



## Original Research

# Transgenic overexpression of the miR-200b/200a/429 cluster inhibits mammary tumor initiation

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## ABSTRACT

The miR-200 family consists of five members expressed as two clusters: miR-200c/141 cluster and miR-200b/200a/429 cluster. In the mammary gland, miR-200s maintain epithelial identity by decreasing the expression of mesenchymal markers leading to high expression of epithelial markers. While the loss of miR-200s is associated with breast cancer growth and metastasis the impact of miR-200 expression on mammary tumor initiation has not been investigated. Using mammary specific expression of the miR-200b/200a/429 cluster in transgenic mice, we found that elevated expression miR-200s could almost completely prevent mammary tumor development. Only 1 of 16 MTB-IGFIRba429 transgenic mice (expressing both the IGF-IR and miR-200b/200a/429 transgenes) developed a mammary tumor while 100% of MTB-IGFIR transgenic mice (expressing only the IGF-IR transgene) developed mammary tumors. RNA sequencing, qRT-PCR, and immunohistochemistry of mammary tissue from 55-day old mice found *Spp1*, *Saa1*, and *Saa2* to be elevated in mammary tumors and inhibited by miR-200b/200a/429 overexpression. This study suggests that miR-200s could be used as a preventative strategy to protect women from developing breast cancer. One concern with this approach is the potential negative impact miR-200 overexpression may have on mammary function. However, transgenic overexpression of miR-200s, on their own, did not significantly impact mammary ductal development indicating the miR-200 overexpression should not significantly impact mammary function. Thus, this study provides the initial foundation for using miR-200s for breast cancer prevention and additional studies should be performed to identify strategies for increasing mammary miR-200 expression and determine whether miR-200s can prevent mammary tumor initiation by other genetic alterations.

## Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate mRNA translation by binding to mRNAs and in turn, prevent mRNA translation [1,2]. miRNAs are originally transcribed as long primary transcripts and these primary transcripts are processed in two cleavage steps mediated by Drosha and Dicer that produce a mature miRNA of 19–25 nucleotides [3–7]. Mature miRNAs are incorporated into a complex known as the RNA-induced silencing complex (RISC) [4]. Most miRNAs direct RISC to target mRNAs where they induce mRNA degradation or repress translation [3,4,8,9]. miRNAs incorporated into RISC bind primarily to 3'-UTRs of target mRNAs using the miRNA seed sequence found between nucleotides 2 and 8 of the miRNA [3,4,8–10]. However, several reports indicate that miRNAs can also bind mRNAs independent of the seed sequence [11–13]. miRNAs have been

implicated in regulating a number of cancers including breast cancer. One family of miRNAs implicated in breast cancer is the miR-200 family. This family consists of 5 members organized into two clusters, the miR-200b/200a/429 cluster and the miR-200c/141 cluster [14–16]. These five miRNAs have highly similar seed sequences and the miR-200b, miR-200c, and miR-429 share a common seed sequence (AAUACUG) while miR-200a and miR-141 share the same seed sequence (AACACUG) [17]. The most completely characterized function of the miR-200 family is their role in maintaining epithelial identity. miR-200 family members reduce the expression of mesenchymal transcription factors such as *Zeb1/2*, *Twist1/2*, *Snai1/2* [16,18–20] and increase the expression of epithelial genes such as E-cadherin [21,22].

The most studied function of the miR-200 family in breast cancer is the suppression of epithelial to mesenchymal transition (EMT) and thus tumor migration and metastasis. Work from our lab and others showed

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that increased expression of miR-200s in murine and human mammary tumor cells inhibited migration and invasion *in vitro* and metastasis *in vivo* [23–29]. miR-200s have also been reported to influence proliferation, stem/progenitor cell number and the expression of immune regulatory molecules [23,25,27,30–37].

While the role of miR-200s in reducing breast cancer progression is well established, less is known about the role of miR-200s in inhibiting mammary tumor initiation. *In vitro* culture systems with normal mammary epithelial cells can be utilized to study tumor initiation however, the artificial environment including the lack of appropriate cell-cell and cell-ECM contacts as well as the loss of natural fluctuations in hormones, growth factors, nutrients and oxygen may influence cellular transformation. Thus, tumor initiation studies are typically performed in animal models with well-established tumor onset characteristics. Only 8 studies have examined miR-200 alterations *in vivo* using transgenic ([13, 38]) or knockout ([39–44]) models and none of these models altered miR-200 expression in the mammary gland. Thus, the impact of miR-200s on mammary tumor initiation have not been investigated in a relevant, *in vivo* environment.

In this study we have shown that overexpression of the miR-200b/200a/429 cluster in mammary epithelial cells almost completely suppressed mammary tumor development induced by the type I insulin-like growth factor receptor (IGF-IR). *Saa1*, *Saa2*, and *Spp1* were identified as genes that may contribute to mammary tumor initiation that were suppressed by miR-200b/200a/429 overexpression. miR-200 overexpression in the mammary gland did not impair mammary ductal development suggesting that therapeutic use of miR-200s would not significantly impact normal mammary gland function.

## Methods

### Animals and ethics

Animals were housed and cared for following guidelines established by the Central Animal Facility at the University of Guelph and the guidelines established by the Canadian Council of Animal Care. This study was approved by the Animal Care Committee at the University of Guelph (AUP #3994).

TRE-200ba429 transgenic mice were generously donated by Dr. Ri Yiu (Northwestern University Feinberg School of Medicine, Chicago, IL, USA) and have been previously characterized [13]. As the background of the TRE-200ba429 mice was not pure, they were backcrossed into an FVB background. All tumor mice reported in this study were from backcross 6–8 which represents 98.45–99.6% FVB background. All mice used for the mammary gland development study were from backcross 8 or later.

MTB-IGFIR mice that overexpress the IGF-IR in mammary epithelial cells in a doxycycline inducible manner have been previously characterized by our lab [45,46]. MTB-IGFIRba429 mice that overexpress both IGF-IR and the miR-200b/200a/429 cluster in mammary epithelial cells in a doxycycline inducible manner were created by mating MTB-IGFIR mice with TRE-200ba429 mice. MTB-IGFIR and MTB-IGFIRba429 mice were placed on food supplemented with 2 g/kg of doxycycline when the mice were weaned at 21 days of age. Mice were monitored 2 times per week by palpating the mammary glands. Once a palpable mammary tumor was identified tumor size was measured using digital calipers. Mammary tumors were collected once they reached ~10% of the mouse's body weight and mammary glands were collected from mice that did not develop mammary tumors 300 days after IGF-IR induction.

### RNA extraction and RNA sequencing

RNA extraction was completed as previously described [23], and RNA sequencing and analysis was performed by Arraystar (Arraystar Inc, Rockville, MD). Fastq files were analyzed using Genialis software

(Genialis Inc, Houston, TX) following the standard RNA-seq pipeline as previously described [30]. Hierarchical clustering was performed using Genialis software (Genialis Inc, Houston, TX) with all genes and Pearson distance measure and average linkage clustering. Pathway analysis was performed using Enrichr [47,48]. The data has been uploaded to GEO as GSE180264.

### Real-time PCR

Quantitative real-time PCR was performed as described in Jones et al. [23] using primers for *Saa1* (qMmuCID0007991), *Saa2* (qMmuCED0026710), *Spp1* (qMmuCED0040763), and *Hprt* (qMmuCED0045738). The expression of *Saa1*, *Saa2*, and *Spp1* was presented relative to *Hprt* using CFX Maestro software version 2.2 (Bio-Rad Laboratories, Mississauga, ON). Primer efficiency for *Saa1* was 109.1%, for *Saa2*, 109.7%, for *Spp1*, 104.5%, and for *Hprt*, 105.0%.

### Histology and immunohistochemistry

Mammary glands were collected, fixed in 10% formalin overnight and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin for histologic analysis. Immunohistochemistry was performed as previously described [49] using a 1:200 dilution of the anti-Spp1 antibody (cat# ab218237, Abcam, Toronto, ON) or a 1:200 Dilution of the anti-SAA1+SAA2 antibody (cat# ab199030). Slides were scanned using a Motic Easyscan digital slide scanner (Motic Richmond, BC).

### Wholmount analysis

Wholmount analysis was performed on mammary glands from 55-day old and 75-day old mice as described in Moorehead et al. [50] except images were captured with a Canon EOS 6D camera (Canon Canada, Mississauga, ON) equipped with a 100 mm Canon marco lens (Canon Canada, Mississauga, ON). Duct length was determined by importing the images into Aperio ImageScope (Leica Biosystems, Concord, ON) and averaging the distance measured from the edge of the lymph node (closest to the nipple) to the tips of the three longest ducts.

### miR-200b and miR-200c *in situ* hybridization

*In situ* hybridization was performed using a miR-200b (SR-mmu-miR-200b-3p-S1) or miR-200c (SR-mmu-miR-200c-3p-S1) probe from Advanced Cell Diagnostics (Newark, CA). Positive (SR-Mm-Snord85-S1) and negative control (SC-Scramble-S1) probes were also used but images using these probes were not included. *In situ* hybridization was performed as described in the miRNAscope HD (RED) Assay user manual using a 15 min incubation at 99 °C in 1x target retrieval reagent and a 30 min incubation with protease IV at 40 °C. Slides were scanned using a Motic Easyscan digital slide scanner (Motic Richmond, BC).

### Statistics

For analysis comparing the means of two different groups, a Student's *t*-test was performed. For analyses comparing the means of three or more groups, an ANOVA followed by a Tukey's test was performed using GraphPad Prism 8.4.3 software (San Diego, CA). To compare survival curves a Log-rank (Mantel-Cox) test was performed using GraphPad Prism 9.1.2 software (San Diego, CA). Means were considered statistically different when  $p < 0.05$ .

## Results

### *miR-200b, miR-200a and miR-429 are overexpressed in mammary epithelial cells of MTB-200ba429 transgenic mice*

MTB-200ba429 mice are transgenic mice that overexpress the miR-200b/200a/429 cluster in a doxycycline inducible manner in mammary epithelial cells. When MTB-200ba429 mice are fed chow containing 2 g/kg of doxycycline beginning at 21 days of age, the levels of miR-200b, miR-200a and miR-429 are approximately 7-8-fold higher than the levels of these miRNAs in normal mammary glands of 55-day old mice (Fig. 1A). The re-expression of the miR-200b/200a/429 cluster did not significantly impact the re-expression of the other two miR-200 family members, miR-200c and miR-141 (Fig. 1A).

The increased expression of miR-200b (used as a surrogate for the miR-200b/200a/429 cluster; Fig. 1B,C) but not miR-200c (used as a surrogate for the miR-200c/141 cluster; Fig. 1D,E) was confirmed in mammary epithelial cells of MTB-200ba429 transgenic mice (Fig. 1C,E) compared to control mice (Fig. 1B,D) using *in situ* hybridization.

### *Transgenic expression of the miR-200b/200a/429 cluster prevents tumor initiation*

To determine whether miR-200s inhibit mammary tumor initiation and progression, TRE-200ba429 transgenic mice were mated with MTB-IGFIR transgenic mice. MTB-IGFIR transgenic mice overexpress the type I insulin-like growth factor receptor (IGF-IR) in mammary epithelial cells in a doxycycline inducible manner and IGF-IR overexpression induces rapid mammary tumor development in 100% of mice Fig. 2A and [45,46]. Crossing MTB-IGFIR and TRE-200ba429 transgenic mice produced mice (MTB-IGFIRba429 mice) that overexpressed both the IGF-IR transgene and the miR-200b/200a/429 transgene in the same mammary epithelial cells since both transgenes are driven by the reverse tetracycline transactivator and doxycycline. While MTB-IGFIR transgenic mice developed mammary tumors with 100% frequency and an average latency of ~43 days post IGF-IR induction, overexpression of miR-200b/200a/429 in mammary epithelial cells overexpressing IGF-IR almost completely suppressed tumor incidence (Fig. 2A). Only 1 of 16 MTB-IGFIRba429 mice (6.25%) developed a mammary tumor and this tumor was first detected at 130 days post IGF-IR induction. The other 15 MTB-IGFIRba429 mice failed to develop mammary tumors by the study endpoint (300 days post IGF-IR induction). In addition, there were no hyperplastic lesions visible upon histologic analysis of mammary glands

from MTB-IGFIRba429 300 days post IGF-IR induction. Mammary tumors that arose in MTB-IGFIR mice were typically solid sheets of epithelial cells with varying amount of necrosis (Fig. 2B) while the one tumor that arose in a MTB-IGFIRba429 mouse had tumor cells that appeared less densely packed (Fig. 2C). However, with only one tumor in the MTB-IGFIRba429 mice it is difficult to compare mammary tumor histology between MTB-IGFIR and MTB-IGFIRba429 mice.

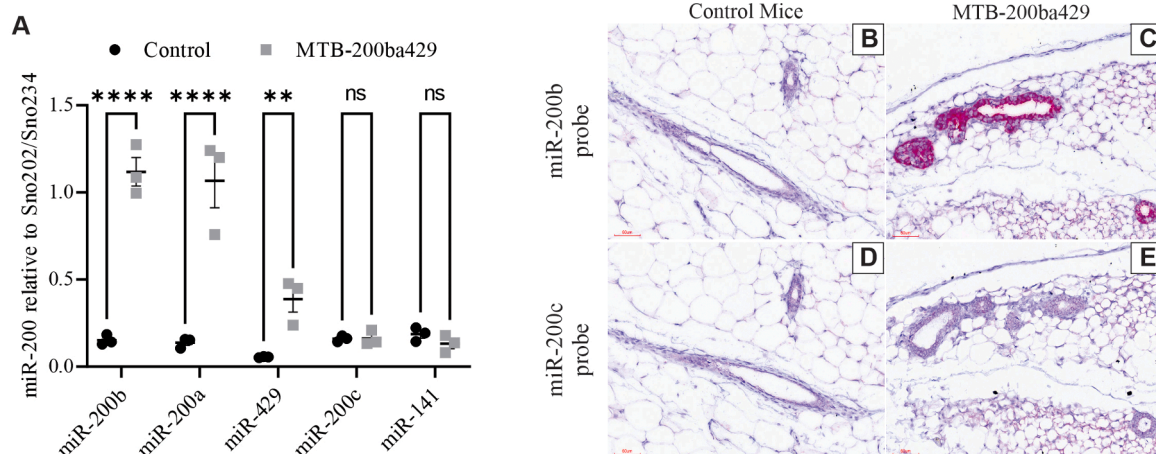
### *Overexpression of miR-200b/200a/429 prevents IGF-IR induced hyperplasia but does not restore terminal end bud structure*

To better understand how overexpression of miR-200s inhibit mammary tumor initiation, mammary glands of 55-day old and 75-day old control, MTB-200ba429, MTB-IGFIR, and MTB-IGFIRba429 mice were evaluated using wholemount analysis and histology. By 55 days of age, or 34 days after induction of the IGF-IR transgene, MTB-IGFIR mice have hyperplastic lesions that are visible in mammary wholemounts and histologic sections (Fig. 3A,B, arrows indicate hyperplasia while LN indicates the lymph node found in the fourth mammary gland). In contrast, mammary glands from 55-day old MTB-IGFIRba429 mice did not display hyperplasia (Fig. 3C,D). The absence of hyperplastic lesions in the MTB-IGFIRba429 mice is consistent with the lack of tumor development observed in almost all MTB-IGFIRba429 mice.

Mammary ductal morphogenesis was significantly inhibited in both MTB-IGFIR (Fig. 3A,I) and MTB-IGFIRba429 mice (Fig. 3C,I) compared to control (Fig. 3E,I) and MTB-200ba429 mice (Fig. 3G,I) at both 55 and 75 days of age. Mammary wholemounts of both the MTB-IGFIR (Fig. 3A) and MTB-IGFIRba429 (Fig. 3B) mice revealed terminal end buds (TEBs) with irregular shapes compared to the smooth, bulbous shape of the TEBs in control (Fig. 3E) and MTB-200ba429 (Fig. 3G) mice. Therefore, miR-200b/200a/429 overexpression in IGF-IR transgenic mice can prevent mammary epithelial hyperplasia but cannot restore TEB structure or ductal elongation. However, overexpression of miR-200b/200a/429 in mammary epithelial cells on its own, does not significantly impair TEB structure or ductal morphogenesis.

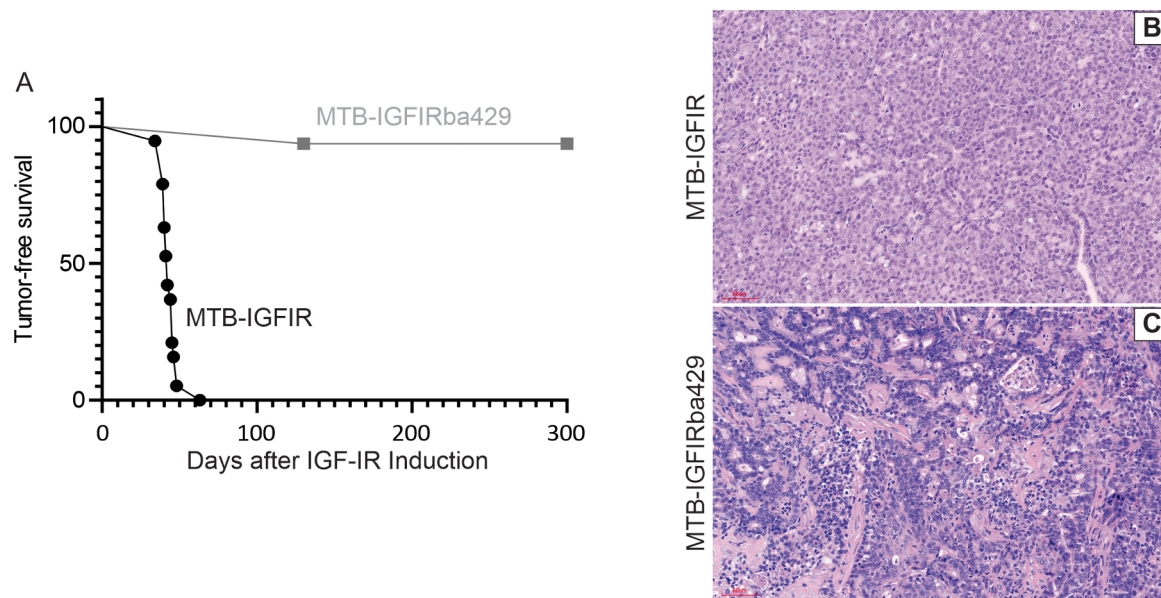
### *RNA sequencing reveals genes potentially regulated by miR-200b/200a/429 that inhibit mammary tumor development*

To investigate genes potentially regulated by miR-200b/200a/429 overexpression that prevented IGF-IR induced mammary tumor development, RNA sequencing was performed. Gene expression analysis was performed on day 55 mammary glands from control, MTB-200ba429,



**Fig. 1.** miR-200 expression in 55-day old control and MTB-200ba429 transgenic mice. (A) Expression of miR-200b, miR-200a, miR-429, miR-200c or miR-141 in virgin mammary glands from MTB-200ba429<sup>+</sup> transgenic mice or control mice as determined by qRT-PCR,  $n = 3$ . (B) *in situ* hybridization for (B,C) miR-200b or (D,E) miR-200c in (B,D) control mammary glands or (C,E) mammary glands from MTB-200ba429<sup>+</sup> mice. Positive staining for miR-200b or miR-200c appears red while the sections were counterstained with Gill's hematoxylin. Scale bars are 60  $\mu$ m. \*\* indicates  $p < 0.01$  and \*\*\*\* indicates  $p < 0.0001$  (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).





**Fig. 2.** Overexpression of the miR-200b/200a/429 prevents mammary tumor development induced by the IGF-IR transgene. (A) Tumor free survival curve for MTB-IGFIR ( $n = 19$ ) and MTB-IGFIRba429<sup>+</sup> ( $n = 16$ ) transgenic mice. (B,C) Hematoxylin and eosin stained sections from (B) MTB-IGFIR tumor and (C) the only MTB-IGFIRba429 tumor. Scale bars are 100  $\mu$ m.

MTB-IGFIR and MTB-IGFIRba429 mice. Four mammary glands were collected from mice of each genotype. One of the MTB-IGFIRba429 mammary glands (mouse KW1084) was removed from the analysis as it consistently expressed lower levels of most transcripts compared to the remaining three mammary glands or had 0 reads for transcripts abundantly expressed in the mammary glands from the other three MTB-IGFIRba429 mice. For example, the counts per millions (CPM) for *Krt8*, a transcript expressed in mammary epithelial cells, was 0.39 CPM in mammary tissue from mouse KW1084 but exceeded 135 CPM in the other three mammary gland samples.

Hierarchical clustering revealed that mammary transcript expression did not segregate the mammary glands based on the genotype of the mouse from which they were derived (Supplemental File 1). Pairwise comparison of differentially expressed transcripts (log FC  $\geq 1$ , FDR < 0.01) from mammary tissue from the various genotypes revealed a relatively small number of differentially expressed transcripts. For example, only 7 transcripts (*miR200b*, *Cidea*, *Adtrp*, *Egfl6*, *Ces1f*, *Luzp2*, *Cox8b*) were significantly upregulated and two transcripts (*Sfrp2*, *Isr2*) significantly downregulated in the MTB-200ba429 mammary glands compared to control mammary glands (Supplemental File 2) suggesting that overexpression of miR-200b/200a/429 only impacts a small number of genes during ductal morphogenesis. The fact that miR-200b was the most significant differentially expressed transcript (Supplemental File 2) further confirmed that miR-200b had been overexpressed in MTB-200ba429 mammary tissue.

The greatest number of differentially expressed transcripts (log FC  $\geq 1$ , FDR < 0.01) was observed when MTB-IGFIR mammary glands were compared to control mammary glands with 252 transcripts upregulated and 38 transcripts downregulated in MTB-IGFIR mammary glands compared to control mammary glands (Supplemental File 2). The fourth ranked differentially expressed transcript, based on FDR, in MTB-IGFIR mammary glands compared to control mammary glands was *Igf1r* (logFC 3.2, FDR  $1.3 \times 10^{-15}$ ) confirming that the IGF-IR transgene was overexpressed in MTB-IGFIR mice. Pathway analysis using only the 252 transcripts elevated in MTB-IGFIR mammary glands showed that the top Encode and ChEA pathway was SUZ12 while the top KEGG pathways was ECM-receptor interaction (Fig. 4A–C). The top Molecular Function was insulin-like growth factor I binding (Fig. 4C) which is consistent with hyperactivation of the IGF-IR due to transgenic overexpression.

Transcripts differentially expressed in MTB-IGFIR mice compared to

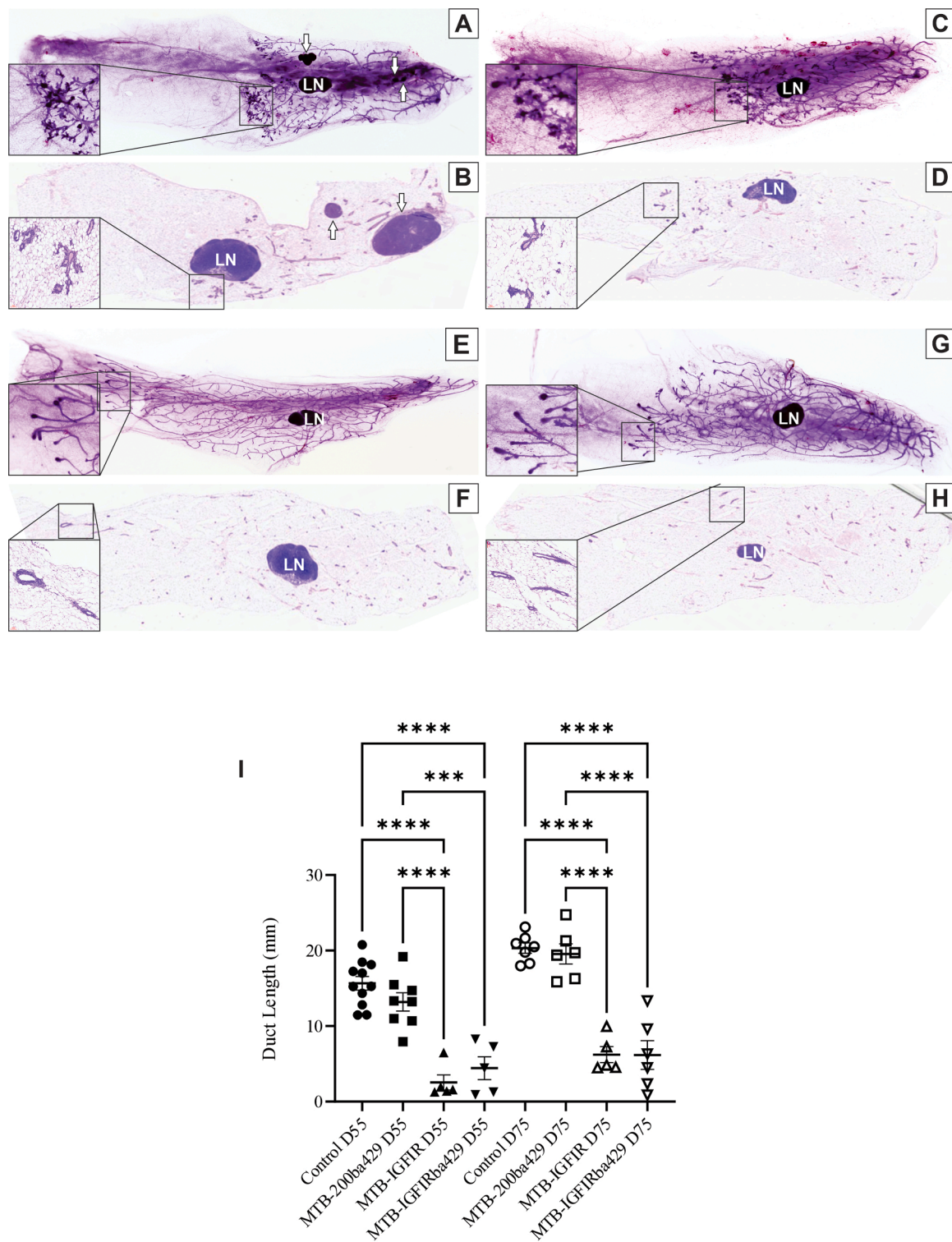
MTB-IGFIRba429 mice were also examined. The mammary glands from MTB-IGFIR mice contained 47 upregulated and 47 downregulated transcripts compared to MTB-IGFIRba429 mammary glands (Supplemental File 2). Pathway analysis of these 94 transcripts revealed the top Encode and ChEA pathway was SUZ12 while the top KEGG pathway and Molecular Function was Parathyroid hormone synthesis, secretion and action and death receptor activity, respectively (Fig. 4D–F).

The differentially expressed genes in the MTB-IGFIR vs control mammary glands were then compared to differentially expressed genes in the MTB-IGFIR vs MTB-IGFIRba429 mammary glands to identify genes consistently altered in mammary glands of mice that developed mammary tumors (MTB-IGFIR) compared to mammary glands of mice that rarely developed mammary tumors (control, MTB-IGFIRba429). Eighteen transcripts were identified, all of which were expressed at significantly higher levels in the MTB-IGFIR mammary glands compared to mammary glands from control or MTB-IGFIRba429 mice (Table 1). Of these 18 transcripts, 15 encoded genes, two encoded unclassified genes (GM42793, GM47585) and one coded for a long non-coding RNA (lncRNA, GM10384).

Three databases (TargetScan, miRDB and miRWalk) were investigated for potential miR-200b, miR-200a or miR-429 binding sites. TargetScan uses the seed region of each miRNA to predict mRNA targets [51–55] and miRDB uses the target prediction tool MirTarget [56,57] while miRWalk searches for potential binding sites in 3'-UTR, 5'-UTR and coding regions using TarPmiR [58,59]. Only *Proser2* was identified as a miR-200a target in all three databases while *Tmprss4*, *Spp1*, and *Slc30a2* were identified as potential miR-200a or miR-429 targets in two of the databases (Table 1). The remaining transcripts were only identified as potential miR-200b, miR-200a or miR-429 targets by miRWalk. Transcript *9130230L23Rik*, the two unclassified genes and the lncRNA were not recognized by TargetScan, miRDB or miRWalk and thus information regarding their regulation by miR-200s was unavailable (Table 1).

We then focused on *Saa1*, *Saa2* and *Spp1* since *Saa1* and *Saa2* were the two transcripts with the lowest FDR in Table 1 and we have previously shown that *Spp1* was the most significant, differentially expressed gene in a DNA microarray analysis comparing MTB-IGFIR mammary tumors to mammary glands of control mice [60,61]. Quantitative real-time PCR confirmed the elevated levels of *Saa1*, *Saa2*, and *Spp1* in mammary glands from 55-day old MTB-IGFIR mice compared to control,

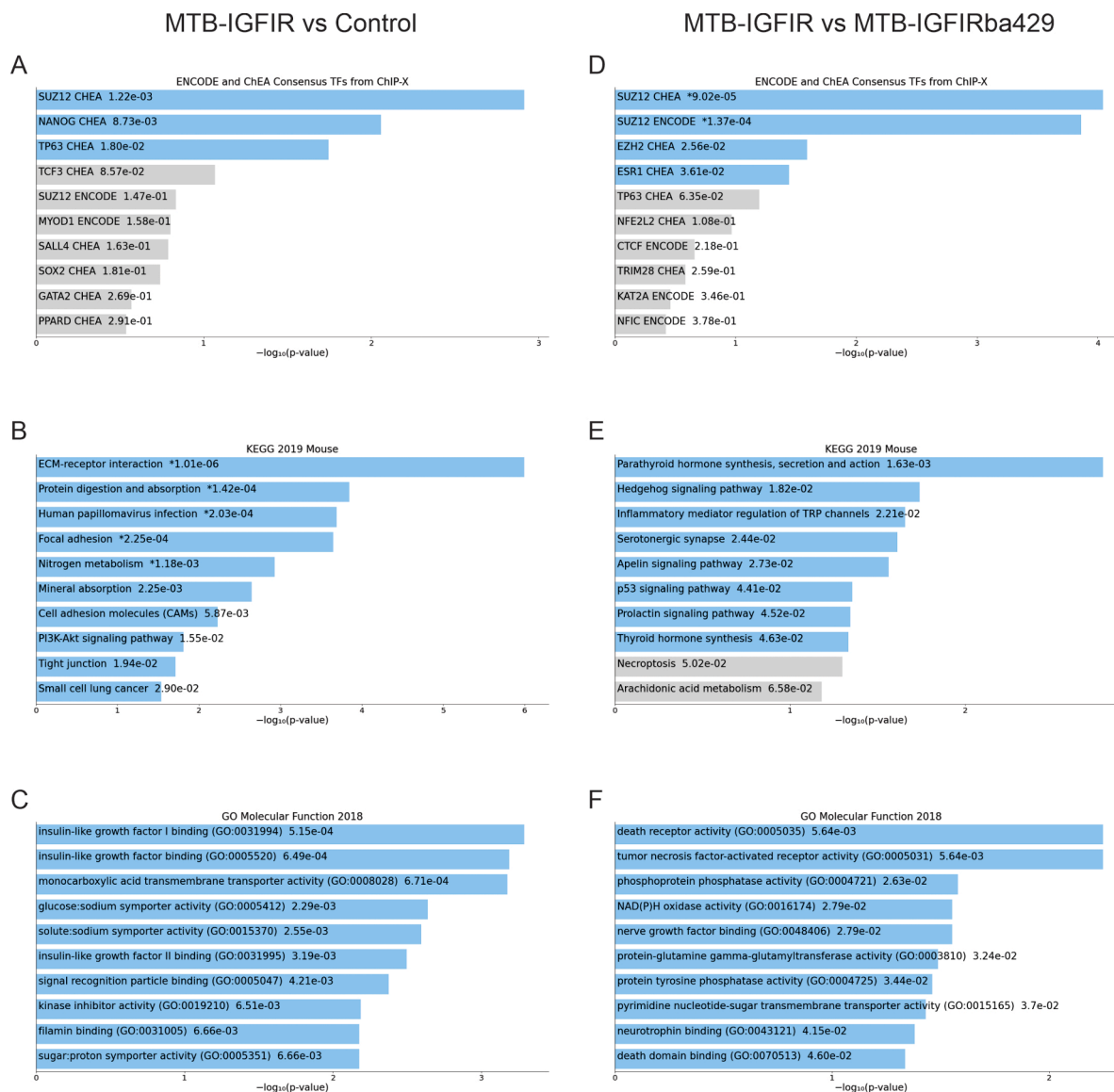




**Fig. 3.** Mammary ductal development in control, MTB-IGFIR, MTB-200ba429 and MTB-IGFIRba429 mice. (A,C,D,G) Wholemount analysis and (B,D,F,H) histologic analysis of 55-day old mammary glands from (A,B) MTB-IGFIR, (C,D) MTB-IGFIRba429, (E,F) control, and (G,H) MTB-200ba429 mice. Insets show a magnified version of the terminal end buds. White arrows indicate hyperplastic lesions while LN indicates the lymph node found in the 4th mammary gland. (I) Quantification of the ductal length in 55-day old (D55) and 75-day old (D75) mice. \*\*\* indicates  $p < 0.001$  and \*\*\*\* indicates  $p < 0.0001$ .

MTB-200ba429, and MTB-IGFIRba429 mammary glands (Fig. 5AC). Immunohistochemistry with a Spp1 antibody showed high levels of Spp1 staining in MTB-IGFIR hyperplastic lesions and very low levels in mammary epithelial cells of MTB-IGFIRba429 mice (Fig. 5A,B,D,E). Mouse kidney tissue served as a positive control for the Spp1 antibody (Fig. 5C,F). The antibody that detects both Saa1 and Saa2 did not detect

Saa1/Saa2 protein in mammary ducts of MTB-IGFIRba429 mice or hyperplastic lesions of MTB-IGFIR mice. While the Saa1/Saa2 antibody did stain liver tissue, this antibody failed to detect Saa1/Saa2 in MTB-IGFIR hyperplastic lesions or control mammary epithelial cells (Supplemental File 3). The level of Saa1 and Saa2 transcripts were much lower (Fig. 5A, B) than the levels of Spp1 transcript (Fig. 5C) and thus the amount of



**Fig. 4.** Pathway analysis of RNA sequencing data using Enrichr. Top (A,D) ENCODE and ChEA Consensus TFs from ChIP-X, (B,E) KEGG 2019 Mouse, and (C,F) GO Molecular Function 2018 in (A-C) MTB-IGFIR 55-day old mammary glands compared to (D-F) control 55-day old mammary glands.

*Saa1* and *Saa2* protein may be below the detection limit of this antibody in formalin-fixed, paraffin embedded mammary tissue.

## Discussion

The function of miR-200s in mammary tumor growth and metastasis have been extensively studied using human and murine mammary tumor cell lines. These studies from our lab [23–25] and others [62–64] generally show that miR-200s can inhibit the growth of primary mammary tumors and suppress metastatic spread. However, the ability of miR-200s to prevent mammary tumor initiation, has not been explored.

In this manuscript we have shown for the first time that overexpression of the miR-200b/200a/429 cluster in mammary epithelial cells significantly suppressed mammary tumor development induced by transgenic overexpression of IGF-IR. This finding is significant as although improving breast cancer therapy is an important clinical achievement, the ultimate goal in cancer biology is to prevent cancer development. Remarkably, an approximate 7-fold increase in the miR-200b/200a/429 cluster was sufficient to prevent mammary tumor development induced by a potent oncogene (IGF-IR) in ~94% of the

mice. Importantly, overexpression of the miR-200b/200a/429 cluster did not significantly impair mammary ductal development. The reason this observation is important is that although most women develop breast cancer after ductal development has been completed, the optimal window to administer preventative strategies remain unclear. Our lab and others have shown that mammary epithelial cells are particularly susceptible to transformation during puberty [65–69] and thus preventative strategies may need to be initiated during, or prior to the onset of, puberty for maximal efficacy. Two MTB-200ba429 and two control female mice were administered doxycycline supplemented food throughout their lifetime including during mating and lactation. The MTB-200ba429 females had the same litter size as the control mice and successfully nursed their offspring (unpublished observations). Thus, our data suggests that overexpressing miR-200s should not negatively affect mammary gland development or function.

Exactly how miR-200s prevents mammary tumor initiation remains incompletely defined however, our data suggest and important roles for *Saa1*, *Saa2* and *Spp1*. These three genes were significantly elevated 34 days after the induction of the IGF-IR transgene when small, multifocal hyperplastic lesions were forming and co-expression of the miR-200b/

**Table 1**

Predicted miR-200b, miR-200a, or miR-429 sites in transcripts differentially expressed in mammary glands from MTB-IGFIR vs Control and MTB-IGFIR vs MTB-IGFIRba429 mice.

Transcript	MTB-IGFIR vs Control		MTB-IGFIR vs MTB-IGFIRba429		Predicted target of miR-200b/200a/429		
	LogFC	FDR	LogFC	FDR	TargetScan	miRDB	miRWalk
Saa2	3.9	9.2E-13	3.2	1.2E-21	No	No	miR-200b, miR-200a
Saa1	3.6	1.4E-11	2.7	4.6E-10	No	No	miR-200b
Slc26a9	3.1	9.4E-09	2.0	9.3E-03	No	No	miR-200b, miR-200a, miR-429
Gm42793	4.6	2.1E-07	4.2	1.9E-03	–	–	–
Tmprss4	2.2	2.4E-07	1.7	6.2E-04	miR-200a	No	miR-200b, miR-200a, miR-429
Kctd14	1.6	1.2E-04	1.6	8.9E-03	No	No	miR-200b, miR-200a, miR-429
Spp1	2.8	3.3E-04	2.4	3.3E-05	miR-200a	No	miR-200b
Car6	1.8	4.8E-04	3.2	6.3E-06	No	No	miR-200b, miR-429
Muc4	2.2	6.9E-04	1.8	7.8E-06	No	No	miR-200b, miR-429
Slc30a2	2.4	7.4E-04	2.1	8.9E-03	miR-429	No	miR-200b, miR-200a, miR-429
Gm47585	3.5	1.4E-03	4.5	8.5E-03	–	–	–
Proser2	1.3	1.6E-03	1.4	9.2E-04	miR-200a	miR-200a	miR-200a
Rem2	1.6	1.7E-03	2.5	6.3E-06	No	No	miR-200b, miR-200a
9130230L23Rik	1.8	2.1E-03	2.0	6.7E-03	–	–	–
Slc35d3	2.2	2.2E-03	3.8	2.7E-05	No	No	miR-200b, miR-429
Duox1	2.0	3.2E-03	2.5	1.7E-03	No	No	miR-200b, miR-200a
F2r11	1.1	3.6E-03	1.3	2.8E-03	No	No	miR-200b, miR-200a
Gm10384	1.8	3.8E-03	2.6	3.4E-08	–	–	–

200a/429 cluster with IGF-IR prevented the increase in *Saa1*, *Saa2* and *Spp1* expression. *Saa1* and *Saa2* genes encode serum amyloid A proteins *Saa1* and *Saa2*, both of which are acute phase proteins [70]. *Saa1* and *Saa2* have been associated with pancreatic, renal, lung, colorectal, ovarian, oral, gastric, and breast cancer as well glioblastoma [71–78]. Although the increase in *Saa1* and *Saa2* proteins are typically attributed to tumor-associated inflammation, studies have shown that *Saa1* can regulate Akt signaling in tumor cells and siRNA knockdown of *Saa1* in pancreatic cells inhibits migration/invasion and epithelial to mesenchymal transition [71,79]. In breast cancer, *Saa1* was found to promote invasion [80,81]. However, it is also possible that *Saa1* and *Saa2* are elevated as part of the acute phase protein response [82,83] and thus may serve as biomarkers for mammary tumorigenesis but play no direct role in mammary tumor initiation.

*Spp1* is secreted phosphoprotein 1 and is also known as osteopontin. This protein is an important regulator of bone but has also been implicated in breast cancer. *Spp1* can be expressed by tumor cells as well as immune cells and fibroblasts where it can regulate process like EMT, angiogenesis and metastasis as well as modulate immune cell function [61,84–87]. *Spp1* contains an Arg-Gly-Asp (RGD) sequence allowing it to bind to several integrin receptors [88]. Another receptor for *Spp1* is CD44 [89]. Following binding to either of these receptors, *Spp1* can initiate signaling via the PI3K/Akt and Raf/MEK/ERK signaling pathways [90–92]. We have previously shown the importance of *Spp1* in mammary tumorigenesis in MTB-IGFIR mice and in murine mammary tumor cell lines [60,61].

There is some indirect evidence that *Spp1* regulates breast cancer initiation in women. Conditions that increase a women's chance of developing breast cancer such as high breast density, obesity and BRCA1 mutations have been associated with elevated *Spp1* expression. Women with dense breasts have a 4-fold increase of developing breast cancer [93,94] and it has been shown that *Spp1* is significantly elevated in dense breast tissue compared to normal breast tissue [95]. Similarly, obesity has been associated with an increased risk of developing breast cancer. One of the proteins elevated in the plasma of obese individuals is *Spp1* [96]. Breast cancer risk is also elevated in women with BRCA1 mutations. Since BRCA1 can suppress *Spp1* expression [97], women with BRCA1 mutations frequently express elevated levels of *Spp1* and this increase in *Spp1* expression may contribute to the elevated breast cancer risk associated with BRCA1 mutations.

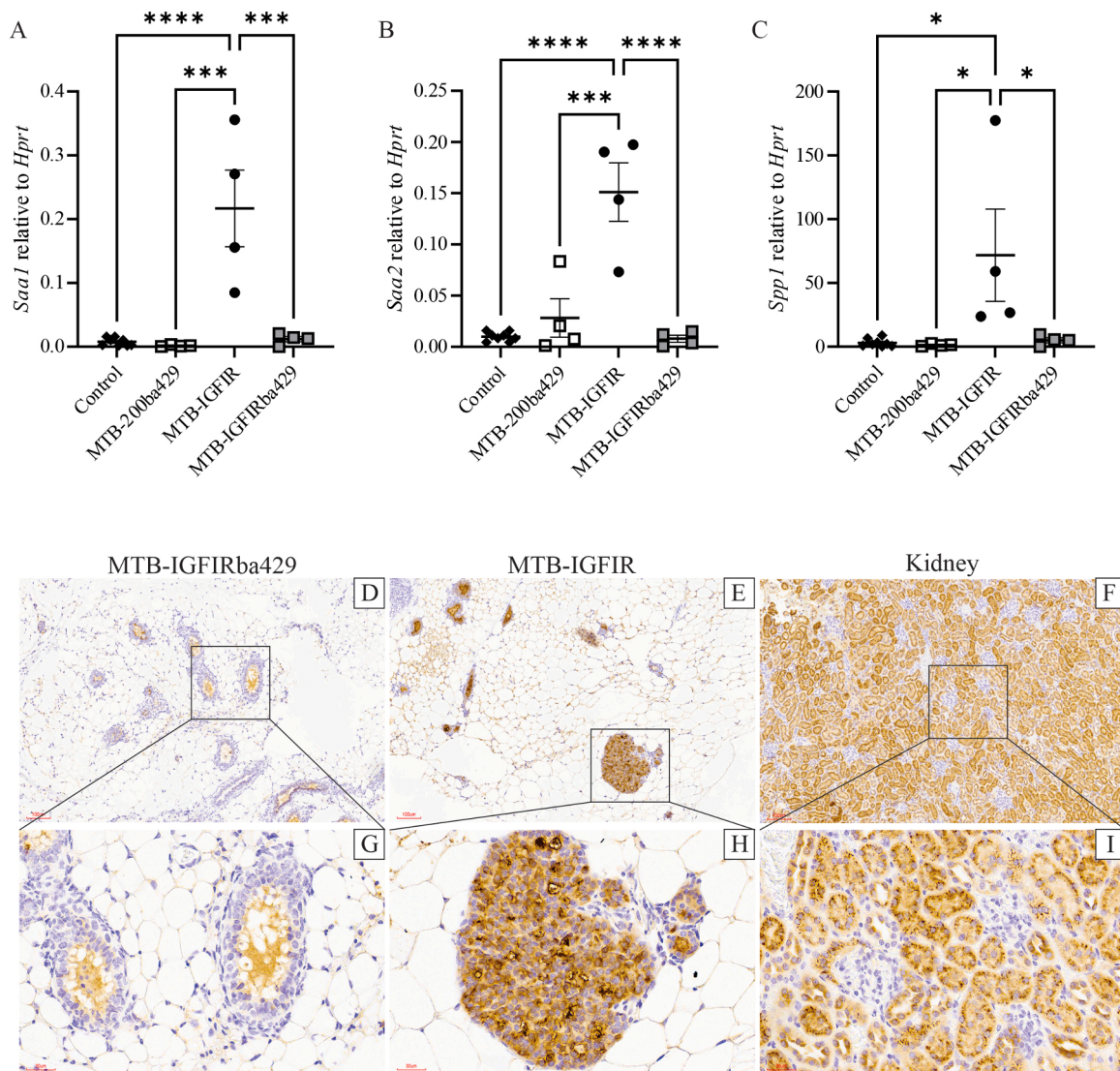
There are multiple ways miR-200s could inhibit *Saa1*, *Saa2* and *Spp1* expression including, direct binding to *Saa1*, *Saa2* and *Spp1* mRNA and regulation of DNA or histone methylation in the promoters of these

genes. *Saa1*, *Saa2* and *Spp1* have predicted miR-200b, miR-200a or miR-429 binding sites and thus miR-200s are predicted to directly regulate mRNA levels of these transcripts. With respect to methylation, our pathway analysis implicates SUZ12. SUZ12 is a component of the polycomb repressor complex 2 (PRC2) that mono-, di-, and trimethylates histone H3 on lysine 27 (H3K27) leading to chromatin compaction and suppression of transcription [98–100]. *Saa1* [77] and *Spp1* [101] have been shown to be regulated by H3K27 methylation and our previous study found that increased expression of miR-200s in murine and human breast cancer cells resulted in an elevation of H3K27me3 [24]. Therefore, miR-200s may directly target genes critical for tumor initiation and/or influence histone methylation to regulate gene expression.

Clinical application of increasing miR-200 expression to prevent breast cancer may be challenging, however, our work with transgenic mice overexpressing the miR-200b/200a/429 cluster did not find any significant impact on mammary ductal development or lactation suggesting that increasing miR-200s in the mammary gland will not impair mammary gland function. The biggest hurdle comes from identifying ways to increase miR-200 expression as our understanding of miR-200 regulation is poor. The expression of miR-200 family members do increase in mammary epithelial cells during pregnancy and lactation [102] and thus, lactogenic hormones potentially increase miR-200 expression. This is especially intriguing considering a full-term pregnancy and lactation reduced breast cancer risk [103–105]. An alternative way to clinically manipulate miR-200 expression or the expression of miR-200 regulated genes specifically in mammary epithelial cells would be through the injection of viral vectors or target inhibitors into the mammary ducts via the nipple. Mammary epithelial cells line the mammary ducts and administration of substances into the mammary duct will interact with mammary epithelial cells. This approach would also induce a localized increase in miR-200 or gene expression, eliminating potential side effects of systemically increasing miR-200 levels or miR-200 target genes.

In summary, our data shows that miR-200s can inhibit mammary tumor development potentially through the regulation *Saa1*, *Saa2* and *Spp1*. Future studies will need to determine the exact mechanism through which miR-200s regulate the expression of these genes and whether miR-200s can suppress mammary tumor development induced by other oncogenes. Before miR-200s can be considered as a preventative strategy, approaches that can induce miR-200 expression in the mammary gland need to be identified. Fortunately, our data suggests that miR-200 overexpression in mammary epithelial cells should not significantly impair mammary development or function.





**Fig. 5.** Expression of *Saa1*, *Saa2* and *Spp1* in 55-day old mammary glands. Expression of (A) *Saa1*, (B) *Saa2*, and (C) *Spp1* in mammary glands from 55-day old control, MTB-200ba429, MTB-IGFIR and MTB-IGFIRba429 mice as determined by quantitative real-time PCR. \* indicates  $p < 0.05$ , \*\*\* indicates  $p < 0.001$  and \*\*\*\* indicates  $p < 0.0001$ . Immunohistochemistry for *Spp1* in mammary glands from 55-day old (D,G) MTB-IGFIRba429 or (E,H) MTB-IGFIR mice. (F,I) Kidney tissue from wild type mice served as a positive control. Scale bars for (D-F) are 100  $\mu$ m and for (G-I) are 30  $\mu$ m.

#### Ethics approval and consent to participate

Animals were housed and cared for following guidelines established by the Central Animal Facility at the University of Guelph and the guidelines established by the Canadian Council of Animal Care. This study was approved by the Animal Care Committee at the University of Guelph (AUP #3994).

#### Consent for publication

Not applicable.

#### Availability of data and materials

RNA sequencing has been uploaded to GEO under accession number GSE180264.

#### CRediT authorship contribution statement

**Katrina L Watson:** Data curation, Formal analysis, Investigation,

Methodology, Writing – review & editing. **Rui Yi:** Resources, Writing – review & editing. **Roger A Moorehead:** Writing – original draft, Project administration, Formal analysis, Funding acquisition, Methodology, Supervision.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101228.

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