Argonaute-2-null embryonic stem cells are retarded in self-renewal and differentiation

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RNA interference (RNAi) pathways regulate self-renewal and differentiation of embryonic stem (ES) cells. *Argonaute 2* (*Ago2*) is a vital component of RNA-induced silencing complex (RISC) and the only Ago protein with slicer activity. We generated *Ago2*-deficient ES cells by conditional gene targeting. *Ago2*-deficient ES cells are defective in the small-RNA-mediated gene silencing and are significantly compromised in biogenesis of mature microRNA. The self-renewal rate of *Ago2*-deficient ES cells is affected due to failure of silencing of *Cdkn1a* by ES-cell-specific microRNAs (miRNA) in the absence of *Ago2*. Interestingly, unlike *Dicer*- and *Dgcr8*-deficient ES cells, they differentiate to all three germ layers both *in vivo* and *in vitro*. However, early differentiation of *Ago2*-deficient ES cells is delayed by 2–4 days as indicated by persistence of higher levels of self-renewal/ pluripotency markers during differentiation. Further, appearance of morphological and differentiation markers is also delayed during the differentiation. In this study we show that *Ago2* is essential for normal self-renewal and differentiation. Also, our data suggest that self-renewal and differentiation of ES cells are regulated by both siRNA and miRNA pathways.

1. Introduction

Self-renewal and pluripotency are the defining properties of embryonic stem (ES) cells. The delicate balance between self-renewal and differentiation is a result of complex interactions among various self-renewal and differentiation factors (Silva and Smith 2008; Chen and Daley 2008). Adding a new dimension to these complex interactions is the regulation of the pluripotency factors by miRNAs (Tay *et al.* 2008). Differentiation is accompanied by sharp changes in the mRNA and protein constitution. Suppression of pluripotency factors is prerequisite for the differentiation processes as evidenced from the reprogramming of terminally differentiated cells in the presence of these factors (Takahashi and Yamanaka 2006). Suppression of the pluripotency factors is achieved during differentiation by different mechanisms at various levels of gene expression, such as transcriptional repression (Gu *et al.* 2005), epigenetic modifications (Lagarkova *et al.* 2006) and post-transcriptional silencing by miRNAs (Tay *et al.* 2008). Among these, miRNAs can effect acute and rapid suppression of gene expression by target mRNA degradation or translational inhibition (Valencia-Sanchez *et al.* 2006).

Ago subfamily proteins form the core components in the RNA interference (RNAi) effector pathways. The mouse genome encodes four Ago subfamily proteins (AGO1–4), of which, AGO2 is unique in catalysing the cleavage of target mRNA guided by perfectly complementary small RNAs (Liu *et al.* 2004; Meister *et al.* 2004; Rand *et al.* 2004). The function of AGO2 in miRNA pathway is redundant with the function of other AGOs (Su *et al.* 2009), but its catalytic function in siRNA pathway cannot be compensated by other AGOs (Liu *et al.* 2004). It is also involved in biogenesis of mature miRNA from

pre-miRNA and post-transcriptional regulation of miRNA expression (O'Carroll *et al.* 2007; Diederichs and Haber 2007). The arrest of *Ago2*-knockout embryos at early stages of embryogenesis (Morita *et al.* 2007) is reminiscent of arrest in *Dicer1*- (Bernstein *et al.* 2003) and *Dgcr8*-knockout (Wang *et al.* 2007) embryos, suggesting an indispensable role in early post-implantation development. *Dicer1*- and *Dgcr8*-deficient ES cells self-renew but differentiate poorly (Wang *et al.* 2007) or not at all (Kanellopoulou *et al.* 2005; Murchison *et al.* 2005). All the four *Ago*s have an overlapping function in miRNA silencing, which is essential for survival of ES cells (Su *et al.* 2009). However, the function of *Ago2* – the only mammalian *Ago* that is essential for the siRNA pathway – is yet to be understood in the regulation of self-renewal and differentiation of ES cells. To explore this aspect, we generated *Ago2*-deficient ES cells by conditional gene targeting.

2. Materials and methods

2.1 *Generation of Ago2-null ES cells*

A targeting vector was constructed with 7.4 kb isogenic DNA comprising exon 12–14 from the R1.9 ES cell (Alex *et al.* 2005) genomic DNA. Exon 13 was floxed by inserting a *lox*P site upstream of exon 13 and a floxed Neomycin gene downstream of exon 13 into *Pst*I sites. HSV *tk* gene was placed at the 3′ end of the homology region to enrich for targeting events. R1.9 ES cells were electroporated with 40 μg of linearized vector and selected against 0.25 mg/ml of G418 (GIBCO) and 2 μM gancyclovir (Sigma). The resulting subclones were analysed by Southern hybridization with a probe external to the 5′ sequences included in the targeting vector. Resulting wild-type/floxed clones were cultured at higher levels – 1.5 mg/ml – of G418 to enrich for loss of heterozygosity events and analysed by Southern hybridization. The homozygous targeted clones obtained from the loss of heterozygosity experiments were analysed for karyotype, and only the clones with normal karyotype were used for further studies. The homozygous targeted clones were transfected with NLSCre-recombinase expressing plasmid and seeded at very low density to ensure formation of isolated colonies. The subclones were plated in replicates and selected for G418 sensitivity. G418-sensitive clones were analysed by Southern hybridization for deletion of the exon 13 and Neomycin gene (figure 1B).

2.2 *Tissue culture and teratoma assay*

ES cells were cultured on mouse embryonic feeder (MEF) cells in ES cell medium (Nagy *et al.* 2003). RNA isolation, embryoid body (EB) and monolayer differentiation were performed by depleting feeder cells by differential adherence to uncoated plates for 1 h, and ES cells were cultured for five passages on gelatinized plates without feeders. Monolayer differentiation was carried out by culturing ES cells on gelatinized plates in ES cell medium without leukemia inhibiting factor (LIF). For ES cells colony formation assay, 600–700 ES cells were cultured per well in gelatinized six-well plastic plates for 6 days and stained for alkaline phosphatase (Chemicon), and colonies were scored manually. ES cells were cultured in hanging drops for 2 days and then in low attachment culture dishes in ES cell medium without LIF to culture them as EBs. For embryoid growth curve, 2 days onwards, individual EBs were cultured in separate wells in 96-well plates and imaged every alternate day. A diameter of only spherical embryoid bodies was measured using image-pro express software. For teratoma assay, approximately 5 million cells were subcutaneously injected into nude mice and allowed to develop into teratomas. Five nude mice for each cell line were analysed. After 21 days, the teratomas were dissected, sectioned and stained with Haemetoxylin and Eosin.

2.3 *Dual luciferase assay*

Dual luciferase assay (Promega) was carried out by growing $15–18 \times 10^3$ cells in 48-well plates for 16 h before transfection. 140 ng of renilla luciferase plasmid (pSV40 – Promega) and 20 ng of firefly luciferase plasmid (pGL3 – control vector – Promega) were co-transfected in each well using Effectene reagent (Qiagen). 2.5 pmol of siRNA against renilla luciferase (Ambion) was transfected per well. The luciferase assay was performed as per the manufacturer's instructions.

2.4 *RNA analysis*

Small RNA was isolated using mirVana miRNA isolation kit (Ambion) as per the manufacturer's instructions and the Northern blot analysis was performed as described earlier (Lau *et al.* 2001). The probes used for pre-miRNA and mature miRNA were nucleotide probes complementary to the respective miRNAs. Blots were stripped in 1% SDS at 70°C for 1 h and then probed with U6 small nuclear RNA as the loading control. The densitometric quantification of the Northern blots was performed using ImageJ software (*www.rsbweb.nih.gov/ij/*) by gel analysis method as described in the user's guide. Expression levels of miRNAs, U6 RNA or 5S RNA of *Ago2*-null ES cells was plotted relative to the wild-type levels.

2.5 *Cell cycle and proliferation analysis*

Cell doubling time experiments were performed by plating 0.5×10^6 cells per 25 cm^2 flask, by counting after 30 and 60 h. The cell doubling time was obtained with the equation,
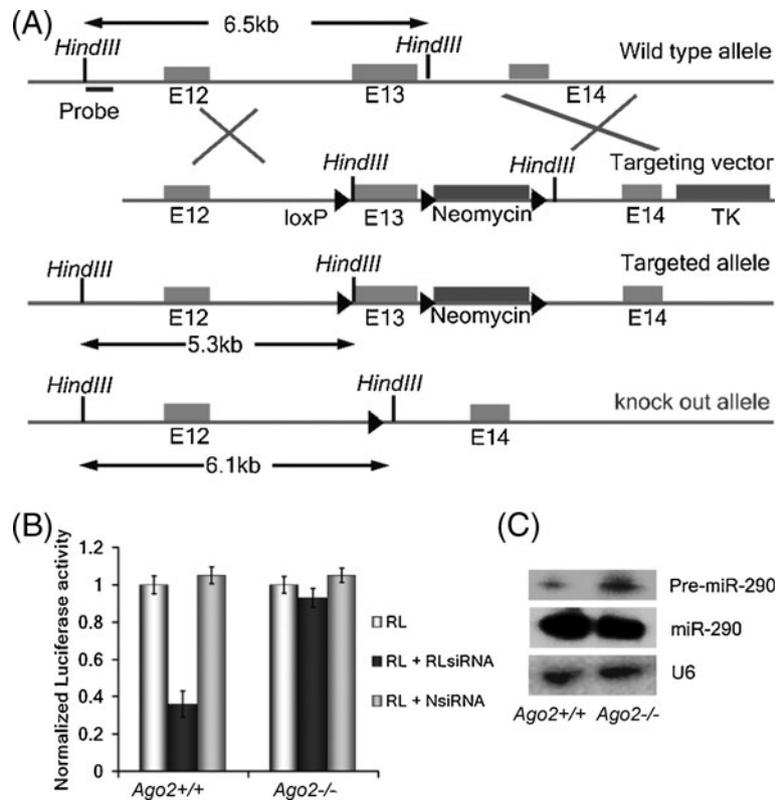
$$\text{doubling time} = \ln 2 / (\ln (A/A_0)/t),$$

Figure 1. *Ago2*-null ES cells are defective in small-RNA-mediated gene silencing and miRNA biogenesis. (**A**) Conditional targeting of *Ago2* gene by homologous recombination. Upon targeting, exon 13 is floxed by placing a Neomycin gene immediately downstream of exon 13. Conditionally targeted homozygous ES cells were obtained by loss of heterozygosity. *Ago2*-null ES cells were derived by deletion of exon 13 and Neomycin gene using transient expression of Cre recombinase. (**B**) Dual luciferase assay for gene silencing. ES cells transfected with renilla luciferase vector alone, or along with a non-specific siRNA (NsiRNA), did not show any decrease in the renilla luciferase activity. *Ago2*-null ES cells transfected with Renilla luciferase and siRNA against renilla luciferase (RLsiRNA) showed reduced levels of renilla luciferase activity in comparison with the wild-type ES cells. Renilla luciferase activity was normalized to firefly luciferase activity, which was used as transfection control. (**C**) Northern blot analysis revealed higher levels of pre-miR-290 and lower levels of mature miR-290 in *Ago2*-null ES cells in comparison with the wild-type ES cells. U6 RNA is used as loading control.

where A_0 is the initial ES cell number and A is the ES cell number after growth period t. ES cell growth rate was analysed by counting cells at different time points in a haemocytometer. Cell cycle analysis was performed by treating the ES cells with PI and RNaseA for 30 min at room temperature. The cell cycle profile was acquired in FACSCalibur. For apoptosis analysis, cells were labelled with and Annexin V – FLUOS (Roche Diagnostics), and analysed by flow-cytometry (FACSCalibur). Analysis of FACS data was performed with Flowjo software.

2.6 *Immunoblot analysis*

Total proteins were extracted with EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) containing 1× protease inhibitor cocktail and resolved on a 12% SDS–PAGE. Proteins were transferred onto PVDF membrane and hybridized with Cdkn1a and β-actin antibodies (Abcam) and processed for immunodetection.

2.7 *Q-PCR*

Total RNA was isolated from ES cells and EBs at various time points. Six micrograms of total RNA was treated with TURBO DNase (Ambion) and purified with RNeasy mini kit (Invitrogen). cDNA was synthesized with superscriptII (Invitrogen) from 2 µg of purified RNA. Quantitative PCR (Q-PCR) was performed with SYBR GreenER qPCR supermix (Invitrogen) in 7300 Real Time PCR System (Applied Biosystems). PCR was done in triplicate to minimize the variation, with *Hprt* as an endogenous control.

3. Results

3.1 *Ago2-deficient ES cells are defective in siRNA-mediated target gene silencing and miRNA biogenesis*

Ago2-deficient ES cells were generated by deletion of exon 13 (figure 1A; supplementary figure 1). The RT-PCR analysis of the total RNA from the *Ago2*-knockout ES cells revealed the absence of *Ago2* mRNA, confirming the loss of expression of *Ago2* in these cells. Further, the ability of the *Ago2*-deficient ES cells to silence the target mRNA in the presence of specific siRNA was analysed by dual luciferase assay (figure 1B), where *Ago2*-deficient cells were unable to silence the target gene in response to target specific siRNA. We performed RNA blot analysis with probes complementary to ES-cell-specific and non-ES-cell-specific miRNAs (Houbaviy *et al.* 2003). In *Ago2*-null ES cells, the expression levels of mature miRNA were significantly reduced with an increase in the expression levels of pre-miR-290 in comparison with the wild-type ES cells (figure 1C; supplementary table 1), indicating defects in pre-miRNA processing. This observation is reminiscent of the loss of slicer-independent activity of *Ago2* in the

hematopoietic lineage (O'Carroll *et al.* 2007). The defects in miRNA biogenesis were observed both in miRNAs expressed exclusively in ES cells, i.e. miR-290, miR-292, miR-293 and miR-294, and in those expressed in ES cells as well as in other differentiated cell types, i.e. miR15a, miR16 and miR21 (supplementary figure 2). *Ago2*-null ES cells are neither capable of gene silencing in presence of small RNA nor are they efficient in miRNA biogenesis.

3.2 *Loss of Ago2 affects the rate of self-renewal of ES cells*

Ago2-deficient ES cells were cultured without any overt changes in their morphology, and these cells continued to express typical ES cell markers, indicating that *Ago2*-null cells can self-renew. However, *Ago2*-null ES cell colonies appeared to be smaller in comparison with the wild-type ES cell colonies (figure 2A). The population doubling time of the null ES cells was found to be longer (~14.5 h) than that of wild-type ES cells (~12 h) (supplementary figure 3). This increase may either result from differential apoptosis or cell cycle delays or both. Apoptosis was ruled out as the null ES cells did not show significantly higher number of cells
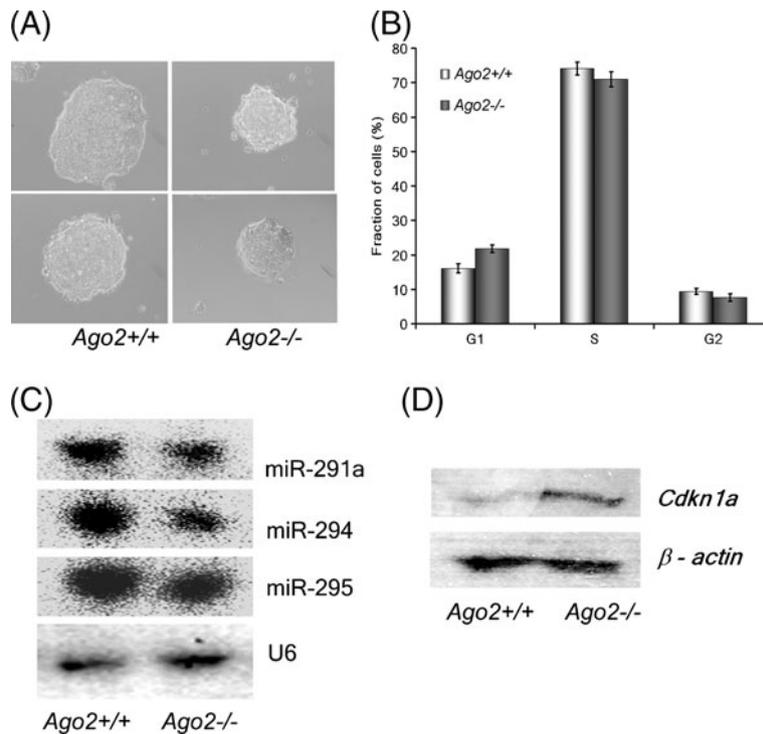

Figure 2. *Ago2*-null ES cells show slow self-renewal. (**A**) Five-day-old colonies of wild-type and *Ago2*-null ES cells. Images were taken at 10× magnification. (**B**) Cell cycle analysis of *Ago2*-null ES cells (*n*=4). (**C**) Northern blot analysis of ES-cell-specific miRNAs- miR-291a, miR-294 and miR-295; U6 was used as loading control. (**D**) Western blot analysis of Cdkn1a; β-actin used as loading control.

positive for early apoptotic marker, Annexin V, and negative for PI (data not shown). On the other hand, a higher percentage of null ES cells was found in the G1 phase of cell cycle as compared to that of the wild type (figure 2B). This observation is comparable to cell cycle defects reported in *DGCR8*-deficient ES cells (Wang *et al.* 2007) and *Dcr-1* mutant germline stem cells in *Drosophila melanogaster* (Hatfield *et al.* 2005) In both *Dgcr8* and *Dcr-1* mutants, mature miRNAs were absent. We observed lower levels of miR-291a, miR-294 and miR-295 (figure 2C; supplementary table 1). These miRNAs are known to promote rapid proliferation in ES cells by silencing *Cdkn1a* (Wang *et al.* 2008). We also observed upregulation of *Cdkn1a* in the *Ago2*-deficient ES cells (figure 2D). Thus, the increased population doubling time of *Ago2*-deficient ES cells can be attributed to delayed G1-S phase transition resulting from upregulation of *Cdkn1a*. This apparent upregulation in turn is an outcome of failure of ES-cell-specific miRNAs (miR-291a, miR-294 and miR-295) mediated silencing of *Cdkn1a*. These results suggest that the self-renewing ability of the *Ago2*-deficient ES cells may not be affected *per se*; however, the rate of their self-renewal is adversely affected.

3.3 *Ago2-null ES cells express higher levels of pluripotency markers under monolayer differentiation conditions*

To analyse *in vitro* differentiation ability, clonal differentiation assays were performed by plating wild-type and *Ago2*-null ES cells at clonal density in the absence of MEFs, with or without LIF. Wild-type ES cells showed varying level of differentiation depending upon the presence or absence of LIF. Most wild-type ES cell colonies were characterized by flattening around the rim and loss of staining for alkaline phosphatase at the edges of the colony. Differentiation was much more pronounced in the absence of LIF as indicated by unstained colonies. In contrast, very few *Ago2*-deficient ES cell colonies showed flattening around the rim and relatively less unstained colonies were seen in the absence of LIF (figure 3A). During monolayer differentiation experiments, the expression of pluripotency markers like *Oct4*, and the self-renewal marker *Nanog* in *Ago2*-deficient ES cells, was not suppressed to the same extent as in wild-type ES cells (figure 3B). Silencing of *Oct4* and *Nanog* by specific miRNAs (miR-134, miR-296 and
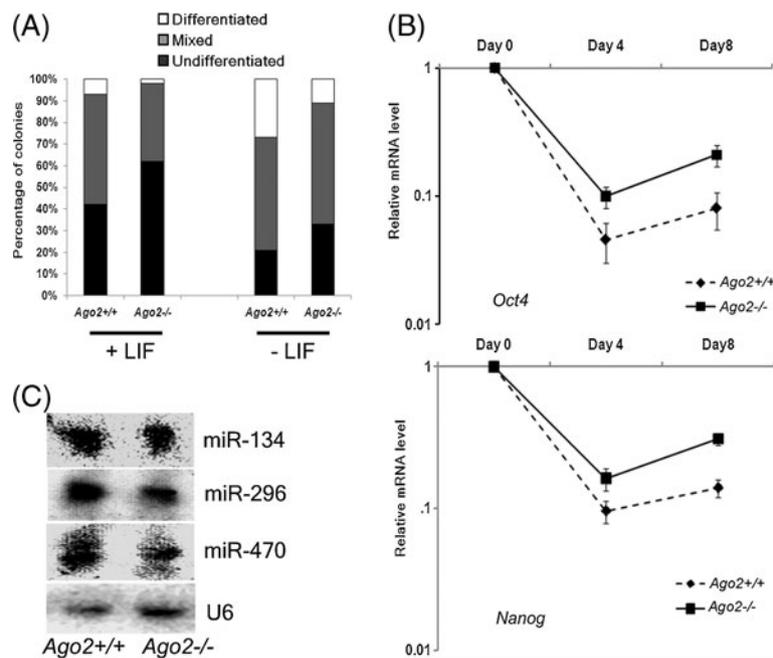

Figure 3. Inefficient silencing of self-renewal factors in *Ago2*-null ES cells during monolayer differentiation. (**A**) Clonal differentiation assay (feeder free) in the presence or absence of leukemia inhibiting factor (LIF). The bar diagrams indicate the percentages of undifferentiated, mixed and differentiated colonies in the presence or absence of LIF after 6 days of culture scored for alkaline phosphatase staining. (**B**) Q-PCR analysis of pluripotency markers – *Oct4* and *Nanog* at day 4 and day 8 of ES cells grown without LIF and mouse embryonic feeders (MEFs). *Hprt* was used as a reference. For each gene the expression levels were normalized to their expression levels at day 0. (**C**) Northern blot analysis of miR-134, miR-296 and miR-470; U6 was used as loading control.

miR-470) is followed by appearance and increase in expression of differentiation markers. We observed reduced levels of miR-134, miR-296 and miR-470 in *Ago2*-knockout ES cells (figure 3C; supplementary table 1). The higher levels of *Oct4* and *Nanog* observed in *Ago2*-deficient ES cells reflect the inability of the null cells to silence these genes during differentiation in the absence of LIF.

3.4 *Ago2-null embryoid bodies exhibit persistence of higher levels of pluripotency markers during differentiation*

To further study the differentiation potential of *Ago2*-null ES cells, we differentiated them into EBs in the absence of LIF. EBs from *Ago2*-null ES cells were smaller in size and had retarded growth in comparison with the wild-type EBs (figure 4A). The *Ago2*-null EBs differentiated to cystic embryoid bodies; however, the appearance of cysts was delayed by 2–3 days as compared with the wild-type EBs (figure 4B). In contrast to the *Dgcr8*-null ES cells, which do not form cystic EBs at all, *Ago2*-deficient EBs showed atypical expression levels of self-renewal, pluripotency and differentiation markers in comparison with the wildtype EBs (figure 4C; supplementary figures 4 and 5). The self-renewal marker *Nanog* and the pluripotency markers *Oct4, Sox2, Stella* (Hayashi *et al.* 2008) and *Rex1* (Rogers *et al.* 1991) were sharply suppressed during the growth and

differentiation of wild-type EBs. Although suppression of these genes was observed in *Ago2*-null EBs, it was neither sharp nor complete (figure 4C; supplementary figure 4). Further, the equilibrium between self-renewal and differentiation appeared to be skewed in favor of self-renewal. Inhibition or reduction of ERK and GSK3 activity is known to promote self-renewal (Silva and Smith 2008). In the case of *Ago2*-null EBs, the expression level of *Erk2* and *Gsk3* are relatively very low up to day 6 (supplementary figure 6). Suppression of self-renewal and pluripotency factors is essential for normal differentiation. It appears that *Ago2*-deficient EBs are inefficient in silencing the self-renewal and pluripotency factors during the early phase of differentiation of ES cells into EBs. Given this phenotype, we predicted absence or late appearance of differentiation markers for various lineages in *Ago2*-null EBs.

3.5 *Appearance of differentiation markers is delayed in Ago2-null EBs*

The primitive ectoderm arises from the inner cell mass of the blastocyst and gives rise to three germinal layers of the embryo (Rathjen *et al.* 1999). The expression of primitive ectoderm marker *Fgf5 (*Hebert *et al.* 1991) is sharply upregulated by day 2 in wild-type EBs. In contrast, *Ago2*-null EBs showed only slight induction of *Fgf5* by day 2, attaining wild-type levels only by day 4 (figure 4C). Similarly, markers of endoderm (*Gata6*) and mesoderm (*Bmp2, T,* also known as
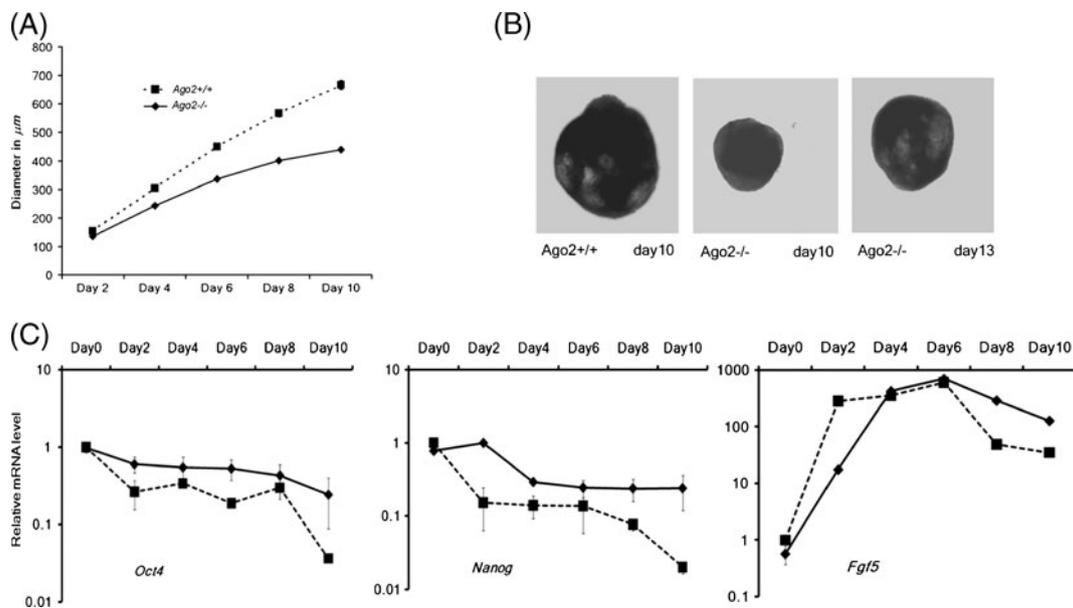

Figure 4. *Ago2*-null ES cells exhibit slower rate of differentiation. (**A**) Growth curve of embryoid bodies (EBs), mean diameter of spherical EBs in μm (*n*=90–05) is plotted against time. Error bars represent s.d. (**B**) Ten- and thirteen-day-old EBs with or without cysts. (**C**) Q-PCR analysis of pluripotency and differentiation markers – *Oct4, Nanog* and *Fgf5* at different stages of EB formation. *Hprt* was used as a reference. For each gene the expression levels were normalized to mRNA levels at day 0 of the wild type.

Brachyury) were delayed. *Gata6, Bmp2* and *Brachyury* were upregulated in the wild-type EBs by day 2 as expected, but *Ago2*-deficient EBs showed levels comparable to that of undifferentiated ES cells. In *Ago2*-deficient EBs, *Brachyury* attained wild-type levels by day 4, where as expression of *Gata6* and *Bmp2* reached wild-type EBs levels only after day 6 (supplementary figure 5). Morphology and expression of several early differentiation markers suggest that the *Ago2*-deficient ES cells eventually differentiate into EBs. However, the delayed appearance of cysts and the slow induction of differentiation markers clearly indicate the lag in differenti-ation of *Ago2*-deficient EBs.

3.6 *Ago2-null ES cells give rise to poorly differentiated teratomas*

Teratomas were generated by subcutaneous injection of wild type and *Ago2*-deficient ES into immunodeficient mice. Consistent with our observations in the EBs, the cells in the *Ago2*-deficient teratomas were largely undifferentiat-ed. Although *Ago2*-deficient teratomas show glandular structures lined by epithelium, but the extent of cell types and differentiation structures are not comparable to that of wild-type teratomas (figure 5). The differentiation defects in EBs and teratomas suggest that *Ago2*-mediated siRNA pathway regulate some aspects of ES cell differentiation independent of miRNA pathway.

4. Discussion

In this study we show that AGO2 – the mammalian Ago protein catalysing the siRNA pathway – is essential for normal self-renewal and differentiation of ES cells but dispensable for ES cell survival. *Ago2*-deficient ES cells did not undergo apoptosis, unlike ES cells lacking all the four *Ago*s which undergo apoptosis (Su *et al.* 2009). The ES cells lacking all the four *Ago*s undergo apoptosis due to loss of both miRNA pathway and the siRNA pathways. In contrast, in *Ago2*-null ES cells, only the siRNA pathway is defective due to loss of *Ago2*; the miRNA pathway is still functional suggesting that only the miRNA pathway is essential for ES cell survival and the siRNA pathway catalysed by the *Ago2* is dispensable for the survival of ES cells.

Our study also provides evidence that siRNA pathway is essential for normal self-renewal and differentiation of ES cells. Although loss of *Ago2*-catalysed siRNA pathway does not affect the ability of ES cells to self-renew in culture, it adversely affects the rate of self-renewal. The cell cycle defect observed by us is reminiscent of the cell cycle defects in *Dgcr8*-deficient ES cells. Unlike *Dgcr8*-deficient ES cells, where both miRNA-mediated siRNA and miRNA pathways are nonfunctional, *Ago2*-deficient ES cells are defective in the siRNA pathway alone. It appears that the cell cycle defect in *Dgcr8*-deficient ES cells resulting from
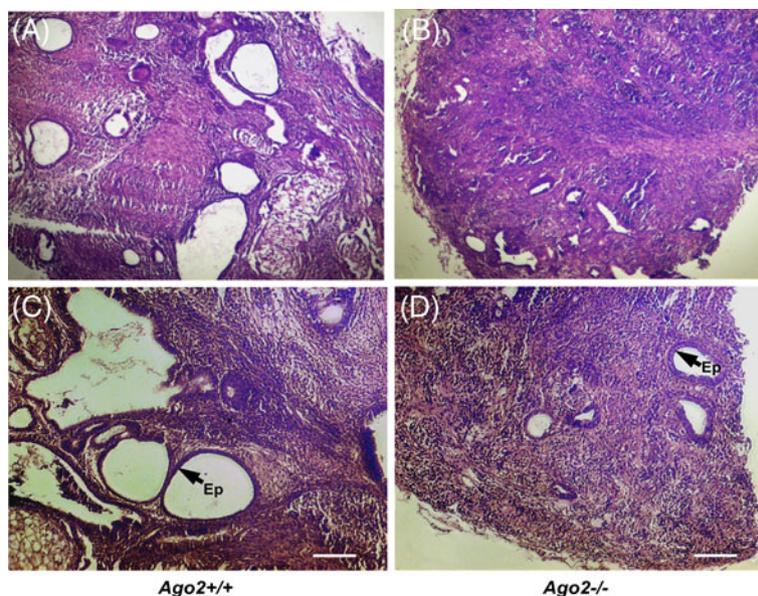

Figure 5. *Ago2*-null ES cells differentiate poorly into teratomas. *Ago2*-deficient ES cells form teratomas and differentiate when injected subcutaneously into nude mice. (**A** and **C**) Haematoxylin and Eosin stained sections of wild-type teratomas at 5× and 10× magnification, respectively, showing various cell types and tissue organisation. (**B** and **D**) Similarly treated sections of *Ago2*−/− teratomas at 5× and 10× magnification, respectively, showing a few cell types and differentiated tissue structures. *Ago2*-null teratomas were largely composed of undifferentiated cells and relatively very few cell types compared with that of the wild-type teratomas. Ep – epithelial tissue.

failure of ES-cell-specific miRNA silencing of *Cdkn1a* is mediated by siRNA pathway, as in case of *Ago2*-deficient ES cells. Its plausible that the shortening of G1 phase of cell cycle in ES cells is mediated by siRNA and miRNA pathway is dispensable for it.

Higher expression levels of pluripotency markers during differentiation is similar to the *Tcf3*- and *Gcnf*-knockout ES cells, which show persistently higher levels of pluripotency markers associated with slower differentiation as a result of loss of inhibitory effect of TCF3 and GCNF on *Nanog* and or *Oct4* during differentiation (Gu *et al.* 2005; Pereira *et al.* 2006). In the absence of AGO2, specific miRNAs fail to silence *Nanog* and *Oct4* by siRNA pathway. This results in persistence of higher levels of pluripotency markers during differentiation. The persisting pluripotency factors delay differentiation, as evident from very low levels of various differentiation markers until day 4 to day 6 in EBs.

The *Dicer1*-null ES cells fail to differentiate (Kanellopoulou *et al.* 2005), whereas ES cells that lack *Dgcr8* express some differentiation markers, most of which never attain the wild-type expression levels in the EBs, suggesting defective differentiation (Wang *et al.* 2007). The differentiation defect in *Ago2*-null ES cells is subtle in comparison with the *Dicer1*- and *Dgcr8*-null ES cells. Loss of DGCR8 or DICER1 results in complete absence of miRNA, confirming that they are at the base of miRNA processing pathway. This may in turn render the miRNA-mediated silencing by all the miRNA effector pathways disabled. In *Ago2*-null ES cells, mature miRNA are present, albeit at lower levels, and *Ago2*-null ES cells are defective in silencing the target genes in presence of specific miRNA due to loss of slicer activity. In *Ago2*-null ES cells, the siRNA pathway in which *Ago2* is indispensable is disabled. The effector pathways involving other *Argonaute*s (Azuma-Mukai *et al.* 2008) and the redundant function of Ago proteins in ES cell survival (Su *et al.* 2009) are still intact. However, the differentiation defects in *Ago2*-deficient ES cells are more subtle and different from that of *Dicer1*- and *Dgcr8*-deficient ES cells. It appears that unlike the rate of self-renewal of ES cells, which is regulated by the siRNA pathway, differentiation of ES cells is regulated by both siRNA and miRNA pathways to a large extent in a non-overlapping manner. Hence, the absence of the siRNA pathway leads to a subtle phenotype of delayed differentiation. It also suggests that the miRNA pathway may be essential for the formation of cystic EBs, which are absent in *Dicer1* and *Dgcr8* EBs. The subtle phenotype of *Ago2*-null ES cells in comparison with the *Dicer1*- and *Dgcr8*-null ES cells makes it a good ES cell model system to study the roles of small-RNA-mediated by the siRNA pathway in regulation of self-renewal and differentiation ES cells into various lineages.

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