**Mga is essential for the survival of pluripotent cells during peri-implantation development**

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**ABSTRACT**

The maintenance and control of pluripotency is of great interest in stem cell biology. The dual specificity T-box/basic-helix-loop-helix-zipper transcription factor Mga is expressed in the pluripotent cells of the inner cell mass (ICM) and epiblast of the peri-implation mouse embryo, but its function has not been investigated previously. Here, we use a loss-of-function allele and RNA knockdown to demonstrate that Mga depletion leads to the death of proliferating pluripotent ICM cells in vivo and in vitro, and the death of embryonic stem cells (ESCs) in vitro. Additionally, quiescent pluripotent cells lacking Mga are lost during embryonic diapause. Expression of Odc1, the rate-limiting enzyme in the conversion of ornithine into putrescine in the synthesis of polyamines, is reduced in Mga mutant cells, and the survival of mutant ICM cells as well as ESCs is rescued in culture by the addition of exogenous putrescine. These results suggest a mechanism whereby Mga influences pluripotent cell survival through regulation of the polyamine pool in pluripotent cells of the embryo, whether they are in a proliferative or quiescent state.

**KEY WORDS:** Mga, Pluripotency, ESCs, T-box, Basic-helix-loop-helix-zipper, Transcription factor, Mouse, ODC

**INTRODUCTION**

By the time the mouse embryo implants in the uterus, three cell lineages have been established: first, the outer layer of trophoderm (TE) separates from the inner cell mass (ICM), and then the ICM segregates into the inner pluripotent epiblast (EPI) and the primitive endoderm (PE), adjacent to the blastocoeal cavity. The PE and the TE undergo initial stages of differentiation through the actions of the transcription factor genes *Gata6* and *Cdx2*, respectively (Chazaud et al., 2006; Jedrusik et al., 2008; Johnson and McConnell, 2004; Silva et al., 2009; Strumpf et al., 2005), whereas the EPI remains pluripotent, largely through the action of the transcription factor genes *Pou5f1* (also known as *Oct4*), *Nanog* and *Sox2* (Avilion et al., 2003; Loh et al., 2006; Nichols et al., 1998; Silva et al., 2009). The maintenance of pluripotency in the ICM and EPI is essential for embryonic development, as the EPI will eventually differentiate into all of the tissues of the embryo.

The basic-helix-loop-helix-leucine-zipper (bHLHZip) domain genes of the MAX-interacting network are thought to play crucial roles in the development of the ICM and EPI (Grandori et al., 2000; Hurlin and Huang, 2006). This network of genes includes *Max* and *Mga*, as well as members of the Myc, Mad and Mnt families of transcription factors (Ayer et al., 1993; Hurlin et al., 1999, 1995; Meroni et al., 1997; Zervos et al., 1993). Only by forming heterodimers with MAX are MAX-network proteins able to bind DNA at the E-box sequence and to activate or repress transcription of E-box-containing target genes (Baudino and Cleveland, 2001; Hurlin et al., 1999; Meroni et al., 2000; Walker et al., 2005). Despite its near-universal expression in oocytes and throughout development, the role of zygotic MAX has been elucidated only in the peri-implantation period, in which it is essential for embryonic survival: embryos lacking *Max* die shortly after implantation, as maternal stores of MAX are depleted (Shen-Li et al., 2000). Embryos lacking Myc, Mnt and Mad family genes die after implantation. The binding partners of the MAX-network that are crucial for early development are unknown.

MGA, the least studied of the MAX-network transcription factors, is a dual-specificity transcription factor that contains both a bHLHZip domain and a T-box domain and is able to bind to and regulate transcriptional targets through both E-box sites as well as T-box-binding elements (TBEs). Heterodimerization with MAX is required for MGA to bind to E-box target gene promoters. On TBEs, MGA is able to bind alone, although activation or repression is modulated by heterodimerization with MAX (Hurlin et al., 1999).

In zebrafish, *Mga* mRNA was detected as a maternal transcript in the fertilized egg and is expressed widely throughout later development (Rikin and Evans, 2010). Morpholino depletion of *Mga* in fertilized zebrafish eggs results in defects in the brain, heart and gut derivatives, although no common transcriptional targets or pathways have been identified (Rikin and Evans, 2010). In mouse, *Mga* mRNA is first detected at E3.5 in the peri-implent ICM (Yoshikawa et al., 2006) and appears to be widely expressed during later organogenesis (Hurlin et al., 1999). Expression of both mRNA and protein is seen in embryonic stem cells (ESCs), the *in vitro* analog of the ICM (Hu et al., 2009; van den Berg et al., 2010). In ESCs, MGA was found in a complex with *Pou5f1*, and *Mga* knockout leads to ESC differentiation, suggesting that MGA plays a role in the maintenance of pluripotency through its interaction with *Pou5f1* (Hammachi et al., 2012; Hu et al., 2009; van den Berg et al., 2010). To address the role of *Mga* in murine development, particularly its possible role in maintenance of pluripotency in the early embryo, we examined the development of mouse embryos lacking functional *Mga* through gene disruption and RNA knockdown.

**RESULTS**

*Peri-implantation lethality of a loss-of-function Mga allele*

A multipurpose, conditional *Mga* mutant allele, *Mga*\(^{E153E01}E153E01\)\(^E\)\(^{W1}\)\(^{W2}\), was generated by the German Gene Trap Consortium (Fig. 1). In its original orientation, here referred to as *Mga*\(^{E1}\), the endogenous
and no homozygous mutants were recovered (Table 2).

splice site of the allele is not fully functional, although both male and female expressing mouse to generate the inverted conditional allele, mice were bred with a constitutively active FLPe recombinase-configuration referred to as β trap cassette, creating a truncated fusion protein that carries a reporter protein. Adapted from Schnutgen et al. (2005).

Fig. 1. The gene trap cassette and Mga mutations produced from the FlpRBB targeting vector. The MgaGr allele orients a splice acceptor-β-galactosidase-neomycin resistance cassette to accept the upstream exon 3 splice site of the Mga locus (top) and to create a mutant truncated reporter protein. After treatment with FLP recombinase (which results in inversion, step 1, and excision, step 2), the splice acceptor is no longer in the proper orientation to accept the upstream splice, and a wild-type transcript is produced by splicing around the inserted cassette. After further treatment with CRE recombinase, inversion and excision (steps 3 and 4) occur to produce the MgaGr-inv allele, which functions like the MgaGr allele producing a truncated reporter protein. Adapted from Schnutgen et al. (2005).

upstream exon donates a splice site that is accepted by the gene trap cassette, creating a truncated fusion protein that carries a β-geo reporter under the control of the Mga promoter. When exposed to FLPe recombinase, the cassette is inverted, a configuration referred to as MgaInv, and the splice acceptor is in the wrong orientation to accept the upstream splice, thus allowing the wild-type transcript to be produced. When subsequently exposed to CRE recombinase, the cassette is flipped once more, referred to as the MgaRe-inv configuration, producing the β-geo fusion protein. The MgaGr and MgaRe-inv alleles act as mutant reporter alleles and the MgaInv allele acts as a conditional-mutation allele (Fig. 1) (Schnutgen et al., 2005).

MgaGr/+ mice were recovered at the expected Mendelian frequency (42/88 from Mga+/×MgaGr/+ matings) and were viable and fertile. However, no MgaGrGr mice were recovered at weaning from inter se matings of MgaGr/+ mice (0/84) (Table 1). MgaGr/+ mice were bred with a constitutively active FLPe recombinase-expressing mouse to generate the inverted conditional allele, MgaInv/+ (Fig. 1). MgaInv/+ mice were born at the expected frequency (7/12 from Mga+/×MgaInv/+ matings), indicating that the conditional MgaInv/+ allele did not have an obvious heterozygous or dominant negative effect (Table 1). Homozygous MgaInv/Inv mice, however, were recovered at only ~50% of the expected frequency (19/150 from Mga+/×MgaInv/+ matings), indicating that the conditional MgaInv/+ allele is not fully functional, although both male and female MgaInv/Inv mice that were recovered had no apparent phenotype and were viable and fertile. This conditional allele was unable to compensate for the MgaGr mutation, as no MgaGrInv/+ mice were recovered at weaning (0/22 from Mga+/×MgaInv/+ matings) (Table 1).

Dissection of the uteri of females from inter se MgaGr/+ matings at E9.5-E11.5 revealed only cellular debris or a few trophoblast giant cells in ~1/4 of the deciduae (6/25). Trophoblast giant cells from one of these proved to be MgaGr/Gr when genotyped by PCR (Table 2). Similarly, at E5.5, ~1/4 of the deciduae were empty (9/44) and no homozygous mutants were recovered (Table 2). Histological examination at E5.5 and E6.5 showed instances of cellular debris with isolated trophoblast giant cells in ~1/4 of deciduae (10/39 at E5.5; 4/10 at E6.5) (Fig. 2A). MgaGr/Gr embryos were recovered from uterine flushes at a rate slightly lower than the expected Mendelian frequency at E4.5 (49/245) and at the expected frequency at E3.5 (21/89), and appeared morphologically normal (Table 2, Fig. 2A). Taken together, these results indicate that MgaGrGr embryos develop to the blastocyst stage but die during the process of implantation.

Mga is expressed in the pluripotent ICM and EPI of the peri-implantation embryo

Embryonic expression of Mga was assessed using the MgaGr reporter allele. There was robust β-galactosidase activity in the EPI of MgaGr/+ embryos at E5.5 and E6.5, to the exclusion of the PE and extraembryonic tissues (Fig. 2B). Standard X-gal staining was insufficient for detection in preimplantation stages, but the more sensitive S-gal method showed staining in the EPI at E4.5 in MgaGr/+ embryos. Although neither X-gal nor S-gal showed staining at E3.5, RT-PCR for Mga in wild-type embryos demonstrated expression at E3.5 but not at E2.5 or E0.5 (one-cell stage) (Fig. 2B).

ICMs of blastocysts lacking Mga fail to thrive in vitro

Embryos were isolated at E3.5 from MgaGrGr/+×MgaGrGr/+ matings, cultured in vitro for 4 days and then genotyped by PCR. Some cultures of MgaGrGr/+ embryos were stained with X-gal to assess β-galactosidase reporter activity. The ICM of the blastocyst cultures, but not the TE, showed strong X-gal staining after two days of culture, which later disappeared, indicating that embryonic expression of Mga was initially recapitulated in the in vitro culture system (data not shown). Whereas MgaGrGr/+ embryos (n=11) were able to attach to the tissue culture substrate and form a normal-appearing outgrowth of trophoblast giant cells, the ICM derivatives failed to thrive and did not form multicellular or cystic structures like their MgaGr/+ and MgaGrGr/+ littermates (n=35) (Fig. 3A). The outgrowth area of the ICM and TE was similar for wild-type and

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>+/+</th>
<th>+/Gr</th>
<th>Gr/Gr</th>
<th>n.d.</th>
<th>P</th>
</tr>
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<tr>
<td>E3.5</td>
<td>27</td>
<td>41</td>
<td>21</td>
<td>0</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>E4.5</td>
<td>83</td>
<td>113</td>
<td>49</td>
<td>0</td>
<td>P=0.004</td>
<td></td>
</tr>
<tr>
<td>E5.5</td>
<td>12</td>
<td>23</td>
<td>0</td>
<td>9</td>
<td>P=0.003</td>
<td></td>
</tr>
<tr>
<td>E9.5-E11.5</td>
<td>6</td>
<td>13</td>
<td>1</td>
<td>5</td>
<td>P=0.04</td>
<td></td>
</tr>
</tbody>
</table>

MgaGrGr embryos are present during preimplantation development (E3.5-E4.5) but are not recovered after implantation. n.d., not determined; n.s., not significant.

Table 1. Number of progeny of each genotype recovered at weaning from the indicated matings

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mating</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
<th>P</th>
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<tr>
<td>MgaGrGr/+ × MgaGrGr/+</td>
<td>46</td>
<td>42</td>
<td>n.a.</td>
<td>n.s.</td>
<td></td>
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<tr>
<td>MgaGrGr/+ × MgaGrGr/+</td>
<td>31</td>
<td>53</td>
<td>0</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>MgaGrGr/+ × MgaGrGr/+</td>
<td>5</td>
<td>7</td>
<td>n.a.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>MgaGrGr/+ × MgaGrGr/+</td>
<td>38</td>
<td>93</td>
<td>19</td>
<td>P=0.001</td>
<td></td>
</tr>
<tr>
<td>MgaGrGr/+ × MgaGrGr/+</td>
<td>N/A</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>Gl/Gr</td>
</tr>
</tbody>
</table>

MgaGrGr/+ mice are not recovered indicating earlier lethality; MgaGrGr/+ mice are recovered at lower than Mendelian frequency and MgaGrGr/+ mice are not recovered, suggesting that MgaGr is a hypomorphic allele. n.a., not applicable; n.s., not significant.

Table 2. Number of embryos of each genotype recovered from MgaGrGr/+ × MgaGrGr/+ matings at different developmental stages
ICM after 3 days of culture (Fig. 4A,C). These results indicate that dsMga embryos precisely phenocopy Mga\textsuperscript{Gt/Gt} embryos, and they strongly support that Mga\textsuperscript{Gt} is a null allele.

**ESCs lacking Mga have a growth defect**

Failure of in vitro ICM growth from Mga\textsuperscript{Gt/Gt} embryos precluded establishment of mutant ESCs and suggested that ESCs would fail to thrive in the absence of Mga. To test this hypothesis, we derived ESCs in serum-containing medium (mES) from embryos homozygous for the conditional allele Mga\textsuperscript{Inv} that also carried the tamoxifen-inducible, ubiquitously expressed ROS426\textsuperscript{creERT2} allele (de Luca et al., 2005), hereafter referred to as creERT2. Mga\textsuperscript{Inv/Inv}; creERT2 ESCs were morphologically indistinguishable from Mga\textsuperscript{Inv/Inv} ESCs, grew at similar rates and expressed pluripotency markers POU5F1 (OCT4) and NANOG (data not shown). Upon the addition of 4-hydroxytamoxifen to the culture media, however, there appeared to be a large amount of cell death, and surviving colonies of Mga\textsuperscript{Inv/Inv}; creERT2 cells, which were normal in appearance (Fig. 3B), were smaller and sparser than Mga\textsuperscript{Inv/Inv}; creERT2 cells without 4-hydroxytamoxifen or Mga\textsuperscript{Inv/+} cells with or without 4-hydroxytamoxifen. Quantification of cell number at 24-h intervals revealed a ~35% decrease in the number of cells in Mga\textsuperscript{Inv/Inv}; creERT2 cultures with 4-hydroxytamoxifen compared with the controls (Fig. 3B). Genotyping of the surviving colonies revealed that there was incomplete CRE-induced inversion of the Mga\textsuperscript{inv} allele regardless of the length of 4-hydroxytamoxifen treatment (Fig. 3B). Increasing the concentration of 4-hydroxytamoxifen beyond 0.5 μM resulted in toxicity for both Mga\textsuperscript{Inv/Inv}; creERT2 and Mga\textsuperscript{Inv/+} cells, but the amount of inversion of the Mga\textsuperscript{inv} allele remained constant (data not shown). Together, these results indicate that in 4-hydroxytamoxifen-treated cultures, the surviving colonies had escaped complete inversion and that at least one functional Mga allele is necessary for the survival of ESCs.

**Embryos lacking Mga have increased apoptosis**

Reduced ICM outgrowth of Mga\textsuperscript{Gt/Gt} blastocyst cultures and reduced growth of 4-hydroxytamoxifen-treated Mga\textsuperscript{Inv/Inv}; creERT2 ESCs suggests an increase in cell death and/or a decrease in cell proliferation in the ICM or ICM derivatives in the absence of Mga. Counts of phosphorylated histone H3 (phospho-H3)-positive cells in the EPI did not reveal any difference in the number of mitotic cells in Mga\textsuperscript{Gt/Gt} embryos (3.1±0.8) compared with Mga\textsuperscript{Gt/+} and Mga\textsuperscript{Gt/+} embryos (2.8±0.3) at E4.5 (t=0.259, P=0.65) (Fig. 5). The number of cells in prophase in Mga\textsuperscript{Gt/Gt} embryos (1.8±0.3) was similar to that in Mga\textsuperscript{Gt/Inv}; creERT2 and Mga\textsuperscript{Inv/Inv}; creERT2 cells, but expression was present at E3.5 and E4.5 (top right). β-galactosidase staining was observed in the EPI of E4.5 embryos. RT-PCR on pooled embryos did not suggest an increase in cell death and/or a decrease in cell proliferation. Counts of phosphorylated histone H3-positive cells, which were normal in appearance (Fig. 3A), were normal in appearance (Fig. 3B), and were not increased in area, whereas the TE areas were similar to wild type (WT) (Fig. 3A). This indicates a failure of ICM survival as a contributing factor in the embryonic lethality of Mga\textsuperscript{Gt/Gt} embryos. Culture of 41 blastocysts from heterozygous crosses that were allowed to attach and were then cultured in 2i medium for two additional days resulted in 9 (22%) outgrowths with little or no ICM. This matched closely the expected number of homozygous mutants (χ²=0.20, P>0.1), indicating that 2i medium was insufficient to overcome the deficiency of Mga.

We also generated blastocysts lacking Mga through RNAi by microinjection of long double-stranded RNA into zygotes, an approach that has been used to elicit gene-specific knockdown (Wianny and Zernicka-Goetz, 2000; Zhang et al., 2012b). Most embryos injected with dsGfp as a control hatched from the zona pellucida (77%; 23/30), and showed robust TE and ICM outgrowth in vitro (60%; 18/30) (Fig. 4A,B). By contrast, only 42% (11/26) of dsMga-injected blastocysts hatched, and whereas most showed evidence of TE cells, only 4% (1/26) had a morphologically evident
Early differentiation of the ICM is not affected by the absence of Mga

At E4.5, the pluripotent ICM differentiates into two cell layers: the EPI, which remains pluripotent, and the PE, which forms an epithelial sheet at the blastocoel surface. Immunofluorescence with antibodies against the pluripotency marker NANOG, which marks the EPI, and GATA4, which marks the PE, showed that both layers form normally in MgaGt/Gt embryos at E4.5 (n=3) (Fig. 5). Together, these results indicate that MgaGt/Gt embryos show the normal spatial and temporal gene expression pattern characteristic of the differentiation of PE and maintenance of pluripotency in the EPI.

Embryos lacking Mga lose pluripotent cells during diapause

Despite the presence of pluripotency markers at E4.5, the death of ICM derivatives in vivo and in vitro suggests that pluripotent cell maintenance is affected in MgaGt/Gt embryos. To test this, we examined embryos in which diapause or implantation delay was induced by tamoxifen and depot medroxyprogesterone 17-acetate (DMPA) injections at E2.5 (Nichols et al., 2001). In diapause, the EPI and PE layers form but cell division ceases. RT-PCR of pooled wild-type embryos shows that one day after induction of diapause, Mga expression is at a level similar to E3.5 embryos and then falls to a low level at 4 and 7 days of diapause (Fig. 6C, and data not shown). Immunofluorescence using antibodies against NANOG and GATA4 was used to assess the persistence of EPI and PE cell populations, respectively, throughout diapause. One day after induction of diapause, MgaGt/Gt embryos showed similar expression patterns of NANOG and GATA4 as well as MgaGt/Gt (n=7) embryos (Fig. 5). Together, these results indicate that MgaGt/Gt embryos show the normal spatial and temporal gene expression pattern characteristic of the differentiation of PE and maintenance of pluripotency in the EPI.
To assess the function of ODC1 in peri-implantation embryos, we knocked down Odc1 by microinjection of dsOdc1 double-stranded RNA into one-cell zygotes. dsOdc1 embryos developed normally to the blastocyst stage. Outgrowth assays revealed that, whereas 59% (13/22) of dsOdc1 blastocysts hatched from the zona pellucida and attached to the culture dish, only 27% (6/22) showed evidence of ICM formation (Fig. 4). Similar to Mga\textsuperscript{GFr/Gr} and dsMga embryos, outgrowth of TE cells in dsOdc1 embryos was not different from control dsGFP outgrowths.

To directly assess ODC levels, immunofluorescence was performed with antibodies against ODC. Projections of confocal stacks showed decreased ODC signal in the EPI of Mga\textsuperscript{GFr/Gr} embryos (4/6) at E4.5 compared with Mga\textsuperscript{+/+} and Mga\textsuperscript{GFr} embryos (3/3) (P=0.006; Fisher's Exact Probability test) (Fig. 7B). There was also strong fluorescence on the TE in all samples, although secondary antibody controls (data not shown) indicated that this was background staining.

Exogenous putrescine rescues the ICM in Mga mutant embryos and survival of Mga mutant ESCs

Because decreased Odc1 expression in Mga\textsuperscript{GFr/Gr} embryos suggests that they lack the ability to produce putrescine and thus lack the end products of the polyamine synthesis pathway, we attempted to rescue Mga\textsuperscript{GFr/Gr} embryos by supplying blastocyst cultures with exogenous putrescine and measuring ICM and TE outgrowth area after 96 h. Putrescine did not affect the outgrowth area of TE, but the ICM area of Mga\textsuperscript{GFr/Gr} embryos was significantly larger in embryos treated with putrescine (n=11) than in untreated embryos (n=7) (t=−4.58, P=0.0003), whereas the area of the ICM in Mga\textsuperscript{+/+} and Mga\textsuperscript{GFr} embryos was similar with (n=29) or without putrescine (n=24) (t=1.4, P=0.18) (Fig. 7C). Notably, the ICM area of the Mga\textsuperscript{GFr/Gr} cultures was not significantly different than the putrescine-treated control cultures (t=−1.83, P=0.08). These results indicate that exogenous putrescine is sufficient to rescue the ICM outgrowth of Mga\textsuperscript{GFr/Gr} embryos.

To determine whether putrescine could also rescue Mga mutant ESCs, Mga\textsuperscript{Inv/Inv; creERT2} ESCs were plated and allowed to attach for 1 day. Cultures were then supplemented with 4-hydroxytamoxifen, 4-hydroxytamoxifen plus 200 µM putrescine or with putrescine alone. After an additional 2 days of culture, cell counts from Mga\textsuperscript{Inv/Inv; creERT2} cultures with 4-hydroxytamoxifen plus putrescine were not as high as untreated Mga\textsuperscript{Inv/Inv; creERT2} embryos (t=1.83, P=0.0001), but not as high as untreated Mga\textsuperscript{Inv/Inv; creERT2} ESCs (t=−6.14, P<0.0001), whereas the ICM area of the Mga\textsuperscript{GFr/Gr} embryos was significantly larger in embryos treated with putrescine (n=11) than in untreated embryos (n=7) (t=−4.58, P=0.0003), whereas the area of the ICM in Mga\textsuperscript{+/+} and Mga\textsuperscript{GFr} embryos was similar with (n=29) or without putrescine (n=24) (t=1.4, P=0.18) (Fig. 7C). Notably, the ICM area of the Mga\textsuperscript{GFr/Gr} cultures was not significantly different than the putrescine-treated control cultures (t=−1.83, P=0.08). These results indicate that exogenous putrescine is sufficient to rescue the ICM outgrowth of Mga\textsuperscript{GFr/Gr} embryos.

Expression of the Myc target gene ornithine decarboxylase 1 (Odc1) is decreased in embryos lacking Mga

Based on a similar expression pattern and embryonic mutant phenotype as well as its regulation by the bHLHZip factor MYC (Pendeville et al., 2001), we identified Odc1, the gene coding for ornithine decarboxylase (ODC), as a candidate downstream target of MGA. ODC catalyzes the decarboxylation of ornithine to form ornithine decarboxylase (ODC), as a candidate downstream target (Pendeville et al., 2001), we identified the phenotype as well as its regulation by the bHLHZip factor MYC. Based on a similar expression pattern and embryonic mutant (\textit{Odc1}) expression in \textit{Mga}\textsuperscript{GFr/Gr} embryos is decreased in embryos lacking \textit{Mga}. Conversely, the lack of an early effect on GATA4-expressing cells reflects a lack of requirement for \textit{Mga} in initial PE differentiation and in differentiated cells.

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Fig. 4. Knockdown of Mga or Odc1 results in ICM failure. (A) Results of outgrowth assays following injection with dsGfp, dsMga or dsOdc1. All injection groups developed to the blastocyst stage at similar rates (~90%, not shown). Percentage of total embryos (N) that hatched and formed ICM outgrowths is shown for each group. (B) Control embryos (dsGfp) form obvious ICM colonies and TE outgrowths (yellow arrows). (C,D) Examples of the failure to hatch and to form ICM outgrowth after microinjection of dsMga or dsOdc1. The numbers in the panels refer to the number of embryos out of the total that failed to hatch (as shown) or the number of embryos out of the total that hatched and outgrew that failed to form an ICM (as shown).

Fig. 5. Mga\textsuperscript{GFr/Gr} embryos showed more apoptosis, but cell proliferation and differentiation were normal at E4.5. Cell proliferation, as measured by phospho-H3 immunostaining, was similar in Mga\textsuperscript{GFr/Gr} compared with Mga\textsuperscript{+/+} and Mga\textsuperscript{GFr} embryos. Confocal Z-stacks of cleaved caspase 9 immunofluorescence showed that more Mga\textsuperscript{GFr/Gr} embryos had fragmented nuclei (7/9) than did Mga\textsuperscript{+/+} and Mga\textsuperscript{GFr} embryos (7/47). The pluripotency markers NANOG and POU5F1, as measured by a GFP reporter, are similar in Mga\textsuperscript{GFr/Gr} embryos compared with Mga\textsuperscript{+/+} and Mga\textsuperscript{GFr} embryos, as was the PE marker GATA4. Numbers in the panels indicate the number of embryos out to the total with an appearance similar to that shown.
able to survive but that other factors might be required to completely compensate for the loss of Mga. Putrescine alone had no effect. (t = −0.152, P = 0.88) (Fig. 7C). To test the specificity of putrescine, MgaInv/Inv; creERT2 cultures were treated with cadaverine, a structurally related cationic polyamine that has an additional carbon link in its backbone but is not part of the polyamine synthesis pathway. Cadaverine treatment had no effect on MgaInv/Inv; creERT2 cultures alone (t = −0.26, P = 0.80) or when combined with 4-hydroxytamoxifen (t = 0.05, P = 0.96). None of the supplemented media combinations used had any effect on Mga+/+ or Mga+/+; creERT2 ESC lines (data not shown).

By culturing MgaInv/Inv; creERT2 ESCs with 4-hydroxytamoxifen plus putrescine, two MgaRe-inv/Re-inv; creERT2 subclones were derived. Both of these lines showed a decrease in the number of cells 48 h after the removal of putrescine (t = 3.60, P = 0.023; t = 4.99, P = 0.001) (Fig. 7C). Linear regression of the cell counts on each day of culture showed decreasing numbers of surviving cells in cultures without exogenous putrescine compared with cultures with putrescine (y = −0.06, R² = 0.74; y = −0.06, R² = 0.69). Immunofluorescence with antibodies against ODC was performed on MgaRe-inv/Re-inv; creERT2 ESC lines grown in the presence of exogenous putrescine. Both cell lines showed decreased ODC signal in ESC colonies compared with control MgaInv/Inv; creERT2 ESCs with or without putrescine. All cell lines showed heterogeneous NANOG staining throughout the ESC colonies (Fig. 7B).

ESCs grown in LIF- and serum-containing medium contain both self-renewing cells and a spectrum of cells in transition toward differentiation, whereas 2i conditions favor a more uniform population of core pluripotent cells in a ground state over cells primed for differentiation (Marks et al., 2012). We grew MgaRe-inv/Re-inv; creERT2 ESCs in 2i conditions and tested the effect of exogenous putrescine. Unlike the result for ESCs cultured in mES media, there was no significant difference in cell counts after 48 h with or without putrescine (t = 1.82, P = 0.09) (Fig. 7C). This
indicates that the putrescine-mediated rescue of Mga-deficient ESCs, and thus the requirement for ODC, is more marked in the more heterogeneous EPI-like pluripotent cell population favored by mES culture conditions than on the naïve ICM-like pluripotent cells favored by 2i culture conditions.

DISCUSSION

The insertion of a gene trap cassette into the third intron of Mga results in a fusion protein containing the T-box and a functional β-galactosidase reporter but lacking the rest of the Mga protein, including the bHLHZip domain. The allele lacks a heterozygous phenotype, indicating that it is not acting as a dominant negative, in spite of the presence of the T-box in the fusion protein. Homozygous Mga<sup>Gt/Gt</sup> embryos, on the other hand, have a defect in ICM derivatives in which Mga transcripts have been detected (Yoshikawa et al., 2006). Moreover, embryos in which Mga was knocked down by RNAi had a blastocyst outgrowth phenotype similar to Mga<sup>2i/2i</sup> embryos. Thus, the gene trap allele Mga<sup>2i</sup> appears to be functionally equivalent to a null allele. In addition, the conditional Mga<sup>inv</sup> allele, derived from the Mga<sup>2i</sup> allele, is not fully functional, as mice homozygous for Mga<sup>inv</sup> can survive but are not recovered at Mendelian frequencies, and no Mga<sup>2i/inv</sup> compound heterozygotes were recovered.

Homozygous Mga<sup>2i/2i</sup> embryos implant in the uterus, but ICM derivatives fail to develop beyond E4.5 and show increased apoptosis but no change in cell proliferation. Similarly, in vitro, mutant blastocysts attach and TE outgrows, but the ICM fails to survive. ESCs derived using the conditional Mga<sup>inv</sup> allele also fail to thrive when the conditional allele is inverted to the Mga<sup>Re-inv</sup> configuration. As the expression of Mga is restricted to the ICM of embryos at E3.5 and the EPI of later embryos, these results indicate that embryonic lethality is caused by the defective development of the ICM and EPI. The Mga mutant phenotype is similar to that of mice mutant for ICM- or EPI-specific genes associated with pluripotency, notably Pou5f1 and Sox2 (Avilion et al., 2003; Nichols et al., 1998). However, the EPI of peri-implantation Mga mutants does not appear to lose pluripotency, as judged by expression of Pou5f1 and Nanog. Additionally, the differentiation of PE is normal in Mga mutant embryos at E4.5, indicating that loss of Mga does not affect this early differentiation event within the ICM.

To investigate whether pluripotent cells could be maintained in Mga mutant embryos, we challenged the embryos by inducing diapause to delay implantation (Nichols et al., 2001). Strikingly, despite entering diapause with a similar number of pluripotent cells, mutant embryos rapidly lost NANOG-expressing EPI cells during delayed implantation. The number of differentiated, GATA4-positive PE cells, by contrast, was initially similar to wild-type embryos and declined only after prolonged delay. This suggests that the lack of Mga primarily affects the maintenance of the pluripotent cells, not the survival of differentiated cells. Death of PE later in diapause could be a secondary effect of the loss of EPI.

Based on phenotypic similarity of the mouse mutants as well as the presence of E-box sites in its promoter (Bello-Fernandez et al., 1993; Pendeville et al., 2001), we identified Odc1 as a candidate gene responsible for the death of the pluripotent cells of Mga mutant embryo. Odc1 codes for ODC, the rate-limiting enzyme in the synthesis of putrescine from ornithine in the polyamine synthesis pathway (Fig. 7A). Disruption of this pathway in vitro and in vivo has shown that polyamines have important roles in pluripotency and peri-implantation development. Odc1 mutant embryos die shortly after implantation, with a phenotype remarkably similar to that of Mga mutant embryos, although, unlike Mga mutant embryos, their growth in vitro is not rescued by exogenous putrescine (Pendeville et al., 2001). On the other hand, mutation of Amnd1, a rate-limiting enzyme in synthesis of the polyamines spermidine and spermine from putrescine, causes a similar mutant phenotype in which ICM outgrowth in vitro is rescued by exogenous spermidine (Nishimura et al., 2002). In ESCs, Amnd1 is essential for self-renewal and is downregulated translationally during differentiation to neural progenitor cells (Zhang et al., 2012a). In F9 teratocarcinoma cells, decreased levels of ODC result in differentiation due to the accumulation of excess decarboxylated S-adenosylmethionine (Frostesjo et al., 1997).

We found that ODC was reduced in the EPI of E4.5 day Mga mutant embryos and in Mga mutant ESCs. Moreover, we found that culture with exogenous putrescine partially rescued cell survival in both the ICM outgrowths and in ESCs. Mga<sup>Re-inv/Re-inv; creERT2</sup> ESCs, derived from Mga<sup>inv/inv</sup>; creERT2 ESCs by culturing with tamoxifen and exogenous putrescine, declined dramatically when putrescine was removed from the media. This suggests that a key role of MGA in the peri-implantation development is to regulate the transcription of Odc1, and thus the cellular polyamine pool. Although c-MYC is known to directly activate Odc1 transcription (Bello-Fernandez et al., 1993) in the context of the Mga mutant embryo, ODC levels are still decreased, supporting the theory that MGA regulates MYC-MAX target genes in vivo (Hurlin et al., 1999). This could come about if, for example, MGA and c-MYC compete for available MAX, their obligate heterodimerization partner (Grinberg et al., 2004). Alternatively, c-MYC might serve to amplify transcription of Odc1 after induction of transcription by MGA rather than inducing it on its own. This notion is supported by the fact that, in human colon carcinoma cells and intestinal epithelial cells, c-Myc transcription has been shown to be dependent on ODC function, indicating another factor in initial Odc1 transcription (Celano et al., 1988; Liu et al., 2006). The notion of c-MYC acting as transcriptional amplifier rather than as the transcriptional initiator is supported by the role that c-MYC plays in the global amplification of transcription in ESCs (Lin et al., 2012).

The fact that putrescine does not support full rescue of Mga mutant embryos or of ESCs indicates that other targets might be involved. These could be transcriptional targets of Mga or they could be any number of Myc targets for which Mga competes. Mga/Max has also been found complexed with E2f6 in a repression complex involved in gene silencing, which can bind Myc and T-box-binding elements as well as containing chromatin modifiers (Ogawa et al., 2002).

Our results suggest a possible mechanism for Mga to play a crucial role in peri-implantation development through interaction with other proteins in the MAX network, notably c-MYC, to regulate transcription of Odc1. In the absence of Mga, Odc1 is downregulated, and end products of the polyamine synthesis pathway necessary for the maintenance of the pluripotent cells of the ICM/EPI lineage are not available in sufficient quantities. This regulation of an MYC/MAX target by MGA could also have significance in human cancer, as it has recently been reported that MGA is recurrently inactivated in high-risk chronic lymphocytic leukemia and Richter syndrome, a disease associated with elevated c-Myc levels (De Paoli et al., 2013). Further experiments will provide insight into the mechanism through which disruption of the polyamine pool leads to the loss of pluripotent cell self renewal.
However, loss of control of the cell cycle and subsequent apoptosis seems a likely mechaninism, based on the oncogenic potential of tissues that have aberrant polyamine pools (Erdman et al., 1999; Scquopp et al., 2012). It is also possible that apoptosis is driven by DNA instability or reactive oxygen species, based on the role that polyamines have been shown to play in both processes [reviewed by Seiler and Raul (2005)]. Given the myriad roles that polyamines play in cellular homeostasis, it is possible that loss of pluripotency is the final effect of multiple deregulated processes.

### MATERIALS AND METHODS

#### Mice and genotyping
ESC cells were isolated from E14Tg2a ESCs (Sv129P2) after retroviral infection using rsFlpRosa26 (FlpR; www.genetrans.org). The insertion of FlpR in intron 3 of Mga was identified by splinkerette PCR (Horn et al., 2007). Mice carrying the Mga
t allele were obtained from the German Gene Trap Consortium and crossed with ICR mice (Taconic). The allele and its derivatives are referred to as Mga
t (for gene trap), Mga
t (for FL-P recombinase inverted gene trap) and Mga
t (for CRE recombinase re-inverted gene trap). Mice, embryos and ESCs were genotyped using PCR. Ear punches or tail tips were digested in PB/NDS-lysis buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2-6H2O, 0.1 mg/ml gelatin, 0.45% NP4, 0.45% Tween20] with 100 μg/ml proteinase K (Roche, 03115801001). Cultured embryos were lysed in 15 μl of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA, 100 mM NaCl, 0.5% sarkosyl (sodium lauryl sarcosinate), 100 μg/ml proteinase K] at 55°C for 2 h. Genotyping was performed using three-primer PCR designed to amplify wild-type and mutant bands as indicated in Table 3. PCR conditions were:

- 4 min at 95°C, followed by 32 cycles of 30 s at 95°C, 30 s at 61°C, 40 s at 72°C
- 7 min at 72°C

RT-PCR

For RT-PCR of wild-type embryos, 42 E0.5, 26 E2.5, 35 E3.5 and 29 E4.5 embryos were pooled. RNA was isolated using an RNeasy mini kit (Qiagen, 74104), and RT-PCR was performed using a OneStep RT-PCR kit (Qiagen, 210212) and the primers AJW346 and AJW349 for Mga, and AJW371 and AJW372 for β-actin (Table 3).

- β-galactosidase activity assay

Embryos were fixed for 20 min in 4% paraformaldehyde at 4°C and then washed three times in PBS with 0.1% Tween20 ( Fisher, BP337-500). For whole-mount staining, embryos were incubated in X-gal staining buffer [1 mg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside in dimethyl sulfoxide (Sigma, B4352), 20 mM K3Fe, 20 mM K3Fe, 2 mM MgCl2 in PBS] overnight at 37°C, washed three times in PBS with 0.1% Tween and fixed in 4% paraformaldehyde. For cryosectioning, embryos were transferred to 20% sucrose overnight, embedded in O.C.T. Compound (Tissue-Tek, 4835), sectioned at 10-12 μm, fixed in 4% paraformaldehyde for 10 min at 4°C and incubated in X-gal staining buffer overnight at 37°C. Slides were fixed in 4% paraformaldehyde, counterstained with Eosin Y (Sigma, 318906-500ml) and mounted in Permout ( Fishe, SP15-500). For greater sensitivity using Salmon-gal (6-Chloro-3-indolyl-β-D-galactopyranoside; Lab Scientific, X668), embryos were fixed, washed twice with 5-Gal rinse solution [0.1% sodium deoxycholate, 0.2% IGEPAL CA-630 (Sigma, 88960), 2 mM MgCl2 and 0.1 mM phosphate buffer (pH 7.3)] and incubated with 1 mg/ml Salmon-gal and 6 μg/ml NBT (4-Nitro Blue tetrazolium chloride; Sigma, N6876) dissolved in 70% N, N-dimethylformamide in water at 37°C for one or more days (Sundararajan et al., 2012).

**Blastoctyst outgrowth in vitro**

Blastocyst culture was performed as previously described (Bhatnagar et al., 1995). Briefly, embryos were collected by uterine flushing using M2 media (Sigma, M7167-100ml) at E3.5, zona pelucidae were removed by incubation in acidic Tyrode’s solution (Sigma, T7188) and embryos were plated on tissue culture dishes in 20 μl drops of ES media [DMEM, 13% FBS (Hyclone, Cat #SH30071.03 Lot #ARG27092), 1% penicillin/streptomycin, 1% GlutMax, 1% sodium pyruvate, 1% non-essential amino acids, 0.1% β-mercaptoethanol, LIF derived from CHO cells expressing LIF] and covered in mineral oil. Some embryos were cultured in ES media for 1-2 days until they had attached and started to outgrow, and were then cultured for two additional days in 2i media (Millipore, ESGRO-2i SF016). For rescue experiments, embryos were cultured in ES media with 200 μM putrescine (Sigma, P5780-5g). Cultures were imaged and photographed daily, and the surface area of the blastocoele and trophectoderm were assessed by morphology and/or quantified using ImageJ (NIH). Following culture, embryos were scraped off of the dish and genotyped.

### Immunohistochemistry

Immunohistochemistry was performed as previously described (Artus et al., 2010). Embryos were cultured in DMEM/HEPE ( Gibco, 12430-054) and 10% FBS (Hyclone, Cat #SH30071.03 Lot #ARG27092) for 20 min at 37°C in 5% CO2, fixed in 4% paraformaldehyde with 0.1% Tween20 and 0.01% Triton X-100, and washed three times with PBT with 0.1% Tween-20 and 0.01% Triton X-100 ( Fisher, BP151-500) for 10 min at room temperature or overnight at 4°C, washed in PBT (PBS with 0.1% Triton X-100), permeabilized with 0.5% Triton X-100 for 20 min at room temperature and washed three times with PBT. Antigens were unmasked with NH2Cl ( Sigma, A-4514) in PBT for 10 min at room temperature. Following washes, embryos were blocked in 2% donkey serum in PBT for 45 min at room temperature, incubated with the first primary antibody overnight at 4°C in 2% donkey serum, washed three times with PBT and incubated with the second primary antibody overnight at 4°C in 2% donkey serum. The following day, embryos were washed three times with PBT, incubated with secondary antibody overnight at 4°C in the dark, washed three times with PBT and incubated with Hoechst 33342 (Sigma, B2261) diluted 1:500 overnight at 4°C in the dark. Embryos were analyzed using a Nikon A1R confocal microscope and NIS Elements v4.0 software. Embryos were then lysed and genotyped with Huceral II polimerase. Statistics were analyzed using Fisher’s Exact Probability test. Antibodies used were rabbit anti-NANOG (Abcam, ab80892; 1:50), rabbit anti-FGF (Invitrogen, A11122; 1:50), anti-GATA-4 ( Santa Cruz Biotechnology, sc1237; 1:100) and anti-ODC1 (Developmental Studies Hybridoma Bank, CPTC-ODC1-1-s; 1:10).

### Embryonic diapause

Diapause was induced in pregnant females at E2.5 by subcutaneous injection of 3 mg DMPA suspended in PBS (Medroxyprogesterone...
17-acetate; Sigma, M1629-1g) and intraperitoneal injection of 20 µg tamoxifen in sunflower seed oil (Sigma, T5648-1g). Embryos were flushed from the uterus with M2 1-7 days later.

ESC derivation and culture

Mga

ESC lines were derived using established protocols (Battle-Morera et al., 2008). Briefly, diapause was induced at E2.5, embryos were recovered at E4.5, washed in Ham’s F12 media, incubated in 20% rabbit anti-mouse serum antibody (Sigma, M5774) in Ham’s F12 for 30 min at 37°C in 7% CO2 in air. Embryos were then incubated with 20% guinea pig complement (CalBiochem, 234395) in Ham’s F12 for immunosurgical isolation of ICMs. ICMs were plated on gelatin-coated tissue culture dishes in mES media supplemented with recombinant human 100 µg/ml BMP4 (R&D Systems, 314-BP-010) and 50 mM MEK inhibitor PD98059 (Cell Signaling Technology, 9900S). After 7-10 days, ICM outgrowths were trypsinized with 0.25% trypsin and replated on mitomycin-C (Sigma, M4287)-treated MEFs. Inversion of the Mga allele was induced using 0.5 µM 4-hydroxytamoxifen (Sigma, H6278). Some cultures were supplemented with 200 µM putrescine (Sigma, P5780-5g) or 200 µM cadaverine (Sigma, D22606) dissolved in water. For cell counts, cells were allowed to attach over night and were then treated for 48 h, trypsinized and counted on a hemocytometer. Statistics were analyzed using Student’s t-test.

RNAi embryo production, microinjection and culture

B6D2F1 female mice were superovulated with 5 IU pregnant mare’s serum gonadotropin (PMSG) (Sigma) followed by 5 IU human chorionic gonadotropin (hCG) (Sigma) 48 h later and were mated with B6D2F1 males. Zygotes were collected at 21 h post hCG. Cumulus cells were resuspended in RNase-free water. The concentration of dsRNA was adjusted to allow successful microinjection. Approximately 5-10 pl dsRNA was loaded into a microinjection pipette and constant flow was maintained. 1 µg/µl dsRNA was treated for 48 h, trypsinized and counted on a hemocytometer.

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. In vitro transcription was performed using T7 MEGAscript Kit (Ambion), and 0.5 µl of TURBO RNase-free DNase was added to each 10-µl reaction to remove the DNA template. dsRNA was treated with NucAway Spin Columns (Ambion) to recover the dsRNA. The dsRNA was then extracted with phenol:chloroform, precipitated with 70% ethanol and resuspended in RNase-free water. The concentration of dsRNA was measured by NanoDrop, diluted to 1 µg/ml and stored at −80°C until use.

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Competing interests

The authors declare no competing financial interests.

Author contributions

W.W. and T.F. designed and produced the gene trap line and carried out preliminary experiments. K.Z. and J.M. designed and carried out the dsRNA experiments. A.J.W. and V.E.P. developed the concept, designed and carried out experiments and data analysis, and prepared and edited the manuscript. All authors edited and approved the final manuscript.

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