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Argonaute 2 Is Required for Extra-embryonic Endoderm Differentiation of Mouse Embryonic Stem Cells

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SUMMARY

In mouse, although four Argonaute (AGO) proteins with partly overlapping functions in small-RNA pathways exist, only Ago2 deficiency causes embryonic lethality. To investigate the role of AGO2 during mouse early development, we generated Ago2-deficient mouse embryonic stem cells (mESCs) and performed a detailed characterization of their differentiation potential. Ago2 disruption caused a global reduction of microRNAs, which resulted in the misregulation of only a limited number of transcripts. We demonstrated, both in vivo and in vitro, that AGO2 is dispensable for the embryonic germ-layer formation. However, Ago2-deficient mESCs showed a specific defect during conversion into extra-embryonic endoderm cells. We proved that this defect is cell autonomous and can be rescued by both a catalytically active and an inactive Ago2, but not by Ago2 deprived of its RNA binding capacity or by Ago1 overexpression. Overall, our results suggest a role for AGO2 in stem cell differentiation.

INTRODUCTION

RNA-silencing pathways play an important role in a wide range of biological processes, including regulation of animal development, cell proliferation, and differentiation (Ghildiyal and Zamore, 2009). In mammals, microRNAs (miRNAs) are the main RNA-silencing molecules and are involved in the post-transcriptional regulation of gene expression (Jonas and Izaurralde, 2015). miRNAs are about 22 nt long RNA molecules processed from longer hairpin transcripts by the consecutive cleavage of the RNase III-like enzymes DROSHA and DICER (Ha and Kim, 2014). Mature transcripts by the consecutive cleavage of the RNase III-like enzymes DROSHA and DICER (Ha and Kim, 2014). Mature miRNAs are subsequently loaded into an Argonaute (AGO) protein to form the RNA-induced silencing complex, which leads to the destabilization or translational repression of the target transcripts. Out of the four AGO proteins (AGO1–4) that share structural similarities (Meister, 2013) and have overlapping functions in the miRNA pathway (Su et al., 2009; Wang et al., 2012a), only AGO2 possesses an endonuclease catalytic activity (Liu, 2004). In mice, the deletion of the Ago2 gene leads to embryonic lethality, while Ago1, Ago3, and Ago4 deletions are viable (Alish et al., 2007; Che loufi et al., 2010; Liu, 2004; Modzelewski et al., 2012; Morita et al., 2007; O’Carroll et al., 2007; Van Stry et al., 2012). The developmental arrest observed in Ago2 mutant embryos occurs post-implantation and is not dependent on its catalytic activity (Che loufi et al., 2010).

At implantation, the mouse embryo is composed of three lineages: the trophectoderm that will give rise to the placenta, the primitive endoderm (PrE) contributing to the yolk sac, and the epiblast (EPI), which will form the embryo proper (Arnold and Robertson, 2009; Rossant and Tam, 2009). The EPI and PrE are specified within the inner cell mass (ICM) at the blastocyst stage. This process is characterized by the mutually exclusive expression of lineage-specific transcription factors: NANOG in the EPI-biased cells versus GATA6 in the PrE-biased cells (Chazaud and Yamanaka, 2016). The fibroblast growth factor 4/mitogen-activated protein kinase (FGF4/MAPK) signaling pathway governs this cell-fate choice by promoting the expression of PrE genes and the repression of EPI genes within the initially homogeneous ICM (Chazaud et al., 2006; Kang et al., 2012; Nichols et al., 2009; Yamana ka et al., 2010). In this process, Gata6 is downstream of the FGF-signaling pathway and upstream of secondary extra-embryonic endoderm (ExEn) genes such as Gata4, Pdgfra, and Sox17 (Artus et al., 2010, 2011; Chazaud et al., 2006; Niakan et al., 2010; Plusa et al., 2008). Gata6 mutant embryos die post-implantation and display a compromised PrE differentiation (Bessonnard et al., 2014; Koutsouarakis et al., 1999; Morrisey et al., 1998; Sch rode et al., 2014). In vitro, Gata6 mutant mouse embryonic stem cells (mESCs) cannot induce the expression of ExEn genes and fail to establish ExEn lineage during embryoid body (EB) formation (Capo-chichi et al., 2005).

In order to investigate the roles of AGO2 in pluripotent stem cells, we generated Ago2 knockout (Ago2KO) mESCs and assessed their differentiation potential in vitro and
Figure 1. Characterization of Ago2 Knockout mESCs and Impact on Gene Expression

(A) Top: schematic representation of the CRISPR/Cas9-mediated Ago2 KO strategy. Bottom: representative pictures of the wild-type (WT) and Ago2 KO mESC clones. Scale bars, 50 μm.

(B) Relative expression of Ago2 mRNA in WT, Ago2_KO1, and Ago2_KO2 mESCs, measured by RT-qPCR. The error bars show the SD of three independent experiments.

(C) Western blot analysis of AGO2 and OCT4 protein levels in WT and Ago2 KO mESCs. TUBULIN is used as loading control.

(D) Relative expression of Oct4, Nanog, and Sox2 mRNAs in WT and Ago2_KO cells, measured by RT-qPCR. The error bars show the SD of three independent experiments.

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in vivo. Although Ago2_KO mESCs successfully differentiate toward the three embryonic germ layers, they were unable to give rise to ExEn during EB differentiation and conversion of mESCs to ExEn stem cells (XEN) (Cho et al., 2012; Kunath, 2005). The deletion of Ago2 results in an impaired expression of GATA6 protein and ExEn genes during in vitro XEN conversion, leading to a cell-autonomous differentiation defect. The observed phenotype is specific to Ago2 and overexpression of AGO1 in Ago2_KO mESCs does not rescue the phenotype. Finally, we demonstrate that the catalytic function of AGO2 is not required, whereas its small-RNA binding capacity is essential for the XEN conversion of Ago2_KO mESCs. Our findings provide insights into the understanding of AGO2 function during stem cell differentiation and identify AGO2 as a player in ExEn formation in vitro.

RESULTS

Generation of Argonaute Knockout Mutant mESCs
In order to assess the role of Ago2 in mESC differentiation, we generated two independent mESC knockout clones (Ago2_KO1 and Ago2_KO2) carrying different genomic deletions generated through CRISPR/Cas9 genome editing (Doudna and Charpentier, 2014) (Figure 1A, top). Inactivation of Ago2 was confirmed at the RNA and protein levels (Figures 1B and 1C). Both clones did not show any obvious morphological difference compared with the wild-type (WT) mESC colonies (Figure 1A, bottom) and presented no changes in expression of the core pluripotency factors Oct4, Nanog, and Sox2 (Figures 1C and 1D).

As AGO1 is the only other AGO protein expressed in mESCs, we generated Ago1_KO mESCs (Ago1_KO1 and Ago1_KO2) using the same gene deletion strategy (Figures S1A–S1C). Although the alteration of the miRNA biogenesis pathway may negatively affect the cell cycle (Greve et al., 2013), we observed no significant variation in the cell cycle for Ago2_KO and Ago1_KO compared with WT mESCs (Figure 1E). Interestingly, we noticed an upregulation of AGO1 at the protein level but not at the mRNA level in Ago2_KO mESCs (Figures S1B and S1C), consistent with the notion that increased availability of miRNAs consecutive to the absence of AGO2 may impact the stability of AGO1 (Smibert et al., 2013).

Ago2 Knockout mESCs Show Reduced miRNA Levels with a Limited Impact on the Transcriptome
The generation of small-RNA libraries from WT, Dicer_KO, Ago1_KO, and Ago2_KO mESCs revealed a global reduction of the whole miRNA population (3.8-fold) in Ago2_KO and no significant downregulation of miRNA levels in Ago1_KO compared with WT mESCs (Figures 1F and S1D and Table S1). As expected, the Dicer_KO cells showed a strong reduction of miRNAs (13.4-fold) (Figure 1B). The expression of selected miRNAs was validated by northern blot (Figure S1E, top).

To assess whether all miRNAs could be loaded into AGO1 in the absence of AGO2, we performed immunoprecipitation of small RNAs charged in either AGO1 or AGO2 proteins in WT and Ago2_KO cells. While all miRNAs are bound to both proteins in WT mESCs, we observed a stronger enrichment of some miRNAs loaded in AGO1 in Ago2_KO (defined as group 1), whereas other miRNAs (defined as group 2) were not or were poorly loaded in AGO1 (Figure 1G). Those group 2 miRNAs were more affected by the loss of AGO2, incorporated less into AGO1, and were not detectable by northern blot in Ago2_KO mESCs (Figures 1G and S1E, bottom). Nevertheless, we did not detect any miRNA-specific sequence or length signatures distinguishing the two groups.

To establish the consequences of the global miRNA downregulation in Ago2_KO mESCs, we analyzed the transcriptome of WT and Ago2_KO mESCs by RNA sequencing (RNA-seq). Surprisingly, we identified only 22 genes significantly upregulated in Ago2_KO compared with WT mESCs, which could be potential direct miRNA targets.

(E) Cell-cycle analysis of WT, Ago2_KO1, Ago2_KO2, Ago1_KO1, and Ago1_KO2. Stacked bars represent the percentage of cells in G2/M, S, or G1 phase of the cell cycle. The values were obtained from three independent experiments.
(F) Boxplots depicting the global levels of miRNAs in WT, Ago2_KO, Ago1_KO, and Dicer_KO mESCs measured by small-RNA-seq. The normalized miRNA reads are represented as the mean of two replicates (except for Dicer_KO) in counts per 10 million (CPTM). The significance (****p < 0.0001) was assessed using non-parametrical Mann-Whitney ranked test. The fold change (FC) relative to WT cells was calculated by comparing the mean miRNA levels in each condition.
(G) Scatterplot representing the miRNA levels in CPTM after AGO1 and AGO2 immunoprecipitation compared with WT mESCs. AGO2- and AGO1-loaded miRNAs in WT cells are represented in blue and green. The AGO1-loaded miRNAs in Ago2_KO mESCs from groups 1 and 2 are represented in red. Group 2 defines miRNAs 2-fold less enriched in Ago2_KO compared with WT mESCs. The remaining miRNAs are defined as group 1. Four miRNAs from group 2, validated by northern blot (Figure S1E), are highlighted.
(H) Volcano plots representing the differentially expressed genes in Ago2_KO (left) and Dicer_KO (right) cells compared with WT mESCs. RNA-seq data represent the mean expression of two independent biological replicates. Significantly (fold change >2 and adjusted p value <0.01) up- and downregulated genes are highlighted in red and blue, respectively. See also Figure S1.
Figure 2. Embryoid Body Differentiation of Ago2_KO mESCs
(A) Schematic representation of the EB differentiation protocol. LIF, leukemia inhibitory factor.
(B) Western blot showing the expression of AGO2, FOXA2, OCT4, GATA6, and NANOG proteins after 0, 2, 4, 6, and 10 days of EB differentiation of WT and Ago2_KO1 mESCs. TUBULIN is shown as loading control.

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Impaired Expression of Extra-embryonic Endoderm Markers in Ago2 KO Embryoid Bodies

To assess the role of Ago2 in mESC differentiation, we generated EB from Ago2 KO and WT mESCs and monitored the expression of different markers (Figures 2A–2C). During EB formation, mESCs differentiate into cell types derived from the three germ layers surrounded by an outer layer of ExEn. For both the Ago2 KO and WT cells, we observed a similar downregulation of the pluripotency factor OCT4 (Figure 2B) and upregulation of ectoderm (Nestin, Fgf5, Pax6) and mesoderm (Brachyury, Fgf8, Actc1) markers (Figure 2C) over time. In contrast, the expression of Gata6, Gata4, and Foxa2, expressed in both extra-embryonic and definitive endoderm (DE), was severely impaired in Ago2 KO compared with WT cells (Figures 2B and 2C). Interestingly, expression of Cxcr4, Cldn6, and Foxa1, which are preferentially expressed in DE (Wang et al., 2012b), was similar in Ago2 KO1 and WT differentiating cells (Figure S2A). In contrast, the expression of Dab2, a gene preferentially expressed in ExEn, was strongly reduced in Ago2 KO compared with WT cells (Figure 2C), suggesting that ExEn but not DE differentiation was affected in Ago2-deficient cells. To confirm this observation, we used a direct differentiation protocol of mESCs toward DE (Figure S2B) (Gouon-Evans et al., 2006) and found that Ago2 KO and WT cells were equally competent to give rise to DE precursor cells expressing high levels of CXCR4 and c-KIT (Figures S2C and S2D). We subsequently performed immunostaining on sections of 10 day old EBs (Figure 2D). Contrary to WT EBs, having an outer epithelial layer of ExEn cells expressing GATA6, SOX17, GATA4, and DAB2, the majority of the Ago2 KO EBs were lacking an outer epithelial layer and showed a strongly reduced expression of GATA6, SOX17, and DAB2 (Figure 2D). A variable expression of GATA4 was still observed on the outer layer in some Ago2 KO EBs. Moreover, we noticed a persistent weak expression of NANOG pluripotency factor in Ago2 KO EBs compared with WT, by both western blot and immunofluorescence (Figures 2B and 2D). The defect in ExEn differentiation was specific for the Ago2 KO mESCs, as both normal expression of markers and differentiation of an outer epithelial layer were observed for Ago1 KO mESCs (Figures S2E and S2F). Altogether, our data show that in the absence of Ago2, mESCs can exit from the pluripotency state and differentiate into derivatives of the three germ layers, but are defective in the formation or the maintenance of ExEn.

Ago2-Deficient mESCs Differentiate into Embryonic Germ Layers

To further investigate whether Ago2 KO mESCs were able to differentiate into DE in vivo, we generated chimeric mice with untagged and GFP-tagged WT and Ago2 KO mESCs (Figures S3A and S3B). Chimeras were obtained with similar efficiency following injections of mESCs of both genotypes into C57BL/6N blastocysts (Figures 3A and S3C–S3E). Mouse ESC contribution to internal organs was monitored by simple sequence length polymorphism (SSLP) genotyping PCR on mouse tissues derived from the three embryonic germ layers: ectoderm (brain, eyes, skin), DE (thymus, lung, liver), and mesoderm (heart, spleen, kidney). We took advantage of several microsatellite repeats that are polymorphic between injected mESCs (E14 line, 129/Ola strain) and the recipient blastocyst (C57BL/6 strain) (Figures 3A, S3C, S3D, and S3F). Similar variable contribution to internal organs was observed for both WT and Ago2 KO mESCs. We also monitored the contribution of WT-GFP and Ago2 KO-GFP mESCs to the adult pancreas by fluorescence microscopy (Figure 3B). We observed a clear co-localization of GFP with insulin in beta-islet cells and with pancreatic amylase in acinar/duct cells. In addition, we performed fluorescence-activated cell sorting analysis of dissociated pancreatic cells using the FOXA2 DE marker (Figure 3C). A similar proportion of GFP-positive cells among FOXA2-positive pancreatic cells was observed in both WT and Ago2 KO chimeras, indicating that Ago2 KO-GFP mESCs contributed normally to the formation of DE-derived adult pancreatic lineages. Since FOXA2 marker showed no co-staining with immune cell markers (CD45* and CD11b) (Figure S3G), we can exclude a contribution from infiltrating immune cells of mesodermal origin to the FOXA2 and GFP double-positive

(C) Relative gene expression of ectoderm, mesoderm, and endoderm markers in WT, Ago2 KO1, and Ago2 KO2 mESCs during EB differentiation measured by RT-qPCR. The levels at day 6 (d6) and day 10 (d10) are represented relative to the levels in WT cells at day 0 (d0). Error bars represent the SD of three biological replicates. The dashed line shows levels at d0.

(D) Immunofluorescence on EB sections after 10 days of EB differentiation of WT, Ago2 KO1, and Ago2 KO2 cells. The fluorescence signal of three different sections is shown for each of the proteins: NANOG, GATA6, SOX17, GATA4, and DAB2. The nuclei are stained with Hoechst 33342. Scale bar, 50 μm.

See also Figure S2.
Figure 3. Generation and Analysis of Chimeric Mice

(A) SSLP PCR genotyping on DNA from tissues derived from WT and Ago2_KO chimeras. The contribution of the cells to the tissues was assessed following D19mit19 and D18mit184 microsatellite length. mESCs (129/Ola strain) were injected into recipient blastocysts (C57BL/6 strain). DNA from 129/Ola and C57BL/6 mouse strains was used as control. The pictures of the tested chimeras with various degrees of coat chimerism are presented on the left side.

(B) Immunofluorescence analysis of sections of pancreas extracted from adult Ago2_KO-GFP and WT-GFP chimeras. Sections from WT C57BL/6N are used as GFP-negative controls. As previously, WT and Ago2_KO2 mESCs expressing GFP were injected into recipient C57BL/6 blastocysts. Representative epifluorescence pictures show GFP-expressing cells, insulin-expressing pancreatic beta islets, and amylase-expressing acinar cells in non-fixed sections. Some co-expressing cells are indicated with a white arrow. Nuclei are stained with DAPI.

(C) Flow-cytometry analysis of GFP and FOXA2 definitive endoderm marker expression in pancreatic cells derived from WT-GFP and Ago2_KO-GFP chimeras. Pancreatic cells from WT C57BL/6 mice are used as unstained control.

See also Figure S3.
pancreatic cell population. Taken together our data show that Ago2 is dispensable for the formation of the three embryonic germ layers including the DE.

**AGO2 Is Required for ExEn Stem Cell Conversion**

To better characterize the defect of Ago2 KO mESC differentiation into ExEn in vitro, we treated WT, Ago1 KO, and Ago2 KO mESCs with low doses of retinoic acid and Activin A in order to promote their differentiation into cXEN cells (Cho et al., 2012; Niakan et al., 2013) (Figure 4A). After 5 days of differentiation, we observed the formation of cXEN colonies exhibiting the characteristic epithelial-like morphology for both WT and Ago1 KO cells. In contrast, the two Ago2 KO lines predominantly showed an atypical fibroblast-like morphology (Figure 4A). The induction of secondary ExEn markers was strongly impaired in Ago2 KO compared with WT and Ago1 KO cXENs, while the expression of Gata6 mRNA was only slightly diminished (Figure 4B). Interestingly, NANOG and OCT4 proteins were not detectable after 5 days of XEN conversion in all genotypes, whereas GATA6 protein expression was strongly not detectable after 5 days of XEN conversion in all genotypes (Rugg-Gunn et al., 2010). Strikingly, while the removal of H3K27me3 repressive marks and the deposition of H3K27ac active marks on the Gata6 promoter were similar in both WT and Ago2 KO cells after conversion (Figures 4F and S4C), we observed a reduction of H3K27ac activation marks and an increase in H3K27me3 repression marks on the Gata4, Sox17, and Foxa2 promoters in Ago2 KO compared with WT cXENs (Figures 4F and S4C). These observations suggest a post-transcriptional regulation of Gata6 expression that leads to an impaired transcriptional induction of the secondary ExEn genes during conversion. Taken together, our data indicate that Ago2 KO cells fail to transcriptionally activate the proper cXEN gene expression program downstream of Gata6. They incorrectly engage in a different cell fate where Gata6 post-transcriptional regulation might be mediated by multiple miRNAs (Figure S4D), suggesting a complex cell-type-dependent combinatorial regulation.

**Figure 4. cXEN Conversion of WT, Ago2 KO, and Ago1 KO Cells**

(A) Schematic representation of the cXEN conversion protocol. Representative pictures of WT, Ago2 KO1, Ago2 KO2, Ago1 KO1, and Ago1 KO2 cells after 5 days of cXEN differentiation. cXEN colonies are indicated by white dashed lines. Scale bars, 50 μm.

(B) Relative gene expression of ExEn markers in WT, Ago1 KO, and Ago2 KO mESCs before (ESC) and after conversion (cXEN), measured by RT-qPCR. The error bars represent the SD of three biological replicates. The significance was assessed using a two-way ANOVA followed by Dunnett’s multiple comparison for the set of ExEn markers in Ago1 KO and Ago2 KO mutants compared with the WT condition after cXEN conversion. The calculated p values are WT versus Ago1 KO1 (*p = 0.1655, ns), WT versus Ago1 KO1 (p = 0.1270, ns), WT versus Ago2 KO1 (**p = 0.0061), and WT versus Ago2 KO2 (*p = 0.0417).

(C) Expression of the AGO1, AGO2, GATA6, NANOG, and OCT4 proteins before (ESC) and after (cXEN) conversion of WT, Ago1 KO1, and Ago2 KO1 cells by western blot. TUBULIN is shown as loading control for the two membranes used for blotting.

(D) Western blot analysis of GATA6 and NANOG levels at several time points during cXEN conversion of WT and Ago2 KO1 cells. Coomassie staining (CM) of the membrane is shown as loading control.

(E) Relative expression of ExEn markers during time series of cXEN conversion of Ago2 KO and WT cells, measured by RT-qPCR. The error bar represents the range of three independent experiments. For each gene the statistical significance of the variation caused by the absence of Ago2 over the time of differentiation (two factors) was assessed using a two-way ANOVA. The p values obtained for each gene are p < 0.0001.

(F) ChIP-qPCR experiments on Gata6, Gata4, Sox17, and Foxa2 promoters before and after cXEN conversion of WT (blue/light blue) and Ago2 KO (red/light red) cells. Antibodies against SUZ12, RING1B, H3K27me3, and H3K27ac were used. The enrichment was calculated compared with a repressed (Cd2) and an active (Smad3) promoter. An intergenic region was used as negative control. The error bars correspond to the SD of three independent experiments. See also Figure S4.
20% of the transcripts were misregulated: 2,514 genes were significantly up- and 2,821 downregulated in Ago2 KO compared with WT cXENs (Figure 5B). In accordance with our previous results (Figure 4C), pluripotency markers were strongly downregulated in both WT and Ago2 KO cXENs, while ExEn markers were highly induced only in WT cXENs (Figure 5C). Although both mesodermal and trophectodermal markers were not induced in Ago2 KO cXENs, we noted an induction of mesenchymal markers, ectodermal, and fibroblast-related markers (Figures 5D and 5B). This highly divergent gene expression profile is also translated at the level of several signaling pathways such as WNT, TGF-beta, MAPK, or HEDGEHOG pathways (Figures 5E and 5C and Table S5), critical for regulation
of the cell-fate choice during early development (Frum and Ralston, 2015). Collectively our data indicate that Ago2 KO mESCs do not adopt an ExEn identity and differentiate into an undefined fibroblast-like cell type (Figures 4A and 5D).

**Ago2 KO cXEN Differentiation Failure Is Cell Autonomous**

*In vivo*, the establishment of ExEn is driven by FGF4, which is secreted by EPI-biased cells (Kang et al., 2012). Addition of FGF4 to the conversion medium of Ago2 KO mESCs did not rescue GATA6 protein level, nor the expression of ExEn markers (Figures 6C and S6B), neither the formation of XEN colonies (Figure S6C). Similarly, co-culture of Ago2 KO mESCs with GFP-labeled WT mESCs did not rescue their differentiation defects as shown by the absence of cXEN colony formation (Figure S6D) or expression of the endoderm protein FOXA2 for Ago2-deficient GFP-negative cells (Figures 6B and S6A). These data demonstrate that the differentiation defects of Ago2 KO mESCs are cell autonomous.

**FGF/ERK Pathway Correctly Regulates NANOG Expression in Ago2 KO mESCs**

Similar to WT cXEN, Ago2 KO cells expressed the Fgfr2 receptor and downregulated the expression of Fgf4 and Nanog mRNAs after cXEN conversion (Figure S6D), indicating that the cells normally initiate their differentiation program. Therefore, we reasoned that the defect could be related to the FGF/ERK signaling cascade. Consequently, we verified the functionality of this pathway by treating WT and Ago2 KO mESCs with two FGF receptor inhibitors (AZD4547; PD173074) and one MEK inhibitor (PD0325901) (Figure S6E). Phosphorylation of ERK was strongly reduced in the presence of the inhibitors and correlated with an upregulation of the NANOG protein in both WT and Ago2 KO mESCs. Conversely, the addition of FGF4 slightly reduced NANOG levels (Figure S6E). Notably, we observed a different response to the FGF/ERK inhibitors in the Ago2 KO compared with WT cells during the conversion to cXEN, as NANOG was downregulated even though the FGF/ERK signaling pathway was inhibited (Figure S6F). Taken together these experiments demonstrate that although the FGF/ERK pathway correctly regulates NANOG expression in Ago2 KO mESCs, its inhibition fails to maintain pluripotency under differentiation culture conditions as it does for WT mESCs.

**Ago2 KO cXEN Differentiation Failure Is Bypassed by GATA6 Overexpression**

It has been shown that GATA6 overexpression alone is sufficient to reprogram mESCs into XEN cells (Shimosato et al., 2007). Therefore, we generated mESC WT and Ago2 KO clones expressing GATA6 in an inducible manner. After 3 days of induction, we observed the formation of XEN-like colonies and high expression of ExEn markers in both WT-GATA6 and Ago2 KO-GATA6 cells (Figures 6D and S6G). Therefore, GATA6 expression is sufficient to induce XEN conversion in Ago2 KO mESCs, bypassing the misregulation observed in the context of XEN conversion. Taken together our data show that the XEN conversion impairment of Ago2-deficient mESCs is cell autonomous and can be rescued by exogenous GATA6 expression.

**The AGO2 Small-RNA Binding Capacity Is Required during XEN Conversion**

Finally, we performed rescue experiments by ectopically expressing inducible hemagglutinin (HA)-tagged WT (HA-GATA2), catalytic mutant D699A (CATmut) (O’Carroll et al., 2007), and small-RNA-binding mutant Y311A/Y312A (RNAmut) (Gao et al., 2014) Ago2 cDNA in Ago2 KO mESCs (Figure 7A, top). After 5 days of induction, we observed a strong restoration of AGO2 protein levels with the CATmut Ago2 transgene and a weaker but comparable restoration with both the HA-tagged WT Ago2 and the RNAmut Ago2 transgenes (Figure 7A). During cXEN conversion, induction of the HA-GATO2 and CATmut effectively rescued the impaired expression of ExEn markers (Figure 7B). Moreover, the expression level of groups 1 and 2 miRNAs, as well as the formation of cXEN colonies, was restored, despite the lower level of AGO2 protein in HA-Ago2 compared with WT mESCs (Figures 7A, 7C, and 7D). In contrast, the RNAmut transgene expression did not induce any significant change in ExEn markers nor miRNA stabilization and the cells presented a fibroblast-like morphology similar to that of the Ago2 KO cells after conversion (Figures 7B–7D).

To assess if AGO1 might rescue Ago2 phenotypes, we overexpressed AGO1 in Ago2 KO mESCs (Figures 7E and S7A). No significant induction of ExEn markers and no formation of XEN-like colonies were observed in Ago2 KO-AGO1 cells after cXEN differentiation (Figures 7F and 7G). Nevertheless, we observed a global restoration of the expression of both groups of miRNAs before cXEN conversion (Figures S7B–S7D and Table S6). The functionality of these restored miRNAs was also validated by measuring the level of potential miRNA targets, upregulated in Ago2 KO cells (Figures S1F and S7E). Taken together, our results show that AGO2 but not AGO1 is required for the correct induction of the ExEn markers and the formation of cXEN colonies. We demonstrated that AGO2 small-RNA binding capacity but not its catalytic activity is essential for this differentiation process. Moreover, AGO1 overexpression and miRNA restoration is not sufficient to rescue Ago2 KO XEN conversion defect.
DISCUSSION

In this study, we used mESCs and their differentiation derivatives as a model system to investigate the underlying causes of the post-implantation lethality of the Ago2 mutant mouse embryos. By generating Ago1- and Ago2-deficient mutant mESCs in the same genetic background, we demonstrated that the deletion of either one of these two genes had no significant effect on mESCs, cell cycle, and self-renewal. Deletion of Ago2 compromised miRNA...
Figure 7. XEN Conversion of mESCs Requires the Small-RNA Binding Capacity of AGO2

(A) Schematic representation of the inducible constructs used for the ectopic expression of HA-tagged AGO2, catalytic AGO2 mutant (CATmut), and small-RNA-binding deficient PAZ domain AGO2 mutant (RNAmut) in Ago2 KO mESCs. Expression of the AGO2 constructs after 5 days of induction with doxycycline (dox) is shown. TUBULIN is used as loading control.

(B) cXEN conversion of Ago2 KO complemented mutants with or without doxycycline. The relative expression of ExEn markers was measured by RT-qPCR before (ESC) and after conversion (cXEN). The error bars represent the SD of three independent experiments.

(legend continued on next page)
levels, with a weak impact on the transcriptome, indicating its preponderant role in the miRNA pathway in mESCs and potential compensatory function of the AGO1 protein, in line with previous observations (Su et al., 2009). Indeed, the upregulated AGO1 stabilizes the steady-state miRNA level in Ago2 KO mESCs. This observation is also in accordance with the previously described correlation between AGO abundance and miRNA availability (Martinez and Gregory, 2013; O’Carroll et al., 2007; Wang et al., 2012a; Winter and Diederichs, 2014). Notably, miRNAs moderately (group 1) or strongly affected (group 2) by Ago2 deletion were broadly rescued by AGO1 ectopic overexpression, demonstrating a functional overlap of the two AGO proteins in miRNA loading and stabilization in mESCs.

The miRNA levels have been shown to be important for embryonic development (Greve et al., 2013). Indeed, Dicer mutant embryos deprived of miRNAs die after implantation at 6.5 dpp (Bernstein et al., 2003) and Dicer KO mESCs can be maintained in vitro but cannot exit pluripotency (Bodak et al., 2017). The in vitro differentiation of Ago2 KO mESCs revealed that even with reduced miRNA levels, the cells effectively differentiate toward ectodermal, mesodermal, and DE lineages. The successful generation of chimeric mice using both Ago2 KO mESC clones demonstrated that Ago2 is dispensable for the post-implantation differentiation processes of the embryo. Using a cXEN conversion protocol, we proved that AGO2 is required for ExEn differentiation. Indeed, our study revealed a severe in vitro conversion defect of Ago2 KO mESCs toward XEN cells. The requirement of Ago2 for the ExEn cell-fate choice is relevant in vivo, as it is expressed from the oocyte to the blastocyst stage (Lykke-Andersen et al., 2008; Park et al., 2015; Wu et al., 2016) and has been shown to be present both in embryonic and in extra-embryonic tissues (Cheloufi et al., 2010) at embryonic day E9.5. In previous in vivo studies, Ago2 mutant mice were generated using insertional mutations after exon 12, after exons 3–6 (Alish et al., 2007; Liu, 2004), or after exon 1 (Morita et al., 2007). The three studies observed an early embryonic lethality phenotype and described a developmental arrest ranging from implantation to mid-gestation. Although placenta defects have been shown to contribute to the embryonic lethality of Ago2-deficient embryos (Cheloufi et al., 2010), the cell-autonomous ExEn differentiation defects that we observed in vitro may also contribute to their in vivo developmental defects. Further, in vivo studies are required to clarify the essential role of AGO2 in PrE formation and differentiation during early development.

We demonstrated that the cXEN conversion of mESCs requires AGO2 small-RNA binding capacity but not its catalytic activity, suggesting that AGO2 loaded with a small-RNA molecule may be functionally required for this process or for the stabilization of the AGO2 protein itself. Interestingly, our experimental data indicate that the global level of miRNAs is not critical for ExEn differentiation, as its restoration by AGO1 ectopic overexpression does not rescue the Ago2 KO conversion defect. A previous study analyzed the miRNA content of cells derived from extra-embryonic tissues and highlighted their importance using Dicer KO mutants (Spruce et al., 2010), in which AGO2 is per se downregulated due to the lack of miRNAs (Smibert et al., 2013), making it impossible to dissociate both phenotypes. Our findings point toward the presence of either some AGO2-specific small RNAs or non-canonical AGO2-specific functions during the conversion process. Furthermore, AGO2 subcellular localization may also be altered when the protein has impaired small-RNA loading (Nishi et al., 2015; Schraivogel et al., 2015) and in turn be responsible for the differentiation defect.

The considerable variation of gene expression profiles and morphology between Ago2 KO and WT cXENs indicates that Ago2 KO cells differentiate into a fibroblast-like cell fate expressing mesenchymal markers instead of epithelial markers, normally expressed by XEN cells (Brown et al., 2010). This impaired differentiation of Ago2 KO cells into a different cell lineage is also illustrated by their inconsistent response to the FGF/ERK pathway inhibitors on NANOG and GATA6 expression (Figures S6E and S6F). Although they exit pluripotency during conversion, Ago2 KO cells fail to properly express GATA6.
protein levels and consequently to activate the ExEn gene expression program downstream of GATA6. Importantly, we were able to bypass this defective regulation by ectopically expressing Gata6 cDNA deprived of its 3’ UTR in Ago2 KO cells. Ago2 KO fibroblast-like cells have distinctive mRNA and miRNA expression profiles compared with WT XEN cells. Some of the upregulated miRNAs are loaded into AGO1 and can target the Gata6 3’ UTR, potentially explaining the downregulation of GATA6 protein. Alternatively, GATA6 could be destabilized in Ago2 KO cells due to the downregulation of an unknown cofactor, which would specifically stabilize GATA6 by preventing its degradation, like BMI1 in fully differentiated XEN cells (Lavial et al., 2012).

Finally, the role of AGO2 in XEN differentiation might also be relevant for the de-differentiation or reprogramming of somatic cells, as it has been recently demonstrated that an intermediary XEN-like state is essential for chemical generation of induced pluripotent stem cells (Zhao et al., 2015). Interestingly, our study shows that AGO2 is specifically required for the induction of XEN cell fate from mESCs, which underlines the importance of a specific RNAi pathway in a directed differentiation process.

**EXPERIMENTAL PROCEDURES**

**Chimeric Mice Generation**

Blastocyst micro-injections were performed at the EPIC transgenic facility (ETH, Zurich). For details see the Supplemental Experimental Procedures. All experiments were conducted according to Swiss law of animal protection and with the approval of the local institutional animal care committees (licence nos. 106/2013 and ZH123/16).

**mESC Culture**

The E14 mESC line (129/Ola background) was used and cultured as described previously (Bodak et al., 2017). If required, the medium was supplemented with 1 μg/mL doxycycline, 1 μM retinoic acid, 2 μM PD0325901, 0.1 μM PD173074, 1 μM AZD4547, or 500 ng/mL FGF4 + 1 μg/mL heparin.

**CRISPR/Cas9-Mediated Gene Knockout**

The Ago2 KO and Ago1 KO cell lines were generated using a paired CRISPR/Cas9 strategy on WT E14 mESCs as described previously (Wettstein et al., 2016).

**cXEN Conversion of mESCs**

The cXEN conversion was performed as described in Niakan et al. (2013).

**mRNA and miRNA Expression Analysis**

The mRNA and miRNA extraction and quantification were performed as described in Cirera-Salinas et al. (2017).

**Cross-Linked Immunoprecipitation of AGO1/AGO2-Bound Small RNAs**

The RNA immunoprecipitation was performed as described in Selth et al. (2011). The pulled-down small RNAs were analyzed by deep sequencing.

**ACCESSION NUMBERS**

The accession numbers for all the RNA-seq data reported in this paper are GEO: GSE78971 and GSE80454.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.12.023.

**AUTHOR CONTRIBUTIONS**

R.P.N. and C.C. conceived and performed experiments, interpreted the data, and wrote the manuscript with inputs from M.C.-T.; D.C.-S. performed most of the experiments for the revision of the manuscript; M.B., R.W., J.L., A.G., H.W., and S.V.-P. contributed to experiments; J.Y. and R.P.N. performed bioinformatics analysis; M.C.-T. performed experiments and provided expertise. C.C. supervised the study and secured funding.

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Embryonic stem cells (ES cells) have the potential to differentiate into any cell type in the body, making them a valuable tool for research and potential medical applications. However, ES cells are obtained from early embryos and this process is highly regulated to ensure proper differentiation and self-renewal. The transcription factors Oct4 and Sox2 play a crucial role in maintaining the undifferentiated (pluripotent) state of ES cells, while the expression of classical embryonic antigens like SSEA4 and TRA-1-81 marks the transition towards differentiation.

To further differentiate ES cells into specific lineages, factors such as FGFs and Wnt signaling play key roles. FGFs promote the maintenance of the undifferentiated state, whereas Wnt signaling can lead to differentiation. The balance between these signals is critical for the proper development of ES cells.

The importance of proper protein interactions is also evident in the context of ES cell differentiation. For instance, the importin-β protein interacts with nuclear import factors to facilitate the nuclear import of proteins into the cell nucleus. This process is crucial for the correct localization and function of proteins within the cell.

In summary, the maintenance of ES cell pluripotency involves a complex interplay of transcription factors, extracellular signals, and intracellular protein interactions. Understanding these processes is crucial for the development of therapies that can utilize ES cells for regenerative medicine.