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ORIGINAL ARTICLE

miR-216 and miR-217 expression is reduced in transgenic mouse models of pancreatic adenocarcinoma, knockout of miR-216/miR-217 host gene is embryonic lethal

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Abstract Mice harboring a G12D activating Kras mutation are among the most heavily studied models in the field of pancreatic adenocarcinoma (PDAC) research. miRNAs are differentially expressed in PDAC from patients and mouse models of PDAC. To better understand the relationship that Kras activation has on miRNA expression, we profiled the expression of 629 miRNAs in RNA isolated from the pancreas of control, young, and old P48[+/Cre];LSL-KRAS[G12D] as well as PDX-1-Cre;LSL-KRAS[G12D] mice. One hundred of the differentially expressed miRNAs had increased expression in the advanced disease (old) P48[+/Cre];LSL-KRAS[G12D] compared to wild-type mice. Interestingly, the expression of three miRNAs, miR-216a, miR-216b, and miR-217, located within a ~30-kbp region on 11qA3.3, decreased with age (and phenotype severity) in these mice. miR-216/-217 expression was also evaluated in another acinar-specific ELa-Kras[G12D] mouse model and was downregulated as well. As miR-216/-217 are acinar enriched, reduced in human PDAC and target KRAS, we hypothesized that they may maintain acinar differentiation or represent tumor suppressive miRNAs. To test this hypothesis, we deleted a 27.9-kbp region of 11qA3.3 containing the miR-216/-217 host gene in the mouse's germ line. We report that germ line deletion of this cluster is embryonic lethal in the mouse. We estimate that lethality occurs shortly after E9.5. qPCR analysis of the miR-216b and miR-217 expression in the heterozygous animals showed no difference in expression, suggesting haplosufficiency by some type of compensatory mechanism. We present the differential miRNA expression in Kras[G12D] transgenic mice and report lethality from deletion of the miR-216/-217 host gene in the mouse's germ line.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most lethal type of cancer in the USA, and it is estimated that it will become the second most deadly cancer in the USA by 2030 (Rahib et al. 2014, Siegel, Miller, and Jemal 2015). The last decade or more has been spent trying to understand the molecular and signaling mechanisms that regulate the development of PDAC so that better therapies and methods for early detection may be developed. This work has led to extensive growth of genetically engineered mouse models which have provided experimental systems to better understand and

identify the molecular mechanisms underlying PDAC development. As the Kras G12D oncogene has been identified as the key oncogenic mutation that has been associated with development of PDAC in humans, the majority of genetically engineered mouse models have been based on this particular mutation. Since PDAC typically arises from pre-neoplastic lesions, termed PanINs, it becomes extremely important to use mouse models which allow us to study molecular changes that happen during this phase. Hruban and coworkers (Vincent et al. 2011) provided a framework by which PDAC arises from a series of genetic mutations which involves inactivation of tumor suppressor genes such as p16, DPC4, P53, and BRCA along with the activation of oncogenes such as Kras.

Pioneering work by Hingorani and Tuveson used Ptf1a and PDX-1 promoters to limit expression of Kras harboring the activating G12D mutation to the developing pancreas, restricting expression at E8.5–E9.5 (Hingorani et al. 2003). Popular modifications that combine the $Kras^{G12D}$ mutation with other mutant genes including p53 (Hingorani et al. 2005), Tgfβ (Ijichi et al. 2006), Smad4 (Bardeesy et al. 2006), and Brca1 (Shakya et al. 2013). Combining the double or sometime triple mutations decreases the latency of PDAC progression in mice. Despite its ductal appearance, PanINs and PDAC can develop from pancreatic acinar cells when mutant Kras is restricted using an acinar-specific promoter (Desai et al. 2007, Grippo et al. 2003, Guerra et al. 2007, Habbe et al. 2008); however, the same was not the case when Kras was restricted to the epithelial cells of the pancreas (Brembeck et al. 2003). These studies revealed an underlying role of pancreatic acinar cells in PDAC development.

microRNA (miRNAs) are small noncoding RNAs that recognize and bind to the 3′ UTR of mRNAs resulting in translational repression. Numerous profiling studies over the past decade have shown that the expression miRNAs are altered in cancer, including pancreatic (Bloomston et al. 2007, Lee et al. 2007, Szafranska et al. 2007, Volinia et al. 2006). miRNAs reported as deregulated in human PDAC include overexpressed miRNAs (miR-21, miR-155, miR-181a/b, and miR-221/-222) and those with reduced expression in the tumor (miR-217, miR-216a/b, miR-375, and miR-148a) recently reviewed in Visani et al. (2015). It was further noted that overexpression of certain miRNAs (i.e., miR-155 or miR-21) (Costinean et al. 2006, Medina, Nolde, and Slack 2010) or knockout of other miRNAs such as miR-122 in the liver can contribute to the development of cancer in mice (Hsu et al. 2012, Tsai et al. 2012). However, to the best of our knowledge, we are unaware of any published work on miRNA knockout or knockin mouse models that show PDAC development.

The initial objective of this study was to evaluate the miRNA expression in the $Kras^{G12D}$ transgenic mouse model of PDAC. We report that a large number of miRNAs are deregulated in LSL-Cre $Kras^{G12D}$ transgenic mice, where the majority of them are increased in expression and that only a few miRNAs are reduced in expression. The three miRNAs with reduced expression in the $Kras^{G12D}$ pancreas, (i.e., miR-216a, miR-216b, and miR-217) are contained within a genomic cluster on 11qA3.3. Although, these three miRNAs have been reported in the literature to be downregulated in PDAC patients (Deng et al. 2014, Schultz et al. 2012, Szafranska et al. 2007, Szafranska et al. 2008, Vychytilova-Faltejskova et al. 2015), their role in the pancreas remains completely unknown. To investigate a potential tumor suppressive role for these miRNAs, we developed a germ line knockout of the miR-216/-217 host gene. We conclude that deletion of the 27.9-kbp region that hosts the miR-216/-217 gene is embryonic lethal in mice.

Materials and methods

$Kras^{G12D}$ transgenic mice

Transgenic mice harboring an activating Kras mutation were used. Total RNA from PDX-1-Cre;LSL-KRAS^{G12D}, P48$^{+/Cre}$;LSL-KRAS^{G12D}, PDX-1-Cre/wt (control), LSL-KRAS^{G12D}/wt (control), and P48Cre/wt (control) mice was isolated using miRNeasy (Qiagen) according to the manufacturer's protocol and the RIN was determined using the Agilent Bioanalyzer. The mean age of the mice was 209 days (PDX-1-Cre;LSL-KRAS^{G12D}) and 34 days (young) and 239 (old) days for P48$^{+/Cre}$;LSL-KRAS^{G12D}. In the P48$^{+/Cre}$;LSL-KRAS^{G12D} model, young mice are representative of early disease where only some PanINs have developed, where old mice are advanced in tumor progression. The mean age of all the control mice was 52 days. Detailed information on the mice may be found (Jiang et al. 2016). The mean age of the acinar-specific ELa-Kras^{G12D} mouse was 240 days compared to that of wild-type mice which was 220 days.

RNA isolation and qPCR

For the transgenic mice and controls, RNA was supplied to us from the Tuveson lab. All RNA samples had a RNA Integrity Number (RIN) of 5 or greater (Jiang et al. 2016). Pancreatic RNA was isolated from the ElasCre and miR-216/-217$^{+/-}$ mice as described (Azevedo-Pouly, Elgamal, and Schmittgen 2014). All RNA samples had a RIN of 8 or greater.

qPCR

Total RNA was treated with DNase I and cDNA was made using the Life Technologies assays to 629 mature miRNAs as described (Schmittgen et al. 2008) or by priming with random hexamers as described (Tirmenstein et al. 2000) for the analysis of the miR-216/-217 host gene expression. qPCR was

performed on an Applied Biosystems 7900 HT real-time PCR instrument that was equipped with a 384-well reaction plate using standard conditions. The 18S rRNA was used as an internal control and data were analyzed using the comparative C_T method. To validate the normalizer for the qPCR, we report the C_T values for the various test and control groups for all experiments performed in the study. The expression of the internal control gene did not significantly change between the test and control groups, and there were no situations where a significant change in the internal control between test and control groups exists (FC < 1.5, P > 0.05). A miRNA was considered not expressed in a particular sample if the mean $C_T \geq$ 36 in both the old and control groups, and they were considered differentially expressed if the fold change between the comparative groups was greater than 1.5-fold and P < 0.05 (Student's t test). Primer sequences are available upon request.

Mouse miR-216a, miR-216b, and miR-217 targeting vector design and cloning

A targeting vector including two homology arms of 4.8 kbp upstream and 5.0 kbp downstream of the 27.9-kbp region containing the miR-216/-217 cluster was synthesized by Vega Biolab (Philadelphia, PA). Bacterial colonies positive for this construct were selected by kanamycin resistance and DNA plasmid was purified according to standard phenol:chloroform extraction. Figure 3 shows the targeting strategy: successful homologous recombination leads to replacement of 27,863 bp (28,735,939–28,763,801; GRCm38 mm10, UCSC database) containing the miR-216/-217 cluster with a ~2.0-kb neomycin cassette resulting in a germ line knock-out (KO).

Generation of chimeras and miR-216/-217 KO F1 breeding

The Genetically Engineered Mouse Modeling Core of the Ohio State University introduced the targeting vector into S1B6 mouse embryonic stem (mES) cells according to standard procedure (Piovan et al. 2014). Briefly, early passage exponentially growing mES cells were electroporated in the presence of 25 μg of linearized miR-216/-217 KO targeting vector. After a recovery period of 24 h, cells were transferred to culture medium containing 200 μg/ml G148 and ganciclovir (2 × 10-6 M) to select for specifically targeted clones. Two positively recombined clones were then microinjected into C57Bl/6 blastocyst-stage embryos to generate chimeric mice. High percentage chimeras (>90 %) in turn were breed to C57Bl/6 wild-type female mice. Heterozygous F1 offspring were initially genotyped using the same Southern blot probes used for mES cell selection.

miR-216/-217 KO mouse colony maintenance

All mouse work was performed prior approval by the Institutional Animal Care and Use Committee (I-ACUC) and according to guidelines established by the University Laboratory Animal Resources (ULAR) of The Ohio State University (OSU). Breeding was done with two females and one male per cage. Breeders were started between 6–8 weeks of age and retired at 8 months of age. Weaning was performed at 21 days and tail snips were collected for genotyping.

miR-216/-217 KO genotyping PCR

Genotyping PCR was optimized in the F1 generation and used to confirm genotype in F1s and subsequent litters. Primers were designed for the miR-216b locus in the genome to yield a 286-bp wild-type band and for the Neo cassette to yield a 351-bp mutant band. Internal primer to amplify a 200-bp fragment of the *Fabpi* gene was also used to control the quality of each PCR reaction. Tail snips were digested with 300 μl Direct PCR Lysis Reagent (Viagen Biotech, Los Angeles, CA) according to the instructions of the manufacturer. PCR was performed with Crimson Taq (NEB, Ipswich, MA), 0.7 μM primer pairs, and 1 μl of lysate in 15 μl reactions. Cycling conditions were as follows: 94 °C incubation for 30 s, 30 cycles of 94 °C-20 s/60 °C-30 s/68 °C-40 s, followed by 10 min at 68 °C. Bands were detected with a 2 % agarose gel stained with ethidium bromide. PCR genotyping of experimental mice was confirmed by Southern blotting. Sequence of primers are available upon request.

Southern blotting

Southern blotting was performed according to a previously published protocol (Piovan et al. 2014) to confirm homologous recombination in mES cells, F1, and selected mice of subsequent generation. DNA was extracted from mES in 96-well plates or tail snips by phenol:chloroform extraction and digested with BglII or Hind III for Southern blotting analysis of 5′ or 3′ recombination, respectively. 5′ and 3′ Southern probes were amplified by PCR from genomic regions not included in the vector homology arms corresponding to 28,730,511–28,731,035 (5′) and 28,769,000–28,769,799 (3′) (GRCm38 mm10, UCSC database) of mouse chromosome 11, respectively.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) from the Broad Institute was used to evaluate the relationship between the miRNA and acinar gene expression. Gene expression data (GSE33322) on 6–8-month-old Pdx1-cre;KrasLSL^{G12D} mice was accessed and converted into a GCT file using the Microsoft Excel.

GSEA was performed by comparing three sample data sets for normal pancreas (high miR-216/-217 expression) to three data sets for PDAC (low miR-216/-217 expression). A list of 30 acinar genes as reported by Morris et al. were used for the enrichment analysis (Morris et al. 2014).

Results

miRNAs are differentially expressed in transgenic mouse models of PDAC

The expression of 629 miRNAs was profiled in RNA isolated from the pancreas of young and old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice, PDX-1-Cre;LSL-KRAS^{G12D} mice, and controls. Of the 629 miRNAs profiled, 229 were expressed as defined in the "Materials and methods" section. The differential expression of the miRNAs was compared in the old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice versus the control mice. Of the 229 expressed miRNAs, 103 were differentially expressed, with 100 having increased expression and three miRNAs with reduced expression (Fig. 1) and STable 1. In a comparison of young P48$^{+/Cre}$;LSL-KRAS^{G12D} mice versus control mice, only five miRNAs were differentially expressed (all increased expression, STable 1). Also in the PDX-1-Cre;LSL-KRAS^{G12D} vs control, 21 were differentially expressed with 19 increased and 2 miRNAs decreased (STable 1).

Interestingly, only three miRNAs had reduced expression in the old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice versus the control mice comparison; miR-216a, miR-216b, and miR-217 (Fig. 1, STable 1). miR-216a, miR-216b, and miR-217 are contained within a 17.6-kbp segment on chromosome 11qA3.3. The expression of miR-216a, miR-216b, and miR-217 sequentially decreased from control to young and old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice (Fig. 2a–c). The expression of these three miRNAs in the PDX-1-Cre;LSL-KRAS^{G12D} mice was in between that of the young and old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice (Fig. 2a–c). The findings of intermediate miR-216/-217 expression in the old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice are not surprising due to the mosaic expression of Kras in these mice. A recent review of the differential miRNA expression in human PDAC tissues compiled an 18 miRNA signature of PDAC from the differentially expressed miRNAs (13 increased and 5 decreased) reported in at least three studies (Visani et al. 2015). Our data from miRNA expression profiling in the KRAS^{G12D} transgenic mouse model of PDAC report that 8 of the 18 miRNAs in the human signature were also differentially expressed in the mouse model of PDAC (5 increased and 3 decreased). These include miR-100, miR-181a, miR-181b, miR-222, and miR-223 (increased) and miR-216a, miR-216b, and miR-217 (decreased).

The Pdx and Ptf1a promoters drive the differentiation of early pancreas progenitors from the endoderm during
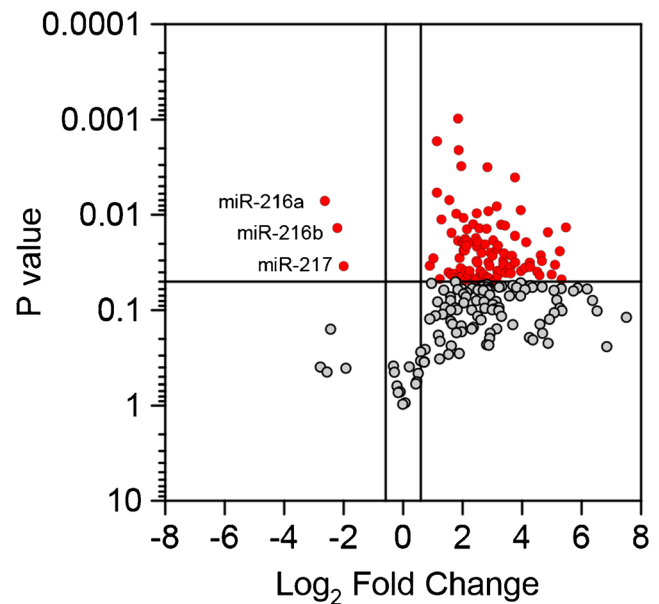

Fig. 1 Volcano plot, miRNA expression in KC (p48) mice. The expression of 629 miRNAs was profiled in control and old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice. Of the expressed miRNAs shown, 100 had increased expression and 3 had reduced expression in the old P48$^{+/Cre}$;LSL-KRAS^{G12D} mice compared to the control

development. These progenitors give rise to both exocrine and endocrine populations of the pancreas (Kawaguchi et al. 2002). As miR-216a, miR-216b, and miR-217 were reported to be highly enriched in rat acini (Bravo-Egana et al. 2008) and the current belief is that pancreatic ductal adenocarcinoma arises from pancreatic acinar cells (Guerra et al. 2007), we wished to determine the pancreatic expression of the three reduced miRNAs in a third transgenic mouse, the Ela-Kras^{G12D} mouse (Grippo et al. 2003). The mutant Kras in these mice are driven by the acinar-specific elastase promoter (Grippo et al. 2003). We found that the expression of the three miRNAs reduced with age in the Ela-Kras^{G12D} mice (Fig. 2d). To establish a relationship between expression of miR-216/-217 and acinar genes, GSEA was performed using a publically available data set of on pancreatic gene expression in Pdx1-cre;KrasLSL^{G12D} mice. A set of 30 acinar-specific genes were positively enriched in the normal pancreas with high miR-216/-217 expression compared to the PDAC with low miR-216/-217 expression (Fig. 3). We conclude that miR-216a, miR-216b, and miR-217 are downregulated in the Pdx-1, Ptf1a, and Ela-Kras^{G12D} transgenic models of PDAC and that lower levels of the miRNAs correlates with lower expression of acinar-specific genes.

Germline knockout of miR-216a, miR-216b, and miR-217

The reduced expression of miR-216a, miR-216b, and miR-217 with increasing age of the mice was of interest to us for several reasons. Firstly, these three miRNAs are reduced
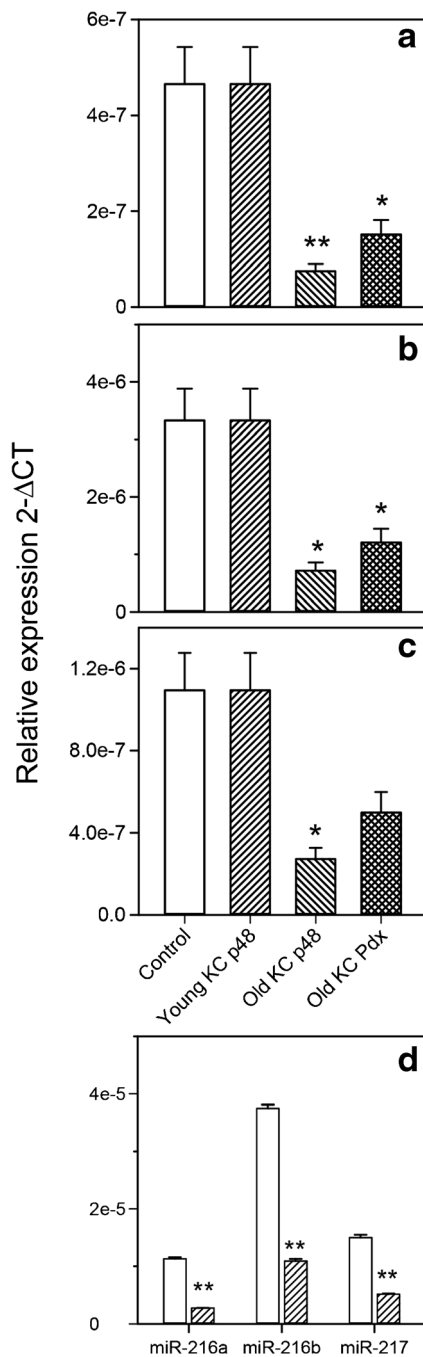
Fig. 2 miR-216a, miR-216b, and miR-217 expression in control and transgenic mice. The miR-216a (**a**), miR-216b (**b**), and miR-217 (**c**) expression is shown in young and old P48$^{+/Cre}$;LSL-KRAS^{G12D}, old PDX-1-Cre;LSL-KRAS^{G12D}, and control mice. **d** Pancreatic expression of miR-216a, miR-216b, and miR-217 in Elas Kras^{G12D} mice. Mean ± S.E.M. *$P < 0.05$, **$P < 0.01$ compared to control

Fig. 3 Gene set enrichment analysis in normal pancreas and PDAC. The gene set enrichment of expression data from 6–8-month-old Kras^{G12D} or control mice obtained from data set GSE33322 were studied. A set of 30 acinar-specific genes were used for the enrichment

during PanIN progression, (Yu et al. 2012; Xue et al. 2013), pancreatitis, and PDAC in both patients (Deng et al. 2014, Schultz et al. 2012, Szafranska et al. 2007, Szafranska et al. 2008, Vychytilova-Faltejskova et al. 2015, Xue et al. 2013, Yu et al. 2012) and transgenic mice (Ali et al. 2012, Rachagani et al. 2015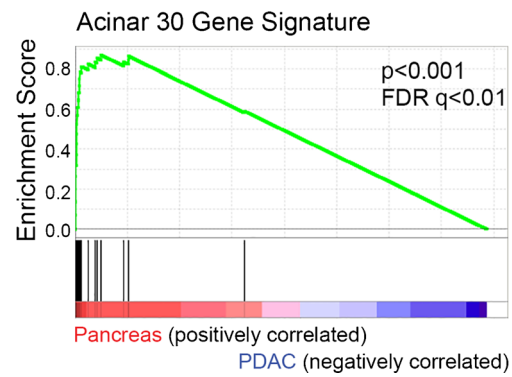). Secondly, miR-216b targets KRAS in nasopharyngeal carcinoma (Deng et al. 2011), miR-217 targets KRAS in PDAC cells, and miR-217 oligo reduces proliferation of PDAC cell lines (Zhao et al. 2010). Thus, it is possible that these three miRNAs function as tumor suppressors in pancreatic acini and their repression contributes to both increased KRAS expression as well as tumorigenesis.

To test this hypothesis in vivo, we deleted a 27.9-kbp region on chromosome 11 of the mouse genome containing the miR-216a, miR-216b, and miR-217 host genes in the germ line of mice (Fig. 4a). At the time the mouse was generated, the targeting was engineered for the complete germ line ablation of the cluster deemed the fastest approach possible in just one targeting event due to the large size of locus (~28 kbp). Following transduction with the targeting vector and in vitro selection, a successful recombination was demonstrated by Southern blot analysis of G418/gancyclovir-resistant mES clones (Fig. 3b). In addition to a ~19.9-kbp wild-type fragment, mES DNA digested with Bgl II and surveyed with a locus-specific probe designed outside the 5′ region of homology to the targeting vector showed a mutant 6.8 kbp that results from the additional Bgl II site introduced by the neomycin cassette (Fig. 4b).

We performed a serial breeding of time of heterozygous F1 and heterozygous F2 animals for an extended period. Since the genotyping of over 200 pups did not yield any homozygous mice, we suspected embryonic lethality and set out to perform embryo analysis to establish the occurrence of lethality during development. First, we collected wild-type embryo RNA to determine miR-216a, miR-216b, and miR-217 expression during development. Figure 5 shows that expression of the miRNAs in whole wild-type embryo peaks between 11.5 and 13.5 days post coitus (d.p.c.). Based on this observation, we collected embryos at 7.5, 9.5, 11.5, 13.5, and 18.5 d.p.c. from multiple heterozygous mating cages. We were able to collect a viable homozygous embryo at 9.5 d.p.c. However, collection at later embryonic days did not show any viable
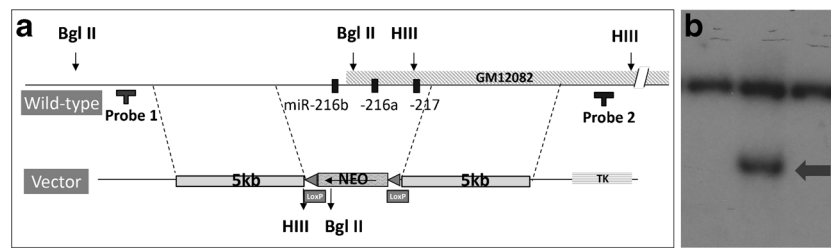
Fig. 4 Targeting strategy to generate miR-216/-217 knockout mice. **a**. Wild-type miR-216/-217 locus (*top*) and miR-216/-217 targeting vector (*below*). Targeting homologies are illustrated as *gray boxes*. Designed probes and restriction enzymes are also included. **b**. Representative Southern blot of three G418-resistant clones following Bgl II digestion of extracted DNA and survey with a 5′ locus-specific probe. In addition to a 19.9-kbp wild-type band, correctly recombined clones (middle lane) showed a mutant 6.8-kbp fragment (*arrow*) due to the presence of a Bgl II site in the neomycin cassette replacing the miRNA cluster

homozygous animal, suggesting that complete loss of the miR-216/-217 cluster causes embryonic lethality around 9.5 days of gestation or shortly thereafter (Fig. 6).

Haplosufficiency in F2 miR-216/-217 KO mice

Due to the triple miRNA knockout mouse being embryonic lethal, we shifted out efforts to phenotyping the miR-216/-217 heterozygous (het) mice. We first measured miR-216b and miR-217 levels in the pancreas of miR-216/-217 het ($n = 18$) and wild-type mice ($n = 11$). Surprisingly, there was no significant reduction of miR-216b or miR-217 in the miR-216/-217 het mice. If anything, we observed a slight increase in mature miR-216b and miR-217 expression in the heterozygous mice (Fig. 7a). To see if any differences exist between the expression of the miR-216/-217 host gene in wt and het mice, we analyzed two different segments, the 3′ miR-216/-217 host gene transcript (HGT), and the 5′ HGT, by qPCR. The pattern of expression for the host gene miR-217/-217 was similar to the mature miRNA (Fig. 7b). The change in the mouse body weight was compared between the wild-type control mice and miR-216/-217 het mice but found no significant difference (data not

shown) nor were there any significant changes on the plasma biochemistry between the het and wild-type mice with the exception of a slight increase in total bilirubin (Fig. 7c). Finally, microscopic evaluation of pancreatic sections of the miR-216/-217 het mice showed no remarkable changes compared to the wild-type controls (data not shown).

Discussion

We profiled the expression of over 600 mature miRNAs in KRAS[G12D] transgenic mouse models of PDAC. A large percentage of miRNAs had increased expression in the transgenic mice compared to controls. We also profiled the transcribed ultraconserved region RNA (T-UCR) expression in the identical RNA samples from this study along with the RNA isolated from patient's PDAC specimens (Jiang et al. 2016). The T-UCRs had primarily increased expression in both the mouse and human pancreases. We used qPCR profiling of clinical specimens of PDAC to show a similar trend as reported here (predominate miRNA upregulation with reduction in

Fig. 5 miR-216a, miR-216b, and miR-217 expression levels in the mouse embryo. Wild-type embryos were collected for RNA and analyzed for miR-216/-217 expression by qPCR
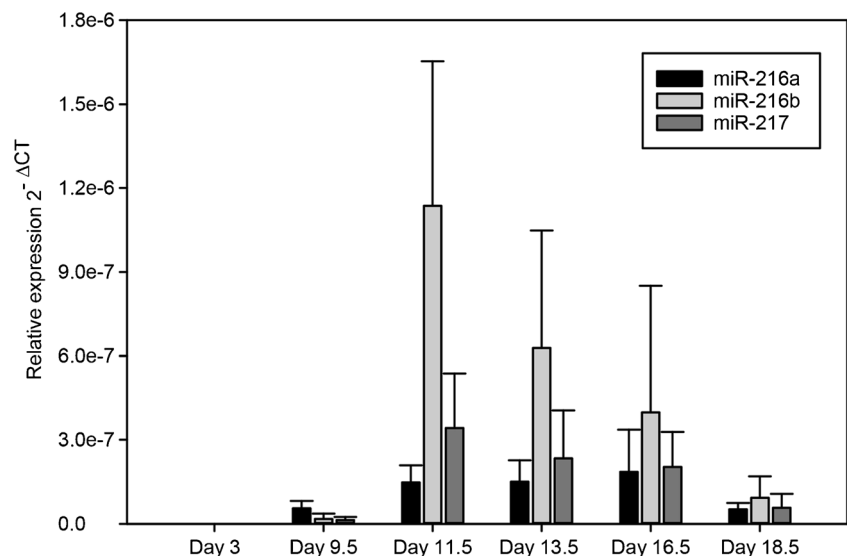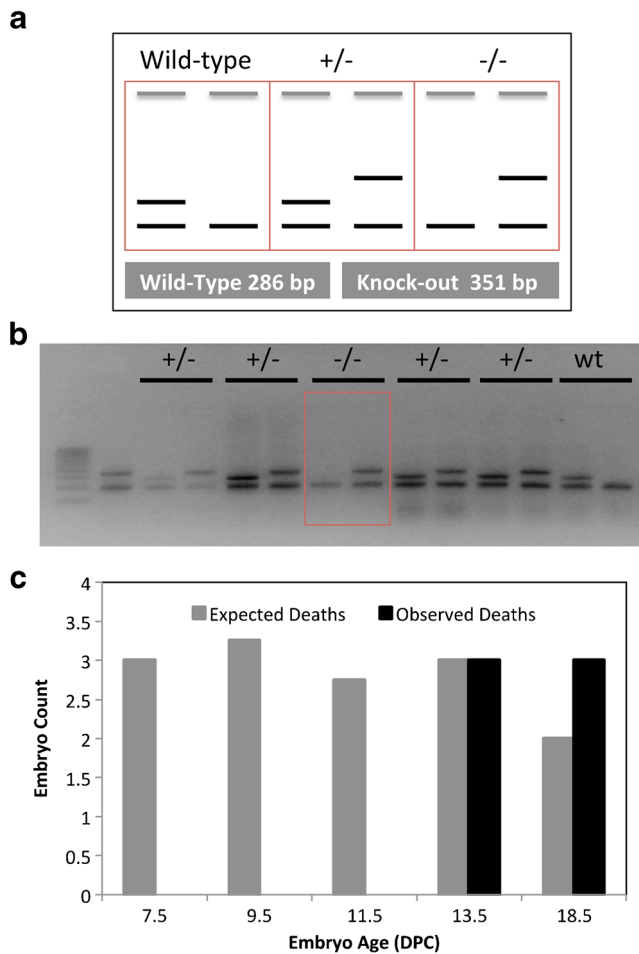
Fig. 6 Embryonic lethality in miR-216/-217 homozygous mice. **a**. The miR-216a, miR-216b, and miR-217 expression was measured in the mouse embryos at various stages of development by qRT-PCR. **b**. miR-216/-217 expression during development at E3.5, E9.5, 13.5, and 17.5. **c** (Revise based on new). Genotyping of the E9.5 litter revealed a viable homozygous embryo

Fig. 7 Analysis of haploinsufficient mice. **a** Mature miR-216b and miR-217 and (**b**) miR-216/-217 3′ and 5′ host gene transcript (HGT) expression in wild-type (*open bars*) and miR-216/-217[+/−] (*hatched bars*) mice. **c**. Full blood biochemistry panel results for wild-type and heterozygous mice. (*n* = 18) Mean ± S.E.M.

miR-216/-217, unpublished data). It is interesting that two classes of noncoding RNAs (miRNAs and T-UCRs) have predominately increased expression in both mouse models and clinical specimens of PDAC.

The expression of three miRNAs transcribed from the miR-216/-217 miRNA family was reduced in the P48[+/Cre];LSL-KRAS[G12D], PDX-1-Cre;LSL-KRAS[G12D], and ELa-Kras[G12D] mice. Our data confirm the recent findings of Rachagani et al. (2015) who reported a reduction in the expression of miR-216a, miR-216b, and miR-217 in the pancreas P48[+/Cre];LSL-KRAS[G12D] mice (Rachagani et al. 2015). The results of our study parallels those miRNA profiling studies in human PDAC showing an increased expression of several miRNAs yet reduction in the acinar miRNAs (Visani et al. 2015). Similar patterns of miRNA expression in both transgenic mice and human PDAC further supports the use of these models for experimental pancreatic cancer research.
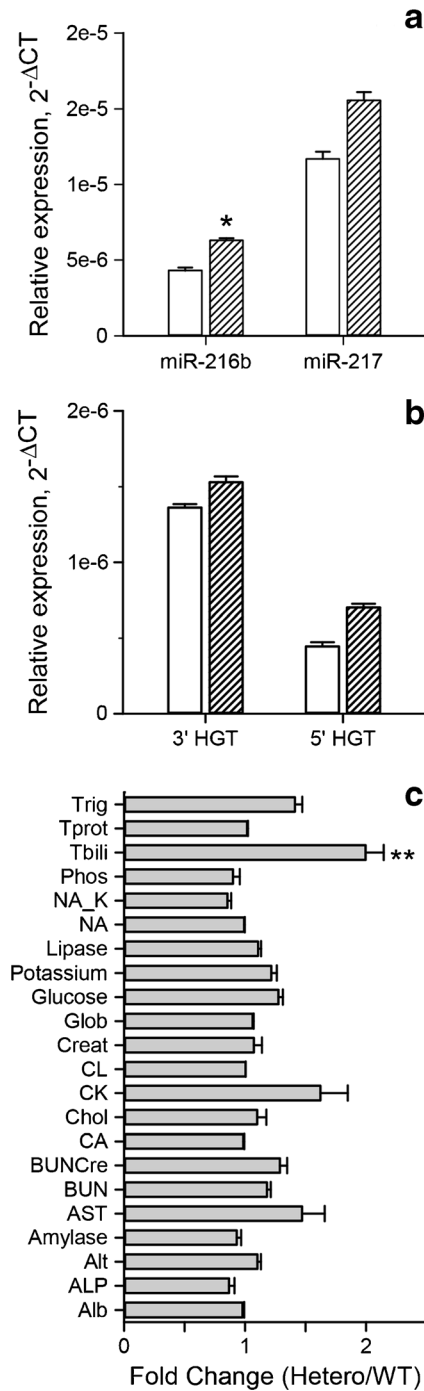
Deletion of 27.9 kbp from the germ line of the mice was embryonic lethal. This was determined by the presence of no viable homozygotes after genotyping over 200 pups. One homozygote was identified at E9.5 but not at later time points suggesting that the deletion caused lethality shortly after E9.5. At E9.5, the developing pancreas undergoes the primary

transition. The primary transition is characterized by the very first appearance of low levels of digestive enzymes and glucagon and insulin gene expressing cells (Cleaver et al. 2010). Although lethality occurred about the time that the pancreas would begin secreting digestive enzymes, others have found that failure to develop a pancreas does not result in lethality in utero (Hale et al. 2005, Kawaguchi et al. 2002). It is more likely, therefore, that interruption of other crucial developmental milestones is responsible for the lethality phenotype.

Several miRNA gene knockout mice have been known to induce lethality in mice (Farmer et al. 2013, Kuhnert et al. 2008, Liu et al. 2008, Ventura et al. 2008, Wang et al. 2008, Zhao et al. 2007). Knockout of miR-205 was embryonic lethal in mice (Park et al. 2012). Deletion of the primary transcript of brain expressed miR-137 was also embryonic lethal, the authors estimate that lethality occurred between E4.5 and E11.5 (Crowley et al. 2015). There was no difference in the mature miR-137 expression between the wild-type and miR-137 heterozygotes (Crowley et al. 2015). Our data (Fig. 6a) showed no decrease in miR-216b and miR-217 expression in the heterozygotes and in fact there was a slight increase in the expression (Fig. 7). Possibly, some type of compensatory mechanisms such as increased transcription occur in these heterozygotes.

miR-216a and miR-217 are located within the mouse ENSMUST00000138164, a 64-kbp transcript on 11qA3.3. This transcript has been annotated as a lncRNA. miR-216b is located on a separate transcript, 5.4 kbp upstream of GM12082 (Fig. 3). We deleted 27.9 kbp portion of 11qA3.3 which contained both miR-216b as well as a large portion of the lncRNA GM12082 (Fig. 3). Ongoing studies have developed individual, germline knockouts of miR-216a, miR-216b, and miR-217 (Sutaria, Coppola, and Schmittgen, unpublished data). We report no lethality from the knockout of each individual miRNA. Thus, the lethality observed here is a result of either the simultaneous knockout of all three miRNAs or loss of the miR-216/-217 host gene/lncRNA. If the latter is true, then this would add GM12082 to small but growing list of published studies that show lethality from the germline knockout of lncRNAs (Bassett et al. 2014).

In summary, we confirm published data in both human and mouse PDAC (Rachagani et al. 2015, Visani et al. 2015) that miR-216a, miR-216b, and miR-217 are reduced during the mouse's age when PDAC would progress. We also demonstrate that the germ line deletion of the miR-216/-217 host gene is embryonic lethal. As miR-216/-217 are acinar enriched, it will be of interest to develop either germ line or conditional knockout mice of the individual miRNAs to evaluate any potential tumor suppressor roles for these miRNAs during PanIN or PDAC development in transgenic mice.

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