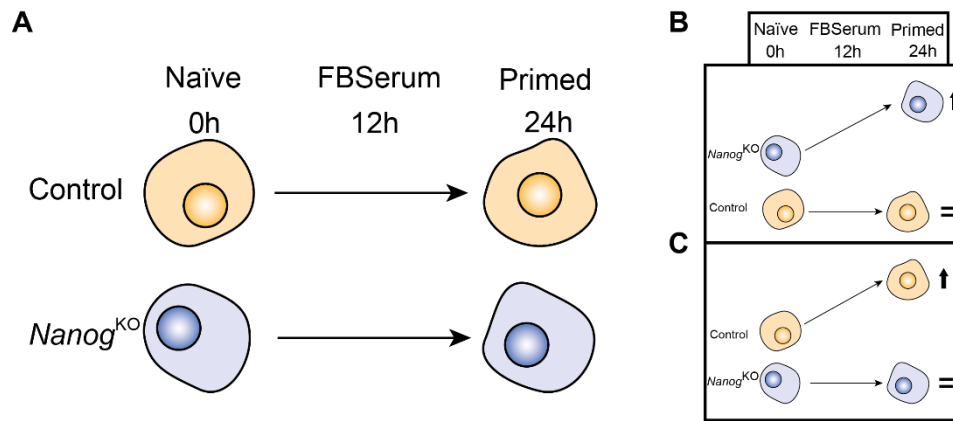


Figure 35. ES cells differentiation model and set up: (A) Three time points were taken during ES cells priming: 0h, 12h and 24h per triplicate. (B,C) Graphical model of the different group of genes we expect: (B) Genes upregulated in KO conditions during priming of ES cells and (C) genes upregulated in control conditions during priming of ES cells.

At these early stages of ES cell differentiation, changes in the patterns of expression could point us towards genes directly regulated by NANOG. To analyze the differences we looked for genes that changed their expression dynamics from zero to 24 hours (Table S1). Genes with a stable trend of expression in control ES cells that show an increase in *Nanog* KO cells would point to genes that are repressed by *Nanog* (Fig 35B), while genes with the opposite behaviour (that is, activated in controls but that do not change in mutant cells) would be positively regulated by *Nanog* (Fig 35C).

Principal component analysis (PCA) of the RNA-seq data showed a clear separation of the samples based on genotype of the cells (PC1, that explains close to 40% of variability; Fig 36), and timing of differentiation (PC2, 25% of variability; Fig 36). It is also interesting to observe that time 0h samples differentiate more from their respective 12h and 24h, indicating that a major change in the transcriptome is occurring in the first 12 hours.

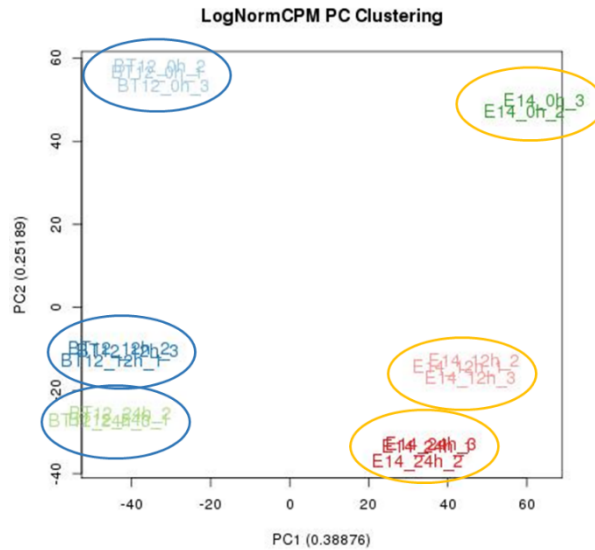


Figure 36. PCA showing sample and time distribution. Barcode of each sample goes as follows: 1. BT12 and E14 are *Nanog* KO ES cells and the parental ES cell line respectively. 2. Times are marked as 0h, 12h and 24h. 3. Different replicates are assigned numbers from 1 to 3. Samples circled in blue are *Nanog* KO ES cells and in orange are parental ES cells

Interestingly, the comparison at time 0 between control and mutant ES cells showed minimal if any differences in the expression of core pluripotency genes (Fig 37), in line with previous observations on the dispensability of *Nanog* at the pluripotent state (Chambers et al., 2007a).

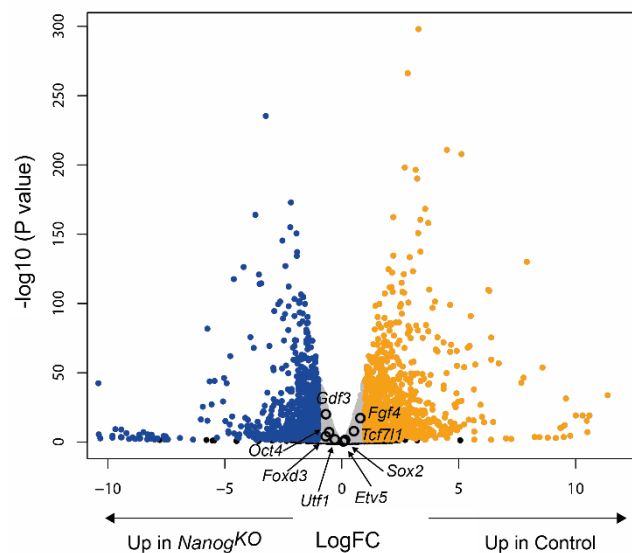


Figure 37. Volcano plot of the comparison at 0h between *Nanog*^{KO} and control ES cells: In blue, we find genes upregulated in *Nanog*^{KO} cells. In orange, genes upregulated in control cells. In grey genes that have less than [0.5] Log Fold Change (LogFC). In black genes with more than 0.05 adjusted P value. Core pluripotency factors are indicated.

We analyzed changes to gene expression taking into account their behaviour over time (Fig 35B, C), identifying two clear clusters with the predicted pattern of change (Fig 38A,B,C). Upregulated genes in mutant cells (89 genes) were mostly related to anterior neural development, such as *Pou3f1* or *Sox8* (Bell et al., 2000; O'Donnell et al., 2006; Zhu et al., 2014) (Fig 38A, D). On the other hand, genes that lost their upregulation in mutant cells (55 genes) were mainly involved in mesoderm as for example *Mef2c* or *Hand2* (Fig 37B, E) (Reichenbach et al., 2008; Skerjanc et al.).

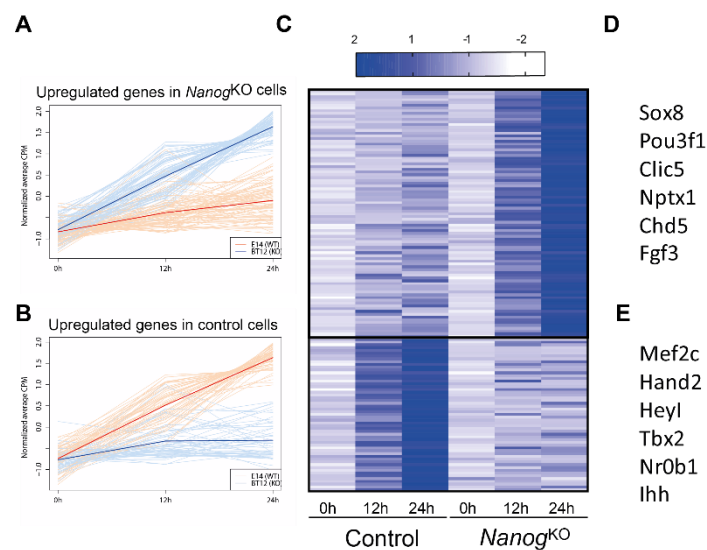


Figure 38. RNAseq analysis of the differences between control and *Nanog*^{KO} cells across time: (A) Genes upregulated in *Nanog*^{KO} across time. (B) Genes upregulated in control across time. Blue lines represent *Nanog*^{KO} ES cells, red lines represent control cells. Thicker lines represent the mean of normalized average CPM of said genes. (C) Heatmap comparing both set of genes. (D) Representative genes of the (A) set of genes. (E) Representative genes of the (B) set of genes.

When we examine genes downregulated in one condition (controls of mutants) that do not change in the other, we do not find any relevant set of genes. Only 13 genes are downregulated in the *Nanog*^{KO} and do not change in controls, and vice versa 15 genes downregulated in the control do not change expression in *Nanog*^{KO} cells (Fig 39).

This analysis indicates that *Nanog* is involved in repression of anterior genes while promoting posterior fates, what suggests that *Nanog* might be important for the establishment of the anterior-posterior axis of the embryo at the exit of pluripotency.

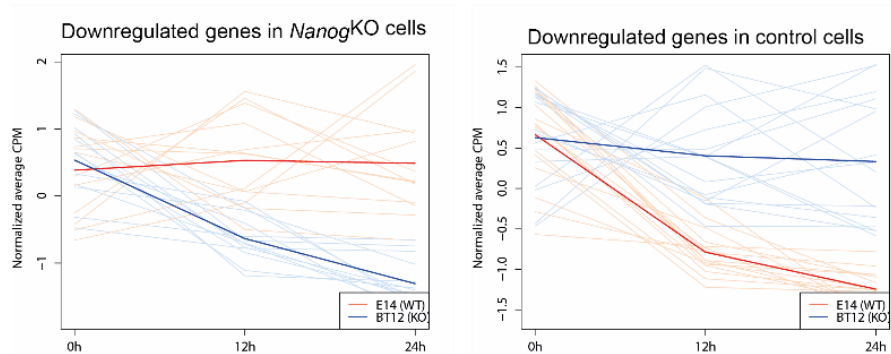


Figure 39. Set of genes downregulated for either *Nanog*^{KO} or control cells across time: Blue lines represent *Nanog*^{KO} ES cells, red lines represent control cells. Thicker lines are the mean.

7.2 Integration of different RNA-seq data reveals *Pou3f1* as a primary target for repression by NANOG in gastrulating mouse embryos

To more specifically address the putative role of *Nanog* in the post-implantation epiblast, we again took advantage of published E6.5 embryo single-cell RNA-seq datasets (Mohammed et al., 2017b; Scialdone et al., 2016).

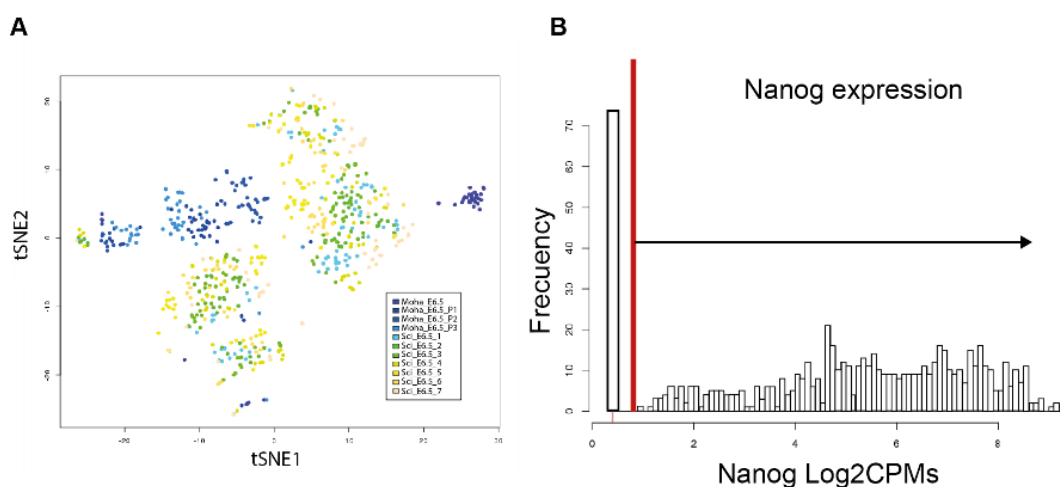


Figure 40. Representation of cells from the different experiments and their *Nanog* levels: (A) tSNE of all the experiments coming from two different datasets: Moha (Mohammed et al., 2017a) and Sci (Scialdone et al., 2016). Cells from each experiment are represented in different colours according to the legend. (B) Levels of expression of *Nanog* in Log2 counts per million. Red bar and black arrow represent the point from which we start counting cells expressing *Nanog*.

In this case we used two different E6.5 datasets given that we want to look at the earlier process of axis formation, and merging these datasets would help to give robustness to our analysis. This is the stage at which *Nanog* is re-expressed in

the posterior part of the embryo (Hart et al., 2004b), and anterior genes such as *Sox2* already show a restricted expression pattern in the epiblast. We merged both expression datasets (Fig 40) and selected those single cells expressing *Nanog* above a certain threshold (see Methods).

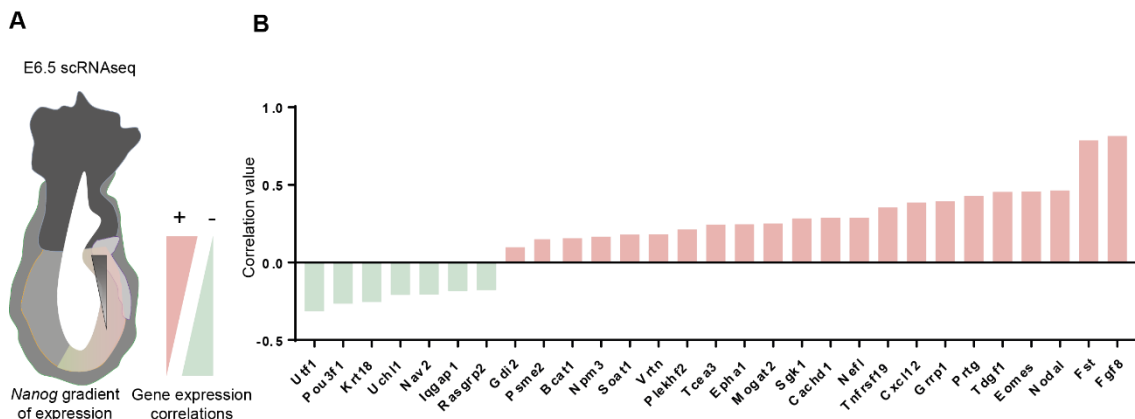


Figure 41. Correlation between *Nanog* and all the other genes expressed: (A) Schematic representation of an E6.5 embryo. Light red indicates *Nanog*'s expression pattern. Black triangle on the embryo explains the diminishing levels of *Nanog* towards the distal region of the embryo. Red and green triangles represent the positive (red) and negative (green) correlations between *Nanog* and any other given gene. (B) Correlation values of the genes that show highest statistical correlation (negative in green and positive in red).

We then established the correlation of all expressed genes to *Nanog* (Fig 41; Table S2). The analysis of those genes whose expression in single cells showed the highest statistical correlation (both positive and negative) with the expression of *Nanog* confirmed our previous observations on cultured cells. Genes that correlate positively with *Nanog* are related with gastrulation and mesoderm formation, such as *Fgf8* (Fig 42A), *Nodal* or *Eomes* (Fig 41B). On the other hand, among the genes that correlate negatively with *Nanog* we found *Pou3f1* (Fig 42B) together with other neural genes such as *Nav2* (Fig 41B). Unexpectedly, *Sox2* did not show any correlation with *Nanog* levels (Fig 42C). Interestingly, in this group we also find *Utf1*, a pluripotency associated gene that during gastrulation is restricted to the anterior region of the embryo and to extraembryonic tissues (Okuda et al., 1998). Hematopoietic genes did not come up in this analysis, most possibly due to the merged data set we used, and that these genes are not expressed in the epiblast of E6.5 embryos.

We induced *Nanog* by administering dox to pregnant females in the drinking water from E4.5 to E7.5 *Nanog*^{tg} embryos and examined changes in gene expression by RNA-seq as compared to controls of the same genotype but that

had not been treated with dox (Lopez-Jimenez et al.). In this dataset, many genes involved in early aspects of embryo patterning were downregulated, such as Hox genes, but the most downregulated gene was *Pou3f1* (Fig 43). Moreover, we found important hematopoietic genes downregulated in this RNAseq such as *Tal1* or *Klf1*, confirming our previous observations. Other anterior neural genes, as for example *Sox2*, *Hesx1* or *Zic3*, were not changed. We confirmed these observations by whole mount *in situ* hybridization on E7.5 embryos from the *Nanog^{tg}* line treated or not treated with dox. Induction of *Nanog* led to a downregulation of *Pou3f1* in the anterior epiblast of treated embryos, while expression of *Sox2* was unchanged (Fig 44). Interestingly, this effect was limited to early stages, as treatment up to E8.5 increases the expression of both *Pou3f1* and *Sox2* in the anterior neural tube (Fig 44).

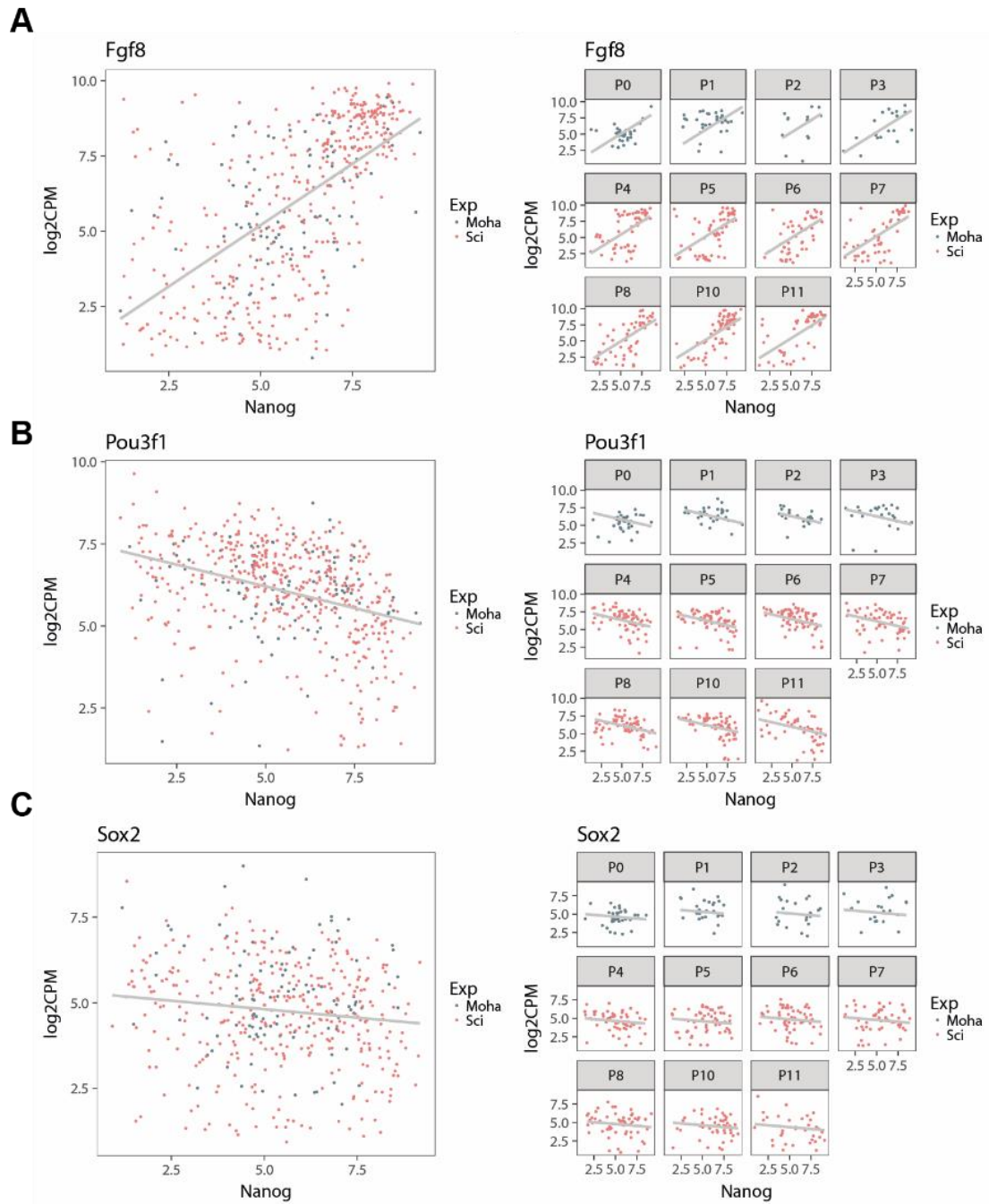


Figure 42. Graphic representation of examples of the correlations with *Nanog* on mixed scRNAseq experiments: On the left we find all the cells that express both *Nanog* and the interrogated gene. On the right correlation with *Nanog* of each experiment separately. Red cells come from Scialdone experiments (Scialdone et al., 2016) and blue cells come from Mohammed experiments (Mohammed et al., 2017a). (A) Correlations with *Fgf8*. (B) Correlations with *Pou3f1*. (C) Correlations with *Sox2*.

We then merged the data from all previous transcriptomic analysis, finding only three genes shared by those upregulated in *Nanog* KO ES cells in early differentiation, showing a significant negative correlation with *Nanog* in E6.5 single cell transcriptomics, and downregulated in E7.5 *Nanog* gain-of-function embryos: *Pou3f1*, *Lrp2* and *Clic6* (Fig 45). *Lrp2* and *Clic6* are expressed in primitive endoderm and later derivatives (Gerbe et al., 2008; Sherwood et al., 2007), lineages where *Nanog* has a well-defined negative regulatory role.

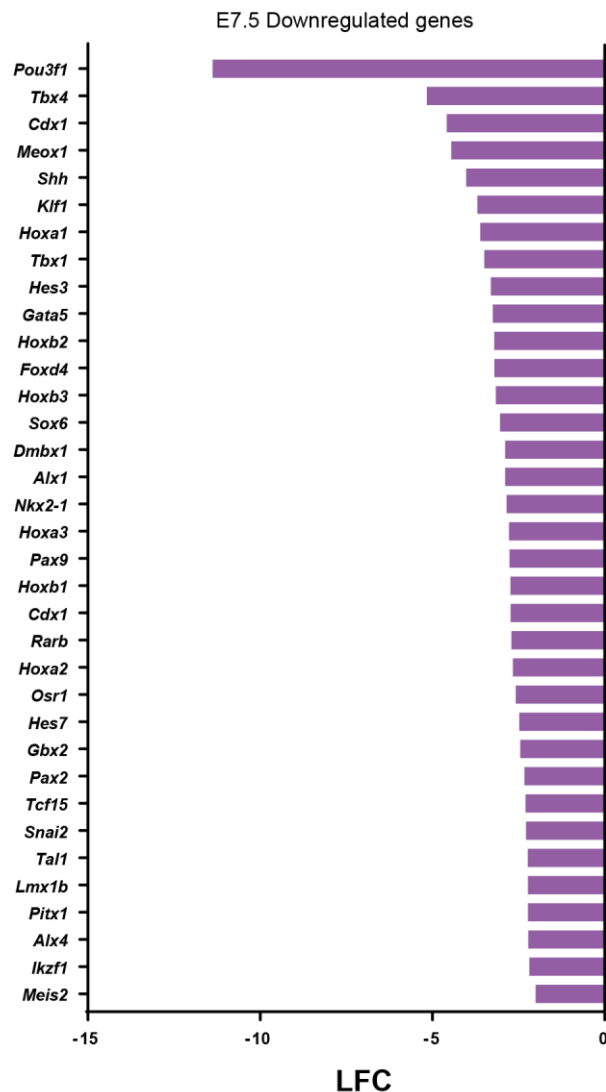


Figure 43. List of the most downregulated genes in E7.5 embryos with ectopic expression of *Nanog* when compared with control embryos: Bars mark the log fold change of the difference between *Nanog* induced and control embryos.

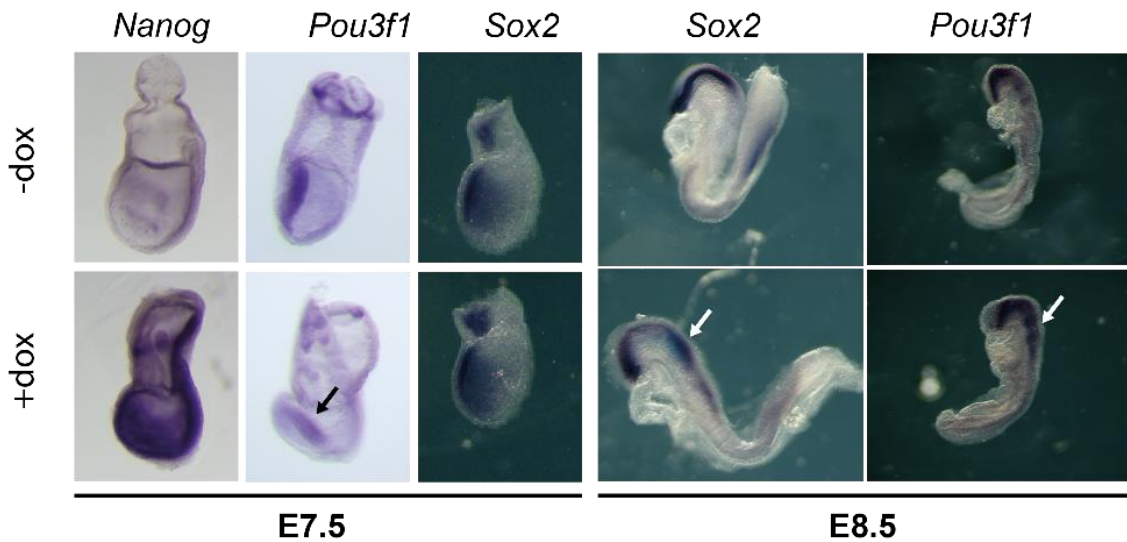


Figure 44. *In situ* hybridization of *Nanog*^{tg} embryos with and without doxycycline: Black arrow means decrease in expression upon *Nanog* ectopic expression. White arrows increase in expression upon *Nanog* ectopic expression.

Therefore, *Pou3f1* is the prime candidate to be a direct target of *Nanog* mediating its role in suppressing anterior epiblast fate. Interestingly, genes that were positively correlated with *Nanog* in E6.5 single cells and that were upregulated in dox-treated *Nanog*^{tg} embryos were in its majority related with early gastrulation and mesoderm specification, such as *Eomes*, *Fgf8*, *Tdgf1* (*Cripto*) or *Mixl1* (Table 5).

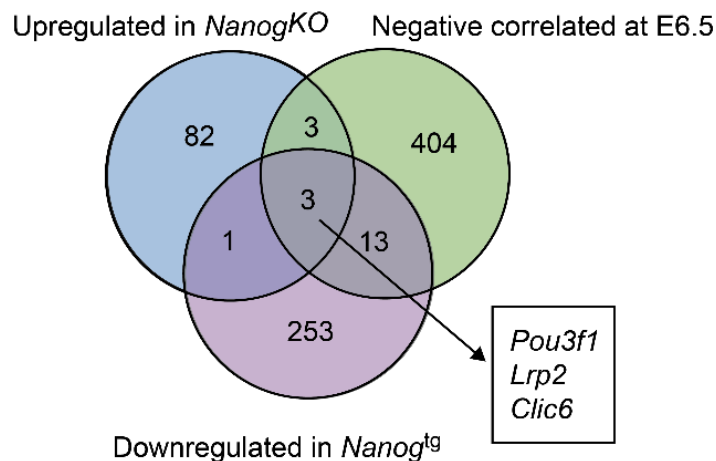


Figure 45. Venn diagram of the different RNAseq analyzed. In blue are all genes significantly upregulated upon *Nanog* loss of function in ES cells priming. In green genes that are negatively correlated with *Nanog*. In purple genes downregulated upon *Nanog* ectopic expression. Boxed genes are the only related to all three groups.

Intersections	total	Genes	Intersections	total	Genes
down in 7.5 neg corr up in ko	3	Pou3f1	neg corr up in ko	3	Gclm
		Lrp2			Mt3
		Clic6			Dusp4
up in 7.5 up in ko	3	Serpib9f	down in 7.5 up in ko	1	Ctsh
		Serpib9e	neg corr up in wt	1	Ccdc64b
		Rassf4	down in 7.5 up in wt	1	Maf
neg corr up in 7.5	9	Nid1	pos corr up in 7.5	16	Dact1
		Krt19			Fes
		Scamp5			Ahcy
		Tmem14c			Eomes
		Nxn			Nefl
		Skil			Dppa5a
		Enc1			TdGF1
		Car2			Palm
		Lgals1			Rps2
down in 7.5 neg corr	13	Rbp4			Rpl21
		311002H16Rik			Upp1
		Crabp2			Fgf8
		Tmem181b-ps			Epha2
		Npl			Mixl1
		Gstm1			Socs2
		Gnb4			Pmepa1
		Hnf4a	Bbx		
		Slc7a8	Wls		
		Ddah1	Hmga1-rs1		
		Igf2	Tdh		
		Cubn	Slc25a36		
		Cd59a			
		down in 7.5 pos corr	5		

Table 5. List of genes included in the different overlap of the venn diagram (Fig 45). Intersections columns establish the gene sets that intersect. Total columns regard the total number of genes that intersect. Gene's columns contain the gene symbols of the gene belonging to a specific intersection.

7.3 *Nanog* expression impairs neural differentiation in vitro

To confirm that *Nanog* has a direct role in negatively regulating differentiation to the early neural lineage, we derived ES cells from the *Nanog*^{tg} and performed a differentiation towards anterior neural fate (Gouti et al., 2014, 2017), sampling the culture with or without dox for up to six days (Fig 46A). Analysis of gene expression by RT-qPCR showed that upon *Nanog* overexpression, pluripotency related genes such as *Rex1* followed the same pattern in untreated or treated cells, despite a slight increase in *Nanog* gain-of-function cells along the time course. On the other hand, anterior neural specific genes were downregulated during the differentiation process, among them *Pou3f1*, *Otx2* and *Pax6*. *Sox2*,

that qualifies as both a pluripotency and early neural gene did not change (Fig 46B). Thus, upon neural differentiation, *Nanog* does not affect core pluripotency gene expression, but it does downregulates genes important for neural specification.

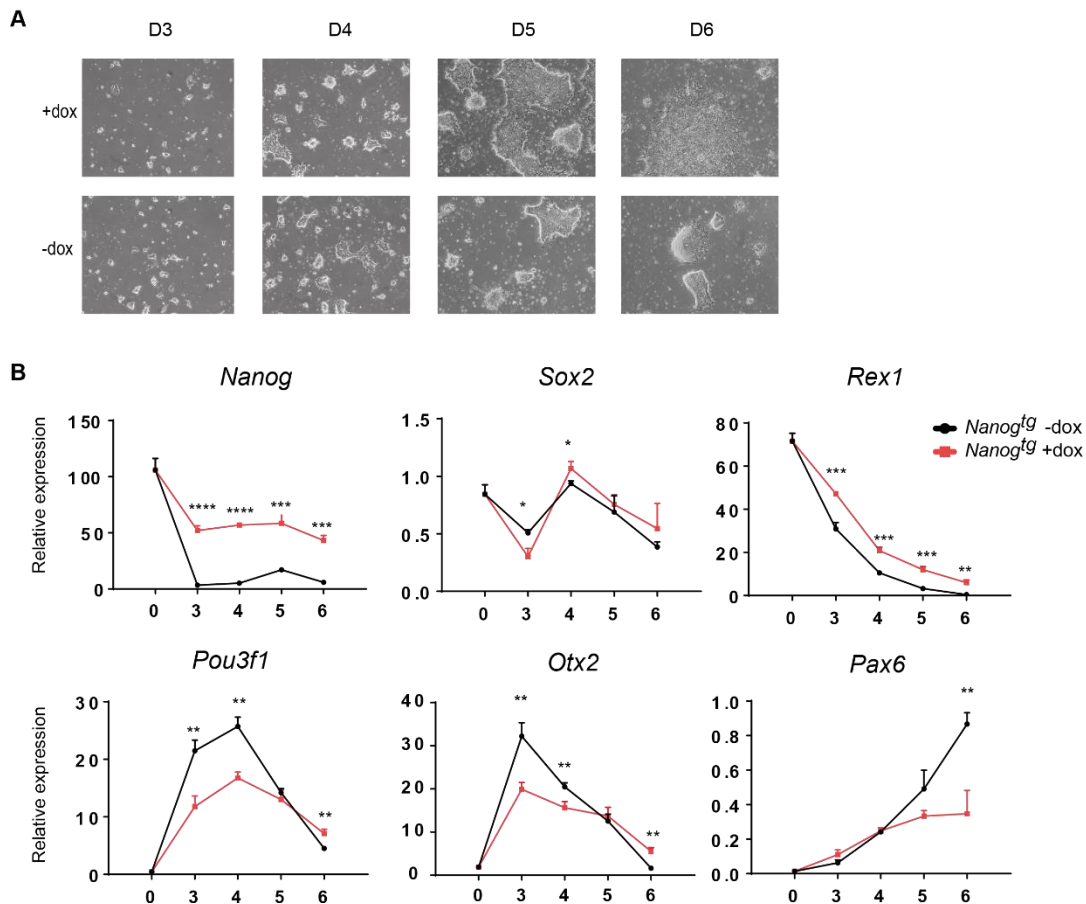


Figure 46. RT-qPCR screening of a neural differentiation of *Nanog*^{tg} ES cells. (A) Representative pictures of *Nanog*^{tg} differentiation with and without doxycycline. Pictures taken at day 3,4,5 and 6. (B) RT-qPCR of different genes with timepoints at day 0, 3, 4, 5 and 6. In red differentiation with doxycycline. In black differentiation without doxycycline (See methods).

7.4 A distal NANOG-binding element represses *Pou3f1* expression in the posterior epiblast

All the above evidence indicates that *Pou3f1* is likely a target of NANOG during anterior-posterior axis specification in the epiblast.

Taking advantage of the already analyzed ChIP-seq data for NANOG binding in ES and epiblast-like cells (EpiLCs) (Murakami et al., 2016), we examined the

Pou3f1 locus and identified three prominent regions of NANOG binding at 11.5 and 9.5 kilobases (kb) upstream and 9 kb downstream (Fig 47A). Interestingly, these regions were bound in EpiLC but not in ES cells. This is suggestive of a specific input of *Nanog* on *Pou3f1* in the epiblast but not at earlier pluripotent stages (Fig 47A).

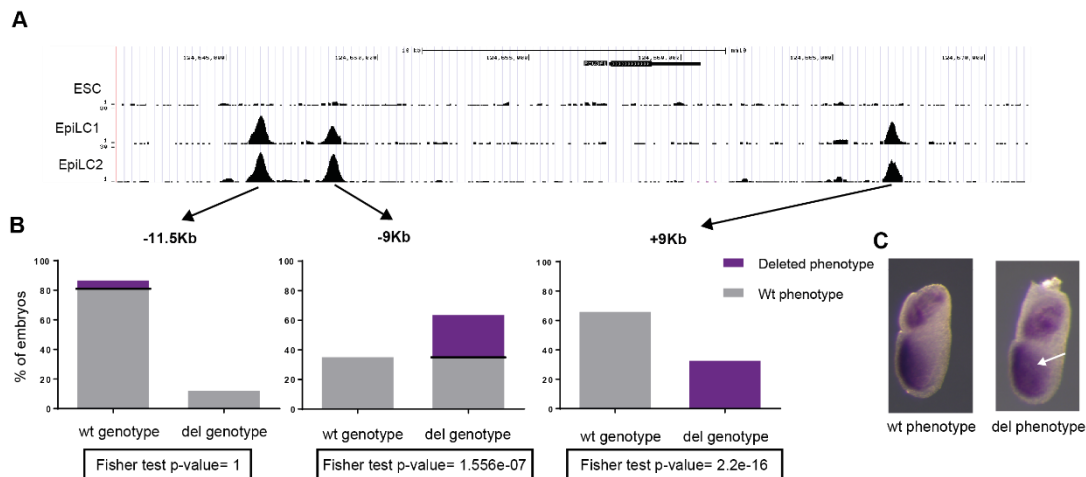


Figure 47. NANOG-BS deletion in E6.5 by CRISPRCas9 injection in the zygote. (A) Locus of *Pou3f1* containing three NANOG-BS in the EpiLCs but not in the ES cells. Arrows points towards (B). Graphical representation of the phenotypic observations in embryos recovered at E6.5 and displaying the fisher test p value below each graph that belongs to the specific deletion. (C) In situ hybridization of E6.5 with wt phenotype and deleted (del) phenotype, this presenting and expanded pattern of expression of *Pou3f1* towards the posterior region of the embryo's epiblast as indicated by the white arrow.

We deleted each of the three binding site separately by CRISPR/Cas9 genome editing in a transient transgenic embryo assay. Embryos microinjected with Cas9-gRNA ribonucleoproteins were recovered at E6.5, processed for whole mount *in situ* hybridization, and subsequently genotyped for the projected deletion. Since our hypothesis was that deletion of the NANOG bound region would de-repress *Pou3f1* expression, we looked for an expansion of the pattern of expression of *Pou3f1* towards the posterior epiblast. This assay showed that only deletion of the +9 kb downstream region caused a reproducible expansion in *Pou3f1* expression (Fig 47B, C). Therefore, we generated a stable mouse line carrying this deletion for a more detailed analysis (Fig 48 A). Mice homozygous for the deletion were viable and fertile. This was not completely unexpected, as homozygous null *Pou3f1* survive up to birth (Birmingham et al., 1996), and we have shown that the effect of *Nanog* on *Pou3f1* is limited to early stages of

epiblast patterning (see above). We crossed mice heterozygous and homozygous for the deletion, and compared littermates for the expression of *Pou3f1* by whole mount *in situ* hybridization. We observed that 3 out of 5 homozygous embryos presented a phenotype of posterior expansion while none of the heterozygous embryos did so (Fig 48 B). These results show that the +9 kb NANOG bound region is important for the restriction of *Pou3f1* expression to the anterior epiblast.

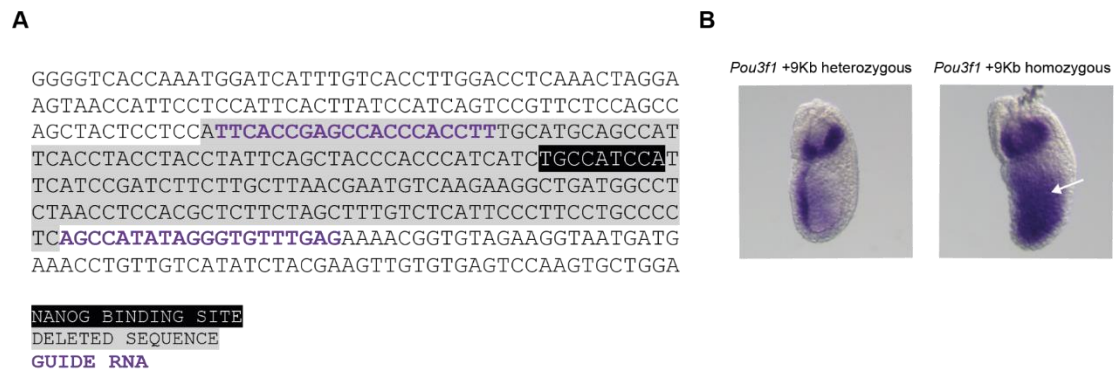


Figure 48. Representative *in situ* hybridization of *Pou3f1* showing the differences between heterozygous and homozygous E6.5 embryos for the deletion of *Pou3f1*. (A) Sequence of the deleted locus. In purple the gRNAs, in grey deleted region and in black NANOG binding site. (B) *Pou3f1* ISH. White arrows show the expansion of the expression pattern of *Pou3f1* towards the posterior region of the embryo in the embryo homozygous for the deletion.

Taken all together, we can conclude that *Nanog* repress *Pou3f1* when it is expressed ectopically in the whole epiblast at the onset of gastrulation. Physiologically we observe the same trend by reanalysis of scRNAseq. This not only happens in the whole embryo, but also during directed differentiation towards neural fates where we see a downregulation of anterior genes upon *Nanog* induction such as *Otx2* and *Pou3f1*. In addition, deletion of one out of three different NANOG bound regions in the *Pou3f1* locus is enough to expand the expression pattern of *Pou3f1* to the posterior region of the epiblast of the embryo.

In all, with this thesis we have demonstrated that *Nanog* is not only necessary for embryonic pluripotency or PGCs formation, but it also plays a fundamental role in tissue differentiation during early postimplantation development, blocking red blood cell fate during mesodermal fate choices, and restricting anterior gene programs in the epiblast of the gastrulating embryo.

DISCUSSION

DISCUSSION

A matter of disentanglement

The pluripotency gene regulatory network (GRN) in ES cells and in the inner cell mass of the blastocyst is crucial for the activation and maintenance of pluripotency (Fig 6). However, this GRN is not only important at these stages, but also at the exit of pluripotency, in which it acquires different roles during differentiation (Iwafuchi-Doi et al., 2012). Upon implantation, changes in chromatin and in the transcriptional and epigenetic landscape make the core of this GRN formed by SOX2-OCT4-NANOG interactions change. While *Oct4* is still expressed in the whole epiblast, *Sox2* becomes restricted to the anterior epiblast and *Nanog* to the posterior epiblast (Fig 7). The entanglement between these TFs that was previously crucial in pluripotency, is finally untying to allow for tissue specification. This event change the way these TFs were interacting with each other and force them to interact with different partners. While *Sox2*, activated by OCT4 and POU3F1, is expressed in anterior (Iwafuchi-Doi et al., 2011b), *Nanog* is activated in posterior regions by SMAD2/3 and OCT4 (Sun et al., 2014). In the anterior epiblast SOX2 activates neural differentiation, and NANOG triggers mesoderm induction in the posterior epiblast (Mendjan et al., 2014; Wang et al., 2012). Actually, one of the first cells to differentiate, the primordial germ cells (PGC), express all three core pluripotency TFs, but during PGC specification, *Sox2* and *Nanog* show contrasting roles. *Nanog* induces PGC-like cells while *Sox2* represses them (Murakami et al., 2016). Our results further reveal a role for pluripotency factors at post-implantation stages, as we show that *Nanog* has a repressive role in the posterior epiblast of the gastrulating embryo. We also demonstrate that *Nanog* is able to block red blood cell lineage differentiation in a cell autonomous manner and that *Nanog* might directly suppress *Tal1* in the gastrulating embryo. Likewise, we have described how *Nanog* is able to repress *Pou3f1* in the posterior embryo without affecting *Sox2* expression, which opens the way to a broader GRN for the establishment of the anteroposterior axis of the epiblast.

Nanog in pluripotency and beyond

The pluripotent potential of stem cell relies on the gene pluripotency network placed around *Oct4*, *Sox2* and *Nanog* (Navarro et al., 2012; Tay et al., 2008; Trott and Martinez Arias, 2013). Up to date, there are two different and well defined steady state stages of pluripotency that have been termed naïve and primed. They have been extensively described *in vitro* and have clear transcriptional and epigenetic differences (Joo et al., 2014; Nichols and Smith, 2009b). The main functional difference is that the primed state cannot give rise to blastocyst chimeras. Beyond that, both can be maintained *in vitro*: with LIF and 2i for the naïve state and with Activin and FGF for primed ES cells (Tesar et al., 2007). These two states can be interconverted *in vitro* although reprogramming towards pluripotency comprise a much complex and costly process than differentiation (Festuccia et al., 2013).

Overexpression of *Nanog* in ES cells make them able to self-renew without LIF, BMP or 2i (Chambers et al., 2003; Hall et al., 2009; Ying et al., 2003). Although *Nanog* is essential for epiblast formation in the preimplantation embryo (Mitsui et al., 2003b) it can be deleted in ES cells that maintain their pluripotency, although they are more prone to differentiate (Chambers et al., 2007a). Therefore, it has been well established that *Nanog* is important for pluripotency maintenance both *in vitro* and *in vivo*. Yet little is known about the role of *Nanog* at the exit of pluripotency.

Exit of pluripotency and embryo axis formation

Although most of the axis formation experiments have been performed *in vivo* (Arnold and Robertson, 2009; Beddington and Robertson, 1999; Hiramatsu et al., 2013), ES cells allow us to study this process *in vitro* (Deglincerti et al., 2016; Morgani et al., 2018; Stryjewska et al., 2017). In the postimplantation embryo, anterior-posterior patterning is one of the first differentiation events to occur. At this stage both anterior –such as *Otx2* and *Pou3f1*- and posterior genes –like *Zeb2* or *Brachyury*- are upregulated. Due to the parallelisms between naïve ES cells and pluripotent epiblast, and between primed ES cells and the

postimplantation epiblast, it is expected that in the first stages of differentiation in ES cells we would have upregulation of these anterior and posterior markers. (Acampora et al., 2016; Buecker et al., 2014; Stryjewska et al., 2017).

In this thesis, we wanted to capture the first steps in the priming of naïve ESC so that we could assess the role of *Nanog* in early pluripotency exit. With this aim in mind, we maintained *Nanog*^{KO} ES cells in naïve conditions to later culture them in a permissive medium for differentiation and look closely at the very first step of differentiation, just 24 hours (Fig 35).

Accordingly, from our *Nanog*^{KO} experiments in ES cell priming we have been able to interrogate what is the role of *Nanog* in promoting such first stages of differentiation. We observed that *Nanog* was repressing anterior fate while promoting posterior differentiation. As pluripotent cells transit towards a primed state, it is obvious that the absence of *Nanog* will generate an imbalance in the lineage fate of the cells, that reflects the equilibrium in which *Nanog*, *Oct4* and *Sox2* are in this state transition (Pfeuty et al., 2018). We could expect that in the absence of *Nanog*, *Sox2* would drive the cells towards a more anterior fate. Also it has been recently shown that *Nanog* antagonizes the anterior gene *Otx2 in vitro* (Acampora et al., 2017). However in this thesis we have established that another anterior gene more consistently regulated by *Nanog* is *Pou3f1*, both *in vitro* as well as *in vivo*.

Pou3f1 has been identified as an activator of *Sox2 in vivo* and *in vitro* through the N2 enhancer (Iwafuchi-Doi et al., 2011b; Zhu et al., 2014). *Pou3f1* is expressed in the ExE and the anterior epiblast (Zwart et al., 1996). It is expressed at E5.5 (when *Nanog* is switched off) in the mouse epiblast and at E6.5 its expression is restricted to the anterior epiblast. In later embryos its expression restricts to a more anterior position (Zhu et al., 2014; Zwart et al., 1996). This localization to the anterior epiblast coincides with the start of the re-expression of *Nanog* (Hoffman et al., 2013b). Additionally, when we deleted a specific NANOG bound site in the locus of *Pou3f1*, this gene starts expressing in the posterior region of the embryo.

Therefore *Pou3f1* is not just a marker of the exit of pluripotency upregulated when *Nanog* is downregulated (Acampora et al., 2016), but instead a direct target that

is repressed by *Nanog* during embryo differentiation. This not only occurs in the first stage of differentiation, as when we differentiate the cells towards anterior neural fate we also observe a downregulation of *Pou3f1* upon *Nanog* activation *in vitro* (Fig 46). A question that emerge from our experiments and the pattern of expression of both genes is whether physiological disappearance of *Nanog* expression at E5.5 is important for *Pou3f1 de novo* expression in the epiblast at that same stage (Festuccia et al., 2013).

Anteriorization of the embryo: causes and consequences

We have generated a mouse line with a deletion in the locus of *Pou3f1*, 9Kb downstream of the transcription start site, that correspond to a NANOG bound region as detected by ChIP-seq (Murakami et al., 2016). This bound region of NANOG is only present in EpiLCs, which indicates a shift in the GRN that accompanies NANOG in this cell state similar to the postimplantation/pre-gastrulation epiblast (Hayashi et al., 2011). The phenotype we observe is a posteriorization of the expression of *Pou3f1*. However, this expansion in the domain of expression of *Pou3f1* does not occur in all embryos analyzed, what poses some interesting questions. Does expression of *Pou3f1* in the posterior epiblast anteriorizes this region? Does this jeopardize later embryo viability, or only those embryos that do not gain *Pou3f1* expression make it through gastrulation? The answer to these questions will shed some light on the boundaries between anterior TFs and posterior TFs and their external inputs.

In addition, it is important to highlight that according to our results the effect of *Nanog* on *Pou3f1* appears to be stage specific. The repression we have observed in *Pou3f1* upon *Nanog* activation is only observed in early gastrulation stages. Later, when *Nanog* is no longer expressed but we activate its expression we observed an ectopic expression of *Pou3f1* and of *Sox2* as well, which could be triggered by the former. Understanding this change in the response of *Pou3f1* to *Nanog* from repression to activation sure merits further investigation.

Analysis of co-expression and correlations of well studied genes in scRNAseq data is going to be crucial for answering questions on embryo patterning during gastrulation. In this thesis we have used two different data sets of E6.5 scRNAseq

to correlate expression with *Nanog* (Mohammed et al., 2017a; Scialdone et al., 2016). As data keeps on growing, we will be able to build a denser map with higher resolution of the physiological interactions between different genes. This will help us understand how the pluripotency GRN is dismantled at the exit of pluripotency and how new ones come together during gastrulation.

The very first red blood cell

Moving to the other end of the embryo, red blood cell (RBC) precursors are the first cell type to be specified from nascent mesoderm during mouse gastrulation (Baron et al., 2012, Kinder et al., 1999). While the genes and networks that determine primitive hematopoietic cells are well understood (Isern et al., 2011, Kingsley et al., 2013), much less is known about how precursors are specified during the early stages of primitive streak formation (Padrón-Barthe et al., 2014). Actually, studies of scRNAseq have helped us discover some genes that are involved in this process such as *Tal1* (Scialdone et al., 2016). Differentiation from epiblast to RBC occurs in a very short time frame (Padrón-Barthe et al., 2014). Hence, it is only logical to hypothesize that even before gastrulation there must be a priming of the cells that are going to migrate through the primitive streak up to the extraembryonic region where they will become RBC.

Role of *Nanog* in mesoderm-to-RBC fate choice

Here, we show that the pluripotency factor NANOG regulates the transition from multipotent mesodermal progenitors to red blood cell precursors in these early steps, at least partially through the direct regulation of the lineage specifier *Tal1*. Our results suggest that a similar situation may occur during specification of the first mesodermal lineages. *Nanog* expression in *Brachyury*-positive cells maintains them in a pan-mesodermal multipotent state, whereas its downregulation would allow the expression of early hematopoietic lineage specifiers, driving their differentiation to primitive red blood cells. This process, however, occurs during a limited time window during the initial phases of gastrulation, as *Nanog* is quickly downregulated in all cells by E8.0-8.5 (Hart et al., 2004a; Scialdone et al., 2016). By this stage, mesodermal progenitors have

ingressed through the primitive streak and are no longer able to activate the early hematopoietic program, a process that also involves restricted spatial signaling through the Wnt and Bmp pathways (Cheng et al., 2008; Mimoto et al., 2015; Myers and Krieg, 2013). Therefore, this *Nanog*-mediated switch would act to control the rapid specification of blood precursors, the first lineage determination event in gastrulation, and required to supply the embryo with oxygen to support its subsequent exponential growth.

Early postimplantation embryo regulation of *Tal1* expression

We also show that *Nanog* directly represses the master hematopoietic regulator *Tal1* (Porcher et al., 2017) through an upstream regulatory element located in an intron of the neighboring *Stil* gene. Interestingly, this site is occupied by NANOG only during the differentiation of ES cells to EpiLCs (Murakami et al., 2016). This change in binding site usage during this transition again suggests that *Nanog*, as it does in the locus of *Pou3f1*, has specific roles in the postimplantation pre-gastrulating epiblast (the *in vivo* equivalent of EpiLCs (Hayashi et al., 2011)) that are distinct from those operating during the pluripotent state. *Tal1* is certainly a prime candidate for mediating the effects of *Nanog* on erythropoiesis, as we found that it is consistently repressed at different embryonic stages and in adult erythroid progenitors. However, we do not rule out that other genes involved in early erythroid development will be also direct *Nanog* targets.

A new model for myelodysplasia?

In the adult, *Nanog* expression leads to defective erythroid-cell maturation, as also occurs in the embryo, and to an accumulation of MEPs showing downregulation of *Tal1*. This can be explained by a defect in the differentiation of said progenitors, and the phenotype we observe is reminiscent of the adult-specific *Tal1* knockout (Hall et al., 2005). It is therefore tempting to speculate that the regulatory circuit acting in the early embryo is reenacted in the adult solely by induction of *Nanog*. This could be a way of inducing a myelodysplasia in adult mice, however adult mice with global *Nanog* expression start dying from day 10 of treatment because of epithelial tumors and problems in the esophagus

(Piazzolla et al., 2014) and therefore we would lack important data on circulating cells that last for longer times. Another way of generating this disease model would be through transplantation of bone marrow, but as we have seen in this thesis, *Nanog* induced MEPs are prone to disappear if any host's HSC remains, which would make it difficult to obtain consistent results. Nevertheless, the effect of *Nanog* in bone marrow precursors is severe. It is precisely this strong effect what could help us gain more insight in the dynamics of the different precursors populations, not only in relation with each other but also with hematopoietic stem cells in the context of myelodysplasia.

***Nanog* is a physiological barrier in red blood cell differentiation**

Hematopoietic differentiation of *Nanog*^{-/-} ES cells (Chambers et al., 2007) confirms the proposed role for *Nanog* in erythroid development. Although *Nanog*^{-/-} cells show an initial delay in the activation of early pan-mesodermal markers such as *Brachyury*, once this occurs they show a faster and more coherent expression of erythroid genes. Directed differentiation reveals that the lack of *Nanog* promotes the red blood cell potential of these cells, which show a marked increase in both primitive and more mature erythroid colony formation. Our results show that *Nanog* acts as a barrier to red blood cell development. Controlled downregulation of *Nanog* during the initial phases of differentiation may present a novel approach to boosting the generation of red blood cells from pluripotent stem cells, a major clinical need (Kaufman, 2009).

***Nanog*: a Swiss knife for lineage specification during gastrulation**

Along gastrulation, numerous differentiation processes must take place in an orderly and controlled manner. Upon the formation of the primitive streak epiblast cells will give rise to mesoderm, endoderm and ectoderm (Tam and Behringer, 1997). At this stage, epiblast cells also differentiate towards PGCs. The crucial role of *Nanog* in PGCs differentiation and migration has been studied in detail (Murakami et al., 2016; Sánchez-Sánchez et al., 2010), although recent work has demonstrated that *Nanog* can be replaced completely by *Esrrb* (Zhang et al., 2018). Through the work presented in this thesis, we suggest two novel roles for

Nanog: blocking anterior fate through *Pou3f1*, and red blood cell differentiation through *Tal1*. Thus, we propose that the transcription factor NANOG acquires different functionalities after implantation that were not previously related with each other. It is yet to determine how has *Nanog* gained these properties throughout evolution and how *Nanog* regulation works at the onset of gastrulation. Further understanding of the epigenetic variations at these stages, as well as the changes in partners and target genes of NANOG, as well as SOX2 or OCT4, will broaden our understanding of how a pluripotent cell is able to jump into the void of differentiation.

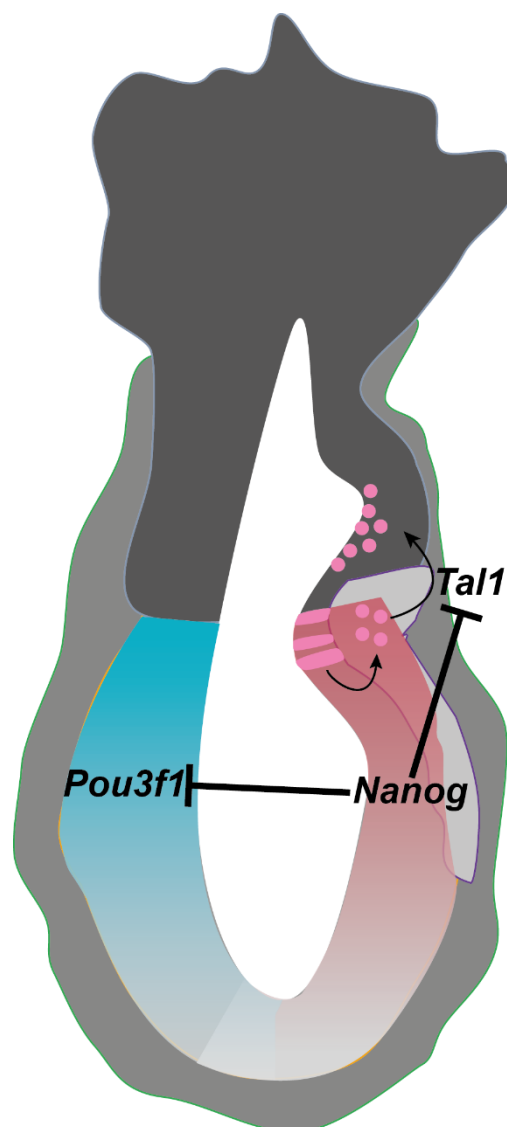


Figure 49. *Nanog* repress *Tal1* in red blood cell specification and *Pou3f1* during anteriorization of the epiblast. Representation of an E6.5 mouse embryo. The expression domain of *Nanog* is indicated in red, and that of *Pou3f1* in blue. Pink cells are lineage-fated red blood cells. Blunt arrows indicate repression. Arrows indicate direction of differentiation and migration.

CONCLUSIONS

CONCLUSIONS

1. *Nanog* blocks erythroid differentiation in the embryo at gastrulation stages in a cell autonomous manner.
2. Loss of function of *Nanog* in embryonic stem cells leads to increased differentiation potential towards erythroblast progenitors.
3. Gain-of-function of *Nanog* in the adult bone marrow leads to blockade of erythroid differentiation, closely resembling the early embryonic hematopoietic phenotype.
4. *Tal1* is a direct transcriptional target of NANOG during gastrulation.
5. Loss of function of *Nanog* during the exit of naïve pluripotency and in the transit to the primed state leads to the upregulation of anterior-neural genes.
6. *Nanog* inhibits neural specification in the early embryo and *in vitro*.
7. *Nanog* directly repress *Pou3f1* in the posterior epiblast of the gastrulating embryo.

CONCLUSIONES

1. *Nanog* bloquea la diferenciación eritroide durante la gastrulación de forma autónoma.
2. La pérdida de función de *Nanog* en células madre embrionarias conlleva un incremento del potencial de diferenciación de estas células a progenitores eritrocíticos
3. La ganancia de función de *Nanog* en médula ósea adulta comporta un bloqueo de la diferenciación eritrocítica, muy parecido al fenotipo observado en la hematopoesis embrionaria.
4. *Tal1* es una diana transcripcional directo de NANOG durante la gastrulación del embrión.
5. La falta de función de *Nanog* durante la salida de la pluripotencia naïve y en la transición hacia un estado preparado para la diferenciación propicia un aumento en la expresión de genes relacionados con linaje anterior-neural.
6. *Nanog* inhibe la especificación neural en el embrión temprano e *in vitro*.
7. *Nanog* reprime directamente *Pou3f1* en la región posterior del embrión durante la gastrulación.

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