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14. ABSTRACT

RNA interference (RNAi) is a gene silencing pathway with roles in mRNA stability, translational control, chromatin organization and genome regulation. MicroRNAs (miRNAs) are a set of small RNAs produced by the RNAi machinery that play important functions in tissue organization and maintenance of cell identity. Several miRNAs have been shown to collaborate with oncogenes in the progression of cancer, and in addition, miRNA expression profiling has revealed widespread miRNA misregulation in cancer. To address the role of miRNAs in the onset and maintenance of breast cancer, we have created embryonic stem (ES) cells and mice in which Dicer, a key enzyme in miRNA biogenesis, can be conditionally inactivated. Using these systems we have demonstrated that Dicer is required for the continued proliferation of ES cells, and that there is indeed a loss of all miRNAs and RNAi-related functions in Dicer null cells. We are using a transplantation model to test the requirement of Dicer for the proliferation of mammary stem cells, and in addition we are cloning small RNAs from mammary stem cells in order to determine the regulatory niches that miRNAs may fill in this cell type. Our ultimate goal is to assess the role of Dicer in mammary tumor stem cell maintenance.

15. SUBJECT TERMS
Dicer, RNAi, mammary stem cells, microRNA

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Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNA genes found in plants and animals that negatively regulate genes by binding consensus sequences in target mRNA transcripts and leading to translational repression or mRNA cleavage. The biogenesis of miRNAs requires successive processing of primary miRNA transcripts by the nuclear RNase III enzyme Drosha followed by nuclear export by Exportin-5 and cleavage by the cytoplasmic nuclease Dicer (Murchison & Hannon 2004). Mature miRNAs are subsequently passed to Argonaute proteins and are incorporated into RNA-induced silencing (RISC) complexes that effect miRNA-mediated target inhibition. Several recent studies have strongly implicated miRNAs as potential oncogenes in a number of cancer models, including breast cancer (Gartel & Kandel 2006, Iorio et al 2005, Volinia et al 2006).

Dicer is a large multidomain nuclease with specificity for double-stranded RNA (dsRNA). It contains an N-terminal helicase domain of unknown function, a PAZ domain, two C-terminal RNase III catalytic domains and a dsRNA-binding motif. Structural studies of Dicer have revealed that it binds the end of its pre-miRNA substrate with its PAZ domain and that the length of the miRNA is determined by the length of a helical region that separates the PAZ from the RNase III domains (Macrae et al 2006). It is a ubiquitously expressed protein whose function is required for mouse embryonic development (Bernstein et al 2003).

Dicer is encoded by a single gene in mouse that is required for the maturation of miRNAs. We have taken the approach of ablating Dicer in mouse in order to study the role of miRNAs in breast cancer and particularly in breast cancer tumor stem cell maintenance. Several studies of the role of Dicer in specific tissues and compartments have revealed that Dicer is often required for morphogenesis, but not patterning of developing tissues, and that in the absence of Dicer, tissues become disorganized and are prone to massive apoptosis (Andl et al 2006, Cobb et al 2005, Giraldez et al 2005, Harfe et al 2005, Harris et al 2006, Muljo et al 2005, Yi et al 2006).

As Dicer is required for the maturation of miRNAs, a Dicer conditional system allows the temporally-controlled ablation of all miRNAs from a tissue of interest. During the last year I have incorporated the Dicer conditional allele into mouse embryonic stem (ES) cells, and used these cells to study the role of Dicer in the maintenance of stem cells in vitro. I have also injected Dicer conditional targeted ES cells into embryos in order to create chimeric mice to found a Dicer conditional mouse colony. This colony is currently being crossed with mammary-specific cre transgenic lines. We are also taking the approach of cloning small RNAs from mammary stem cells to determine the spectrum of miRNAs and other small RNAs that contribute to the mammary gland.

Body

Gene targeting

After the Dicer conditional targeting construct was assembled, sequenced and confirmed for responsivness to Cre, it was linearized and electroporated into embryonic stem (ES) cells. I targeted two lines of 129-derived ES cells (AB2.2 and R1), as well as a line already heterozygous for Dicer (E4) (Bernstein et al 2003). ES cell clones were screened for homologous recombination at the Dicer locus, and nine correctly targeted clones were recovered, five AB2.2, one R1 and three E4 (Fig.1). Several clones were injected into C57BL/6 blastocysts to create chimeric animals, and progeny of male chimeras were subsequently screened for germline transmission by coat color. One R1-derived clone (3A8) transmitted the targeted allele through the germline and was used to establish a Dicer conditional mouse colony.

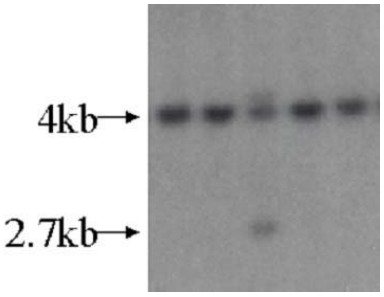


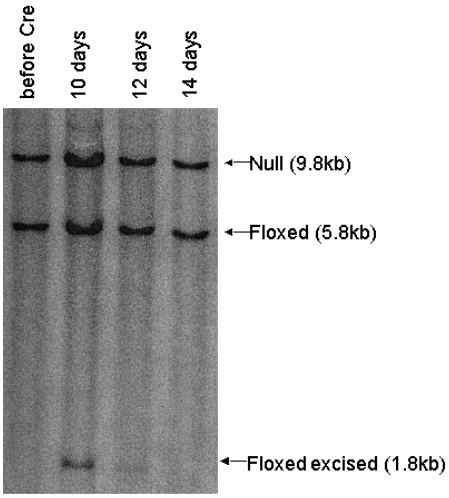
Figure 1: Gene targeting. Southern blot showing targeting of the Dicer locus with a Dicer conditional allele in ES cells.

Replacement of the only remaining endogenous allele of Dicer in E4 ES cells with the conditional allele allowed me to establish several Dicer conditional ES cell lines and mouse embryonic fibroblast (MEF) lines.

ES cells

My initial experiments transfecting Cre into Dicer conditional ES cells, and tracking the Dicer deficient cells in the population by the presence of the flox excised allele immediately revealed that Dicer deficient ES cells are rapidly lost from a mixed population (Fig.2). This result was unsurprising, given that (1) we had previously failed to double target Dicer heterozygous cells with a second null allele or to convert the second allele to a null allele by high G418 selection; (2) blastocyst outgrowth failed to yield Dicer null ES cell lines; (3) Dicer null embryos are depleted of Oct-4 positive ES cell derivatives in the epiblast (Bernstein et al 2003). Together, these results indicated that ES cells depleted of Dicer are either inviable or at a severe growth disadvantage compared to wild-type ES cells, or that loss of Dicer favors differentiation towards an unproliferative lineage.

To distinguish between these possibilities, I derived ES cell clones shortly after loss of Dicer. Conditional ES cells were transfected with Cre and plated at clonogenic densities; colonies were allowed to grow for six to seven days, at which time they were picked and transferred to a 96-well plate, with some material being retained for



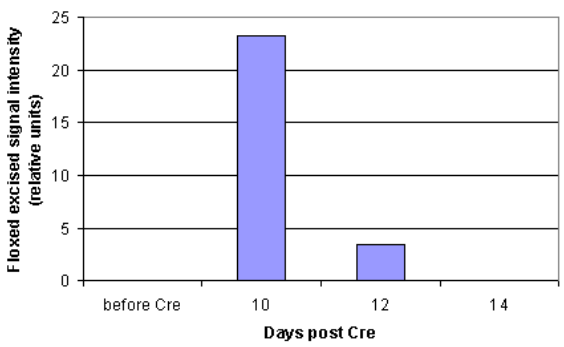


Figure 2: Dicer null ES cell clones are lost from a mixed population. (A) Southern blot showing disappearance of Dicer null allele over time. Shown quantitatively in (B).

genotyping. Dicer null and Dicer heterozygous colonies were indistinguishable at picking, but in the subsequent week all the Dicer null clones appeared to stop proliferating. While Dicer heterozygous clones could be transferred to a larger dish within five days, Dicer null clones failed to outgrow, accumulated dead cells, differentiated and many were completely lost (Fig.3). Patient passaging of these clones eventually lead, after four to twelve weeks, to the establishment of several cell lines deficient for Dicer (Fig.4).

The Dicer deficient cell lines established in this manner were presumably the result of compensatory mutations allowing some cells to escape the proliferation arrest or crisis encountered by Dicer null clones. A microarray analysis of mRNAs in Dicer null cells revealed changes in 3000 transcripts compared to the Dicer heterozygous 'sibling' clones, while SKY revealed a number of karyotypic abnormalities in Dicer null cells (data not shown). That it took up to three months to establish Dicer null lines underscores the enormity of adaptive changes that permitted their growth. Although the Dicer deficient cells retain expression of several ES cell markers, their morphology and cell cycle profile suggest that they may have undergone differentiation (data not shown)

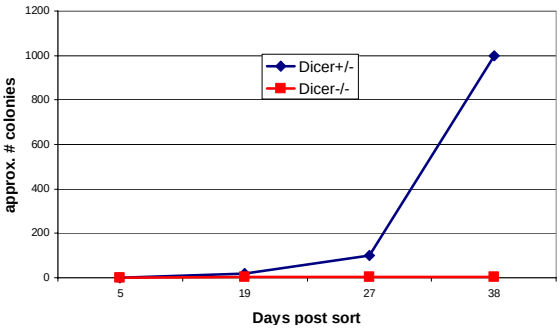


Figure 3: Dicer null ES cell colonies fail to proliferate. Colonies were counted a timepoints after cells plated at clonogenic densities post Cre transfection.

Characterization of Dicer conditional cells

The establishment of Dicer deficient cell lines allowed a more detailed characterization of the Dicer conditional allele. While the null allele present in Dicer heterozygous E4 ES cells ablates exon 23, encoding much of the second RNase III domain, the excised conditional allele removes both exons 22 and 23, encoding parts of the first and second RNase III domains including two of the three active site motifs. However, the loss of exons 22 and 23 leaves the remaining downstream exons in frame, and Dicer transcripts (excluding exons 22 and 23) can be readily detected in Dicer deficient cells. However, antibodies recognizing either the N- or C-termini of Dicer did not detect any protein, either full-length or truncated, in Dicer deficient cells (Fig.5). These results suggest that the Dicer transcript present in the Dicer deficient cells is either not efficiently translated, or that it is translated but results in a highly unstable protein.

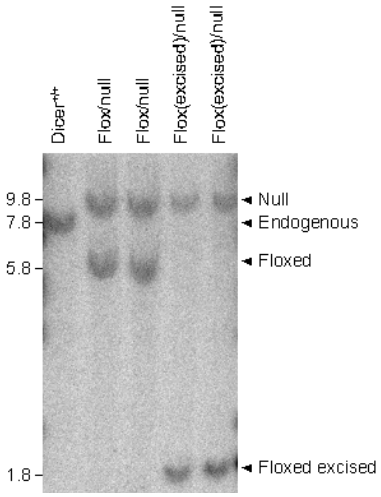


Figure 4: Establishment of Dicer null ES cell lines. Southern blot showing the creation of stable Dicer null ES cells.

Dicer is necessary for the cleavage of dsRNA during initiation of post-transcriptional gene silencing (PTGS), as well as for the excision of mature miRNAs from their hairpin precursors. As expected, these functions are absent in Dicer deficient cells, confirming that the cells are truly deficient for Dicer, and that a single Dicer enzyme is indeed responsible for these activities in mouse (Fig.6).

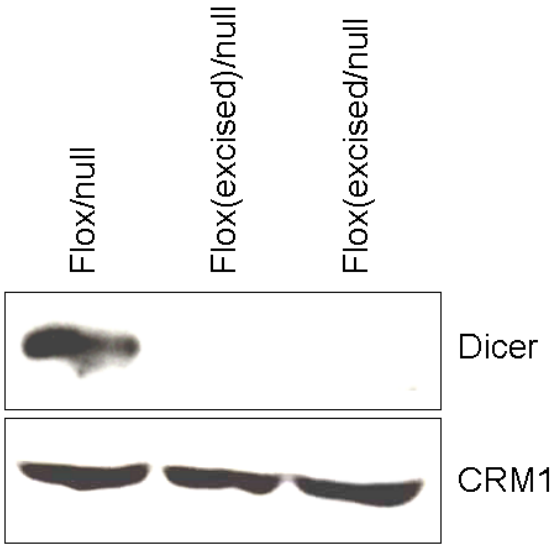


Figure 5:Dicer protein is not detectable in Dicer null ES cells. Western blot using C-terminal antibody showing absence of Dicer in null clones. CRM1 is a loading control.

Dicer is not required for heterochromatin maintenance in ES cells

In addition to its role in the biogenesis of miRNAs, in many organisms Dicer and the RNAi machinery has been implicated in the establishment and maintenance of heterochromatin (Bernstein & Allis 2005). To test whether loss of heterochromatin may be a contributing factor in the loss of Dicer null ES cells, we used chromatin immunoprecipitation and methylation sensitive southern blotting to test whether heterochromatin structure is disrupted at centromeric satellites or at interspersed repeats. Although transcripts derived from heterochromatin could be readily detected in ES cells (data not shown), we found no evidence for loss of heterochromatin in Dicer null cells, and conclude that Dicer is not required in this context for maintenance of heterochromatin integrity (Fig.7).

Cloning small RNAs from mammary stem cells

To determine the complement of small RNAs in the mammary gland, we are cloning small RNAs from mammary stem cells and Comma-D cells. To purify mammary stem cells we are using a Hoechst exclusion protocol to identify the mammary stem cell subpopulation, and we are using a sensitive small RNA cloning protocol that can recover small RNA sequences from as little as 300ng starting RNA. The small RNA spectrum of mammary stem cells will allow us to pinpoint miRNA pathways that may regulate these cells, and will help us to understand the phenotype of Dicer knockout mammary stem cells.

The role of Dicer in the mammary gland

Experiments are currently in progress to (1) reconstitute mammary glands using Dicer null mammary stem cells and (2) knock out Dicer in the developing mammary gland using Keratin-14 Cre.

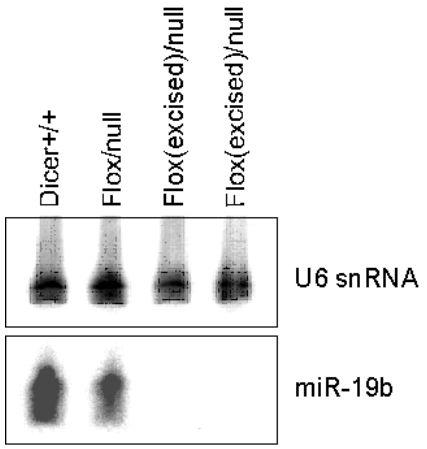


Figure 6: mature miRNAs are not detectable in Dicer null clones. MiRNA northern for miR-19b together with U6 loading control.

Key Research Accomplishments

- The creation and characterization of Dicer null embryonic stem cells, notably the finding that Dicer is required for the proliferation and viability of ES cells in vitro
- The generation of a Dicer conditional mouse model

Reportable Outcomes

Manuscripts

Murchison EP, Partridge JF, Tam OH, Cheloufi S and Hannon GJ (2005) Characterization of Dicer deficient murine embryonic stem cells *PNAS* Aug 23;102(34):12135-40

Murchison EP and Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery *Curr. Opin. Cell Biol.* Jun;16(3):223-9

Conference presentations

Murchison EP, Partridge JF, Tam OH, Cheloufi S and Hannon GJ. Characterization of Dicer deficient murine embryonic stem cells. "RNAi" Cold Spring Harbor Laboratory 2005 (poster)

Murchison EP, Hannon GJ. Proliferation defects in Dicer deficient mouse embryonic stem cells "Diverse roles of RNA in gene regulation" Beaver Run Resort, Breckenridge, Colorado 2005 (poster)

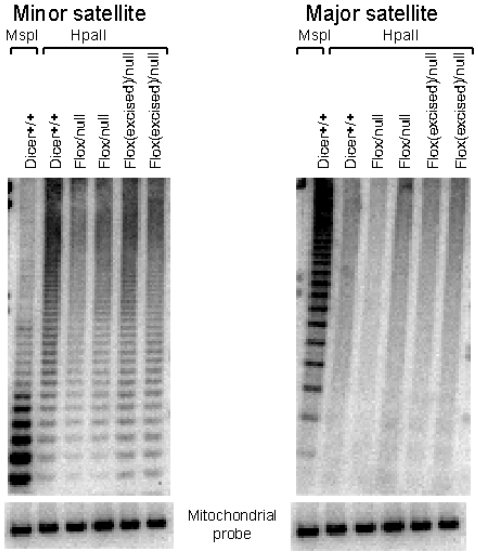


Figure 7: Pericentric heterochromatin is unperturbed in Dicer null clones, shown by methylation sensitive southern blot analysis of the major and minor satellites. MspI is a methylation insensitive isochizomer to HpaII. Mitochondrial DNA is unmethylated and a mitochondrial probe is included as a loading control.

Conclusions

Towards the goal of determining the role of Dicer in the maintenance of mammary tumor stem cells, we have created ES cells and mice carrying a conditional allele for Dicer. In order to investigate the requirement of Dicer in stem cells, we ablated Dicer in the conditional ES cells by transfecting Cre recombinase. Interestingly, we discovered that Dicer is required for the continued proliferation of ES cells despite the presence of key pluripotency regulators Nanog and Oct-4. The proliferation defect observed in Dicer null ES cells was not due to a global change in genome stability, as heterochromatin at centromeres and dispersed repeats remained intact. We propose that Dicer is required for the maintenance of proliferation of ES cells by regulating transcripts involved in cell cycle regulation at the post-transcriptional level. Interestingly, the proliferation of other stem cells has been reported to be dependent on Dicer (Hatfield et al 2005), and the maintenance of stem cells may be a conserved function of Dicer and the RNAi machinery. It will be of great interest to extend this study to the requirement for Dicer in mammary tumor stem cells, and are presently crossing the Dicer conditional mice to animals expressing Cre in the mammary gland. Together

with data on the small RNA profile of mammary tumor stem cells, we hope that this model with give significant insight into the role of Dicer in maintaining this stem cell compartment.

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