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MiRNA-mediated regulation of cell signaling and homeostasis in the early mouse embryo

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At the time of implantation the mouse embryo is composed of three tissues the epiblast, trophectoderm and primitive endoderm. As development progresses the epiblast goes on to form the fetus whilst the trophectoderm and primitive endoderm give rise to extra-embryonic structures with important roles in embryo patterning and nutrition. Dramatic changes in gene expression occur during early embryo development and these require regulation at different levels. miRNAs are small non coding RNAs that have emerged over the last decade as important post-transcriptional repressors of gene expression. The roles played by miRNAs during early mammalian development are only starting to be elucidated. In order to gain insight into the function of miRNAs in the different lineages of the early mouse embryo we have analysed in depth the phenotype of embryos and extra-embryonic stem cells mutant for the miRNA maturation protein Dicer. This study revealed that miRNAs are involved in regulating cell signaling and homeostasis in the early embryo. Specifically, we identified a role for miRNAs in regulating the Erk signaling pathway in the extra-embryonic endoderm, cell cycle progression in extra-embryonic tissues and apoptosis in the epiblast.

Key words: Dicer, miRNA, mouse embryo, trophectoderm, primitive endoderm, epiblast, Erk, cell cycle, apoptosis

Abbreviations: miRNA, microRNA; dpc, days post coitum; ES cells, embryonic stem cells; Epi cells, epiblast stem cells; RISC, RNA induced silencing complex; FGF, fibroblast growth factor; AVE, anterior visceral endoderm; XEN, extra-embryonic endoderm stem cells; TS cells, trophoblast stem cells; cdk, cyclin dependent kinase; ICM, inner cell mass; MEF, mouse embryonic fibroblast

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The first cell fate decisions that occur in the mammalian embryo specify two extra-embryonic lineages, the trophoblast and the primitive endoderm and an embryonic one, the epiblast.[1] The cells of the epiblast are pluripotent, retain the capacity to give rise to all the embryo lineages and are named embryonic stem (ES) cells if isolated from 3.5 days post coitum (dpc) embryos or epiblast stem (EpiS) cells if derived from 5.5 dpc embryos.[2,3] Between 5.5 dpc and 7.5 dpc the epiblast receives signaling from the surrounding extra-embryonic tissues and starts a differentiation process that leads to the establishment of the embryo axes and the specification of the three germ layers (endoderm, mesoderm and ectoderm) in a process termed gastrulation.[4]

The correct specification and development of embryonic and extra-embryonic lineages requires precise temporal and spatial control of gene expression at epigenetic, transcriptional and post-transcriptional levels.[4] During the last decade endogenously produced 20–22 nucleotide long non-coding RNA molecules, termed microRNAs (miRNAs), have emerged as important post-transcriptional regulators of gene expression. miRNAs are transcribed as long precursor molecules that are cleaved into hairpin stem loop intermediate structures in the nucleus by a complex of the proteins Drosha and Dgcr8. Following this the hairpin intermediates are exported from the nucleus and undergo a further cleavage event in the cytoplasm, this time mediated by the RNase Dicer, to form mature miRNAs. Mature miRNAs form a complex with Argonaute proteins, termed the RNA induced silencing complex (RISC), which inhibits the translation of target mRNAs and is often accompanied by mRNA degradation.[5]

Table 1. miRNA families involved in the regulation of cell signaling and homeostasis during mouse early embryo development

Representative miRNA	Family members	Seed region
miR-20a	miR-17-5p, miR-20a, miR-20b, miR-106a, miR-106b, miR-93	AAAGUGC
miR-291a-3p	miR-291a-3p, miR-294, miR-295, miR-302a, miR-302b, miR-302d	AAGUGCU
miR-92a	miR-25, miR-32, miR-363, miR-367, miR-92a, miR-92b	AUUGCAC
miR-19	miR-19a, miR-19b	GUGCAAA
miR-30b	miR-30a, miR30b, miR-30c, miR-30d, miR-30e, miR-384-5p	GUAAACA
miR-669a	miR-669a	GUUGUGU

The family members and seed sequence are taken from the database TargetScan release 5.1 (www.targetscan.org).

Target identification is mediated by base pairing interactions between miRNAs and mRNAs. The majority of targeting is due to interactions between the 7–8 nt at the 5' end of a miRNA (named the "seed" region) and the mRNA; for this reason miRNAs can be functionally grouped into families that share the same seed region and therefore potentially target the same genes.[6] In this article the name of a representative miRNA will be used to refer to the whole miRNA seed family it belongs to. The full list of miRNAs belonging to a given family can be found in **Table 1**.

miRNAs are integrated in regulatory networks that govern a wide variety of processes ranging from the maintenance of tissue homeostasis to the specification of cell fate during development. The generation of embryos lacking mature miRNAs through the deletion of proteins necessary for their processing has revealed that these regulators play an essential role in early embryo development in numerous species.[7-11] In zebrafish the deletion of maternal and zygotic *Dicer* provokes a developmental delay but does not affect the establishment of the body axes or specification of the germ layers during gastrulation.[8] However morphogenesis is affected in these embryos in that there is a failure to elongate the body axes and defects in neural development and heart formation.[12] The effect of loss of miRNAs from the mouse embryo is much more severe. In mouse, zygotic deletion of either *Dgcr8*, *Dicer* or *Ago2* results in developmental arrest at around the time of gastrulation, with mutant embryos appearing morphologically abnormal and much smaller than their wild-type littermates.[9-11] Interestingly, maternal and zygotic deletion of *Dgcr8* in mouse has shown that miRNAs are neither required for early pre-implantation development nor trophectoderm specification.[13]

Given the major role that extra-embryonic tissues have on the development of the mammalian epiblast it is necessary to consider them when analysing the effect of miRNAs deletion on embryo development. For this reason we recently carried out an extensive analysis of the effect of *Dicer* deletion on both embryonic and extra-embryonic tissues of the mouse embryo.[14,15] This study has revealed different functions for miRNAs in each lineage and represents a step forward in our understanding of the regulatory networks governing early mammalian development and the role of miRNAs within them.

MiRNAs as Cell Signaling Modulators in Early Embryo Development

In the majority of cases miRNA-mediated gene silencing results in less than a 50% reduction in gene expression.[16] For most genes such a decrease in expression would be expected to have little biological impact judging from the scarcity of described mouse haploinsufficiency phenotypes. However cells can be very responsive to small changes in signaling levels and for this reason it has been proposed that signaling pathways are excellent candidates for meaningful miRNA based regulation.[17] Numerous signaling pathways play important roles in controlling the patterning, specification and maintenance of embryonic and extra-embryonic tissues during mouse embryo development.[1,4] A number of these have been shown to undergo miRNA based regulation in various species; however the impact of miRNAs on the regulation of signaling pathways during early mammalian development is only starting to be elucidated.

Control of nodal signaling by miRNAs during early mammalian development. Signaling by the TGFβ factor Nodal plays widely conserved roles in regulating events taking place during early embryo development including formation of the body axes and the induction of mesendoderm.[18] A role for miRNAs in conferring robustness to the Nodal signaling pathway during early embryo development has been shown in zebrafish and xenopus embryos.[12,19,20] In zebrafish the miR-430 family of miRNAs modulates expression of both the Nodal homologue *Squint* and the nodal antagonist *Lefty*, thus helping to maintain the agonist/antagonist balance. As a result, deletion of *Dicer* in zebrafish results in overexpression of both *Squint* and *Lefty*.[12] Similarly in xenopus embryos miR-427, a homolog of miR-430, has been shown to regulate the expression of both Nodal agonists (*Xnr5* and *Xnr6b*) and antagonists (*LeftyA* and *LeftyB*). However in this case miR-427 appears to have a stronger effect on antagonist than agonist inhibition, as inhibiting expression of this miRNA results in a failure to differentiate mesoderm, a result also seen on either *Lefty* overexpression or Nodal signaling inhibition.[20]

In mouse Nodal is expressed in the epiblast from the blastocyst stage and requires processing in the trophoblast by the convertases Spc1 and Spc4 to become fully active.[21,22] Its diffusible nature allows it to play important roles in the patterning and development of all three tissue lineages. Our analysis of zygotic *Dicer* mutant embryos revealed that Nodal is robustly expressed in the mouse epiblast in the absence of miRNAs and gets correctly restricted to the posterior of these embryos at the onset of gastrulation, although this restriction is delayed by around a day in comparison to wild-type embryos (**Fig. 1**).[15] Additionally we found that the genes coding for the Nodal processing proteins *Spc1* and *Spc4* are expressed in the trophoblast, the Nodal co-activator *Cripto* is robustly expressed in the epiblast and *Lefty1* expression is not expanded in *Dicer*

null mouse embryos (**Fig. 1**).[15] In agreement with there not being any major perturbation in Nodal signaling in embryos lacking miRNAs, mesoderm formation is initiated, although with a one day delay, in the posterior epiblast and there is no expansion of neuroectoderm markers. The delay in mesoderm formation mimics that of Nodal restriction in these embryos (**Fig. 1**).[15]

Although in *Dicer* mutant embryos we could not detect major defects in Nodal signaling, in both human and mouse ES cells the miR-291a-3p family, that is homologous to the miR-427 and miR-430 miRNA families, inhibits *Lefty* expression to promote mesendodermal cell fates and block neuroectodermal ones.[20,23] Interestingly, in this case miRNAs only target Nodal antagonists but not Nodal itself, pointing to a difference in the way miRNAs modulate this pathway in mammals. These studies in ES cell suggest that miRNAs may indeed be regulating Nodal signaling in the early stages of mouse embryo development. Such modulation could be relatively subtle and therefore difficult to detect in the *Dicer* mutants due to their severe proliferative and apoptotic phenotype (see below).

Control of Erk signaling by miRNAs during early embryo development. Erk is a classical mitogen activated effector kinase involved in the regulation of numerous cellular processes including proliferation, differentiation and development. Canonical activation of Erk occurs through a signaling cascade triggered by binding of one of a number of diffusible ligands to an extracellular receptor. Ligand binding leads to the activation of the small GTPase RAS by SOS, which, in turn, leads to the activation of RAF, which phosphorylates and activates MEK1/2, which then phosphorylates ERK, leading to its activation.[24] This signaling cascade has an amplification effect and is tightly controlled by numerous negative and positive regulators, and mis-activation of Erk can lead to numerous defects including cancer.[25] Recently it has been found that miRNAs are involved in the control of Erk activation in cardiac fibroblasts where miR-21 negatively regulates expression of the FGF signaling inhibitor, Sprouty.[26]

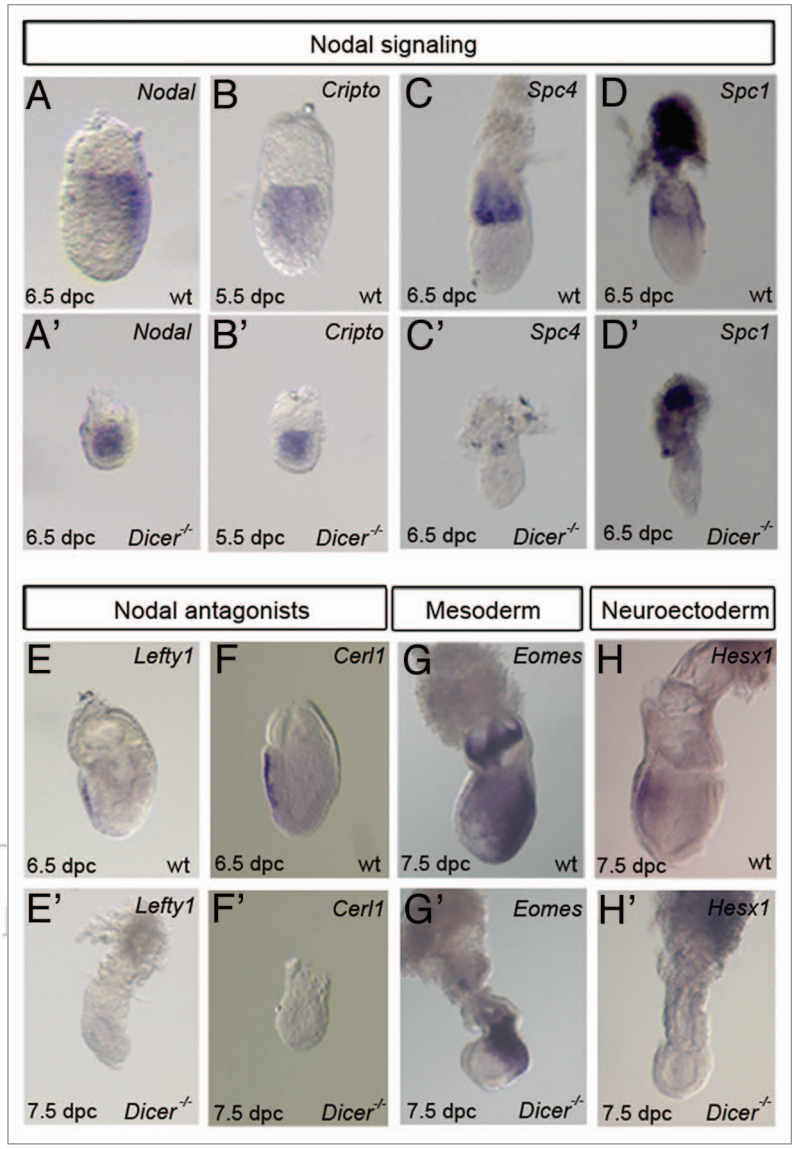

Figure 1. miRNAs are not major regulators of Nodal signaling at mouse peri-gastrulation stages. (A–D') *Dicer*$^{-/-}$ embryos express normal levels of *Nodal*, the Nodal co-activator *Cripto* and the Nodal convertases *Spc1*,[15] and *Spc4*. (E–F') The expression of the Nodal antagonists *Lefty1* and *Cerl1* is not expanded but lost or reduced in *Dicer* null embryos.[15] (G and G') Mesoderm is correctly specified and positioned in the absence of miRNAs, indicating a correct specification of the antero-posterior axis.[15] (H–H') The neuroectoderm marker *Hesx1* is not expanded but lost in *Dicer* null embryos.

When we deleted *Dicer* and thus depleted *miRNAs* in multipotent cells derived from the primitive endoderm (XEN cells), one of the first alterations we observed was a decrease in the levels of phosphorylated Erk1/2.[15] This was followed by reduced cellular proliferation, a decrease in the expression level of a number of AVE markers and an increase in the expression level of a number of extra-embryonic visceral endoderm and parietal endoderm markers. Further investigation revealed that the reduced Erk signaling was contributing to the reduced proliferation and decreased AVE marker expression seen upon *Dicer* deletion as these phenotypes could at least be partially rescued by over stimulating the Erk pathway through the addition of excess ligand. Additionally we found that three negative regulators of the Erk activation cascade were upregulated in *Dicer* deleted XEN cells. These were Rasa2, a GTPAse activating protein that inactivates Ras, Dusp1, a phosphatase that targets Erk and Sulf2, a sulfatase that can remove 6-O-sulfate groups

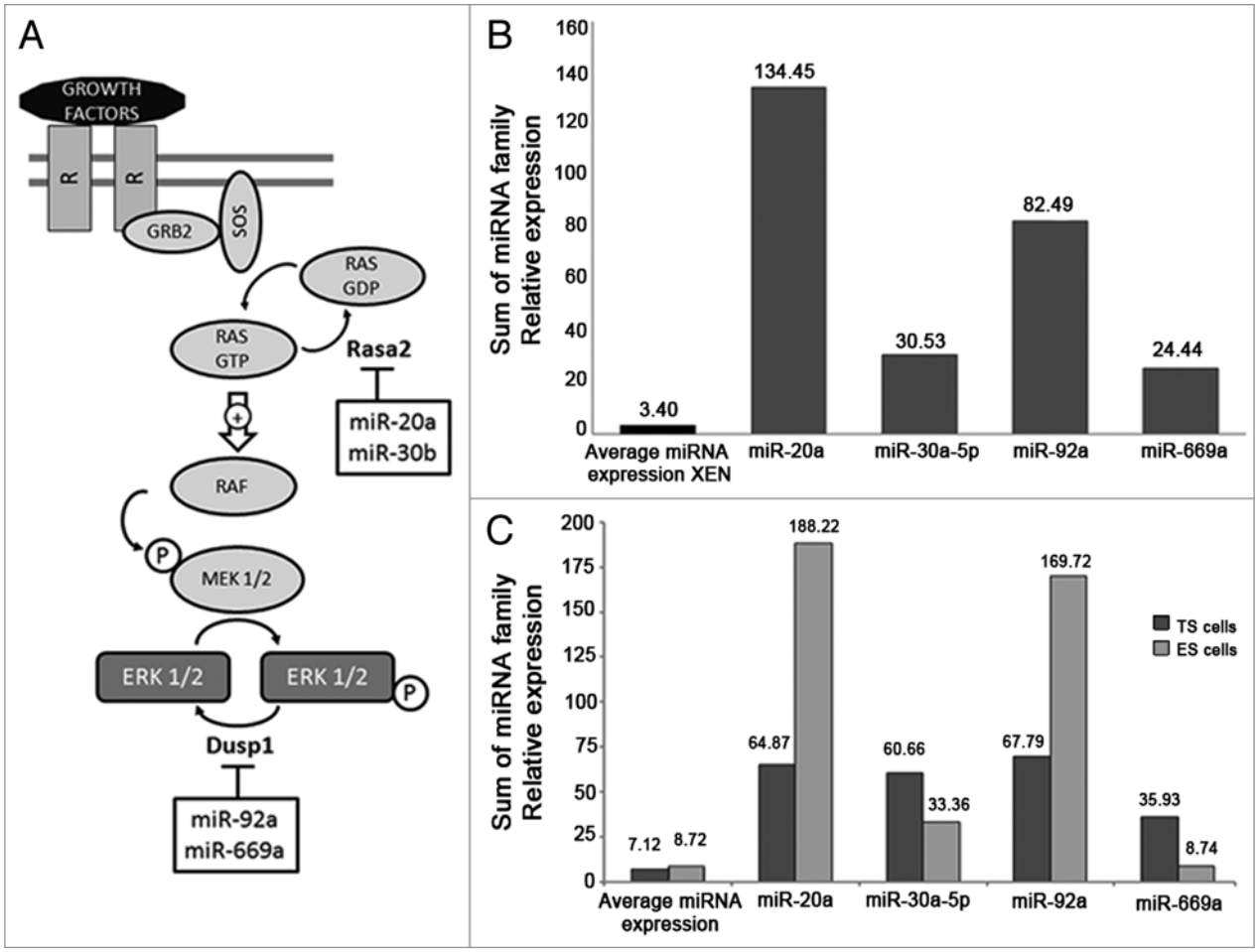

Figure 2. Erk signaling is regulated by miRNAs in extra-embryonic endoderm stem cells. (A) Different miRNA families collaborate in the control of Erk activation in XEN cells by repressing the Erk activation inhibitors Rasa2 and Dusp1. This maintains a tight control of Erk signaling, which is required for XEN cell identity and proliferation. (B) The miRNA families regulating Erk signaling in XEN cells are amongst the most highly expressed miRNA families in this cell type, reflecting their impact on XEN cell homeostasis.[15] (C) The miRNA families miR-20a, miR-30a-5p, miR-92a and miR-669a are also highly expressed in TS and ES cells. In (B and C) the data shown represents the sum of the relative expression of all the members of the miRNA families in each cell type.[15]

from growth factor co-receptors and that has been shown to negatively regulate Fgf signaling (**Fig. 2**).[27-29] Interestingly these genes all have long 3'UTRs and are predicted to be targeted by many of the miRNA families we found to be highly expressed in XEN cells.[15] Indeed we demonstrated that the 3'UTR of both *Rasa2* and *Dusp1* could be targeted by at least two miRNA families strongly expressed in XEN cells: these were the miR-20a and miR-30b miRNA families for *Rasa2* and the miR-92a and miR-669a miRNA families for *Dusp1* (**Fig. 2**).[15] The fact that both genes are being targeted by different miRNAs may reflect that multiple binding sites are required to achieve significant silencing. Additionally the fact that numerous miRNA families appear to control several inhibitors of the Erk activation pathway highlights a level of redundancy common to miRNA-mediated regulation. Such redundancy is likely to provide robustness to biological systems ensuring correct fine-tuning of signaling pathways.

Erk signaling has been found to be vital for specification of the PE lineage, with high levels of Erk activation seeming to drive ICM cells towards a PE fate and low levels towards an epiblast fate.[30] Additionally a number of studies conducted in embryoid bodies suggest that Erk signaling may play important roles in the subsequent maturation and development of the VE.[31,32] In our study, in addition to seeing aberrant differentiation in Dicer deleted XEN cells, we also observed a loss of AVE marker expression in the visceral endoderm of *Dicer* null embryos.[15] These observations suggest that miRNA-mediated regulation of Erk signaling is not only necessary in cultured multipotent cells, but also involved in the molecular control of the AVE in vivo. This is the first hint of a role for Erk signaling in AVE specification in the embryo.

Interestingly we found many of the miRNA families involved in regulating inhibitors of the Erk pathway in XEN cells to be highly expressed in TS and ES cells (**Fig. 2**).[15] In the trophectoderm lineage Fgf/Erk signaling has been found to be vital for TS cell maintenance.[33] However, despite a requirement for Dicer to maintain expression of TS cell markers, we did not detect any change in the phosphorylation status of Erk in either the trophoblast

of *Dicer* null embryos or in *Dicer* null TS cells.[15] These findings indicate that Erk activation is regulated by different mechanisms in the trophoblast and the extra-embryonic endoderm.

Studies conducted in ES cells suggest a role for Erk signaling in the first steps of differentiation of the epiblast lineage during early embryo development.[34] Unfortunately we were unable to investigate whether miRNAs, either through regulation of Erk signaling or some other mechanism, have a role in the early stages of epiblast development due to the presence of maternal Dicer derived miRNAs. However it is interesting to note that ES cells mutant for either *Dicer* or *Dgcr8* show defects in their ability to differentiate.[11,35] These defects have previously been attributed to a failure in *Oct4* methylation due to the reduction in *Dnmt3* expression that is seen in these mutants.[23] It would be interesting to investigate whether defective Erk signaling is also contributing to the differentiation failure of these cells.

MiRNAs as Regulators of Cellular Homeostasis in the Embryo

Every cell maintains at any time a balance between proliferation and death. The cell cycle and apoptosis machinery are linked together so damaged cells either arrest or are eliminated. A strict control of these processes at transcriptional, post-transcriptional and post-translational levels is required to maintain the homeostasis of an organism. Therefore it is not surprising that increasing evidence situates miRNAs as major players in the control of cell cycle progression and cell death in different species during development and disease. In accordance with this, miRNAs also play a major role in the control of proliferation and apoptosis both in the early mammalian embryo and in the stem cells derived from it.

Regulation of the cell cycle by miRNAs in the early mouse embryo. Since their discovery numerous studies have revealed important roles for miRNAs in the regulation of cell cycle progression in many developmental systems. Indeed the first miRNA ever discovered, *lin-4,* was found to play a role in the regulation of cell division in *Caenorhabditis elegans.*[36] In mammals several cyclins, cdks and cdk inhibitors are targeted by miRNAs and alterations in the expression of these miRNAs is thought to contribute to deregulation of the cell cycle during tumor progression.[37]

Our studies found that deletion of *Dicer* in TS and XEN cells leads to greatly reduced levels of cell division.[15] Previous work had shown that miRNA deficient ES cells also have reduced levels of proliferation.[11,23] In *Dgcr8* mutant ES cells this effect has been attributed to increased expression of inhibitors of the G_1-S transition (p21, Rbl2 and Last2) as a result of loss of the miR-291-3p and miR-20a families.[38] We found that both these miRNA families are highly expressed in TS and XEN cells (**Fig. 3**),[15] and that p21, Rbl2 and Last2 are upregulated upon *Dicer* deletion in these cell types.[15] Our results suggest that the same miRNAs play similar roles in regulating cell cycle progression in the different blastocyst-derived pluripotent cells (TS, XEN and ES). Furthermore, other recent studies have found the miR-291-3p family to be expressed in primordial germ cells and adult male germ cells,[39,40] pointing to a widespread role for this miRNA family in regulating proliferation in numerous cell types with "stem" like self-renewing properties. Interestingly, those members of the miR-291-3p family most highly expressed and that better rescue the proliferation defect in *Drgcr8* null ES cells (miR-294 and miR-295) are also the most highly expressed in extra-embryonic stem cells (**Fig. 3**).[15,38] Previous studies have shown that the core pluripotency transcription factors (Oct4, Sox2 and Nanog) regulate expression of members of this miRNA family in ES cells.[41,42] Our finding that these same miRNAs are expressed in other stem cell types raises interesting questions about what regulatory networks are controlling their expression, which will require further investigation.

When we examined *Dicer* null embryos, we observed that the rates of cell proliferation were reduced in the trophectoderm and visceral endoderm at 6.5 dpc.[15] Therefore, miRNA-mediated mechanisms controlling cell division in TS and XEN cells are also operating in extra-embryonic tissues in vivo. In contrast to this, we did not observe any change in the rate of cell division in the epiblast of mutant embryos at the same stage. Given the control of proliferation by miRNAs that has been observed in ES cells,[11,23] proliferation must be regulated differently in the post-implantation epiblast than in ES cells. As mouse ES cells are derived from blastocyst stage embryos this difference may relate to differences in the mechanisms of cell cycle regulation in the epiblast before and after implantation. An additional or alternative explanation is offered by a recent study, which showed that the miR-20a family and numerous cell cycle regulators are more highly expressed in mouse ES cells compared to the ICM, the tissue from which ES cells are derived from.[43] This would suggest that ES cells have significantly altered mechanisms of cell cycle regulation to those seen in vivo.

Regulation of cell death by miRNAs in the early mouse embryo. Increasing evidence points towards miRNAs having important roles in the regulation of cell death in normal and transformed cells. Many miRNAs have been shown to regulate and/or to be regulated by pro-apoptotic and anti-apoptotic factors and their mis-expression has been linked to variations in cell death in many tissues.[44]

During mammalian embryo development miRNAs appear to have a common role in the inhibition of apoptosis, as specific deletion of *Dicer* from diverse tissues results in increased cell death.[45-47] In our study increased apoptosis was the first defect observed in the embryonic tissues of *Dicer* knockout embryos. This defect was independent from any extra-embryonic defects, as it was also observed in embryos with an epiblast-specific *Dicer* deletion. In contrast to what occurs in the epiblast, no increase in apoptosis was seen in either the visceral endoderm or trophectoderm of *Dicer* null embryos. The observed difference correlates with the wild-type situation, where much lower levels of endogenous apoptosis are seen in extraembryonic tissues than in the epiblast.[15] Together this suggests that the mechanisms of apoptosis regulation are different between embryonic and extra-embryonic tissues in the developing embryo. A possible explanation for this could be that mechanisms and check-points for cell quality control are

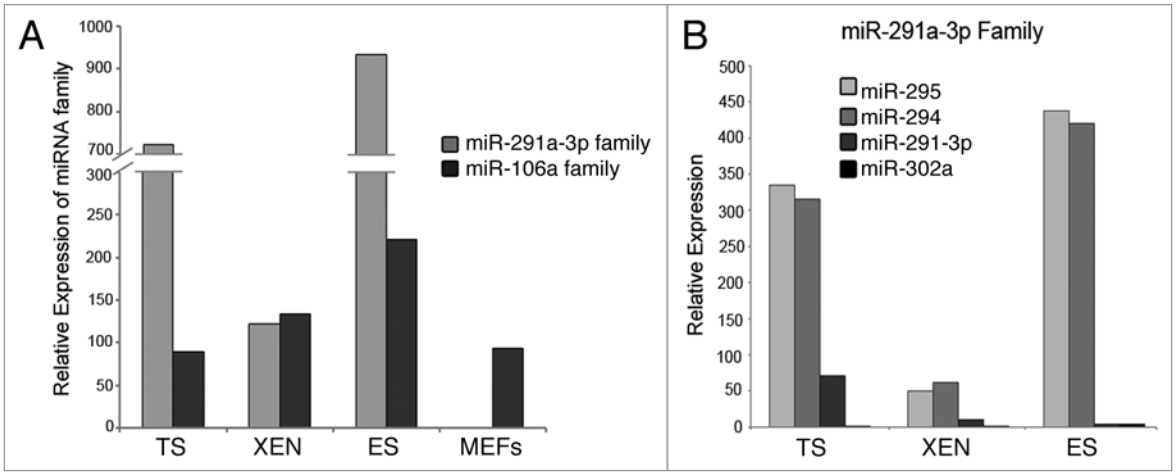

Figure 3. Stem cell specific control of cell cycle progression by miRNAs. (A) The miRNA families miR-20a and miR-291a-3p, known to regulate cell cycle progression in ES cells,[38] are also highly expressed in TS and XEN cells, with the miR-291a-3p family being specific to blastocyst derived stem cells and the miR-20a family also being highly expressed in MEFs.[15] (B) The members of the miR-291a-3p family expressed most highly in TS, ES and XEN cells are those previously shown to have the strongest effect on cell cycle regulation in ES cells.[38]

less necessary in extra-embryonic tissues due to their transient nature.

A central player in the control of apoptosis by miRNAs appears to be Bim, a pro-apoptotic protein that can trigger cell death by neutralizing pro-survival Bcl2-like molecules and/or by activating the Bcl2 inhibitor Bax.[48,49] When the RNA induced silencing complex (RISC) components *Ago1-4* are removed from ES cells, a cell type that bears many similarities to the early epiblast, increased apoptosis is observed as a consequence of raised *Bim* expression.[50] Similarly increased *Bim* expression has been found to account for much of the apoptosis seen in *Dicer* null lymphocytes.[51] When we examined levels of Bim protein in epiblast-specific *Dicer* knockout embryos we found them to be elevated in comparison to wild-type embryos.[15] Additionally we found that miRNAs that directly target *Bim* are highly expressed in wild-type embryos at stages when apoptosis is severely increased in full- and epiblast-specific *Dicer* mutant embryos.[15,52] These miRNAs (miR-17-5p, miR-19a, miR-92 and miR-25) are members of the miR-17-92 and miR-106b-25 clusters. Together these results suggest that one way in which miRNAs may be inhibiting apoptosis in embryonic tissues in the developing embryo is by targeting Bim. However it is worth noting that miRNAs belonging to the miR-17-92 and miR-106b-25 clusters have also been shown to target a number of other pro-apoptotic genes including PTEN.[53,54] Whether further members of the apoptotic pathway are similarly regulated by miRNAs in the early embryo remains to be investigated.

A recently published study has found that the miR-17-92 and miR-106b-25 clusters are highly expressed in the embryo throughout a large period of development and then show reduced expression in adult tissues.[55] A high rate of cell proliferation occurs during embryonic development and this is likely to require mechanisms by which damaged or un-fit cells resulting from these extremely rapid cell cycles can be eliminated.[56,57] Additionally programmed cell death plays crucial roles in shaping the embryo during development.[58] Therefore the apoptosis machinery could be more sensitive during development. If this was the case the ability of miRNAs from these clusters to target several components of the apoptotic pathway could allow them to act as a buffer that ensures that cell death during development only occurs when the necessary stimulation has occurred. In this scenario in the adult organism the apoptosis machinery would be less sensitive and therefore not require the precise control provided by miRNAs.

Concluding Remarks

The work described here provides some of the first insights into the roles miRNAs play in early mammalian embryo development. We have found that miRNAs modulate the Erk signaling pathway in extra-embryonic endoderm stem cells. Such regulation may be required in vivo for primitive endoderm maintenance and specification of AVE identity at peri-implantation stages, a time when Erk signaling has been shown to have crucial roles in this lineage. The fact that we could not detect any obvious alteration in signaling within the epiblast or trophectoderm upon *Dicer* deletion does not necessarily mean that miRNAs do not regulate signaling pathways in these lineages. It may instead reflect miRNAs having more subtle regulatory roles that would be difficult to detect given the severity of the Dicer null phenotype. In addition our studies revealed a role for miRNAs in the control of cell cycle progression and apoptosis in both embryonic and extra-embryonic tissues and stem cells. Indeed emerging evidence suggests regulation of these processes to be a common function of miRNAs both in numerous tissues during development and in adult organisms. Altogether the analysis of *Dicer* mutant embryos and stem cells has provided us with valuable information about some of the functions that miRNAs have during early mammalian embryo development. Further work in this area should expand on our results and provide interesting insights as to how this novel class of

regulators modulate mammalian developmental processes.

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