MED20 is essential for early embryogenesis and regulates NANOG expression

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Abstract

Mediator is an evolutionarily conserved multi-subunit complex, bridging transcriptional activators and repressors to the general RNA polymerase II (Pol II) initiation machinery. Though the Mediator complex is crucial for the transcription of almost all Pol II promoters in eukaryotic organisms, the phenotypes of individual Mediator subunit mutants are each distinct. Here, we report for the first time, the essential role of subunit MED20 in early mammalian embryo development. Although Med20 mutant mouse embryos exhibit normal morphology at E3.5 blastocyst stage, they cannot be recovered at early post-gastrulation stages. Outgrowth assays show that mutant blastocysts cannot hatch from the zona pellucida, indicating impaired blastocyst function. Assessments of cell death and cell lineage specification reveal that apoptosis, inner cell mass, trophectoderm and primitive endoderm markers are normal in mutant blastocysts. However, the epiblast marker NANOG is ectopically expressed in the trophectoderm of Med20 mutants, indicative of defects in trophoblast specification. These results suggest that MED20 specifically, and the Mediator complex in general, are essential for the earliest steps of mammalian development and cell lineage specification.


Introduction

Mammalian preimplantation development refers to the period from fertilization to implantation, during which the fertilized oocyte progresses through a number of cleavage divisions and three major transcriptional and morphogenetic events that lead to a blastocyst capable of implantation (Cockburn & Rossant 2010). The first well-defined event is the maternal-to-zygotic transition, which includes degradation of maternal mRNAs and replacement with zygotic transcripts. This dramatic reprogramming of gene expression is indispensable for establishment of totipotency and embryo development (Latham et al. 1991). The second major event is embryo compaction and polarization, and inheritance of cell polarity in daughter cells during subsequent divisions has been demonstrated as critical for solidification of cell-fate acquisition (Leung et al. 2016). The third critical event is blastomere outer/inner configuration and the first cell-fate allocation when the outer polar cells differentiate exclusively into the trophectoderm (TE), whereas the apolar cells located inside the morula give rise to the inner cell mass (ICM) (Arnold & Robertson 2009). Well-characterized gene expression patterns occur within these two distinct lineages. For example, the transcription factor (TF) OCT4 (also known as POU5F1) is enriched in ICM, while the TF CDX2 becomes highly expressed in TE (Nichols et al. 1998, Niwa et al. 2005). After the first cell-fate determination, when blastocysts reach more than 32 cells, the second cell-fate determination occurs, to segregate the ICM into epiblast (EPI) and primitive endoderm (PE). Well-defined profiles of gene expression demarcate these two populations. For example, NANOG only localizes to EPI cells, while TF SOX17 is expressed exclusively in PE lineage (Frum & Ralston 2015, Molotkov & Soriano 2018, Morgani et al. 2018). Finally, these three lineages EPI, PE and TE will contribute to the embryo, parietal yolk sac and placenta, respectively.

Although distinct localizations of TFs within ICM/TE and EPI/PE/TE lineages have been well illustrated, their upstream regulatory networks are not fully delineated (Lokken & Ralston 2016, Cui & Mager 2018). Among multiple signaling pathways involved in early cell-fate decisions, Hippo signaling was demonstrated to play a critical role through the analysis of mutant mouse embryos lacking TF TEA domain family member 4
(TEAD4) (Yagi et al. 2007). Other experiments also showed the indispensable role of Hippo signaling in the regulation of TE-specific TF CDX2 (Strumpf et al. 2005) and GATA3 (Ralston et al. 2010). Interestingly, recent studies indicate that Hippo signaling promotes ICM fate acquisition as well, though the regulatory mechanisms are still unknown (Wicklow et al. 2014). In addition to the Hippo pathway, the function of Notch signaling in TE lineage specification was also recently uncovered (Rayon et al. 2014). By using double knockouts for Tead4 and the Notch effector Rbpj, Rayon et al. demonstrated Hippo and Notch signals converge on Cdx2 to cooperatively promote TE lineage specification. Additional TFs continue to emerge as crucial regulators of early cell-fate decisions. For example, TFAP2C can directly regulate Cdx2 expression through an enhancer in intron 1 during early cleavage stages to promote TE lineage specification (Cao et al. 2015). Although many other mechanisms, such as epigenetic regulation (Paul & Knott 2014, Marcho et al. 2015) and newly discovered genes (Cui et al. 2016a), contribute to and dictate these unique cellular identities, the full cadre of cellular mechanisms that controls these events remains unresolved.

Lineage specification and cell differentiation are complicated and highly regulated processes relying on the differential expression of various genes within distinct cell populations. For all eukaryote organisms and nearly all RNA polymerase II (Pol II) promoters, a crucial pathway to finely tune these regulatory signals and appropriate transcripomte activation is through the following: enhancer – activator – Mediator – Pol II – promoter (Kornberg 2005). As a core molecular signaling mechanism, the Mediator complex was originally identified in budding yeast (Kelleher et al. 1990), with subsequent identification of many protein subunits (25 in yeast and 30 in human) (Tsai et al. 2014). Functioning as the bridge, Mediator can convey regulatory signaling information to the basal RNA Pol II transcription machinery, eliciting both positive and negative regulation of gene transcription (Beyer et al. 2007). While the core Mediator complex seems to be universally required in all genes, subunit phenotypes can be distinct from one another, suggesting functional redundancy and specificity (Risley et al. 2010). Furthermore, although Mediator is evolutionarily conserved at the protein level, mutants in the same Mediator subunit can display dissimilar phenotypes in different organisms (Hentges 2011). Additionally, studies have confirmed that Mediator can interact with diverse TFs and co-factors to ensure that specific genes are expressed with appropriate temporal and cell type specificity (Yin & Wang 2014).

In the present study, we explored the role of MED20, one of the most conserved proteins in the Mediator complex, during mouse embryo development, using both knockout (KO) and knockdown (KD) strategies. Our data show that MED20 is essential for hatching of the blastocyst from the zona pellucida. Moreover, outgrowth, apoptosis and lineage specification assays revealed that mutant blastocysts exhibit severe ectopic expression of NANOG, an epithelial marker, in the outer putative trophectoderm cells, demonstrating a failure to appropriately implant and establish the trophectoderm lineage.

Material and methods

Generation of Med20 mutants

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst (2015-0011, 2018-0003). Med20 KO allele (C57BL/6NJ-Med20<em1J>/J, Stock #: 027272) was generated on C57BL/6NJ background in the Jackson Laboratory (JAX) using CRISPR-Cas9 system, with two guide RNAs targeting AGGAACCTTTGGGACTGGT and GCTTAGATATTTCAGTAA. The founder with 348bp deletion beginning in intron 1 at GGGGACTGATGGGTGGGGAT and ending after GCTTAGAGTATTTACGTTA at position 47,613,174bp in intron 2 (Fig. 1A), which causes a short truncated protein with only 11 amino acids, was selected to establish the colony. To expand the colony, heterozygous (Het) mice from JAX were backcrossed again with C57BL/6NJ WT for the following heterozygous intercrosses to generate Med20 mutants (Mut). Genotyping primers are used as follows (Fig. 1A): common forward primer for both WT allele and Mut allele: TGATGCCCTTTGATCCACA; WT reverse: CACCTAATCCACAGGTT; Mut reverse: CCCTTGACAGAAAAGGACGC.

Embryo recovery, culture and genotyping

Med20 heterozygous females aged 8–14 weeks were caged with Med20 heterozygous males for natural matings, and the presence of a vaginal plug was defined as embryonic day 0.5 (E0.5). Embryos were then collected from uteri of heterozygous females by dissection or flushing to collect E7.5 or E3.5 embryos, respectively. Embryos were imaged as a group and carefully collected into individual tubes in the order presented, and then lysed for PCR genotyping using the primers mentioned above.

To prepare zygotes for siRNA microinjection or in vitro culture, B6D2F1 female mice aged 8 to 10 weeks were induced to superovulate with 7.51U pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich), followed 48h later by 7.51U human chorionic gonadotropin (hCG, Sigma-Aldrich). Females were mated with B6D2F1 males and killed at 20h post hCG injection. Oviductal ampullae were dissected to release zygotes, and cumulus cells were removed by pipetting in M2 medium containing hyaluronidase (EMD Millipore). Zygotes were then washed in M2 medium (EMD Millipore) and cultured in KSOM medium (EMD Millipore) at 37°C in a humidified atmosphere of 5% CO2/5% O2 balanced in N2.
Med20 represses Nanog expression in TE

Outgrowth assay

Blastocysts were collected and transferred gently into culture plates (Nunclon Delta, Thermo Fisher) and cultured in DMEM (Lonza, Allendale, NJ, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 1X GlutaMAX (Thermo Fisher). Outgrowth assay was conducted at 37°C in a humidified atmosphere of 5% CO₂ for 3 days. Outgrowths were then imaged and genotyped.

Immunofluorescence and confocal microscopy

Immunofluorescence was performed as previously described (Cui et al. 2016a,b). In vivo derived blastocysts were flushed at E3.5, and then cultured overnight before fixation and immunofluorescence (to ensure embryos had undergone EPI/PE/TE specification). In vitro blastocysts were harvested at 4 days post microinjection. Primary antibodies used in this study include: mouse anti-CDX2 (BioGenex, MU392A-UC); rabbit anti-NANOG (abcam, ab80892); rabbit anti-TRP53 (Cell Signaling Technology, #9284); goat anti-SoX17 (R&D Systems, AF1924); goat anti-OCT4 (abcam, ab27985). After secondary antibodies (Alexa Fluor, Life Technologies) and DAPI (Sigma) staining, embryos were transferred to chambered slides (BD Falcon) with one embryo per well for imaging. Embryos were imaged using Nikon A1 Spectral Detector Confocal with FLIM Module. Z-stacks (20X objective, 8µm sections) were collected and maximum projection was applied. Blastocysts collected from heterozygous intercrosses were imaged prior to knowledge of their genotypes. After imaging, embryos were individually recovered and lysed for genotyping.

Microinjection

Microinjection was performed as previously described (Cui et al. 2016a,b). A volume of 5–10 µL of 50 µM Scrambled Control (5'-CAGGGTATCGACGATTACAAA, Qiagen) or Med20 siRNA (siRNA1 target: 5’-CGCAAGCTTAAATCTGAAA, siRNA2 target: 5’-TACAGACCATTTAAACAAA, siRNA3 target: 5’-CTCGGGAAAGCTTGAATCTA, Qiagen, Fig. 1A) was microinjected into the cytoplasm of zygotes.

RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA extraction was performed with a Roche High Pure RNA Isolation Kit (#11828665001), cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories, 170-8891). Specific intron-spanning primers were used for RT-PCR (Actb: 5’-GGCCCAGAGCAAGAGAGGTATCC and 5’-ACGCACGATTTCCCTCAGC; Med20: 5’-AGTGGACTCCTCACCAAGA and 5’-CCTTGGCACTCTGGAAGAAG, Fig. 1A).

Simultaneous extraction of RNA and DNA from single blastocyst

Blastocysts collected from heterozygous intercrosses were lysed individually (10 µL lysis buffer per embryo) following the manual of Roche Kit (#11828665001), with DNase treatment step skipped. A volume of 13 µL Elution Buffer was applied and the eluted mixture of RNA and DNA was used as follows: 6 µL mixture for genotyping PCR with Platinum SuperFi Green PCR Master Mix (Thermo Fisher) and genotyping primers as listed above; the other 6 µL mixture for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, 170-8891). Regarding the resultant 8 µL cDNA, 2 µL was used for Actb RT-PCR and 6 µL was used for Med20 RT-PCR, with Platinum SuperFi PCR Mix (Thermo Fisher) and RT-PCR primers listed above.

Statistical analysis

All experiments were repeated at least three times. Percentage data were analyzed by ANOVA, and a value of P<0.05 was considered statistically significant. Data are expressed as mean±standard error of the mean.

Results

Med20 mutants cannot be recovered in vivo after E3.5

A CRISPR-mediated Med20 knockout allele was generated for the Knockout Mouse Phenotyping Program (KOMP2) at The Jackson Laboratory. During

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the initial phenotyping pipeline of The International Mouse Phenotyping Consortium (IMPC, http://www.mousephenotype.org), no homozygous Med20 mutants were born, nor found at E15.5 or E9.5. Considering this, we first dissected embryos at E7.5 early post-gastrulation stage. Twenty-nine embryos were recovered from five heterozygous intercrosses and were genotyped. Genotyping results showed 20 Het and 9 WT embryos all with normal gastrulation morphology (Fig. 1B). No Med20 mutant embryos were found at E7.5, nor were excessive empty decidua (n = 6), suggesting Med20 mutants failed to implant. Therefore, we switched to collection and genotyping at the E3.5 blastocyst stage, where we recovered mutant Med20 embryos at the expected Mendelian ratios. A total of 24 WT, 69 Het and 18 Mut embryos were found in 15 litters. Compared with WT and Het littermates, Mut blastocysts could not be identified by morphology alone (Fig. 1C). Combined, the normal blastocyst morphology plus the complete absence of mutants during gastrulation suggested implantation failure.

We next performed in vitro outgrowth assays, a technique used as a model for implantation (Armant 2005, Qin et al. 2005, Cui et al. 2016a). Another 42 blastocysts collected from six females were subjected to a 3-day outgrowth assay where each outgrowth was individually cultured, imaged and subsequently genotyped. As expected, successful hatching and outgrowth rates were high in both WT blastocysts (8/10, 80%) and Het blastocysts (21/23, 91.3%), displaying a distinct ICM colony surrounded by robustly proliferating trophoblast cells after 72 h in culture (Fig. 1D). However, all the mutant blastocysts failed to hatch or grow normally (0/9). Three of nine mutant blastocysts failed to hatch out of the zona pellucida (Fig. 1D, type I), two arrested during the process of hatching (type II), and four hatched free of the zona pellucida but did not form a monolayer of attached trophoblast cells (type III). Each of these subtle phenotypes suggested impaired function of TE cells in mutant blastocysts, which lead to failures of hatching or implantation. These results are also consistent with a complete absence of mutant embryos at E7.5.

Med20 mutants have normal expression of OCT4 and CDX2
To explore the cause of the outgrowth and implantation failure, we first examined markers for apoptosis (TRP53) and first cell lineage choices including OCT4 for ICM and CDX2 for TE using immunofluorescence (IF). Blastocysts were collected, imaged and then genotyped. Of 25 blastocysts examined (5 WT, 15 Het, 5 Mut), all genotypes showed no apoptosis and normal robust expression of OCT4 in ICM and mutually exclusive expression of CDX2 in TE (Fig. 2). These results show that Med20-null blastocysts are not dying via apoptosis, and their ICM and TE have been appropriately specified.

Ectopic NANOG in Med20 mutant TE
We next investigated the second cell-fate specification, the segregation of ICM into epiblast (EPI) and primitive endoderm (PE). Blastocysts were collected, assessed for NANOG and SOX17 localization (EPI and PE markers, respectively) via IF and then genotyped. From 31 blastocysts (8 WT, 16 Het, 7 Mut), all genotypes showed regular expression and localization of both SOX17 and CDX2. However, the majority of Med20 mutant blastocysts (5/7, 71.4%) exhibited widespread ectopic expression of NANAOG in TE cells (Fig. 3) compared with WT (1 from 8, 12.5%) and Het (2 from 16, 12.5%), suggesting that MED20 regulates early embryo development in part through repression of NANO-G in CDX2-positive TE cells. Combined with the outgrowth failure of mutant embryos, we can conclude...
that deletion of Med20 results in defective function of TE cells.

**Med20 knockdown embryos phenocopy genetic knockout mutants**

Due to the lack of available antibodies suitable for immunofluorescence, we examined the expression of Med20 by RT-PCR to verify the success of both knockout and knockdown approaches (location of primers is shown in Fig. 1A). RT-PCR using cDNA from different stages of WT embryos shows that Med20 is expressed at all preimplantation stages, from oocyte to blastocyst (Fig. 4A). We then extracted both RNA and DNA from single blastocysts to both genotype embryos and assess Med20 expression (details in Material and methods section). As expected, genotyped mutant blastocysts contain no Med20 mRNA (Fig. 4B), confirming functional knockout of the deletion allele. To further verify the deletion phenotype was due to lack of MED20 and to establish a more efficient system to study MED20 function in embryos, three distinct commercial Med20 siRNAs (Fig. 1A) were individually microinjected into zygotes. Satisfactory KD efficiency was confirmed at early morula stage (2 days after microinjection) with each siRNA (Fig. 4C). Similar to KO phenotype, KD of Med20 did not affect blastocyst formation or morphology, but resulted in overall outgrowth failure (Fig. 4D).

**KD of Med20 also induces severe ectopic NANOG expression in TE cells**

Considering the overlapping expression of NANOG+CDX2 observed in Med20 KO blastocysts, we also performed

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**Figure 3** Knockout of Med20 did not affect the expression or localization of either SOX17 (marker of primitive endoderm) or CDX2 (marker of trophectoderm); however, KO of Med20 led to severe ectopic expression of NANOG (marker of epiblast) in outside CDX2-positive TE cells. Blastocysts in this experiment were flushed at E3.5, and then cultured overnight before fixation and immunofluorescence. Scale bar, 50 μm.

**Figure 4** (A) Expression pattern of Med20 in WT preimplantation embryos. Actb was used as loading control. Oo, metaphase II oocyte; Zy, zygote; 2C, 2-cell embryo; 4/8C, mix of 4- and 8-cell stage embryos; Mo, morula; Blas, blastocyst. (B) Simultaneous extraction of both RNA and DNA from single blastocyst to perform both genotyping PCR and Med20 RT-PCR, confirming KO was successful. Actb was used as loading control. (C) Endogenous Med20 mRNA was significantly depleted by three distinct siRNAs after microinjection. (D) KD of Med20 using distinct siRNAs did not affect blastocyst formation or morphology, but significantly altered embryo outgrowth potential. Red and blue dashed lines indicate ICM colony and trophoblast cells, respectively. Control: scrambled siRNA. n, number of embryos; *, P < 0.05. Scale bars, 100 μm.

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lineage specification assessment in KD blastocysts. Compared with control embryos (Fig. 5), KD of Med20 does not alter SOX17 (PE) or CDX2 (TE) localization. However, as with KO embryos, ectopic NANOG in outer TE cells was detected in all KD groups (ectopic NANOG rate: Control, 2 from 19, 10.5%; siRNA1, 16 from 20, 80%; siRNA2, 16 from 18, 88.9%; siRNA3, 15 from 16, 93.8%), indicating that phenotypes of CRISPR-Cas9-mediated Med20 KO can be fully recapitulated by microinjection of single Med20 siRNAs. Importantly, these results confirm that loss of MED20 results in TE defects and outgrowth failure in vivo and in vitro.

**Discussion**

Cell differentiation and lineage specification is a complex and highly regulated process during development of all multicellular eukaryotic organisms. To obtain unique profiles of gene expression and distinct cellular identity, complexity in transcriptional control between regulatory elements and RNA polymerase must exist. Among these complicated networks, the Mediator complex is a key component of the RNA polymerase II (Pol II) transcriptional machinery. It can convey distal regulatory information to basal Pol II transcription machinery, playing a crucial role in not only activation, but also repression of eukaryotic mRNA synthesis (Beyer et al. 2007). The Mediator complex was originally detected in yeast, and then it was identified in mammalian species comprising up to 30 subunits (Tsai et al. 2014). Though the whole Mediator complex is required for all tissues and cell lineages, different Mediator subunits have distinct target genes, and phenotypes of individual subunit mutants can be distinct from each other (Westerling et al. 2007), indicating a multifaceted role of Mediator complex during organismal development.

Though it is generally believed that the head module of Mediator is involved in interactions with the core Pol II machinery, studies have demonstrated that some regulators can directly target subunits in the head module, for example, Med17 (Park et al. 2001). Med20, another core component in head module, has not been well studied yet. The function of MED20 in mammalian development has not been studied. In this study, we took advantage of both KO and KD strategies to demonstrate that MED20 is essential for early mouse development. Consistent with our findings, other Mediator subunits also exhibit specific embryonic lethal phenotypes; however, only CDK8 and MED21 have similar early lethality with all other documented mutants showing post-gastrulation phenotypes (reviewed in Yin & Wang 2014).

Our studies revealed ectopic NANOG (epiblast marker) expression in CDX2-positive TE cells of Med20 KO and KD blastocysts. It is well established that CDX2 is essential for segregation of ICM and TE lineages at the blastocyst stage by repressing OCT4 and NANOG in the TE, although the detailed mechanism underlying this repression is still largely unknown (Strumpf et al. 2005, Niwa et al. 2005, Wang et al. 2010, Carey et al. 2015, Piliszek et al. 2017, Basset et al. 2018). Interestingly, in our study, both KO and KD of Med20 only causes ectopic NANOG in...
the outer TE cells, without effects on OCT4 expression or localization, suggesting that CDX2 represses OCT4, at least partially, through different pathways. Indeed, previous studies have illustrated that OCT4 can bind to the Nanog promoter, and this cis-regulatory machinery is essential for Nanog pluripotent transcription, suggesting OCT4 is at the top of this regulatory hierarchy (Kuroda et al. 2005, Rodda et al. 2005). Embryonic stem (ES) cells have been used extensively to explore the functions of Mediator complex in cell lineage commitment. For example, a functional role of MED12 in the regulation of Nanog expression and maintenance of ES cell pluripotency has been debated (Tutter et al. 2009, Rocha et al. 2010), and mounting evidence indicates that MED12 and MED1, together with cohesin complex and loading factor, can contribute to ES cell state through DNA loops that directly link enhancers and promoters (Kagey et al. 2010, Apostolou et al. 2013, Phillips-Cremins et al. 2013). Additionally, many Mediator subunits have been identified as regulators of ES cell maintenance: MED6, MED7, MED10, MED12, MED14, MED15, MED17, MED21, MED24, MED27, MED28 and MED30 (Kagey et al. 2010).

Our results suggest that unlike these other subunits, Med20 is essential for repression of Nanog in TE cells to maintain TE identity and function. However, the exact mechanism by which MED20 and/or Mediator coordinates multiple TFs and co-factors to regulate Nanog expression is unknown. Complicating any mechanistic conclusions are studies that have shown certain Mediator subunits have selective affinity among different activators and TFs, such that the absence of MED20 may allow for Mediator interaction with activators of Nanog and other loci (Niwa 2014, Yin & Wang 2014, Miao et al. 2018). Other novel functions of Mediator have recently been documented. For example, it can interact directly with non-coding RNAs to influence transcription (Carlsten et al. 2013), regulate alternative mRNA processing (Huang et al. 2012) and alter epigenetic silencing of selected genes (Ding et al. 2008). In order to fully understand the mechanism and specificity of MED20 function, we will likely need to assess transcription wide effects specifically in TE cells of KO or KD embryos. Alternatively, trophoblast stem cells may serve as a good model to explore MED20 function; however, in vivo results may differ from in vitro cell line studies.

In summary, using both KO and KD strategies, our study suggests that MED20 plays a significant role in proper trophectoderm development that is essential for hatching and implantation. In addition, MED20 is indispensable for repression of NANOG in TE cells during early murine development to maintain TE identity and function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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