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The *miR-17/92* cluster is targeted by STAT5 but dispensable for mammary development

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Abstract

Genome wide analysis revealed the *miR-17/92* cluster as a STAT5 target. This cluster encodes six microRNAs, which predictably target genes that play a role in mammary gland development. In this study we have deleted the *miR-17/92* cluster in mammary stem cells and evaluated in the mouse its function during mammary gland development. Loss of the *miR-17/92* cluster did not affect mammary development from pre-puberty to lactation. Our studies demonstrated that while expression of the *miR-17/92* cluster is under control of the key mammary transcription factor STAT5, its presence is not required for normal mammary development or lactation.

Keywords

STAT5; *miR-17/92* cluster; mammary gland

Introduction

The transcription factors Signal Transducer and Activator of Transcription 5A and 5B (STAT5) regulate mammary gland development from alveolar proliferation and differentiation to the establishment of lactation (Cui *et al.*, 2004; Miyoshi *et al.*, 2001; Yamaji *et al.*, 2009). Studies from several laboratories have identified protein-encoding genes that are under STAT5 control, some of which execute STAT5 functions in diverse cell types including T cells (Burchill *et al.*, 2007) and mammary epithelium (Bry *et al.*, 2004). The biological complexity of STAT5's function invokes the potential participation of microRNAs, whose expression are under STAT5 control.

MicroRNAs are small non-coding RNA molecules that have the ability to modulate cell functions via posttranscriptional regulation of mRNA (Bartel, 2004; Carrington and Ambros, 2003). More than 1,000 microRNAs are encoded in the mammalian genome (Gao *et al.*). However, little is known about microRNA loci that are under cytokine and STAT5 control. Since the discovery of the regulatory role of microRNAs in *C. elegans* in 1993 (Lee *et al.*, 1993) only a handful of studies have been published on the role of microRNA in

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mammary gland development in general and on the ability of STAT5 to regulate these microRNAs in particular. The *miR-17/92* cluster encodes six microRNAs (*miR-17*, *miR-18a*, *miR19a*, *miR19b*, *miR-20a* and *miR-92a*). All members of this cluster are transcribed as a single primary transcript (Lu *et al.*, 2007). In addition, the *miR-17/92* cluster is considered to be an oncogene as its members are highly expressed in many cancers (Volinia *et al.*, 2006). Deletion of the *miR-17/92* cluster in mice resulted in neonatal lethality with specific defects in heart, lung and B cell development (Ventura *et al.*, 2008). In a study performed in our lab, we identified *miR-17/92* as a potential STAT5 target. To investigate its role during mammary gland development we have now deleted the *miR-17/92* cluster in mammary stem cells of the mouse using Cre-mediated recombination.

Results

Identification of *miR-17/92* as a STAT5 dependent microRNA

ChIP-Seq experiments revealed STAT5A islands in the *miR-17/92* locus. High resolution analysis of the DNA sequence in the STAT5 binding location revealed a highly conserved GAS (γ interferon activated sequence) motif (Figure 1A). Real time PCR analysis of expression levels of the *miR-17/92* cluster in mature virgins and at day 6 of pregnancy showed that, compared to virgin mammary tissue, expression of the *miR-17/92* cluster (except *miR-92a*) was elevated 2 to 3.5 fold. At day 6 of pregnancy *miR-17* was elevated 2.2 fold, *miR-18a* 2 fold, *miR-19b* 3.6 fold and *miR-20a* ~3 fold (Figure 1B). Based these findings we hypothesized that this cluster may have a biological role during mammary development.

Analysis of *miR-17/92*^{fl/fl};MMTV-Cre mammary tissue from pregnancy to lactation

In order to study the role of *miR-17/92* during mammary gland development we used the MMTV CreA transgenic mouse line (Wagner *et al.*, 1997) for the deletion of the *miR-17/92* cluster in mammary epithelial stem cells. *miR-17/92*^{fl/fl};MMTV-Cre dams were able to lactate and nurse pups to weaning at the same rate as controls. No mammary gland defects were detected in the whole mount or the histological sections obtained on the morning of delivery (Figure 2A). In addition, whole mount analysis from an early pregnancy time point (day 6) showed no developmental defects (Figure 2B). Therefore we conclude that *miR-17/92* is not necessary for mammary development and for the establishment of lactation.

miR-17/92 cluster target analysis in primary mammary epithelial cells

We verified the deletion of the *miR-17/92* cluster in the *miR 17/92*^{fl/fl};MMTV-Cre mammary epithelium by examining the expression levels of *miR-17* and *miR-18a* in epithelium enriched primary mammary cells. The levels of *miR-17* were reduced by ~ 5 fold in the *miR 17-92*^{fl/fl};MMTV-Cre primary mammary cells, while the levels of *miR-18a* were reduced by ~ 4 fold compared to control cells (Figure 3A). The low levels of *miR-17* and *miR-18a* in the *miR 17-92*^{fl/fl};MMTV-Cre primary mammary cells are due to low levels of contaminating fibroblast that are present in these single cell preparations. For an independent validation of the reduced levels of *miR-17/92* we analyzed the expression levels of genes that are considered to be *miR-17/92* targets *Cdkn1a* (p21), *Bim* (*Bcl2l1*), *PTEN* and components of the TGF- β pathway, SMAD2, SMAD3, TGF- β receptor-I and TGF- β receptor-II by real time PCR. Only the expression levels of SMAD2 and TGF- β Receptor-II were higher in primary cells from *miR-17/92*^{fl/fl};MMTV-Cre compared to *miR-17/92*^{fl/fl} mice (more than 1.5 fold), by 1.7 and 1.9 fold respectively, (Figure 3B).

Analysis of *miR-17-92^{fl/fl};MMTV-Cre* mammary tissue during puberty

The TGF- β pathway regulates early mammary gland development (Pierce *et al.*, 1993). Based on our findings that the TGF- β pathway is up regulated in the absence of the *miR-17/92* cluster in mammary cells, we decided to study the role of *miR-17/92* in the developing mammary gland during puberty. Analysis of mammary tissue harvested from 5, 10 and 14 week old mice showed that *miR-17/92^{fl/fl};MMTV-Cre* mammary epithelia exhibited fewer secondary branching at 10 and 14 weeks of age compared to controls (Figure 4A). In order to rule out variations in the secondary branching induced during the estrus cycle, we performed transplantations into nude mice. The transplanted mammary tissues were removed after 6 week of growth in the host. As shown in Figure 4B, *miR-17-92^{fl/fl};MMTV-Cre* and control transplants (*miR-17-92^{+/+};MMTV-Cre* mice) exhibited equivalent secondary branching. Therefore we conclude that the *miR-17/92* cluster is not vital for mammary development at early stages as well.

Discussion

Here we have identified for the first time that the *miR-17/92* cluster is a cytokine-induced STAT5-dependent microRNA gene and that expression of the entire *miR-17/92* cluster (except for *miR-92a*) increased during pregnancy. These findings support a report that showed increased levels of miRs encoded in the *miR-17/92* cluster during puberty and gestation (Avril-Sassen *et al.*, 2009). Based on all of the above we hypothesized that some of STAT5's function during mammary gland development may be executed by the *miR-17/92* cluster. However, deletion of the *miR-17/92* cluster from mouse mammary stem cells did not have a negative impact on mammary gland development and function. These mice delivered and lactated normally and no abnormalities were detected in pup growth. Furthermore, analysis of whole mount and histological samples obtained at day 6 of pregnancy did not present any noticeable defects. The lack of any defects in the mammary gland led us to the conclusion that the *miR-17/92* cluster is redundant for mammary gland development during pregnancy.

Up to date, about 30 genes are considered to be *miR-17/92* targets (Grillari *et al.*, 2010). Some have been shown to be involved in mammary development. For example, *miR-17* and *miR-20a* target *Cdkn1a* (p21), a cell cycle regulator), *miR-17* targets *Bcl2l1* (Bim-BCL2 interacting mediator of cell death) and *miR-20* and *miR-19* target *Pten*, which regulates growth, adhesion and apoptosis (Cloonan *et al.*, 2008). It was also reported that the *miR-17/92* cluster is a regulator of multiple components of the TGF- β pathway (Dews *et al.*, 2010; Mestdagh *et al.*, 2010; Petrocca *et al.*, 2008). Expression analysis of these predicted targets revealed that in the absence of *miR-17/92* from mammary epithelium the levels of TGF- β receptor II and SMAD2 are up regulated. Previously it has been shown that excessive activation of the TGF- β pathway in mammary epithelium inhibits proliferation and promotes apoptosis in the epithelial compartment in early mammary development. However, TGF- β transgenic mice eventually developed a normal mammary gland and were able to lactate (Moses and Barcellos-Hoff, 2010; Pierce *et al.*, 1993). On the other hand, TGF- β receptor II deletion in the mammary gland results in lobular-alveolar hyperplasia (Forrester *et al.*, 2005; Moses and Barcellos-Hoff, 2010).

Therefore, we speculated that *miR-17/92* may have a role in pre pubertal development. We decided to analyze the ductal development during puberty. While some ductal branching defects were noticed at early stages, the mammary gland transplantation experiments ruled them out. All of the above led us to the conclusion that the *miR-17/92* cluster does not affect any aspect of mammary gland development. However, the STAT5-miR-17/92 pathway may have a role in the development of other cells type. For examples, in the immune system *miR-17/92* cluster members are highly expressed at the pre-B cell stage. Deletion of the

miR-17/92 cluster results in inhibition of B cell development at the pro-B to pre-B transition (Ventura *et al.*, 2008), a phenotype, which resembles the *Stat5* deletion in the hematopoietic cells, where early B cell development is impaired in the absence of *Stat5* (Dai *et al.*, 2007).

In this study we used the *MMTV-CreA* line, which deletes floxed sequences in the epithelial compartment of the gland (Wagner *et al.*, 1997). However, the stromal compartment also plays a role in the regulation of ductal development. Stromal TGF- β signaling has been shown to regulate epithelial branching (Crowley *et al.*, 2005). Since the *miR-17/92* cluster has been linked to the TGF- β signaling pathway (Dews *et al.*, 2010; Mestdagh *et al.*, 2010) and it has been shown that microRNAs play a role in epithelial-stromal interactions (Ucar *et al.*, 2010), we cannot rule out the possibility that this cluster plays a role in the mammary stromal compartment to regulate epithelial branching.

During evolution, gene duplication resulted in two *miR-17/92* paralogs, *miR-106a/363* and *miR-106b/25* (Petrocca *et al.*, 2008). *MiR-106b* and *miR-93* (members of the *miR-106b/25* cluster) exhibit the same expression patterns as *miR-17/92* cluster (Avril-Sassen *et al.*, 2009). Recent studies have shown that *miR-17/92* and its two paralogs are very similar (Ventura *et al.*, 2008) and some of the microRNAs, such as *miR-17*, *miR-26a* and *miR-106a* share the same targets (Trompeter *et al.*, 2011). This suggests that a potential role of the *miR-17/92* cluster is masked by compensatory paralogous miRs.

Methods

Generation of *miR 17-92^{fl/fl};*MMTV-Cre and *miR 17-92^{fl/fl};*WAP-Cre mice

All animals were handled and housed in accordance with the guidelines of the NIDDK Animal Care and Use Committee and NIH regulations. Transgenic mice carrying the floxed *miR-17/92* gene (Ventura *et al.*, 2008) were purchased from The Jackson Laboratory. *MiR 17-92^{fl/fl}* mice were mated with the MMTV-Cre transgenic mouse line (Line A) (Ventura *et al.*, 2008; Wagner *et al.*, 1997).

Chromatin Immunoprecipitation Coupled with Illumina sequencing (ChIP-Seq)

ChIP-Seq experiments were performed as described (Bing-Mei *et al.*, 2012). In brief, mouse embryonic fibroblasts (MEFs) were infected with a retroviral-expression vector carrying a wild-type *Stat5a* gene. For analysis they were starved for 5 hours and then stimulated for 2 hours with growth hormone (250 ng/ml) or left untreated. The cells were cross-linked with 1% formaldehyde for 10 min and chromatin was extracted. Antibodies against STAT5A (PA-ST5A, R&D System), and IgG (AB-105C, R&D System) were used for precipitation. The ChIP DNA fragments were blunt-ended, ligated to the Solexa paired-end adaptors, and sequenced with the Illumina GAIIx genome analyzer.

Histological analysis

Mammary glands were fixed in 10% formalin, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin and imaged on an Olympus BX51 microscope equipped with a Zeiss Axiocam Cc1 and Axiovision software. For whole mount analysis the mammary tissues were dissected and spread on a glass slide. After fixation for 4 hours in Carnoy's solution, the glands were hydrated, stained with carmalum, dehydrated and mounted with Permount (Fisher Scientific).

Preparation of enriched mammary epithelial cells

Single cells from mammary glands were prepared as previously described (Yamaji *et al.*, 2009). In brief, mammary glands from virgin female mice at 12-14 weeks of age were incubated for 8 hours at 37°C in complete EpiCult-B medium (supplemented with 5% fetal

bovine serum) containing 300 U/ml collagenase and 100 U/ml hyaluronidase. After lysis of red blood cells, single cell suspensions were prepared by sequential treatment with 0.25% trypsin for 1–2 min, followed with 5 mg/ml dispase II and 0.1 mg/ml DNase I for 2 min. The cells were filtered through a 70- μ m mesh before use. All reagents were from StemCell Technologies Inc. (Vancouver, Canada).

RNA extraction and analysis

MiRNA and mRNA were extracted with miRNeasy system (Qiagen). Expression analysis was performed by using TaqMan probes (Applied Biosystems; Foster City, CA) for real-time PCR.

Transplantation of mammary epithelia into the cleared fat pad of nude mice

The transplantation was performed as previously described (Miyoshi *et al.*, 2001). In brief, athymic nude mice (3-wk-old) were anesthetized and the proximal part of the endogenous mammary epithelial compartment was removed surgically. Pieces of mammary tissue from mature virgin donor mice (*miR-17/92^{fl/fl};MMTV^{Cre}* and *miR-17/92^{+/+};MMTV^{Cre}* mice as controls) were grafted into the cleared fat pads of recipients. 6 weeks after transplantation, transplanted fat pads were harvested from virgin hosts. For whole mounts, mammary glands were removed, fixed in Carnoy's fixative overnight, stained in carmine alum and mounted.

Statistics

All statistical analyses were performed using a two-tailed unpaired Student's t test. A p-value of 0.05 or less was considered significant.

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Figure 1. MiR-17/92 cluster is regulated by STAT5

(A) A view of STAT5A binding on the *miR-17/92* locus in MEFs. The insert shows a high resolution view of potential STAT5 GAS sites in the *miR-17/92* locus. (B) Expression levels of *miR-17/92* cluster members were analyzed in virgins and six days pregnant mice. Values are means $\pm$ S.D. for three independent experiments. In each analysis virgin mammary tissue was used as the control sample (fold change = 1).

Figure 2. Whole mounts and histological analyses of *miR 17-92^{fl/fl};MMTV-Cre* mammary tissues harvested on the day following delivery of pups and on day six of pregnancy (A) Whole mount (panel a - *miR 17-92^{fl/fl}*, panel d - *miR 17-92^{fl/fl}:MMTV-Cre*) and hematoxylin and eosin (H&E)-stained sections (panel b, c - *miR 17-92^{fl/fl}*, panel e, f - *miR 17-92^{fl/fl}:MMTV-Cre*) of mammary tissues collected after parturition (B) Whole mount (panel a - *miR 17-92^{fl/fl}*, panel d - *miR 17-92^{fl/fl}:MMTV-Cre*) and hematoxylin and eosin (H&E)-stained sections (panel b, c - *miR 17-92^{fl/fl}*, panel e, f - *miR 17-92^{fl/fl}:MMTV-Cre*) of mammary tissues collected on day six of pregnancy. Mammary epithelia developed to same extents in the *miR 17-92^{fl/fl}* and in the *miR 17-92^{fl/fl}:MMTV-Cre* after parturition and on day six of pregnancy. Scale bar indicates 0.02 mm in a and d, 100 μ m in b and e, 10 μ m in c and f.

Figure 3. *miR-17/92* expression and target analysis in *miR 17-92^{fl/fl};MMTV-Cre* primary mammary cells

(A) Expression of *miR-17* and *miR-18a* in *miR 17-92^{fl/fl};MMTV-Cre* and *miR 17-92^{fl/fl}* primary mammary cells show decreased levels of *miR-17* and *miR-18a* in the *miR 17-92^{fl/fl};MMTV-Cre* primary mammary cells. (B) Among the predicted targets *Bcl2l1*, *Cdkn1a*, *Pten*, *Smad2*, *Smad3*, *Tgfbr1* and *Tgfbr2* only *Tgfbr2* and *Smad2* are upregulated by 1.7 and 1.9 fold, respectively, in *miR 17-92^{fl/fl};MMTV-Cre* compared to *miR 17-92^{fl/fl}* primary mammary cells. In each analysis, the *miR 17-92^{fl/fl}* primary mammary cells were used as the control sample (fold change = 1). Values are means $\pm$ S.D. for three independent experiments.

Figure 4. Whole mount of mammary tissue from at early time points of development (A) Whole mount analyses of mammary tissue from *miR-17/92^{fl/fl};MMTV-Cre* and *miR-17/92^{fl/fl}* at the age of 5 (panel a and b), 10 (panel b and c) and 14 weeks (panel e and f). Red arrow marks a site of secondary branching. Scale bar indicates 0.02 mm (B) Whole mount analyses of transplanted mammary tissue of control (panel a, b and c) and *miR-17/92^{fl/fl}; MMTV-Cre* (panel d, e and f) 6 weeks after transplantation. Higher magnification of the whole mounts shown in middle and lower panels. No differences were detected between the *miR-17/92^{fl/fl};MMTV-Cre* and *miR-17/92^{fl/fl}* mammary gland at all the time points analyzed and in the transplanted mammary gland. Scale bar indicates 100 μ m in a and d, 50 μ m in b and e, 25 μ m in c and f.