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Depletion of Suds3 reveals an essential role in early lineage specification

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ABSTRACT

Preimplantation development culminates with the emergence of three distinct populations: the inner cell mass, primitive endoderm and trophectoderm. Here, we define the mechanisms underlying the requirement of Suds3 in pre/peri-implantation development. Suds3 knockdown blastocysts exhibit a failure of both trophectoderm proliferation as well as a conspicuous lack of primitive endoderm. Expression of essential lineage factors Nanog, Sox2, Cdx2, Eomes, Elf5 and Sox17 are severely reduced in the absence of Suds3. Importantly, we document deficient FGF4/ERK signaling and show that exogenous FGF4 rescues primitive endoderm formation and trophectoderm proliferation in Suds3 knockdown blastocysts. We also show that Hdac1 knockdown reduces Sox2/FGF4/ERK signaling in blastocysts. Collectively, these data define a role for Suds3 in activation of FGF4/ERK signaling and determine an essential molecular role of Suds3/Sin3/HDAC complexes in lineage specification in vivo.

Introduction

The first two lineage decisions occur during mammalian preimplantation development. Upon the formation of blastocoel cavity at embryonic day 3.5 (E3.5) in the mouse, the outer epithelial cell layer of the embryo, termed the trophectoderm (TE), is already specified and differentiating. The TE subsequently gives rise only to extra-embryonic lineages (Rossant and Tam, 2009). The inner cells of the blastocyst, the inner cell mass (ICM), will give rise to all cells of the developing embryo as well as the mesodermal component of extra-embryonic tissues. At approximately E4.5 of embryonic development, the primitive endoderm (PE) becomes specified from the ICM and resides as a single layer of cells adjacent to the blastocoel cavity, in direct contact with the epiblast (EPI) (Artus et al., 2011; Rossant and Tam, 2009). Although several mechanisms required for these early lineage decisions have been defined, comprehensive molecular understanding of specification, commitment and differentiation of TE, PE, and/EPI remains incomplete.

During compaction at the 8 to 16-cell stage, a cascade of molecular events involving transcription factors, signaling pathways and epigenetic events leads to separation of ICM and TE (Cockburn and Rossant, 2010; Takaoka and Hamada, 2012). At the late 8-cell stage, blastomeres become polarized, concomitant with compaction. With each subsequent division, cells on the outside of the morula become polarized while the inner cells remain apolar. Recently it has been established that the transcription factor Tead4 is activated in the polarized outer cells and guide TE lineage specification (Home et al., 2012; Nishioka et al., 2008; Yagi et al., 2007). Furthermore, differential Hippo signaling between inside and outside cells has been shown to regulate *Tead4* transcription (Nishioka et al., 2009). Along with Hippo/Tead4 mediated TE specification, reciprocal inhibition between two other transcription factors, Cdx2 and Oct4, is thought to maintain correct ICM/TE cell fate decisions (Niwa et al., 2005). Many experiments have also identified transcription factors such as Eomes, Gata3 and Elf5 that are essential for TE lineage commitment and differentiation but are not required for the initial TE specification (Donnison et al., 2005; Home et al., 2009; Kuckenberg et al., 2010; Russ et al., 2000; Zhou et al., 2005)

Oct4, Nanog and Sox2 are critical transcription factors that are essential for maintaining the pluripotency of ICM and embryonic stem cells (ESCs). Deletion of each of these genes results in failure of ESC derivation and early embryonic lethality (Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998). Oct4 is ubiquitously present in all blastomeres during cleavage stages, and becomes restricted to ICM cells approximately 20 h after Cdx2 is restricted to the future TE (Dietrich and Hiiragi, 2007). Nanog is detectable beginning at the 8-cell stage when it is expressed stochastically, for which the underlying

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mechanism or biological significance has not yet been determined (Dietrich and Hiiragi, 2007). Surprisingly, Nanog is monoallelically expressed in early preimplantation embryos and becomes biallelically expressed in the late blastocyst (Miyanari and Torres-Padilla, 2012). This biallelic expression is required for proper formation of functional EPI and (PE). Single-cell transcriptome analyses have shown that *Sox2* is the earliest transcript that is upregulated in future ICM cells, suggesting a role in the initial TE/ICM separation (Guo et al., 2010). Additionally, knockdown (KD) of Sox2 results in failure of TE lineage specification (Keramari et al., 2010). Thus, both spatial and temporal regulation of pluripotency factors is crucial for successful preimplantation development.

The molecular control of PE lineage specification is less well understood. Gata6 is the earliest known marker for the PE lineage and is required for PE formation (Cai et al., 2008; Chazaud et al., 2006; Morris et al., 2010; Plusa et al., 2008). Additionally, Sox17, Gata4 and Pdgfα are expressed specifically in PE cells (Artus et al., 2011; Niakan et al., 2010; Plusa et al., 2008). PE formation is dependent on proper *Nanog* expression, through a non-cell-autonomous mechanism, likely mediated by FGF/Tyrosine Receptor Kinase signaling from the ICM to the future PE (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010). Supporting this possibility, FGF4 is absent in Nanog-depleted blastocysts (which fail to specify PE), and treatment of blastocysts with tyrosine kinase inhibitors, which presumably block extracellular signal regulated-kinase (ERK) signaling, suppresses PE formation (Frankenberg et al., 2011).

The Sin3a/HDAC complex is largely thought to act as a transcriptional repressor through epigenetic modification at target loci (Hassig et al., 1997b). The Sin3a/HDAC complex contains a core of eight proteins, conserved from yeast to man. Targeted knockout (KO) of *Hdac1*, *Sin3a* or *Suds3* (each a core complex component) results in peri-implantation lethality, indicating a requirement for Sin3A/HDAC function during early embryonic development (Cowley et al., 2005; David et al., 2003; Lagger et al., 2002; Montgomery et al., 2007). Although this complex has mainly been defined as a repressor of transcription (Hassig et al., 1997a), ChIP-chip analysis reveals that HDAC1 occupies mainly active genes in both ESC and trophoblast stem cells (TSC), including important regulators of ESC and TSC self-renewal (*Oct4*, *Nanog*, *Cdx2, Elf5* and *Eomes)* (Kidder and Palmer, 2012). Recently, it has also been shown that *Sin3a*-depleted embryos exhibit apoptosis specifically in the epiblast at the peri-implantation stage (McDonel et al., 2012). Collectively, these data suggests that the Sin3a/HDAC complex may contribute to the proper specification of early lineages in preimplantation embryos through both silencing and activation of critical genes.

Suds3 (also called Sds3) is an integral component of Sin3a/HDAC complex and is required for HDAC1 activity *in vitro* (Alland et al., 2002; Lechner et al., 2000). *Suds3* null mouse embryos form blastocysts, but die soon after (David et al., 2003). Through an ongoing preimplantation RNAi screen, we identified *Suds3* as being involved in specification or function of TE, PE and EPI lineages.

Here we show that Suds3 is present throughout preimplantation development and is required for specification or function of each of the three early lineages in preimplantation embryos. *Suds3*-deficient embryos form TE but fail to implant, indicating defective TE function. Expression of several critical TE factors is abrogated and Suds3-deficient cells fail to contribute to TE in chimaeras, indicating molecular and functional defects in TE cells that are generated in the absence of Suds3. Furthermore, the molecular signature of ICM, EPI and PE are disrupted, and morphologically evident PE is not present in Suds3 deficient embryos. We also show that FGF4 is not detectable in *Sud3*-deficient ICM, possibly due to loss of active Sox2 and that exogenous FGF4 exposure successfully rescues PE formation in *Suds3*-deficient embryos. The results presented herein suggest that the primary defects observed in the absence of Suds3/Sin3a/HDAC function are likely due to loss of FGF4-ERK pathway signaling.

Materials and methods

Embryo production and culture

8 to10-week-old B6D2F1 female mice (Jax #100006) were superovulated with 10 IU PMSG (Sigma) followed by 10 IU hCG (Sigma) 48 h later. Zygotes were collected at 21 h post-hCG treatment from B6D2F1 female mice mated to B6D2F1 males. For parthenotes, cumulus–oocyte complexes were collected from B6D2F1 mice 15 h post-hCG treatment and cumulus cells were removed with hyaluronidase (ICN Pharmaceuticals, Costa Mesa, CA, USA). Zygotes and oocytes were cultured in KSOM medium (EmbryoMax®, Millipore) and incubated at 37 °C in 5% CO_2/5% O_2 balanced in N_2. dsRNA injected oocytes were activated by incubation in 0.01 mM cytochalasin D, 10 mM SrCl2 in calcium-free Chatot–Ziomek–Bavister medium (CZB) for 5 h. Culture of parthenotes and zygotes was performed in KSOM at 37 °C/5% CO_2/5% O_2 balanced in N_2 in a mixed gas incubator. In terms of rescue experiment with FGF4, 200 ng/ml FGF4 (R&D system) and 1 μg/ml Heparin (Sigma) were supplemented into KSOM from day 3 post injection. Use of vertebrate animals for embryo production was approved by the University of Massachusetts IACUC.

Outgrowth assay

Blastocysts were collected, removed of zona pellucida and cultured individually in DMEM (Millipore) containing 10% FBS (Atlanta Biologicals) on 0.1% Gelatin (Sigma). For ICM culture, blastocysts were treated with anti-mouse antiserum (Sigma), washed and exposed to guinea pig complement (Sigma) as described (Solter and Knowles, 1975). The isolated ICM was then cultured individually in gelatinized wells. Both intact blastocysts and ICMs were incubated at 37 °C/5% CO_2 and were observed daily.

Constructs

Full-length *Suds3* was amplified from with primers: Forward: TAAATAATCGATGCCACCATGAGCGCCGCGGGGCTGCTG and Reverse: TAATACGACTCACTATAGGGCTCCCACTGAGCTGATCACA. For Suds3 localization experiment, a 5′ Flag epitope was added to the *Suds3* cDNA by PCR with forward primer encoding the eight-amino-acid Flag epitope with a linker polypeptide (GGSGG): TAAATAATCGATGCCACCATGGATTACAAGGATGACGACGATAAGGGTGGCAGCGGTGGCATGAGCGCCGCGGGGCTGCTG. PCR products were cloned into pCS2 vector that contains SP6 promoter for mRNA synthesis. After linearization with Not I, *Suds3* was *in vitro* transcribed, capped and poly (A) tailed according to instructions (AM1340, Ambion). mRNA was recovered and cleaned by phenol:chloroform extraction and 200 ng/μl was used for microinjection to zygotes and 100 ng/μl for 2-cell blastomere. All constructs were verified by commercial sequencing prior to use (GENEWIZ).

Double-stranded RNA (dsRNA) preparation

DNA templates for T7-RNA polymerase mediated dsRNA production were amplified from genomic DNA or preimplantation embryo cDNA using primers that contained the T7 binding sequences followed by gene specific sequences as follows: dsGfp Forward: TAATACGACTCACTATAGGGCACATGAAGCAGCACGACTT and Reverse: TAATACGACTCACTATAGGGTGCTCAGGTAGTGGTTGTCG, dsSuds3 Forward: TAATACGACTCACTATAGGGAGACGGAACAGGTGGAGAGGAAT