The pluripotency factor NANOG controls primitive hematopoiesis and directly regulates *Tal1*

Julio Sainz de Aja, Sergio Menchero, Isabel Rollan, Antonio Barral, Maria Tiana, Wajid Jawaid, Itziar Cossio, Alba Alvarez, Gonzalo Carreño-Tarragona, Claudio Badia-Careaga, Jennifer Nichols, Berthold Göttgens, Joan Isern & Miguel Manzanares

**Abstract**

Progenitors of the first hematopoietic cells in the mouse arise in the early embryo from *Brachyury*-positive multipotent cells in the posterior-proximal region of the epiblast, but the mechanisms that specify primitive blood cells are still largely unknown. Pluripotency factors maintain uncommitted cells of the blastocyst and embryonic stem cells in the pluripotent state. However, little is known about the role played by these factors during later development, despite being expressed in the postimplantation epiblast. Using a dual transgene system for controlled expression at postimplantation stages, we found that *Nanog* 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 previous data for *NANOG* binding during stem cell differentiation and CRISPR/Cas9 genome editing, we found that *Tal1* is a direct NANOG target. Our results show that *Nanog* regulates primitive hematopoiesis by directly repressing critical erythroid lineage specifiers.

**Keywords** erythropoiesis; gastrulation; *Nanog*; primitive hematopoiesis; *Tal1*

**Subject Categories** Development & Differentiation; Transcription

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**Introduction**

Blood cells first appear during mouse development in the extraembryonic yolk sac at embryonic day (E) 7.5. These are primarily erythroid cells, needed to provide oxygen for the exponential embryo growth at these stages (Baron et al., 2012). This initial primitive hematopoiesis is not generated by hematopoietic stem cells, which first appear later in development (around E10.5) and provide the basis for definitive hematopoiesis (Jagannathan-Bogdan & Zon, 2013).

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 (Lawson et al., 1991; Huber et al., 2004); moreover, detailed fate mapping suggests that these cells are specified in the epiblast before gastrulation (Kinder et al., 1999; Padron-Barthe et al., 2014). 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 ends in the expression of FLK1 (encoded by *Kdr*), which marks most mesodermal cells at gastrulation (Pfister et al., 2007; Chan et al., 2013; 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 (Dore & Crispino, 2011; Baron et al., 2012).

Despite the knowledge acquired in recent years on the regulation of gastrulation and lineage determination of blood cells, we still do not fully understand how hematopoietic precursors are specified from within the pool of common mesodermal cells present in the posterior-proximal region of the gastrulating embryo. In other words, it remains unclear how the first differentiated cell type to appear in the postimplantation embryo (the primitive hematopoietic cells) is specified from a multipotent population of mesodermal progenitors, and how lineage-specific genes are turned on in this rapid transition. In this study, we provide evidence for an involvement in this process of the homeobox transcription factor gene *Nanog*.

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 & Tomlinson, 2009). Loss of *Nanog* in the early blastocyst results in embryonic...
death at implantation (Mitsui et al., 2003); however, Nanog-deficient ES cells are still able to maintain pluripotency, although they are prone to differentiate (Chambers et al., 2007). In the preimplantation embryo, Nanog is expressed throughout the epiblast. During implantation, Nanog is turned off, only to be re-expressed at E6.0 in the posterior part of the epiblast, where the primitive streak will form and gastrulation takes place shortly after (Hart et al., 2004; Osorno et al., 2012). Later, expression is restricted to primordial germ cells, with Nanog playing a crucial role in their development (Chambers et al., 2007; Yamaguchi et al., 2009; Zhang et al., 2018). Aside from its function in the germline, there is little or no previous evidence for Nanog playing any other role in the postimplantation epiblast or in the gastrulating embryo.

Here, we show that sustained expression of Nanog beyond gastrulation blocks differentiation of red blood cells during primitive hematopoiesis. This phenotype can be recapitulated in the adult, where Nanog leads to an increase in the number of megakaryocyte–erythroid precursors (MEPs), possibly by blocking their differentiation. Hematopoietic differentiation of Nanog-deficient ES cells is enhanced, further supporting the hypothesis that Nanog blocks the erythroid lineage in the epiblast of the gastrulating embryo. Furthermore, by re-analyzing single-cell RNA-seq data from gastrulating embryos (Scialdone et al., 2016) and Nanog ChIP-seq data in ES and epiblast-like cells (Murakami et al., 2016), together with CRISPR/Cas9-mediated genome editing, we found that NANOG directly represses the expression of the erythroid specifier Tal1. Together, these findings suggest that Nanog controls the early specification of hematopoietic cells from mesodermal precursors during gastrulation.

Results

Nanog blocks erythropoiesis in developing mouse embryos

Nanog loss of function is lethal at preimplantation stages (Mitsui et al., 2003), therefore preventing analysis of the putative role of Nanog later in development, when it is re-expressed at the posterior part of the gastrulating embryo (Hart et al., 2004). To overcome this obstacle, we used an inducible TetON transgenic model (Nanog<sup>Lox</sup>) in which Nanog expression is induced by the administration of doxycycline (dox) (Piazzolla et al., 2014). We induced Nanog from E6.5 in order to prolong its expression beyond E7.5, when it is normally turned off (Hart et al., 2004), and examined the embryos at E9.5. Visual examination of freshly dissected dox-treated embryos showed some growth retardation and craniofacial defects, but the most notable effect was a lack of blood (Fig 1A). To confirm this observation, we carried out whole-mount in situ hybridization for Hbb-bh1, which encodes the beta-like embryonic hemoglobin (Wilkinson et al., 1987) and for Redrum, an erythroid-specific long non-coding RNA (Alvarez-Dominguez et al., 2014; Paralkar et al., 2014). In untreated (control) Nanog<sup>Lox</sup> embryos at E9.5, Hbb-bh1 labels primitive red blood cells that are distributed throughout the yolk sac. Expression of Nanog up to this stage resulted in near complete blockade of Hbb-bh1 expression (Fig 1A). Redrum is expressed in the developing aorta-gonad-mesonephros (AGM) region, surely from erythroid cells circulating along the aorta, and in the tail bud. Nanog induction led to loss of Redrum expression in the AGM region, but interestingly not in the tail bud that is not a site of embryonic erythropoiesis (Fig 1A). We also checked if the apparent lack of blood was accompanied by vascular defects. Immunostaining for Endomucin, expressed in embryonic endothelial cells, revealed no substantial differences at E9.5 between dox-treated and untreated Nanog<sup>Lox</sup> embryos, as is observed in the correct patterning of intersomitic vessels (Fig 1B). Furthermore, CD31 staining showed that yolk sac vasculature was equally unaffected in dox-treated embryos (Fig EV1A). We also examined heart morphology at these stages, to address if other mesodermal derivatives showed developmental defects. Hearts of freshly dissected E9.5 dox-treated embryos beat normally, and both overall morphology and histological sections showed no defects (Fig EV1B). Prolonged Nanog expression in the embryo thus 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 or cardiac development.

To characterize the effect of Nanog induction on hematopoiesis, we analyzed progenitors and red blood cells by flow cytometry of dispersed individual yolk sacs from E9.5 embryos using c-Kit (a marker of early uncommitted progenitors), CD41 (erythroid progenitors; Mitjavila-Garcia et al., 2002), CD71, and Ter119 (Borges et al., 2012). Dox-treated Nanog<sup>Lox</sup> embryos showed a dramatic reduction in erythroblast cells (CD71<sup>+</sup> Ter119<sup>+</sup>; Fig 1C and D), which supports the above results. However, the total number of hematopoietic progenitor populations (cKit<sup>+</sup>CD41<sup>+</sup> and CD41<sup>+</sup>, respectively) remained unchanged (Fig 1E and F). We examined the morphology of erythroblasts from circulating blood of E9.5 dox-treated and untreated embryos by Giemsa staining (Fraser et al., 2007) and found that the few remaining primitive erythroid cells showed a normal morphology (Fig EV1C). Taken together, these results suggest that Nanog causes a blockade in hematopoietic progenitors, preventing their differentiation toward erythroblast cells.

Nanog downregulates the expression of key erythroid determination genes

We next investigated how prolonged Nanog expression to E9.5 influences hematopoietic gene expression. For this, we isolated progenitor and mature populations by flow cytometry as described above (Fig 1C and D), and conducted RT-qPCR to examine the expression of core lineage determinants of hematopoietic fate: Tal1, Runx1, Gata1, and Klf1 (Palis et al., 1999; Yokomizo et al., 2002; Kuvardina et al., 2015). Gain of Nanog expression in erythroblasts (CD71<sup>+</sup> Ter119<sup>+</sup> population) resulted in significant downregulation of Tal1 and increase of Runx1 (Fig 1G). However, despite consistent gain of Nanog expression, we did not observe differences of gene expression in earlier progenitors (Fig EV1D).

To examine whether similar 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 EV1E). RT-qPCR of individual dox-treated or control E7.5 Nanog<sup>Lox</sup> embryos showed decreased expression of the core erythroidic genes Tal1, Gata1, and Klf1 but no change in Runx1 (Fig EV1F). 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 (Shalaby et al, 1995; Palis et al, 1999). 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 EV1F). 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 hematopoiesis.

Nanog-induced hematopoietic defects are cell intrinsic

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 (Diaz-Diaz et al, 2017) into Nanog<sup>−</sup> ES blastocysts. 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 of GFP<sup>+</sup> cells were used as controls, whereas embryos containing GFP<sup>+</sup> cells were considered chimeras (Fig 2A and B). Erythroid cells were evaluated in individual embryos by flow cytometry analysis of CD71 and Ter119, as described earlier (Fig 1E and F).

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 2B). Chimeras showed a recovery of erythroid cells, with high contribution from GFP<sup>+</sup> wild-type ES-derived cells (Fig 2C). Quantification of erythroid populations in chimeras showed an increased content of CD71<sup>+</sup> Ter119<sup>+</sup> cells (Fig 2D); this increase did not occur when the experiment was repeated without dox treatment (Fig 2E). The number of GFP<sup>+</sup> cells (derived from Nanog expressing cells) in dox-treated chimeras did not differ from that in controls (with no contribution of GFP<sup>+</sup> cells), demonstrating that the recovery of the erythroid populations in chimeras was entirely due to the wild-type ES cells (Fig 2F). 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.

Loss of Nanog enhances hematopoietic differentiation of 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, 2007) to differentiate into blood cells in culture (Irion et al, 2010). Nanog<sup>−</sup> 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 (DS-D7; Fig 3A). Despite a trend for a decrease in the number of common myeloid progenitors (CFU-GEMM), Nanog<sup>−</sup> 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<sup>−</sup> EBs (Fig 3A). This last observation is possibly due to a decrease in common myeloid progenitors together with a significant increase of erythroid progenitors in the mutants. Nanog<sup>−</sup> ES cells thus have an increased potential for specific differentiation to red blood cells.

To investigate how the absence of Nanog affects the gene networks involved in erythroid specification, we monitored control and Nanog<sup>−</sup> 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 in EBs (Feihling et al, 2003). Brachyury expression markedly increased at day 3 in wild-type cells, as previously described (Robertson et al, 2000), but in Nanog<sup>−</sup> EBs this expression peak was delayed until day 5 (Fig 3B). Nanog is thus likely required for the correct temporal activation of Brachyury. 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<sup>−</sup> 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 3B). Given the requirement of Brachyury expression for hematopoietic differentiation (Feihling et al, 2003), we aligned the expression dynamics of wild-type and Nanog<sup>−</sup> cells to the day of Brachyury induction (Fig EV2A). 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<sup>−/−</sup> EBs. In contrast, erythroid gene activation occurred earlier in Nanog<sup>−/−</sup> EBs than in wild-type controls (Fig EV2B). Thus, although mesoderm induction is delayed in Nanog<sup>−/−</sup> EBs, once it occurs the Nanog<sup>−/−</sup> mesodermal cells show an elevated potential for erythroid differentiation.

To further study the effect of loss of Nanog, we deleted a floxed allele from a heterozygous ES cell line (Nanog<sup>lox/−</sup>; Zhang et al, 2018) by transfecting Cre recombinase and differentiating sorted GFP<sup>+</sup> cells (that is activated upon Cre recombination) from ES to

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Figure 1.
epiblast-like cells (Hayashi et al., 2011; Murakami et al., 2016). This process recapitulates in culture the transition from pluripotent cells of the blastocyst to primed cells of the epiblast (Buecker et al., 2014), a time window during development when Nanog is expressed. Mutant cells (Nanog<sup>del</sup>/C0) upregulate Brachyury following the same dynamics as control heterozygote Nanog<sup>flox</sup>/C0 cells. However, they show precocious activation of erythroid gene expression (Fig EV2C), in line with our previous observations.

Blockade of adult erythrocyte maturation by Nanog

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

Figure 2. Wild-type ES cells rescue erythroid maturation in chimeric embryos.
A Experimental design for chimera generation, and contribution of GFP cells to chimeric embryo (right hand side panels).
B Freshly dissected dox-treated Nanog<sup>tg</sup> E10.5 embryos without (control) and with (chimera) contribution of wt-ES<sup>GFP</sup> cells (left, brightfield; right, GFP). Arrows mark the presence of blood in chimeric embryos that is absent from controls. Scale bar, 500 μm.
C Representative FACS plots showing red blood cell maturation as determined by CD71/Ter119 staining in single dox-treated E10.5 control (left) and chimeric (right) embryos.
D–F Quantification of the CD71<sup>+</sup> Ter119<sup>+</sup> population in single dox-treated E10.5 embryos (D; control, n = 18; chimera, n = 10), untreated embryos (E; control, n = 7; chimera, n = 12), and in GFP<sup>-</sup> cells (not derived from wild-type ES cells) from dox-treated embryos (F; control, n = 18; chimera, n = 10). ***P < 0.0005; Student’s t-test. Horizontal line represents mean values and error bars SD.
Figure 3.

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ES cell-derived embryoid bodies

CFU-GEMM

Ery-P

BFU-E

CFU-GM

CFU-M

CFU-G

ESC-derived embryoid bodies

Nanog−/−

Brachyury
Gata1
Hbb-bh1
Klf1
Tal1

wt

Nanog+/−

Figure 3.
Nanog expression was systemically induced in adult Nanog<sup>Lox/Lox</sup> mice by 5-day treatment with dox in drinking water, and the mice were then sacrificed and bone marrow extracted (dox<sup>+</sup>; Fig 4A). As controls, we used untreated mice of the same genotype (dox<sup>−</sup>). Analysis of erythrocyte maturation with CD71 and Ter119 revealed an increase in immature populations (basophilic and polychromatic erythroblasts; S2 and S3, respectively) together with a decrease in the number of more differentiated erythroblasts (orthochromatic erythroblasts, S4; Fig 4B and C). This result suggested a block in the differentiation of erythrocyte precursors, so we next quantified bone marrow progenitors by flow cytometry using lineage cocktail, c-kit, Sca-1, CD34, and CD16/32 (Fig 4D; Challen et al, 2009).

Induced Nanog expression triggered a decrease in absolute cell numbers of hematopoietic stem cells (lineage-Sca1<sup>+</sup> cKit<sup>+</sup>; LSK) and Nanog<sup>tg</sup> and therefore to an accumulation of their precursors. This leads to defective differentiation of these populations—hematopoiesis together with the downregulation of key erythroid lineage factors, such as Tal1, in Nanog expressing MEPs.

To extend these observations, we next carried out bone marrow transplantation of Nanog<sup>Lox/Lox</sup> mice to wild-type irradiated recipients (Fig 4G). After 3 months of engraftment and recovery, more than 95% of peripheral blood cells were derived from donor mice (n = 7; Fig 4H). 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 reconstitute the bone marrow and contribute to peripheral blood cells (ranging from 20 to 80%; Fig 4H). We then purified bone marrow from the transplanted mice and analyzed chimerism in different progenitor populations. While LSK, CMPs of GMPs show variable degrees of contribution of wild-type cells and Nanog expressing cells, MEPs are almost exclusively derived from the host (Fig 4I). 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.

A distal NANO1-binding element represses Tal1 expression in the embryo

Nanog-mediated downregulation of erythroid specification genes in both the embryo and the adult strongly suggests that some of these genes are likely direct transcriptional targets of NANO1. 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 5A). For all of these genes, we found the expected proportion of co-expressing cells with Nanog with the exception of Tal1 (Fig 5A and B). We confirmed that Nanog can downregulate Tal1 at early stages by culturing Nanog<sup>Lox/Lox</sup> embryos with or without dox from E6.5 to E6.75<sub>ex</sub> utero, which did not alter normal development (Fig 5C). Tal1 failed to be upregulated in dox-treated embryos, whereas other hematopoietic genes such as Klf1 were unaffected (Fig 5D). We further confirmed that Nanog downregulates Tal1 by whole-mount in situ hybridization of E7.5 embryos treated with dox<sub>in</sub> utero (Fig 5E).

This evidence strongly suggests that Tal1 is a direct transcriptional target of NANO1 during early gastrulation at the onset of
hematopoietic determination. To investigate this possibility, we analyzed published ChIP-seq data for NANOG binding in ES and 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 genes involved in hematopoiesis and found that treatment with doxycycline led to a decrease in Nanog expression and an increase in the expression of Runx1, Tal1, Gata1, and Klf1. These changes were accompanied by a decrease in the number of LSK, CMP, MEP, and GMP cells in the bone marrow, as shown in Figure 4. Overall, these results suggest that Nanog regulates primitive hematopoiesis.
Thus, in this experimental setting, increased expression of Cdx2 of genomic loci, detecting binding at the regions surrounding show a significant increase in the embryo. However, if dox is added to the medium, erythroid progenitors (MEP; +dox, P<0.005, Student’s t-test. Horizontal line represents mean values and error bars SD).

C Representative FACS plots showing the distribution of different populations distinguished by CD45/C0 staining in whole bone marrow from untreated (−dox) or treated (+dox) adult mice. Stil contribution of donor 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).

D 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−/− mice.

E 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. Horizontal line represents mean values and error bars SD.

F 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 < 0.0005, Student’s t-test. Horizontal line represents mean values and error bars SD.

G Experimental design for the transplant of Nanog−/− bone marrow to wild-type recipients and treatment of chimeric mice.

H Contribution of Nanog−/− 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 blue. Individual mice are indicated on the x-axis (n = 7).

I Contribution of Nanog−/− 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).

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Figure 4. Induced Nanog expression blocks erythroid maturation in adult mice.

A Experimental design for the treatment of adult Nanog−/− 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. SO (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, Student’s t-test. Horizontal line represents mean values and error bars SD.

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of genomic loci, detecting binding at the Nanog locus itself in both ES cells and EpiLCs (Fig EV4A) and in Cdx2 only in ES cells (Fig EV4B). Neither cell type showed evidence of NANOG bound regions surrounding Runx1 (Fig EV4C) or Klf1 (Fig EV4D). Interestingly, EpiLCs, but not ES cells, showed NANOG binding 22 kilobases upstream of Tal1, in an intron of the neighboring Stil gene (Fig EV4E). We also detected NANOG binding downstream of Gata1 (Fig EV4F); however, these regions could be functionally related to the neighboring Eras and Hdac6 genes, which are associated with pluripotency and early stem cell differentiation (Takahashi et al., 2003; Chen et al., 2013).

Analysis of the Tal1/Stil NANOG bound region in EpiLCs (Fig 5F) revealed bona fide consensus binding sites (Fig EV5A). To investigate the function of this region, we deleted it by CRISPR/ Cas9-mediated genome editing (Ran et al., 2013) by microinjection in one-cell stage embryos 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 EV5B). Tal1 expression was significantly increased in targeted embryos, whereas other genes such as Klf1, Cfl1b, or Runx1 were unaffected (Fig 5G). Deletion of this genomic region did not alter Stil expression, despite the location of the site within this gene (Fig 5G). 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.

In order to further confirm these observations and address the effect of the deletion on Tal1 expression and its dependence on Nanog, we analyzed the transition from ES to EpiLC in culture as above. For this, we generated lines deleted for the distal Tal1 element by genome editing as previously described in vivo, but in ES cells derived from the Nanog−/− mouse (Figs 5H and EV5C). We observe that non-treated Nanog−/− ES cells (non-deleted control) show a significant increase in Tal1 expression when they transit to EpiLCs (Fig 5H), what would be the equivalent of the initial expression of Tal1 in the embryo. However, if dox is added to the medium, this increase of Tal1 between ES and EpiLC is no longer significant. Thus, in this experimental setting, increased expression of Nanog is able to block at least partially the early induction of Tal1, in line with our in vivo results. Nevertheless, when we repeat his experiment but with two independent ES cell lines where the NANOG-bound distal element (dTal1) has been deleted (Nanog−/−dTal1del#1 and Nanog−/−dTal1del#2), EpiLC becomes unresponsive to Nanog upon dox treatment and still upregulate Tal1 as cells not treated with dox. These results show that the distal element we have characterized is necessary for correct initiation of Tal1 expression, and that it mediates the response of Tal1 to Nanog.

Discussion

Red blood cell precursors are the first cell type to be specified from nascent mesoderm during mouse gastrulation (Kinder et al., 1999; Baron et al., 2012). While the genes and networks that determine primitive hematopoietic cells are well understood (Isrn et al., 2011; Kingsley et al., 2013), much less is known about how precursors are specified during the early stages of primitive streak formation (Padron-Barthe et al., 2014). 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.

Despite the well-characterized role of pluripotency factors in embryonic stem cells and the preimplantation embryo (Chambers & Tomlinson, 2009), their function at later developmental stages has received much less attention, even if they are expressed up to gastrulation in mice (Yeom et al., 1996; Hart et al., 2004; Ossorno et al., 2012) and primates (Nakamura et al., 2016). Oct4 is involved in proliferation of the primitive streak (DeVeale et al., 2013), in correct trunk elongation of the trunk (Aires et al., 2016), and some evidence points to it having a role in mesoderm and subsequent hematopoietic specification (Kong et al., 2009). However, no clear function is known for Nanog after implantation apart from the regulation of germline development (Chambers et al., 2007). Single-cell RNA-seq expression data from gastrulating embryos (Scaldone et al., 2016) show that Nanog is expressed in a subset of mesodermal precursors. This situation is reminiscent of the heterogeneities in Nanog expression in the preimplantation embryo, which drives lineage segregation of the epiblast and the primitive endoderm.
**Figure 5.**

A. E7.0 embryo

B. E6.5 embryo

C. E7.5 embryo

D. Relative Expression

E. E6.5 embryo

F. ES to EpiL cell transition

G. Relative Expression

H. NSF

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Figure 5. Direct transcriptional regulation of Tal1 expression by Nanog.

A Expected and observed number of mesodermal (Fik1+) cells of the E7.0 mouse embryonic expressing Nanog and selected mesodermal or hematopoietic gene expression, based on single-cell RNA-seq data (Scialdone et al, 2016). Statistical significance was calculated with a hypergeometric test.

B PCA showing the distribution of Fik1+ 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.

C E6.5 Nanog+ embryos after 8 h ex utero culture in the presence (+dox) or absence (−dox) of doxycycline. Scale bar, 100 μm.

D 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. Horizontal line represents mean values and error bars SD.

E Whole-mount in situ hybridization of Tal1 in E7.5 untreated (−dox) or in utero treated (+dox) Nanog+ embryos. Scale bar, 100 μm.

F UCSC browser view of the Tal1/STIL1 region (mm11: chr11: 7 055 755–114 796 471), 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).

G RT–qPCR determination of relative expression in wild-type and CRISPR-deleted embryos (n = 5) of Tal1 (wt, n = 19, deleted, n = 13), Klf1 (wt, n = 3, deleted, n = 6). Gfi1b (wt, n = 10; deleted, n = 8), Runx1 (wt, n = 13; deleted, n = 5) and Stil (wt, n = 19; deleted, n = 13). **P < 0.005. Student’s t-test. Horizontal line represents mean values and error bars SD.

H Experimental design for ES to EpiL cell differentiation of Nanog+ cells and two independent clones (Nanog+; Tal1lox/lox and Nanog+; Tal1lox/lox) where the binding site for Nanog distal to Tal1 has been deleted (left). On the right, relative expression of Tal1 determined by RT–qPCR for each ES cell line (ESC, n = 9 for all three lines) and EpiL cells without (EpiL, Nanog+ and Nanog+; Tal1lox/lox; n = 8, Nanog+; Tal1lox/lox; n = 6) or with dox treatment (EpiL+; dox; n = 9 for all three lines). The genotype of the cell lines is indicated below. Values were normalized to Nanog+ ESC. *P < 0.05, **P < 0.01, ns = not significant; ANOVA with Fisher post-test. Horizontal line represents mean values and error bars standard error of the mean (SEM).

Our results suggest that a similar situation may occur during specification of the first mesodermal lineages. Nanog expression in Brachyury-positive cells would support 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 by E8.0–8.5 (Hart et al, 2004; 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; Myers & Krieg, 2013; Mimoto et al, 2015). 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.

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 has specific roles in the postimplantation pregastrulating mesoderm cells expressing Tal1 (red). The single cell expressing both genes is shown in yellow and indicated by an arrow.

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 these 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 can be reenacted in the adult solely by induction of Nanog.

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).

Materials and Methods

Animal model

We obtained the Nanog/rtTA mouse line (R26-M2rtTA;Col1a1-tetO-Nanog) (Pizzazzola et al, 2014) from Manuel Serrano (CNIO, Madrid) and Konrad Hohenleinder (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. Mice were genotyped by PCR of tail-tip DNA as previously described (Hohenleinder et al 2005; Pizzazzola et al, 2014). Mice were housed and maintained in the animal facility at the Centro Nacional de Investigaciones Cardiovasculares (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 µl intraperitoneal injection of 25 µg/µl doxycycline was administered to pregnant females at E5.5, followed by dox administration in drinking water as above.

**RT–qPCR assays**

RNA was isolated from ESCs or sorted E9.5 cells using the RNeasy Mini Kit (Qiagen) and then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA from individual E6.5-7.5 embryos 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*. Primers used are listed in Dataset EV2.

**Flow cytometry**

E9.5 and E10.5 whole embryos or dissected yolk sacs 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: anti-CD71-FITC (BD Biosciences), anti-TER119-APC (BD Biosciences), anti-c-Kit-PEcy7 (BD Biosciences), and anti-CD41-PE (BD Biosciences). Samples were analyzed with the BD LSRLFortessa flow cytometer.

Bone marrow of adult mice was obtained from femurs and tibias crushed in a mortar and filtered through a 70-µm mesh. The single-cell preparation was then passed through a 70-µm mesh without doxycycline treatment were lysed for RNA isolation. The rest was depleted of red blood cells by lysis in FACSLysing solution (BD Biosciences). Antibodies used for blood maturation assay were anti-CD71-FITC (BD Biosciences) and anti-Ter119-APC (BD Biosciences). Antibodies for BM precursor sorting were Biotinylated lineage cocktail (BD Biosciences), anti-CD34(RAM34)-FITC (BD Biosciences), anti-c-Kit-PEcy7 (BD Biosciences), anti-CD16/32-BV605 (BD Biosciences), and anti-Sca1-PerCP-Cy5.5 (BD Biosciences).

**Cytospin cell preparation**

For peripheral blood cytospin preparations, E9.5 embryos were dissected in warm PBS with 2% FBS and EDTA 0.5 mM, puncturing the yolk sac and the heart to let blood disperse into the media. All the preparation was passed through a 70-µm filter, centrifuged for 5 min at 135 g, and resuspended in a final volume of 200 µl PBS. Cells were collected on a glass slide on a Thermo Scientific Cytospin 4 Cytocentrifuge for 10 min at 200 rpm and stained with May-Grunwald-Giemsa. Slides were scanned on a NanoZoomer-2.0RS C110730 scanner (Hamamatsu).

**Cell culture**

ESCs were maintained in serum-free conditions with Knock out serum replacement (Thermo Fisher), LIF (produced in-house), and 2i (CHIR-99021, Selleckchem; and PD0325901, Axon). BT12 and E14Tg2a ESCs were kindly provided by Ian Chambers and Austin Smith (Chambers et al., 2007). ESC was differentiated toward hematopoiesis according to published protocols (Sroczynska et al., 2009; Iriion et al., 2010; Lesinski et al., 2012).

For embryoid body formation, 5000 ESCs were plated in StemPro34 medium supplemented with nutrient supplement (Gibco) and 2 mM l-glutamine (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 days, to the cultures were added 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 differentiated day 6 by treatment with 0.05% trypsin-EDTA at 37°C for 2–5 min.

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 days 5, 6, and 7 were plated in Methocult GF M3434 methylcellulose medium for quantification of erythroid progenitor cells (BFU-E), granulocyte–macrophage progenitor cells (CFU-GM, CFU-G, CFU-M), and multipotential 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.

*Nanog* floxed ES cells (*Nanog*^lox/lox-^; Zhang et al., 2018) were transfected with a Cre-expressing plasmid to induce recombinase using Lipofectamine 2000 (Invitrogen). After 48 h, GFP-positive cells (*Nanog*^lox/lox-^) and GFP-negative cells used as control (*Nanog*^lox/^) were sorted using a FACS Aria Cell Sorter. Differentiation toward EpilCs was induced by plating 5 × 10⁴ ES cells on a well of a 24-well plate coated with human plasma fibronectin (10 µg/ml Sigma) in N2B27 medium supplemented with 20 ng/ml Activin A (Peprotech), 12 ng/ml bFGF (Peprotech), and 1% Knock out serum replacement (Thermo Fisher) for 3 days.

Embryonic stem cells from *Nanog*^−/−^ mice were derived following standard procedures (Nagy et al., 2003). Differentiation to EpilCs was performed in *Nanog*^−/−^ ES cells and in two different clones of *Nanog*^−/−^ ES cells where the binding site upstream of *Tal1* was deleted (*Nanog*^del−^; *dTal1del* ^#1^ and ^#2^). Differentiation was induced by plating 3 × 10⁴ ES cells on a well of a 24-well plate and using the same conditions above-mentioned. After 3 days of differentiation, doxycycline (2 ng/ml) was added to the medium of the corresponding wells to induce *Nanog* expression. One day later, EpilCs with or without doxycycline treatment were lysed for RNA isolation.

**In situ hybridization and immunohistochemistry**

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 2× 100%). *In situ* hybridization in whole-mount embryos was performed as described (Ariza-McNaughton & Krümlauf, 2002; Julio Sainz de Aja et al. 2019).
Acloque et al., 2008). Signal was developed with anti-digoxigenin-AP (Roche) and BM-Purple (Roche). Images were acquired with a Leica MZ-12 dissecting microscope. Probes for in situ were obtained by PCR of cDNA with the primers listed in Dataset EV2.

For immunohistochemistry in whole mount, embryos were fixed overnight at 4°C in 4% paraformaldehyde, followed by overnight incubation at 4°C in primary antibody diluted 1:100 (rat monoclonal anti-endomucin, Santa Cruz sc-18916), washed and incubated overnight at 4°C with 1:500 Alexa Fluor 488 goat anti-rat (Termo Fisher Scientific, A-11006) for Endomucin or HRP goat anti-rat (Termo Fisher Scientific, 31470) for CD31. For histology, embryos fixed as above were dehydrated through an ethanol series, cleared with xylene, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

RNA-seq

RNA was isolated from three replicates each of approximately 20,000 MEPs purified by sorting from adult untreated and dox-treated Nanogtg mice. Sequencing was performed by the CNIC Genomics Unit using the GAIIx sequencer. Adapters were removed with Cutadapt v1.14 and sequences were mapped and quantified using RSEM v1.2.20 to the transcriptome set from Mouse Genome Reference NCBI-M37 and Ensembl Gene Build version 67. Differentially expressed genes between the two groups were normalized and identified using the limma bioconductor package. Only P-values < 0.05 adjusted through Benjamini–Hochberg procedure were considered as significant. Hierarchical clustering was performed on Z-scored values of the selected genes to generate an overview of the expression profile. Functional enrichment analysis was conducted using Enrichr (Kuleshov et al., 2016).

CRISPR/Cas9 genome editing

sgRNAs were designed using the CRISPR Design Tool from the Zhang Lab at MIT (http://crispr.mit.edu/). Sequences of guide RNAs are indicated in Fig EV5A. The two guide RNAs at 25 ng/μl were incubated with the Cas9 protein (PNA bio) at 30 ng/μl and microinjected into the pronuclei of (CBAxC57)F1 zygotes (1,490); 1,075 surviving embryos were transferred to CD1 pseudopregnant females. 105 embryos were recovered at E6.5, and after discarding delayed or malformed embryos, 72 were lysed in 100 μl extraction buffer from the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems). Aliquots of 10 μl were used for DNA extraction for PCR genotyping, and the remaining 90 μl was used for RNA extraction for RT–qPCR. Embryos for which we did not obtain a clear genotype were discarded, as well as those for which RT–qPCR of housekeeping genes did not reach a minimal threshold.

Embryonic stem cells from Nanogtg mice were electroporated with Cas9 protein and sgRNAs as above. Individual clones were picked, genotyped as above, karyotyped, and expanded for further use.

Statistical analysis

Statistical analysis was performed with the use of two-tailed Student’s unpaired t-test analysis (when the statistical significance of differences between two groups was assessed) or one-way ANOVAs with subsequent Fisher post-test (when the statistical significance of differences between more than two groups was assessed). Prism software version 7.0 (Graphpad Inc.) was used. For the analysis of the expected proportion of co-expressing cells with Nanog, we used a hypergeometric test in R.

Data availability

Sequencing data have been deposited at GEO under accession number GSE119467 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119467).

Expanded View

For this article is available online.

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Author contribution

JSA and MM designed the research with input from JI, BG, and JN. JSA performed the majority of the experiments, with the help of SM for embryo work and tissue culture; IR for genome editing; AB and MT for ES cell work; WJ and AA for data analysis; IC, AB, and CC-T for analysis of adults; and CB-C for in situ. JSA and MM wrote the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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