A genetic and developmental pathway from STAT3 to the OCT4–NANOG circuit is essential for maintenance of ICM lineages in vivo

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Although it is known that OCT4–NANOG are required for maintenance of pluripotent cells in vitro, the upstream signals that regulate this circuit during early development in vivo have not been identified. Here we demonstrate, for the first time, signal transducers and activators of transcription 3 (STAT3)-dependent regulation of the OCT4–NANOG circuitry necessary to maintain the pluripotent inner cell mass (ICM), the source of in vitro-derived embryonic stem cells (ESCs). We show that STAT3 is highly expressed in mouse oocytes and becomes phosphorylated and translocates to the nucleus in the four-cell and later stage embryos. Using leukemia inhibitory factor (Lif)-null embryos, we found that STAT3 phosphorylation is dependent on LIF in four-cell stage embryos. In blastocysts, interleukin 6 (IL-6) acts in an autocrine fashion to ensure STAT3 phosphorylation, mediated by janus kinase 1 (JAK1), a LIF- and IL-6-dependent kinase. Using genetically engineered mouse strains to eliminate Stat3 in oocytes and embryos, we firmly establish that STAT3 is essential for maintenance of ICM lineages but not for ICM and trophectoderm formation. Indeed, STAT3 directly binds to the Oct4 and Nanog distal enhancers, modulating their expression to maintain pluripotency of mouse embryonic and induced pluripotent stem cells. These results provide a novel genetic model of cell fate determination operating through STAT3 in the preimplantation embryo and pluripotent stem cells in vivo.

[Keywords: embryogenesis, inner cell mass; STAT3; OCT4; NANOG; embryonic stem cell]

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implanting embryonic day 4.5 [E4.5] embryos, NANOG and GATA4/6 are key determinants for the epiblast (EPI) and primitive endoderm (PE) fate, respectively [Koutsourakis et al. 1999; Mitsui et al. 2003]. However, the upstream signals that regulate Oct4 expression and its gene circuitry during early embryogenesis are not well documented.

Many initial attempts to identify the upstream regulators were undertaken in vivo [Rothstein et al. 1992; Ko et al. 2000; Guo et al. 2010; Tang et al. 2010], yet it was from in vitro work that leukemia inhibitory factor (LIF) was found to be the key growth factor for the culture and maintenance of mouse ESCs [mESCs] [Smith et al. 1988; Williams et al. 1988]. LIF, a member of the interleukin 6 (IL-6) family of cytokines, binds to glycoprotein 130 (gp130) in association with a ligand-specific receptor subunit, LIF receptor (LIFR). The binding of LIF to the LIFR results in activation of receptor-associated janus kinases [JAKs] in the phosphorylation of receptor docking sites and, finally, in the recruitment of the Src homology-2 (SH2) domain and phosphorylation on Tyr705 residues of signal transducers and activators of transcription (STAT), a gene family originally described in the interferon-induced regulatory pathways [Fu et al. 1990, 1992; Schindler et al. 1992; Darnell et al. 1994]. The STAT proteins are well conserved through evolution [L. Zhang, CPK Patro, CY Ung, TP Phan, PJ Lin, Y Zheng, G Song, A Jean, JC Tong, YE Chin, et al., unpubl.], regulating gene expression in response to external signals [Darnell 1997]. STAT3, first identified as a transcription factor for the IL-6 family of cytokines [Akira et al. 1994; Zhong et al. 1994], was subsequently found to be crucial for ESC pluripotency [Boeuf et al. 1997; Niwa et al. 1998; Raz et al. 1999; Ying et al. 2003]. However, it is not clear how LIF/STAT3 signaling interacts with the core set of pluripotency factors for ESCs, namely, Oct4, Nanog, and Sox2 [Boyer et al. 2005; Loh et al. 2006]. Using a genomewide approach, STAT3 was initially implicated in the OCT4-mediated gene circuitry in cultured ESCs [Chen et al. 2008]; other studies showed direct regulation of Klif4, which is also a member of this circuitry [Hall et al. 2009; Niwa et al. 2009].

Conventional knockout of Stat3 in mice results in embryonic lethality at E6.5 [Takeda et al. 1997]. However, the ability of zygotic Stat3 knockout embryos to undergo apparently normal EPI expansion is in conflict with the central role of STAT3 in maintaining ESCs [Boeuf et al. 1997; Niwa et al. 1998; Raz et al. 1999; Ying et al. 2003]. To resolve this paradox, we investigated the role of STAT3 in implantation embryos and pluripotent stem cells. By eliminating Stat3 in mouse oocytes and embryos, we found STAT3 to be essential for the maintenance of ICM lineages but not for blastocyst formation or TE maintenance. By working with mouse ESCs and induced pluripotent stem cells [iPSCs], we found that STAT3 directly binds and regulates Oct4 [Pou5F1] expression to maintain their pluripotency. These results lead us to propose that STAT3 has an essential role in ICM lineage specification and maintenance and pluripotent stem cell identity.

## Results

### Presence of activated STAT3 in preimplantation embryos

STAT3 is present exclusively in the cytoplasm of oocytes, zygotes, and two-cell stage embryos but strikingly translocates to the nucleus specifically at the four-/eight-cell stage [Fig. 1A]. Using an antibody against phosphorylated tyrosine-specific [pY705];STAT3, we confirmed that nuclear STAT3 at the four-cell stage embryo is indeed tyrosine-phosphorylated [Fig. 1B]. We also observed OCT4 colocalization with nuclear, phosphorylated STAT3 [pY705; pSTAT3] in most four-cell stage embryos. These results indicate that STAT3, while present in earlier stage embryos, is only functionally activated at the four-cell stage.

### STAT3 phosphorylation is dependent on LIF, IL-6, and JAK1 in preimplantation mouse embryos

To identify the cytokines responsible for the STAT3 activation in the four-cell embryos, we examined STAT3 phosphorylation in Lif-null and E-cadherin [Cdhd1]-null preimplantation embryos. STAT3 is known to be phosphorylated by LIF [Akira et al. 1994; Zhong et al. 1994], and Cdhd1-null ESCs exhibit loss of tyrosine-phosphorylated STAT3 [Hawkins et al. 2012]. We found no discernible tyrosine-specific phosphorylated STAT3 in Lif-null four-cell embryos, while STAT3 phosphorylation is present in Cdhd1-null embryos [Fig. 2A]. This suggests that LIF is the cytokine responsible for STAT3 phosphorylation in four-cell embryos, and in contrast to ESCs, this is E-cadherin-independent in vivo. Interestingly, when we examined the STAT3 phosphorylation in later stage embryos, we found that active STAT3 [pY705; pSTAT3] is nonetheless present at the late morula and blastocyst stages [Fig. 2B]. In addition, tyrosine-phosphorylated STAT3 is present in blastocysts derived from zygotes cultured in KSOM [Fig. 2C]. These results suggest that cytokines other than LIF produced by the blastocysts act in an autocrine manner to stimulate STAT3 phosphorylation. Since IL-6 and IFN-γ are responsible for STAT3 phosphorylation [Akira et al. 1994; Zhong et al. 1994] and appear to be expressed in blastocysts [Rothstein et al. 1992], we examined their role in STAT3 phosphorylation in the blastocyst. While treatment of zygotes with a neutralizing antibody against IFN-γ showed no significant change in STAT3 phosphorylation, embryos treated with a neutralizing antibody against IL-6 showed a significant reduction in phosphorylated STAT3 expression [Fig. 2C,D]. Taken together, these results suggest that IL-6 is the additional cytokine produced by the blastocysts and responsible for STAT3 phosphorylation in an autocrine manner in the absence of LIF.

The JAKs TYK2, JAK1, JAK2, and JAK3 are responsible for the activation of STAT proteins; TYK2, JAK1, and JAK2 are activated by IL-6 [Schindler and Darnell 1995]. We therefore examined RNA sequencing [RNA-seq] data obtained from single preimplantation mouse embryos for expression of these kinases during preimplantation mouse development. We found that Jak2 and Tyk2 mRNAs...
are highly expressed in the four-cell stage, whereas Jak1 is highly expressed in the blastocyst stage (Supplemental Fig. S1A). To determine whether JAK1 is a major kinase responsible for STAT3 phosphorylation in the blastocyst, we cultured zygotes in the presence of different JAK inhibitors: JAK inhibitor I (JAKi), an inhibitor for all four JAK kinases; AG490, an inhibitor for JAK2 and JAK3; and ruxolitinib, an inhibitor for JAK1 and JAK2. JAKi- and ruxolitinib-treated blastocysts showed no discernible expression of tyrosine-phosphorylated STAT3, while AG490-treated embryos were positive for STAT3 phosphorylation, suggesting that JAK1 is responsible for STAT3 phosphorylation in the blastocysts (Supplemental Fig. S1B). In addition, OCT4 expression was reduced in JAKi-treated blastocysts compared with controls (Supplemental Fig. S1C), suggesting that embryos treated with JAK inhibitors recapitulate the loss of OCT4 expression in maternal/zygotic Stat3-null expanded blastocysts [see below].

Maternal and paternal Stat3 knockout E4.5 embryos exhibit loss of the EPI and PE lineages

Since STAT3 is present in oocytes [Fig. 1A], conventional zygotic knockout (Stat3*/*) embryos may be able to use this maternal protein during early embryogenesis. We therefore established maternal Stat3 knockout oocytes using a Zp3-Cre mating strategy [De Vries et al. 2004], rendering STAT3 absent in all eggs. However, when fertilized by wild-type males, the maternal deletion is rescued by expression from the paternal allele at the morula stage. Preimplantation embryos are normal, and pups derived from these matings are viable, indicating that maternal STAT3 is not essential for oogenesis and preimplantation development. In contrast, if maternal-null Stat3 oocytes (Stat3mat*/C0) are fertilized by sperm from heterozygous males (Stat3* or Stat3+), no viable pups or E5.5 post-implantation maternal/zygotic Stat3-null (Stat3mat*//*) embryos were found, although normal Stat3mat*//* embryos are present [Supplemental Fig. S2A]. However, Stat3mat*//* E3.5 blastocysts show normal morphology, express the ICM/TE markers OCT4, NANOG, and CDX2, respectively, and have ICM and TE cell numbers comparable with their Stat3mat*//* littermates [Fig. 3A]. These observations show that STAT3 is not required for the first lineage specification in the preimplantation embryo.

Interestingly, when we compared Stat3+/* implanting E4.5 embryos [Fig. 3B] with Stat3mat*//* embryos, we found severely morphologically challenged embryos, which expressed little to no OCT4 [Fig. 3C].
that these defects are primarily in the ICM lineages, we immunostained E4.5 embryos for the TE marker CDX2 and found almost all cells to be CDX2-positive [Fig. 3D], suggesting that maternal/zygotic deletion of Stat3 causes specific loss of OCT4-positive ICM but not CDX2-positive TE cells. Since there were few CDX2-negative cells in Stat3mat/C0/C0 E4.5 embryos, we performed immuno-

staining of NANOG and GATA6 to detect cells of the EPI and PE lineages, respectively. While ICMs of control E4.5 embryos have two normal and distinct populations of NANOG- and GATA4-positive cells (Fig. 3E), we found no NANOG-positive cells and few to no discernible GATA6-positive cells in ICMs of Stat3mat/-/- embryos [Fig. 3F, Supplemental Fig. S2B]. In addition, while there are no active Caspase3 [CASP3]-positive cells in control E4.5 embryos [Supplemental Fig. S2C], we found active CASP3-positive cells in Stat3mat/-/- mutants, indicating loss of ICM cells, at least in part, through apoptosis [Supplemental Fig. S2D]. Thus, using this genetic approach, we found STAT3 to be essential for ICM but not TE lineage maintenance in the implanting mouse embryo.

**Loss of maternal/zygotic Stat3 results in random lineage marker expression in ICM cells**

While maternal/zygotic null E3.5 embryos show appropriate lineage allocation and marker gene expression, E4.5 embryos are morphologically deranged, and ICM cells are lost. To further understand the nature of this rapid, progressive loss of lineage allocation and maintenance, we performed coimmunostaining of OCT4, NANOG, and GATA4 in expanded blastocysts at E4.0. In control blastocysts, OCT4, NANOG, and GATA4 were appropriately expressed in the ICM domain, with NANOG and GATA4 labeling two intermingled cell
Figure 3. Maternal and paternal Stat3 knockout embryos isolated at E4.5 exhibit loss of the EPI and PE lineages but not TE. [A] (Panel 1) Immunodetection of NANOG and CDX2 in Stat3+/+, Stat3mat−/−, and Stat3mat−/− early blastocysts isolated at E3.5. [Panel 2] Immunodetection of OCT4 in Stat3mat−/− and Stat3mat−/− E3.5 embryos. [Panel 3] Bar graphs showing the average cell number in ICM and TE lineages of Stat3mat−/− (n = 3) and Stat3mat−/− (n = 3) early blastocysts isolated at E3.5. ICM and TE cells are identified as NANOG+/CDX2− and CDX2+ cells, respectively. (B,C) Immunodetection of pSTAT3 and OCT4 in Stat3+/- and Stat3mat−/− E4.5 embryos. (D) Immunodetection of CDX2 in Stat3mat−/− E4.5 embryos. White arrow indicates the presence of CDX2-negative cells in the knockout embryos. (E,F) Immunodetection of NANOG and GATA6 in Stat3+/- and Stat3mat−/− E4.5 embryos. White arrows indicate the presence of remaining GATA6-positive cells in the knockout embryos. Bar, 20 μm.
populations in a “salt-and-pepper-like” fashion [Fig. 4A, top row]. In contrast, we found an abnormal phenotype in the ICM lineages of Stat3mat−/− embryos, showing either highly variable ICM marker gene expression or none at all [Fig. 4A; Supplemental Fig. S3A,B]. For instance, few ICM cells in embryo 1 are positive for OCT4 and NANOG (Fig. 4A, white arrows) or OCT4 and GATA4 (Fig. 4A, red arrows), while others are OCT4-, NANOG−, and GATA4-negative. This variability is pronounced, with extreme arrows), while others are OCT4-, NANOG-, and GATA4-negative. For instance, few ICM cells in embryo 1 are positive for OCT4 and NANOG (Fig. 4A; Supplemental Fig. S3A,B). For instance, few ICM cells in embryo 1 are positive for OCT4 and NANOG (Fig. 4A; Supplemental Fig. S3A,B). To confirm that these defects are primarily in the ICM, we immunostained Stat3mat−/− expanded blastocysts with CDX2 antibody and found the CDX2-positive TE lineage intact [Supplemental Fig. S3B]. To further characterize the defect in ICM lineage formation in Stat3mat−/− expanded blastocysts, we counted the number of the EPI-committed [NANOG+/CDX2−], PE-committed [GATA4+/CDX2−], and TE-committed [CDX2+] in mutant embryos. Consistent with the immunostaining results, we found highly variable cell numbers for the EPI and PE lineages but not the TE lineage (Fig. 4B). In addition, the average cell numbers in the EPI and PE lineages were reduced, but this was not the case in the TE lineage when Stat3mat−/− expanded blastocysts were compared with Stat3mat+/− embryos. However, because of the high variation among individual mutant embryos, this reduction is not statistically significant [Fig. 4B]. Consistent with the protein expression results, quantitative real-time PCR (qRT-PCR) also showed variable mRNA expression of ICM and TE marker genes in individual immunosurgically isolated ICMs of Stat3mat−/− E3.5 blastocysts [Fig. 4C]. Taken together, our results suggest that Stat3 is required for ICM lineage maintenance in the expanded blastocyst after initial lineage segregation, most likely by modulating ICM gene expression.

STAT3 directly regulates Oct4 and Nanog expression to maintain ESC pluripotency

Although Stat3mat−/− early blastocysts present a normal morphology, these embryos fail to form typical ICM-derived, OCT4-positive outgrowths [Fig. 5A,B], and ESCs cannot be derived. To examine a possible molecular link between Stat3 and OCT4, we used two pluripotent in vitro cell systems: mESCs carrying an Oct4-GFP reporter [Yamagata et al. 2010] and mouse iPSCs [Takahashi and Yamanaka 2006]. As in preimplantation embryos, the presence of tyrosine-phosphorylated (pY705) Stat3 correlated with GFP expression [i.e., Oct4] in ESCs [Fig. 5C] and iPSCs [Supplemental Fig. S4A]. When we reduced Stat3 transcript levels by using Stat3 shRNA knockdowns, Oct4-Gfp mRNA expression and OCT4 levels in ESCs were reduced [Fig. 5D–F]. Similarly, Oct4 and Nanog mRNA and protein levels were reduced in iPSCs after Stat3 knockdown [Supplemental Fig. S4C–F]. Furthermore, Stat3 knockdown in either ESCs or iPSCs resulted in failure to form distinct alkaline phosphatase-positive colonies [Fig. 5G; Supplemental Fig. S4B], although differentiated cells were observed [Fig. 5G, Supplemental Fig. S4B]. These results suggest that the STAT3–OCT4–NANOG link is important for maintenance of pluripotent, self-renewing cells.

To determine whether Stat3 regulates Oct4 directly, we treated iPSCs with the Stat3 inhibitor STA-21, which resulted in a significant reduction of Oct4 mRNA after 1 h but did not change the mRNA expression of Sox2 and Tbx3 (Stat3 non-target genes) [Fig. 5H; Supplemental Fig. S5]. Treatment with actinomycin D or actinomycin D plus STA-21 did not produce any additional effects. This indicates that Stat3 directly regulates Oct4 expression without affecting mRNA stability [Fig. 5H]. To examine whether there is direct binding of phosphorylated Stat3 to either the Oct4 or Nanog promoter, including the proximal enhancer or distal enhancer region [Fig. 5I, PE and DE, respectively, Supplemental Fig. 3S; Chen et al. 2008], we performed chromatin immunoprecipitation (ChIP) assays using the pY705-STAT3-specific antibody. With Oct4, the highest enrichment of Stat3 was found in a region [−3161 base pairs (bp)]−3010 bp] of the distal enhancer [region 2], and less was found in the promoter region [region 8 in Fig. 5I]. In addition, with Nanog ChIP, the highest enrichment of Stat3 was found in a region [−4320 bp]−4116 bp] of the distal enhancer [region 1], with less binding in the promoter region [region 7 in Supplemental Fig. S3G]. ChIP using a STAT1-specific antibody shows no binding of Stat3 at the Stat3-binding sites of either Oct4 or Nanog [Supplemental Fig. S6]. These results show that Stat3 directly and specifically interacts with the regulatory regions of Oct4 and Nanog. Finally, to assess the transcriptional activation of the Oct4 promoter/enhancer, we prepared an Oct4-luciferase reporter construct containing the 4.6-kb upstream promoter enhancer region of Oct4. For controls, we used luciferase reporter constructs M67 and M67Δ, containing Stat3- and mutated Stat3-binding sites, respectively. Stat3-overexpressing 293T cells [Supplemental Fig. S7] transfected with the Oct4 promoter/enhancer luciferase construct displayed high levels of luciferase activity after treatment of oncostatin M (OSM), a Stat3 activator [Fig. 5J]. The specificity of this activation was confirmed by transfection of Stat3-overexpressing 293T cells with the M67 construct, which produced high luciferase activity after OSM treatment, while those transfected with M67Δ did not. Taken together, the evidence from mutant embryos, shRNA knockdowns in ESCs/iPSCs, Stat3 inhibitors, ChIPs, and luciferase assays demonstrates that pStat3 directly and positively regulates Oct4 and Nanog expression in pluripotent cells in vivo and in vitro.

Discussion

In this study, we investigated the molecular mechanisms of Stat3 action and whether Stat3-targeted genes are involved in both the regulatory circuitry of preimplantation embryos and pluripotent stem cells. Strikingly, we found that Stat3 is highly expressed in mouse oocytes, becomes tyrosine-phosphorylated, and translocates to the nucleus in the four-cell stage embryo, where it is coex-
Figure 4. Maternal and paternal Stat3 knockout, expanded blastocysts exhibit a variable abnormal phenotype. (A) Immunodetection of OCT4, NANOG, and GATA4 in Stat3<sup>mat−/−</sup> and Stat3<sup>mat−/−</sup> expanded blastocysts. Embryo 1 has NANOG-positive (white arrow) and GATA4-positive (red arrow) cells in ICM. Embryo 2 has GATA4-positive cells (red arrow) but no OCT4- and NANOG-positive cells in the ICM. Embryo 3 has Nanog-positive cells in either the ICM or TE (white arrow) and no OCT4- and GATA4-positive cells. Bar, 20 μm. (B) The bar graphs show the average and individual cell number in ICM, PE, and TE lineages in the Stat3<sup>mat−/−</sup> (blue bars) and Stat3<sup>mat−/−</sup> (red bars) expanded blastocysts. The EPI, PE, and TE cells are identified as NANOG<sup>+</sup>/CDX2<sup>+</sup>, GATA4<sup>+</sup>/CDX2<sup>+</sup>, and CDX2<sup>+</sup> cells, respectively. (C) Real-time PCR shows mRNA expression of Nanog, Cdx2, Eomes, and Tead4 in individual ICMs that were isolated by immunosurgery from Stat3<sup>+/+</sup> (yellow bars), Stat3<sup>mat−/−</sup> (blue bars), and Stat3<sup>mat−/−</sup> (red bars) early blastocysts.
pressed with OCT4 in the four- to eight-cell stage. Using Lif-null embryos, we found that STAT3 tyrosine phosphorylation is dependent on LIF in the four-cell embryos, while IL-6, one of the cytokines produced by the blastocyst, acts in an autocrine fashion to ensure STAT3 phosphorylation. We further identified JAK1 as the IL-6 downstream kinase and responsible for STAT3 tyrosine phosphorylation in the blastocyst stage. Using genetically engineered mouse strains designed to eliminate Stat3 in the oocyte and embryo, we demonstrate that STAT3 is essential to maintain the ICM lineages but not TE in late blastocysts (Fig. 6). We further showed that STAT3 directly binds and regulates Oct4 (Pou5F1) and Nanog gene expression to maintain pluripotency of mouse ESCs and iPSCs.

The complexity of STAT3 signaling in preimplantation embryos

Studies on knockout mouse models indicate that the LIF signaling pathway is dispensable during preimplantation development. For instance, the Lif-null embryos develop normally into adulthood, but maternal Lif-null blastocysts cannot implant due to the absence of LIF production by the uterus (Stewart et al. 1992). Lifr mutant embryos die by E12 with placental, cardiac, hematopoietic, and neuronal defects (Yoshida et al. 1996; Nakashima et al. 1999). Jak1-null mice die within the first 24 h after birth.
and jak2-null mice die around E12.5 (Neubauer et al. 1998; Rodig et al. 1998). However, no evidence for early embryo loss has been reported from any of these mutants. In contrast, mESc derivation and maintenance is dependent on LIF [Smith et al. 1988, Williams et al. 1988], LIFR [Ware et al. 1995], and gp130 [Nichols et al. 2001], suggesting that alternative pathways might be involved in maintaining pluripotency in vivo and in vitro. Our results, showing that phosphorylated STAT3 is present in E3.5 despite the absence of LIF, support this suggestion. We further provide a possible explanation for the differential phenotypes of maternal/zygotic Lif- and Stat3-null embryos, showing IL-6 to be involved in STAT3 tyrosine phosphorylation in the blastocyst. This is consistent with the fact that the combination of IL-6 and soluble IL-6 receptor can be used to derive and maintain ESCs without involvement of LIFR [Nichols et al. 1994, Yoshida et al. 1994]. Il6-deficient mice can develop normally [Kopf et al. 1994], suggesting that either LIF or additional, redundant cytokines such as IL-6 are required for STAT3 phosphorylation in vivo. Taken together, our results suggest that multiple cytokines are used for STAT3 tyrosine phosphorylation to ensure proper ICM lineage formation in the blastocyst in vivo. In this light, it would be interesting to examine STAT3 phosphorylation and the developmental capacity of embryos deficient in both LIF and IL-6.

Strikingly, we also identified JAK1 as a possible IL-6 and LIF downstream kinase responsible for STAT3 phosphorylation in the blastocyst. Embryos treated with JAK inhibitors in vitro show loss of STAT3 tyrosine phosphorylation and reduced OCT4 expression in the ICM, a phenocopy of the maternal/zygotic Stat3-null embryos in vivo. Zygotic jak1-null mice develop normally and die within the first 24 h after birth [Rodig et al. 1998], suggesting that maternal JAK1 may play an important role in the development of preimplantation embryos. Overall, we established that an independent, redundant pathway ensuring STAT3 activation is integral to preimplantation development.

Role of maternal STAT3

Absence of STAT3 in the mouse egg appears inconsequential for development, as maternal-null embryos give rise to viable pups. However, maternal/zygotic null embryos display earlier developmental arrest than that reported for zygotic mutants [Takeda et al. 1997], suggesting a maternal gene function revealed only in the absence of zygotic Stat3 expression. A parsimonious explanation for these observations is that transcription from the paternally inherited allele, which occurs at the eight-cell stage in maternal-null embryos, produces sufficient STAT3 for the maintenance of pluripotent cells in the EPI. As a corollary, the presence of maternal STAT3 in zygotic null embryos may be sufficient to support ICM cell maintenance past implantation, yet this protein pool is depleted eventually, causing developmental arrest at E6.5. Finally, in maternal/zygotic null embryos, the complete absence of STAT3 results in early loss of pluripotent cell lineage maintenance, which is somewhat reminiscent of mutations of two other genes essential to the maintenance of pluripotency: Oct4 and Nanog [Nichols et al. 1998; Mitsui et al. 2003; Silva et al. 2009, Messerschmidt and Kemler 2010].

Role of STAT3 in ICM lineages

Our results, showing that maternal/zygotic Stat3 knockout E4.5 embryos exhibit loss of EPI and PE lineages, are consistent with those previously observed from Oct4- and Nanog-null embryos. Oct4 is known to be important in establishing the distinct identity of the ICM in early embryogenesis. Moreover, homozygous Oct4-null em-
bryos, when cultured in vitro, yield outgrowths of trophoblast giant cells but no recognizable ESCs [Nichols et al. 1998]. Formation of the EPI is also dependent on the expression of Nanog, because Nanog-null embryos fail to establish an EPI identity due to degeneration of the ICM [Mitsui et al. 2003; Silva et al. 2009]. Nanog is also required for PE formation through a non-cell-autonomous mechanism [Messerschmidt and Kemler 2010]. Thus, the Stat3mat−/−embryos present phenotypes similar to those from Oct4- and Nanog-null mice, supporting the requirement of STAT3 for development and maintenance of the ICM lineages.

More detailed examination of Stat3-null embryos shows an intact, CDX2-expressing TE epithelium capable of inducing a decidual reaction, an indication that both maternal and zygotic STAT3 is dispensable for TE lineage formation and maintenance (Fig. 6). This finding is consistent with previous studies showing that CDX2 promotes, but OCT4 represses, TE differentiation [Niwa et al. 2005]. Stat3mat−/−embryos appear morphologically normal and contain Nanog-expressing ICM cells. However, 24 h later in the E4.5 embryo, there are very few CDX2-negative cells, none of which expressed pluripotency markers. We speculate that in the absence of STAT3, ICM cells in the expanding blastocysts begin to differentiate inappropriately and fail to proliferate, an effect possibly even potentiated by the absence of STAT3, which has been shown to have anti-apoptotic activity [Hirano et al. 2000].

Molecular interaction of STAT3 with OCT4 and NANOG

It has been reported that STAT3 maintains ESC pluripotency by integrating with the core transcription circuitry OCT4/NANOG through directly regulating Klf4 [Hall et al. 2009; Niwa et al. 2009]. It has also been reported that Stat3−/−ESCs are morphologically indistinguishable from wild-type ESCs, expressing both Oct4 and Nanog in 3i medium, whereas when they are transferred to medium containing 4i-STAT3 inhibitors leads to substantial reduction of ESCs and iPSCs. Short-term treatment of cells with a STAT3 inhibitor is sufficient to reduce the number and percentage of Stat3mat−/−embryos present phenotypes similar to those from Oct4- and Nanog-null mice, supporting the requirement of STAT3 for development and maintenance of the ICM lineages.

We now provide evidence that STAT3 directly regulates Oct4 and Nanog transcription to maintain pluripotency of ESCs and iPSCs. Short-term treatment of cells with a STAT3 inhibitor leads to substantial reduction of Oct4 mRNA, suggesting that Oct4 down-regulation is a direct effect of STAT3 inhibition rather than a consequence of cell differentiation. Furthermore, consistent with the ChIP sequencing [ChIP-seq] data mapping STAT3-binding sites in ESCs [Chen et al. 2008], we now report that STAT3 directly binds to the distal enhancer of Oct4 and Nanog.

Delineation of the role of STAT3 in ICM lineage maintenance in vivo coupled with the demonstration that STAT3 directly interacts with Oct4 and Nanog to maintain pluripotent cells in vitro reveals the essential nature of this regulatory circuit at the outset of mammalian development.

Materials and methods

Cell culture and transfection

iPSCs were generated as described previously [Feng et al. 2009]. Stat3 shRNA was cloned into the pSUPER-puro vector with the target sequence AGGGAGGGCTTAAAGAGACCACAAattcgaagagTGTGAAGTGGAAGGGTATTTTA. Oct4-GFP ESCs [kindly provided by Dr. Kazuo Yamagata] or iPSCs seeded at a density of 4 × 10⁴ cells per well in six-well plates were transfected with 4 μg of pSUPER.puro vector, shLuciferase-pSUPER-puro, or shSTAT3-pSUPER.puro. Puromycin selection [1.5 μg/mL] was applied for 3–5 d before analysis. Alkaline phosphatase staining was detected using the Alkaline Phosphatase Detection kit [Millipore] according to the manufacturer’s recommendations. The specific STAT3 inhibitor STA-21 was used as previously described [Song et al. 2005].

RNA extraction, reverse transcription, and qRT-PCR

RNA was extracted 3 d after transfection, selected in puromycin using TRizol reagent [Invitrogen], and treated with DNase [Amicon]. cDNA synthesis was performed with 1 μg of total RNA and the SuperScript III kit [Invitrogen] according to the manufacturer’s instructions. Endogenous mRNA levels were measured by real-time PCR analysis based on SYBR Green detection with an ABI real-time PCR machine. The sequence of real-time PCR primers is provided in Supplement Table 1. Samples were assayed in duplicate and normalized to endogenous Gapdh or Actin.

Protein extraction and Western blotting

Protein extracts were obtained using cell lysis buffer (20 mM HEPES, 400 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM DTT) with protease inhibitor [Roche] and phosphatase inhibitor. Proteins were separated by SDS-PAGE, transferred to PVDF membrane [Bio-Rad], and probed with specific primary antibody and an appropriate HRP-conjugated secondary antibody. Signals were detected using SuperSignal West Pico chemiluminescent substrate [Pierce]. The following antibodies were used for Western blotting: α-STAT3 [1:1000], Santa Cruz Biotechnology, C-20, α-STAT3 (1:1000), Cell Signal, α-pY705-STAT3 (1:500), Santa Cruz Biotechnology, B-7, α-pY705-STAT3 (1:1000), Cell Signal, α-OCT4 (1:1000), Abcam, ab19857, α-OCT4 (1:500), Santa Cruz Biotechnology, N-19, α-NANOG [1:1000], Abcam, ab21624, and α-Tubulin [1:2500], Santa CruzBiotechnology, sc-5274.

Immunocytochemistry

Cells were seeded at 2.5 × 10⁵ cells per well in four-well plates on gelatin-coated coverslips and cultured for 3 d. The cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.25% Triton X-100 for 15 min. Blocking was performed with PBS containing 0.05% Tween and 1% BSA for 30 min. Cells were reacted with primary antibody rabbit α-OCT4 and Alexa Fluor 488 (1:1000) and rabbit IgG Alexa Fluor 488 (1:1000) and rabbit IgG FITC (1:200) for 1 h at room temperature. Images were captured with a Zeiss AxioVision 4.7.

ChIP assays

ChIP assays with iPSCs were carried out as described previously [Loh et al. 2006]. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and formaldehyde was neutralized by addition of 0.2 M glycine. Chromatin
extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using 5 μg of α-pY705-STAT3 [Cell Signaling]. For all ChIP experiments, qRT-PCR analyses were performed using Stratagene and SYBR Green Master Mix.

**Mice and genotyping**

Stat3<sup>floxed/fox</sup> mice were crossed with Zp3-Cre transgenic mice carrying cre recombinase under the control of the oocyte-specific Zp3 promoter [De Vries et al. 2004] to generate Zp3-Cre, Stat3<sup>floxed/fox</sup>-/+ male mice. These mice were then backcrossed to Stat3<sup>fox/fox</sup> female mice, and Zp3-Cre,Stat3<sup>fox/fox</sup>-/- females were selected. Oocytes from these females were Stat3-deleted. To generate maternal- and zygotic-deleted embryos, Zp3-Cre,Stat3<sup>fox/fox</sup>-/- female mice were mated with Stat3<sup>12/12</sup> males; one-half of their progeny were maternal-zygotic null. Genotyping was done by PCR using DNA extracted from tails tips of 21-d-old mice. The primer pairs used to detect the presence of the Zp3-Cre transgene and the Stat3-floxed alleles were as described [Welte et al. 2003; De Vries et al. 2004]. Genotyping of embryos followed a previous method [Nichols et al. 1998].

**Immunostaining of oocytes and preimplantation embryos**

Embryos were fixed in 2% paraformaldehyde in PBS for 15 min at room temperature or overnight at 4°C. After 48 h, they were mated with male mice. Oocytes and zygotes were harvested from oviducts 17–22 h after hCG injection. Cumulus cells were removed by incubation with 0.3 mg/mL hyaluronidase (Sigma, H4272) in M2 medium. The embryos were recovered in M2 medium, washed in a few drops of KSOM (Millipore), and cultured in microdrops under mineral oil in 5% CO₂ at 37°C. The embryos were collected at different stages for immunostaining. Alternatively, embryos from the two- to eight-cell stage were flushed from oviducts at 1.5 and 2.5 d post-coitum [dpc], and embryos from blastocyst stage were flushed from the uterus at 3.5 dpc.

Zygotes were cultured with neutralizing antibodies against IFN-γ and IL-6 cytokine at 2 ng/mL concentration [ebiScience] and fixed as expanded blastocysts for immunostaining. Zygotes were cultured with 5 μM JAKi (Calbiochem), 5 μM AG490 (Tortis bioscience), and 5 μM Ruxolitinib [InvivoGen] in vitro and fixed as expanded blastocysts for immunostaining.

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