RESEARCH

MicroRNA 204 Mediated Negative Regulation of the IGF2R Promotes Breast Cancer Progression and is a Potential Mechanism Driving Breast Cancer Disparity

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ABSTRACT

In the US, African American women have a significantly higher rate of mortality due to breast cancer compared to Caucasian American women. Molecular differences in tumor biology exist between racial groups; however, their contribution to cancer disparity is not well understood. Our studies have identified a race-specific, mechanistic link between microRNA-204 and the IGF2R. The IGF2R is a tumor suppressor gene in several cancers including breast cancer and IGF2R levels are found at significantly lower levels in African American women with breast cancer when compared to their Caucasian counterparts. We observed elevated levels of miR-204 in serum of African American women with breast cancer when compared to Caucasian American women and identified IGF2R as a direct target of miR-204. We show mechanistically that miR-204 mediated inhibition of IGF2R leads to activation of the IGF1R signaling pathway resulting in increased proliferation, migration and invasion, processes that are required for tumor progression. We developed a unique doxycycline-inducible miR-204 transgenic mouse model to define *in vivo* the oncogenic potential of miR-204 and the mechanism and functional consequences of IGF2R loss. We observed a significant increase in tumor growth and increased metastasis in these animals when compared to non-transgenic controls. This is the first characterization of miR-204 in an *in vivo* model. These data support a mechanism whereby miR-204 promotes tumor aggression through the IGF2-mediated hyperactivation of the IGF1R signaling pathway in response to direct negative regulation of the tumor suppressor IGF2R and that this could be a potential mechanism promoting breast cancer disparity.

KEYWORDS: oncogenes, IGF2, breast neoplasms, transgenic mice, IGF1R

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INTRODUCTION

Breast cancer (BC) is a worldwide health issue as it represents the leading cause of cancer and remains the second leading cause of cancer-related mortality in women. In the US, African American (AA) women have a significantly higher rate of BC mortality compared to Caucasian American (CA) women. Recent studies have highlighted the need for understanding the molecular basis of cancer disparities as these contribute to the observed differences in mortality that we observe in hormonally driven cancers (Albain et al., 2009).

The Insulin-like Growth Factor 2 Receptor (IGF2R) is a transmembrane receptor that binds Insulin-like Growth Factor (IGF-2), resulting in its degradation via internalization and transport to lysosomes (Oka et al., 1985). Removal of IGF-2 from the extracellular environment precludes activation of the insulin-like growth factor 1 receptor (IGF1R); thus the IGF2R is believed to reduce the mitogenic effects of IGF-2. IGF2R is also involved in the activation of transforming growth factor beta (TGF-β) (Dennis and Rifkin, 1991) and may be a high affinity binding receptor for retinoic acid, an agent known to have diverse biological effects in both embryogenesis and oncogenesis (Kang et al., 1997). Thus, important homeostatic controls regulating cell proliferation and apoptosis would be lost with the inactivation of IGF2R, suggesting that this receptor normally functions to inhibit tumor formation. Indeed, loss of heterozygosity (LOH) at the *IGF2R* locus has been reported in breast carcinomas, and somatic missense mutations of the remaining allele have altered ligand binding (Hu et al., 2006; Iwamoto et al., 2006; Tsujiuchi et al., 2004). The IGF2R has been proposed to be a tumor suppressor gene given its antagonist role on cellular growth and evidence of LOH and loss-of-function mutations in several cancers including breast cancer (Chappell et al., 1997; Chen et al., 2002; Hankins et al., 1996; Oates et al., 1998). More recently, studies have shown that reduced IGF2R expression correlates with poor patient prognosis in BC patients (Yu et al., 1996) and that decreased IGF2R expression may contribute to BC disparity (Kalla Singh et al., 2010). This study shows that circulating IGF2 levels, a potent mitogen that signals through the IGF1R, are higher in breast tissue from AA women when compared to CA women. They further went on to show that IGF2R levels were significantly lower in AA breast tumor samples, important as IGF2R acts as a non-signaling sink for IGF2 thereby preventing its pro-survival signaling function. Importantly, IGF2 can also signal through the A isoform of the insulin receptor (IR-A) in many tumor types including breast cancer resulting in tumor promoting effects (Frasca et al., 1999; Ulanet et al., 2010).

MicroRNAs (miRNAs) are small non-coding RNAs that are generally involved in the negative regulation of mRNA through seed sequences present within the 3'UTR of protein coding genes. They have been studied in mammalian species for almost two decades and have been shown to be involved in most all cellular processes. Each miRNA has multiple targets and each 3'UTR can be targeted by multiple miRNAs. Understanding the regulation of these small molecules is complex; however, great strides have been made in a relatively short amount of time.

It should be noted that controversy surrounds the role of miR-204 in breast cancer , with some authors reporting miR-204 as a tumor suppressor (Imam et al., 2012; Li et al., 2014) and others,

including our group, reporting it as an oncogenic miRNA or "oncomir" (Findlay et al., 2008). MicroRNAs, by their nature, have been shown to play many different roles in different cellular contexts including both tumor suppressive and oncogenic roles. Classic examples of other miRNAs that have been shown to have context dependent tumor suppressive and oncogenic effects in breast cancer include miR-205 and miR-27 (Vimalraj et al., 2013). A recent review details the dual nature of miR-204 in cancer (Li et al., 2016).

This study describes a mechanistic link between miR-204 and a novel direct target IGF2R. We developed a unique inducible miR-204 transgenic mouse model to examine for the first time, in an intact system, the oncogenic potential of miR-204 and we show data to support a role for miR-204 mediated activation of the IGF1R signaling pathway and propose that this could be a potential biological mechanism driving aggressive tumor growth.

Materials and Methods

Cell Culture and Reagents

All cell lines were cultured and maintained at 37°C with 5% CO_2. MDA-MB-231 and HEK293 cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and 100 U of penicillin/streptomycin (P/S). BT549 cells were grown in RPMI media supplemented with 10% FBS and 100 U of P/S. MCF10A and MCF12A cells were grown in DMEM:F12 (50:50) media supplemented with 2mM L-glutamine, 5% horse serum, 10µg/mL insulin, 20ng/mL epidermal growth factor (EGF), 500ng/mL hydrocortisone, and 10µg/mL cholera toxin. The MCF10A with (control) and without IGF1R stably overexpressed were a kind gift of Adrian Lee (University of Pittsburgh, PA). All other lines were obtained from ATCC. Ethical approval for our work with human breast cancer cell lines was not required for our *in vitro* studies. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). shIGF1R vectors were obtained from the Hollings Cancer Center genomics/shRNA shared technology resource. The IGF2R construct was a kind gift of Lukas Mach (University of Natural Resources and Life Sciences, Vienna (BOKU)).

Transgenic Mice

Animal care and procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, Approval # ARC-2995.

To generate the tet-regulatable miR-204 transgenic mice, the 0.5-kb region surrounding miR-204 was amplified by PCR from the human genome using the following primers: TetO 204F 5′-gcgcatcgatttggacccaga actattag-3′ and TetO 204R 5′-gcgca ctagtggacagggtgatggagagg-3′ and cloned into the pTetO Splice vector (a kind gift of Dr. Tracy Vargo-Gogola, Indiana University School of Medicine). The clones were screened for miR-204 induction and minimal leaky expression by transfection into the MCF7 TetO cell line (a kind gift of Dr. Tracy Vargo-Gogola, Indiana University School of Medicine). The resultant vector was confirmed by sequencing and was subsequently microinjected into the pronuclei of fertilized FVB/n oocytes by the Medical University of South Carolina Transgenic Mouse Core, yielding 6 potential founder lines. Mice were maintained on an inbred FVB/n background. Bigenic mice were obtained by breeding TetO-204 mice to MTB (MMTV-rtTA) mice (a kind gift of Dr. Lewis Chodosh, Perelman School of Medicine, University of Pennsylvania), which contain the reverse tet

transactivator under the control of the MMTV promoter (Gunther et al., 2002). The TetO-204/MTB bigenic mice were bred to MMTV-Neu transgenic mice (002376; Jackson Laboratories, Bar Harbor, Maine) to obtain trigenic TetO-204/MTB/MMTV-Neu and control mice. All mice were maintained on an FVB/n background. For genotyping, PCR amplification of the MTB and 204 transgenes was performed on genomic DNA prepared from tail cuts using the following oligonucleotide pairs: for TetO-204, 5′-cacgaaattgcttctggtggc-3′ and 5′-tcgaagatgttggggtgttgg-3′; reaction conditions were 98°C for 3 minutes followed by 40 cycles of 98°C for 30 seconds, 62°C for 30 sec., 72°C for 1 min., followed by a 10 min. extension at 72°C; for MTB 5′- TCCAAGGGCATCGGTAAACA-3′ and 5′-GCATCAAGTCGCTAAAGAAG-3′; reaction conditions were 98°C for 3 min. followed by 30 cycles of 98°C for 30 sec., 58°C for 30 sec, 72°C for 30 sec., followed by a 10 min. extension at 72°C. Neu mice were genotyped following protocols supplied by Jackson Laboratories. To induce transgene expression, mice were fed doxycycline-containing chow (2g/kg ad libitum) for the duration of the study.

Immunohistochemistry

IHC was performed as described (Guo et al., 2013). The Ki67 antibody was used at a 1:200 dilution.

Quantitative Real Time PCR

Total RNA from cell lines was extracted using the RNeasyPlus Mini Kit (Qiagen; Valencia, CA). qPCR was performed on a Roche Light Cycler 480 as previously described (Guo et al., 2013). Primer sequences and UPL probe numbers are listed in Table 1. For microRNA analysis RNA was extracted from cell lines using the RNeasyPlus Mini Kit from Qiagen (Valencia, CA). 10ng total RNA was reverse transcribed using miR-204 specific primers using the Applied Biosystems reverse transcription kit as per the manufacturer's instructions. Real time PCR was performed with 1µl of reverse transcribed cDNA using the TaqMan Assay from Applied Biosystems as per the manufacturer's instructions on the Roche LightCycler 480 (Nutley, NJ). Triplicate reactions were run for each sample. The relative expression of each gene was quantified on the basis of Ct value measured against an internal standard curve for each specific set of primers using the software (2^{nd} derivative max) provided by the instrument manufacturer (Roche, Nutley, NJ).

Table 1. Primers (forward (F) and reverse (R)) and probes used in the study.

Primer Name	Primer sequence (5′ – 3′)	Amplicon Length (nt)	Probe #
IGF2R	(F) gagcgatacctctcaagtcaaag (R) gtgaggtctccatccgaatatc	75	4
IGF1R	(F) ttcagcgctgctgatgtg (R) aagttcccggctcatggt	75	7
PUMA	(F) ctgcctcaccttcatcagg (R) gcagagcacaggattcacag	61	79
NOXA	(F) ggagatgcctgggaagaag (R) cctgagttgagtagcacactcg	94	67
GAPDH	(F) agccacatcgctcagacac (R) gcccaatacgaccaaatcc	66	60

Transfection of cell lines

The cloning of miR-204 into pSuppressor-neo vector is already described (Findlay et al., 2008). For the generation of clonal stable MCF12A cells overexpressing miR-204 (204-1; 204-2), pSuppressor-neo vector (Imgenex; San Diego, CA) expressing miR-204 was transfected into MCF12A cells and stable cells were selected in medium containing G418. MCF10A cells were transiently transfected with the pSuppressor-neo vector. For inhibition studies, cells were transiently transfected with antisenseoligoribonucleotides (ASO) specific for miR-204 (ASO-204) or scrambled controls (scr). Cells were transfected with X-tremeGENE^{TM} siRNA transfection reagent (Roche, Nutley, NJ).

Luciferase assays

The IGF2R 3'UTR luciferase reporter vector (pMirTarget) was purchased from Origene (Rockville, MD). The sequence complementary to the seed of miR-204 was deleted with the primers IGF2RmutF 5'-GCTTCTATAACAGAAACTTTCAAGA GTTTTTGTGATGGGGGAGAGGG-3' and IGF2RmutR 5'-CCCTCTCCCCCATCACAAAAAC TCTTGAAAGTTTCTGTTATAGAAGC-3' using a QuikChange Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA). Mutated sequences were validated by sequencing at MWG Operon (Huntsville, AL). MCF12A cells were plated at 50,000 cells per well in a 24-well plate. The pMirTarget reporter constructs (0.5µg, firefly luciferase) were co-transfected with pRL–TK (0.05µg, *Renilla* luciferase) using XtremeGene HP reagent as per the manufacturer's instructions (Roche). Luciferase activity was measured after 48h using the dual luciferase reporter assay system (Promega, Madison, WI). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well.

Western blot analysis

Cell lysate preparation and western blot analysis using enhanced chemiluminescence were performed as described previously (Findlay et al., 2008). Experimental antibodies include IGF2R (Santa Cruz Biotechnology, Dallas, TX), IGF1R, IRS-1, p-AKT, AKT, p-ERK and ERK, p-IGF1R, p-IRS1 (Cell Signaling Technology, Danvers, MA) and p-IR and IR (abcam, Cambridge, MA). GAPDH (Santa Cruz Biotechnology) was used as a loading control.

Transwell migration and invasion assay

Assays were performed as previously described (Guo et al., 2013). Images were taken at a 40X objective for analysis.

Statistical analysis

Sample size for mouse experiments n ≥ 9. For statistical testing, two-sided paired (*in vitro* assays) and two-sample (*in vivo* assays) Student's *t*-tests were performed using Excel (*in vitro* assays) and GraphPad Prism (*in vivo* assays). *p*-values are reported for each individual experiment. Error bars represent standard deviations (SD) of three independent experiments unless indicated otherwise. Time to event outcomes were assessed using Kaplan-Meier curves and distributions compared between groups using the Peto-Peto test which is less sensitive to late differences than the traditional log rank test. For hypothesis testing, the alpha level was set at 0.05.

Results

miR-204 modulates migration and invasion in non-transformed and invasive breast cancer cell lines.

Our group has shown previously that overexpression of miR-204 was able to increase migration and invasion in the non-invasive breast cancer cell line MCF7 (Findlay et al., 2008). However, in order to explore this function further,

we wanted to assess the role of miR-204 as an oncomir in both non-transformed breast cells as well as invasive breast cancer cells. A breast cell line screen demonstrated that non-transformed MCF10A and MCF12A cells had the lowest expression levels of miR-204 and the invasive breast cancer cell lines MDA-MB-231 and BT549 had the highest expression levels of miR-204 (Supplemental Figure 1B). Therefore, we examined migration and invasion in non-transformed MCF10A and MCF12A breast cell lines in which miR-204 had been overexpressed either transiently (MCF10A) or stably (MCF12A) (Supplemental Figure 1D & E). We observed that miR-204 expression was able to significantly increase both migration and invasion of non-transformed breast cells (MCF10A & MCF12A) when overexpressed (Figure 1A & B). We also examined migration and invasion in the invasive breast cancer cell lines MDA-MB-231 & BT549, in which miR-204 was inhibited (Supplemental Figure 1F & G). We observed that migration and invasion was inhibited in two invasive breast cancer cell lines in which miR-204 had been suppressed (Figure 1C & D). These data lend further support to the proposed oncogenic role of miR-204 in breast cancer. We also observed that miR-204 increases proliferation in the non-invasive MCF10A (Figure 1E) and MCF12A (Figure 1F) cells, similar to that observed in MCF7 cells (Findlay et al., 2008).

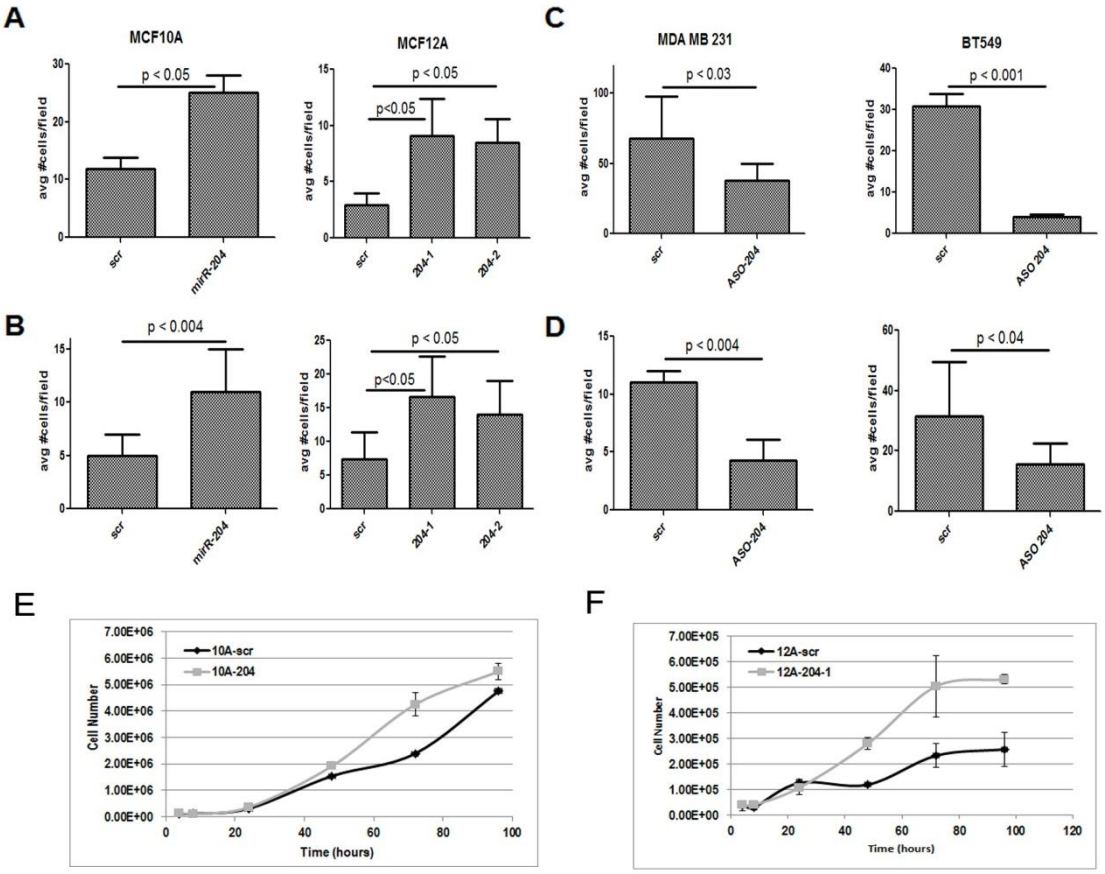

Figure 1: miR-204 regulates migration and invasion. Transwell migration (A & C), invasion (B & D) and proliferation (E & F) assays of various breast cell lines with either overexpression (A, B, E & F) or inhibition (C & D; ASO-204) of miR-204 compared to scrambled (scr) control. MCF10A, MDA-MB-231 and BT549

cells were transiently transfected. MCF12A cells were stably transfected. ASO-204 – antisenseoligoribonucleotides (miR-204 inhibitors).

miR-204 is racially disparate and elevated in breast cancer

To further explore potential targets of miR-204 that may mediate its role as an oncomir in breast cancer, we performed a bioinformatical search of potential targets in publically available databases (Targetscan and miRbase) and identified IGF2R as a predicted target, which has been semi-validated for miR-204 in human trabecular meshwork (HTM) cells (Li et al., 2011). IGF2R is proposed as a tumor suppressor and studies have shown that reduced IGF2R expression correlates with poor patient prognosis in BC patients. Therefore, we wanted to explore its role as a direct target of miR-204 in breast cancer. To examine whether IGF2R expression is repressed by miR-204 through the predicted elements, a luciferase reporter construct containing the 3′ UTR of IGF2R was transfected into breast cancer cells. Cells were co-transfected with either miR-204 or an empty vector (EV) control. The overexpression of miR-204 led to a ~30% decrease in luciferase activity when compared to EV control (Figure 2A). To show that the predicted miR-204 seed sequence within the IGF2R 3'UTR was functional we mutated the seed sequence of miR-204 in the luciferase reporter construct. Co-transfection of cells with the mutated luciferase reporter construct (mut 3'UTR) and miR-204 did not result in a decrease in luciferase activity (Figure 2A) suggesting that miR-204 directly binds to the predicted site within the 3'UTR of IGF2R to negatively regulate its expression.

With respect to cancer disparities, a study showed significantly higher levels of IGF2R in CA compared to AA tumor samples, suggesting that decreased IGF2R expression may contribute to BC disparity (Kalla Singh et al., 2010). Therefore, we wanted to examine whether miR-204 levels differed by AA ancestry in BC patients by performing quantitative PCR (qPCR) for miR-204 on serum samples. We first found that miR-204 levels were elevated in high grade when compared to low grade in both AA and CA women (Figure 2B). However, it was observed that miR-204 levels were significantly elevated in AA compared to CA women in the low grade cohort of BC patients, but not observed in the high grade cohort. We also performed data mining in Oncomine for breast cancer using TRPM3 as a surrogate marker for miR-204, as (1) miR-204 is encoded in the sixth intron of TRPM3, (2) the expression of both genes is driven by the same promoter, and (3) the expression of both genes is positively correlated both *in vitro* (Courboulin et al., 2011; Ying et al., 2013) and *in vivo* (Ding et al., 2015). We found that in this dataset that could be examined by race, TRPM3 levels were higher in AA women when compared to CA women with breast cancer. Of interest, this dataset showed strikingly decreased levels of TRPM3 in women of Asian descent (Figure 2C).

IGF2R is a direct target of miR-204 in breast cancer cells and tissue

To validate IGF2R as a miR-204 target in breast cancer cell lines, miR-204 was overexpressed in MCF10A and MCF12A cells by either transient or stable transfection. In each case the increased expression of miR-204 inhibited endogenous IGF2R expression (Figure 2D). mRNA levels of IGF2R were assessed by qPCR and analysis indicated that the levels of IGF2R mRNA did not decrease, which is what we would expect if miR-

204 was regulating IGF2R by transcriptional degradation. In some cases, we observed an increase in IGF2R mRNA transcript levels, suggesting that miR-204 regulates IGF2R through translational repression (Figure 2E). MDA-MB-231 and BT549 cells, two invasive breast cancer cell lines, were used as a model to inhibit miR-204 expression. Transfection of antisense oligoribonucleotides (ASO) targeted against miR-204 (ASO 204) in MDA-MB-231 and BT549 cells resulted in an increase in IGF2R protein (Figure 2F) and mRNA levels (Figure 2G).

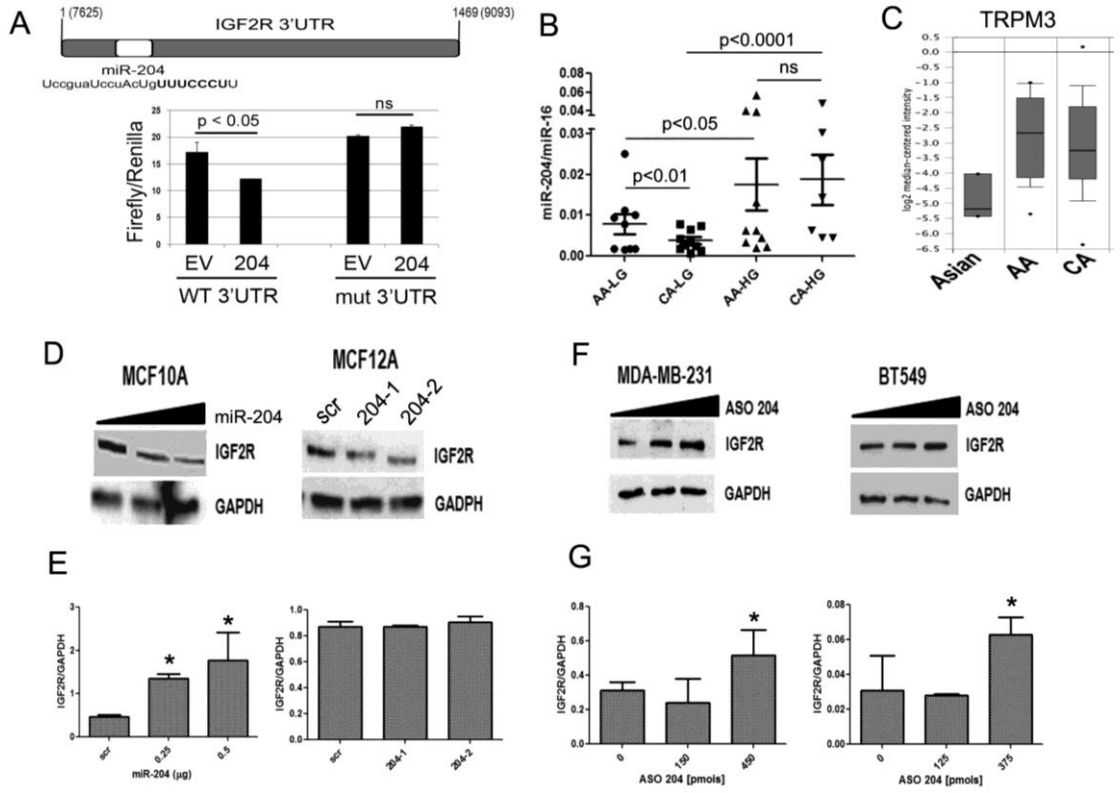

Figure 2: miR-204 directly targets IGF2R and is racially disparate in breast cancer. (A) Upper panel: Schematic representation of the binding site and complementary seed sequence (upper case bold letters) of miR-204 within the 3'UTR of IGF2R. Lower panel: Luciferase activity of breast cells transiently co-transfected with IGF2R 3'UTR (WT 3'UTR) or mutated IGF2R 3'UTR (mut 3'UTR) and miR-204 (204) or EV and Renilla as a control. (B) qPCR analysis of miR-204 expression in serum samples from (n=19) African American (AA) and (n=17) Caucasian American (CA) women with either low grade (LG) or high grade (HG) breast cancer. (C) TRPM3 mRNA levels in breast cancer patients of Asian, AA and CA race/ethnicity (Bittner data set, Oncomine; (Rhodes et al., 2004)). Western blot (D & F) and qPCR (E & G) analysis of IGF2R in breast cells either transiently transfected with miR-204 expression vector (MCF10A), or antisense oligonucleotides to miR-204 (ASO 204; MDA-MB-231 & BT549) or stably transfected with miR-204 (204-1, 204-2) or scrambled (scr) control (MCF12A). *p < 0.05

To examine whether miR-204 regulates IGF2R *in vivo*, we generated a tet-regulatable miR-204 transgenic (miR-204 Tg) mouse (see Methods). Six founder lines were generated and assessed for

germline transmission of the transgene and leaky expression of miR-204 in the absence of dox. We also assessed inducible expression of miR-204 by breeding the miR-204 Tg founder mice to mouse mammary tumor virus (MMTV)-rtTA (MTB) mice that express the reverse tet transactivator (rtTA) in the mammary epithelium under the control of the mouse MMTV long terminal repeat (Figure 3A) (Gunther et al., 2002). Bigenic mice were fed mouse chow containing the tet analog doxycycline (Dox) ad libitum to induce transgene expression. The mice were sacrificed, and the mammary glands were extracted and either fresh frozen for RNA extraction or fixed and embedded for immunohistochemical analysis. qPCR analysis on RNA from the dox-fed mice (for 7 days) showed that we achieved a 15 - 30 fold increase in expression of miR-204 in the miR-204 Tg mice when compared to the control non transgenic (non Tg) mice (Figure 3B). We also examined the mammary tissue for IGF2R expression and observed a significant decrease in IGF2R protein levels in the miR-204 Tg mice when compared to non Tg control mice (Figure 3C), suggesting that miR-204 negatively regulates IGF2R *in vivo*. Interestingly, we observed no significant decrease in IGF2R mRNA levels when miR-204 was overexpressed *in vivo* (Figure 3D), suggesting that miR-204 regulates IGF2R by translational repression, similar to that observed *in vitro*.

miR-204 transgene expression in the mammary epithelium of MMTV-Neu mice increases tumor growth and metastasis

To examine the effects of miR-204 overexpression on mammary tumor growth, miR-204 Tg mice were bred to the MMTV-Neu mice (see Methods). This model was chosen since we have observed a significant increase in the expression levels of miR-204 in tumors derived from these mice when compared to mammary tissue from normal 'non-tumor' mice (Supplemental Figure 1C). Mice were fed dox chow from 3 weeks of age to induce transgene expression in the trigenic mice and to control for any effects of dox on mammary tumorigenesis and progression in the bigenic control groups. Mice were palpated weekly at 5 months of age to detect tumors, and the age of onset and location of the tumor were recorded. We found that miR-204 overexpression did not affect tumor latency (Supplemental Figure 2A). The median time to tumor onset for non Tg mice was 37.5 weeks (95% CI: 35,47) and for miR-204 Tg mice was 39.0 weeks (95% CI: 35,Inf) as determined by Kaplan-Meier analysis (p=0.55). However, once tumors formed (tumor onset), time to sacrifice was shorter in the miR-204 Tg mice (2.0 weeks (95%CI: 2, Inf)) than in the non Tg mice (5.5 weeks (95% CI: 4, Inf)) (p=0.02) (Figure 3E). There was no statistical difference in either multiplicity or tumor location between the groups (Supplemental Figure 2B).

Histological examination of the tumors revealed differences between the non Tg and miR-204 Tg mice (Figure 3F). Tumors from miR-204 Tg mice displayed cells arranged in nests and packets with increased numbers of pseudo-rosettes surrounding blood vessels. These tumors also displayed more abundant vasculature, and peripherally, the cells appeared more spindloid in shape with looser arrangement of cells centrally. Altogether, these changes in the miR-204 Tg derived tumors are consistent with neuroendocrine differentiation. The tumors from the non Tg derived mice are arranged in solid sheets. Few mitoses are present, and most cells are monomorphic with no evidence of differentiation. A few apoptotic cells were observed scattered throughout the tumors. No major differences were

observed in miR-204 expression between the non Tg and 204 Tg tumors as expected at the endpoint of analysis, as we have previously described that this model increases expression of miR-204 during tumorigenesis. As the tumors displayed a more aggressive phenotype once formed, we assessed proliferation by immunostaining with Ki67 (Figure 3F). Although we observed a trend towards an increase in the miR-204 Tg tumors, quantitative analysis showed that the observed increase did not reach statistical significance (p=0.17) when compared to the non Tg controls (Figure 3G). To determine whether miR-204 overexpression altered tumor metastasis, the number of micrometastatic lesions was analyzed in serial sections of lungs from tumor bearing mice. We observed an increase in the total number of lung micrometastases in the miR-204 Tg (27.1 ± 2.0 SD) mice when compared to the non Tg (51.9 ± 5.7 SD) control mice (p<0.001) (Figure 3H).

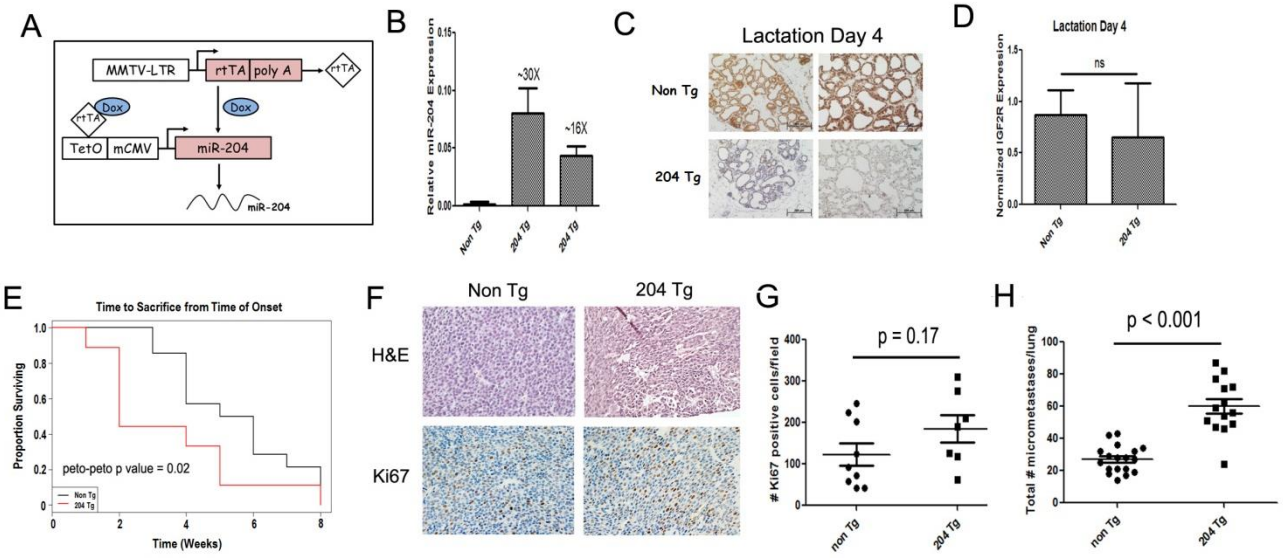

Figure 3: miR-204 drives aggressive tumor growth *in vivo*. (A) A schematic of the two constructs used to generate bigenic tet-regulatable miR-204 transgenic mice (adapted from (Vargo-Gogola et al., 2006)). (B) qPCR analysis of miR-204 transgene expression in mammary glands from control and dox-induced (for 7 days; 6 week old) mice. (C) Immunohistochemistry of IGF2R in non-transgenic (Non Tg) and miR-204 bigenic (miR-204 Tg) mice fed dox for 4 days. (D) qPCR analysis of IGF2R levels in RNA extracted from glands illustrated in panel C. (E) Time to sacrifice from time of onset in non Tg (black lines) and miR-204 Tg (red lines) mice. (F) Representative images of H&E and Ki67 IHC. Quantitation of (G) Ki67 and (H) lung metastases in non Tg (black circles) and miR-204 Tg (black squares) mice. Bars represent the mean ± SEM.

Exogenous IGF2R expression inhibits miR-204-mediated cell migration and invasion

miRNAs have multiple targets, and therefore, the effects observed after miR-204 expression may be the result of the decreased IGF2R protein, as well as non IGF2R-related miR-204 effects. One way to evaluate these possibilities is to examine the phenotypes in cells in which a non-targeted IGF2R is expressed. To test this possibility, the open reading frame (ORF) of IGF2R was transiently transfected into MCF10A cells stably infected with miR-204 (Supplemental Figure 3). As we have previously observed, the protein levels of IGF2R were reduced in the miR-204 expressing cells. In contrast, a smaller

reduction of IGF2R was observed in the cells expressing the non-targetable (ORF) form of IGF2R (Figure 4A). When we assessed migration and invasion in the IGF2R overexpressing cells, we did not observe an increase in either migration (Figure 4B) or invasion (Figure 4C) when miR-204 was co-expressed suggesting that miR-204 does increase migration and invasion through the negative regulation of IGF2R.

IGF2 stimulates the IGF1R signaling pathway when IGF2R is inhibited by miR-204

IGF2 preferentially binds to the IGF2R; however, it is also known to act as an autocrine and paracrine regulator of IGF1R, leading to downstream activation of the PI3K and MAPK/ERK signaling pathways and cell proliferation/survival (Flanigan et al., 2013; LeRoith and Roberts, 2003). Therefore, to determine whether IGF2 stimulates the IGF1R signaling pathway preferentially in the presence of miR-204 (or in the absence of IGF2R), we treated MCF10A cells expressing miR-204 with IGF2 and performed western blot analysis to assess the IGF1R signaling pathway (Supplemental Figure 4A-C). We observed a decrease in IGF2R protein expression when control cells were treated with IGF2; however, no further decrease in IGF2R expression was observed in the miR-204 expressing cells treated with IGF2 (Figure 4D). Importantly, we observed more robust activation of the IGF1R signaling pathway upon IGF2 treatment when miR-204 was overexpressed as illustrated by increased phosphorylation of IRS-1, an intracellular signaling adaptor protein and the main substrate of the IGF1R (Dearth et al., 2007), and AKT (Figure 4E). We did not observe increased activation of the ERK pathway in 204-expressing cells in response to stimulation with IGF2.

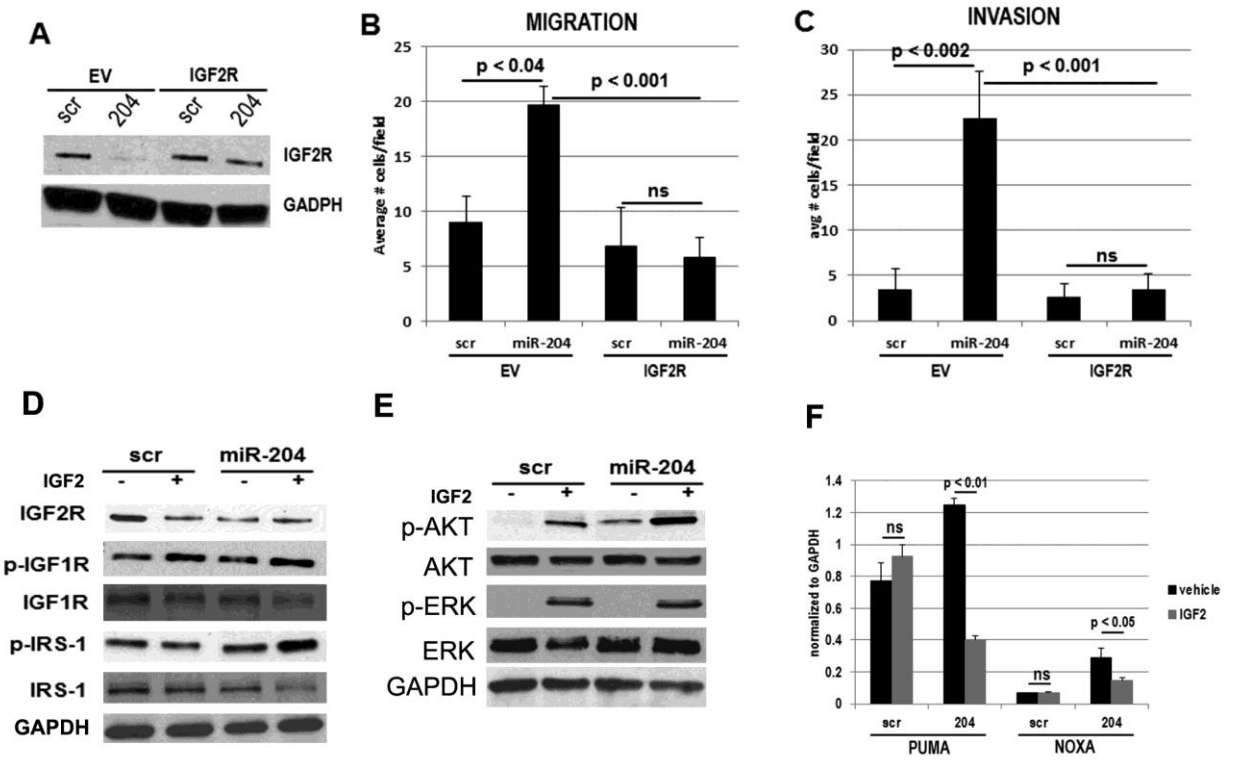

Figure 4: miR-204 drives IGF2 mediated activation of the IGF1R signaling pathway. Western blot (A) and transwell migration (B) and invasion (C) assays of miR-204 stably infected MCF10A cells transiently

transfected with IGF2R or empty vector (EV). Western blot analysis (D & E) of the IGF1R signaling pathway and qPCR analysis of PUMA and NOXA (F) in MCF12A cells stably infected with miR-204 or scr control and treated with (+) or without (-) 50 nM IGF2 for 5 minutes.

PUMA (p53 upregulated modulator of apoptosis) and NOXA (phorbol-12-myristate-13-acetate-induced protein 1) are proteins that play a key role in apoptotic signaling and have been shown to be negatively regulated by IGF1R/IRS-1/AKT signaling pathway in response to certain stimuli to increase cell survival and proliferation (Bean et al., 2013; You et al., 2006). We performed qPCR to assess PUMA and NOXA levels in response to IGF2 stimulation in miR-204 expressing cells. In contrast to cells with an intact IGF2R 'sink' for IGF2, we observe a significant decrease in PUMA and NOXA transcripts when stimulated with IGF2 in miR-204 expressing cells (Figure 4F).

miR-204 mediates migration through activation of the IGF1R signaling pathway

To determine whether miR-204 mediates its functional effects through activation of the IGF1R we transiently transfected IGF1R expressing, or control, MCF10A cells (Kim et al., 2007) with miR-204 (Supplemental Figure 4D & E). We observed activation of AKT in the IGF1R expressing cells alone, but no additional increase when miR-204 was co-expressed (Figure 5A). As expected, both miR-204 and IGF1R expression alone increased migration; however, no additional increase in migration was observed when they were co-expressed (Figure 5B), suggesting that IGF1R and miR-204 function through the same signaling pathway to increase migration. To assess whether IGF1R is required for miR-204 mediated increase in migration, we inhibited IGF1R expression in MCF12A cells with and without miR-204 expression with two short hairpins specific to IGF1R (Figure 5C). As expected, we observed a significant decrease in migration when IGF1R was inhibited in the MCF12A control cells. However, when miR-204 was expressed in the presence of the IGF1R inhibitor, no increase in migration was observed suggesting that IGF1R is required for miR-204 mediated migration in breast cells (Figure 5D).

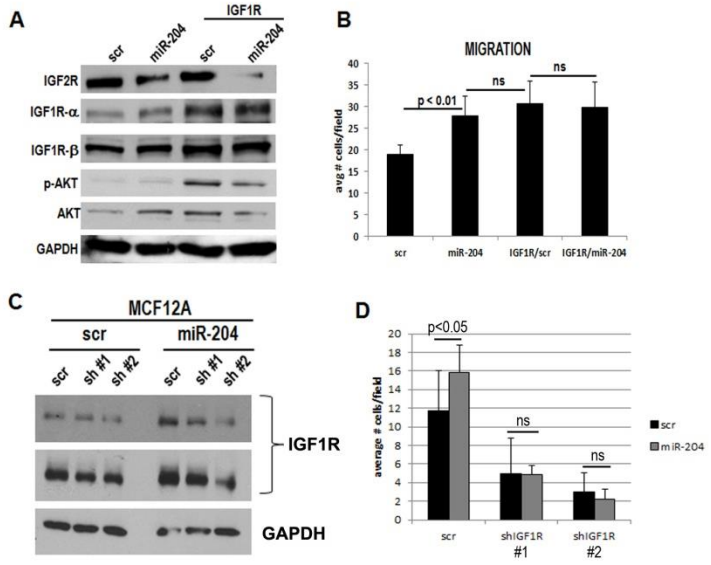

Figure 5: miR-204 mediates migration through activation of the IGF1R signaling pathway. Western blot (**A**) and transwell migration (**B**) assays of IGF1R stably infected MCF10A cells transiently transfected with miR-204 or scr control. Western blot (**C**) and transwell migration (**D**) assays of MCF12A cells stably infected with miR-204 or scr control and transiently transfected with short hairpin constructs to IGF1R (sh #1 & sh #2). IGF1R in panel C is shown for both a short (upper panel) and long (lower panel) exposure time.

DISCUSSION

miR-204, the miRNA of interest in this proposal, is located on chromosome 9q21, a region that is reported to be amplified in cancer (Bussemakers et al., 1999). There are multiple studies that have investigated the role of miR-204 in solid cancers, including melanoma, glioma, NSCLC, bladder cancer, gastric cancer, head and neck cancer, and endometrial cancer. These studies have all reported reduced levels of miR-204 in these solid cancers (Chung et al., 2012; Lam et al., 2011; Schultz et al., 2008; Xia et al., 2014). However, with respect to hormonally driven cancers, e.g., prostate and breast, the story appears to be more complex. Our group and others have shown that miR-204 levels are significantly elevated in breast cancer samples compared to normal controls (Findlay et al., 2008; Mattie et al., 2006). Published studies also suggest its role as a tumor suppressor in breast cancer (Li et al., 2014).

More recently, two studies were published by independent groups that support the role of miR-204 acting as an oncogene, or "oncomir", in cancer (Lee et al., 2016; Todorova et al., 2016). The first was a study aimed at solving the controversy surrounding the seemingly opposing effects of miR-204 in breast cancer (Lee et al., 2016). They performed genome wide pathway analysis and showed definitively that many of the miR-204 target genes are tumor suppressors. It is this characteristic that drives the breast cells towards being oncogenic. However, they agree and support the idea that context is important, and the potential dual activity requires further investigation. The second paper, recently published, investigated specifically the proposed dual role of miR-204 in cancer (Todorova et al., 2016). This study showed that the genomic instability incurred rearrangement could turn tumor suppressor miRNAs into pro-oncogenic ones, using metastatic prostate cancer as a model system. Both studies clearly demonstrate a dual role for miR-204 depending on context.

Furthermore, a study in prostate cancer showed a dual role for miR-204 depending additionally on the subtype of prostate cancer in which it was expressed (Ding et al., 2015). In brief, the authors show that in the context of androgen receptor positive (AR+) prostate adenocarcinoma, miR-204 functions as a tumor suppressor. However, in the context of androgen receptor negative (AR-) neuroendocrine prostate cancer, miR-204 functions as an oncogene. The dual regulatory role of miR-204 in cancer was recently reviewed and highlighted the fact that the cell type in which miR-204 is being expressed, as well as the make-up of that cell, are critical to the function of miR-204 within that cell (Li et al., 2016). More studies are required to determine whether the role of miR-204 in breast cancer is also dependent upon AR expression, an understudied receptor in the breast cancer field. Of interest, a study was recently published showing a positive correlation between AR expression and PDEF in breast cancer (Cao et al., 2018). This is important in the

discussion of miR-204 as (i) PDEF was one of the first identified targets for miR-204 in breast cancer (Findlay et al., 2008); and (ii) we observed a neuroendocrine pathology in mammary tumors derived from our 204 Tg animals and neuroendocrine prostate tumors are known to be AR negative (Tsai et al., 2017).

Our studies support the working hypothesis that miR-204 mediated inhibition of IGF2R frees IGF2 to bind the IGF1R leading to hyperactivation of this pathway, resulting in increased proliferation, migration and invasion, processes required for tumor progression (Figure 6). The generation and utility of an *in vivo* model for miR-204 is a more physiologically representative model than cells grown in culture. Our development of a unique inducible transgenic mouse model has allowed us to investigate the oncogenic potential of miR-204 and furthermore, has provided compelling data to help resolve the controversy surrounding the role of miR-204 in a cellular context. Histologically, the tumors that developed in the miR-204 transgenic mice were distinct from the control non-transgenic mice. We observed increased vasculature and a more spindle-like appearance, indicative of neuroendocrine differentiation. This has potential interest based on the observation mentioned above that miR-204 is specifically expressed and functions as on oncomir in neuroendocrine prostate cancer. Future studies aimed at investigating the potential role of miR-204 in driving neuroendocrine differentiation in tumors may have implications for both prostate and breast cancers.

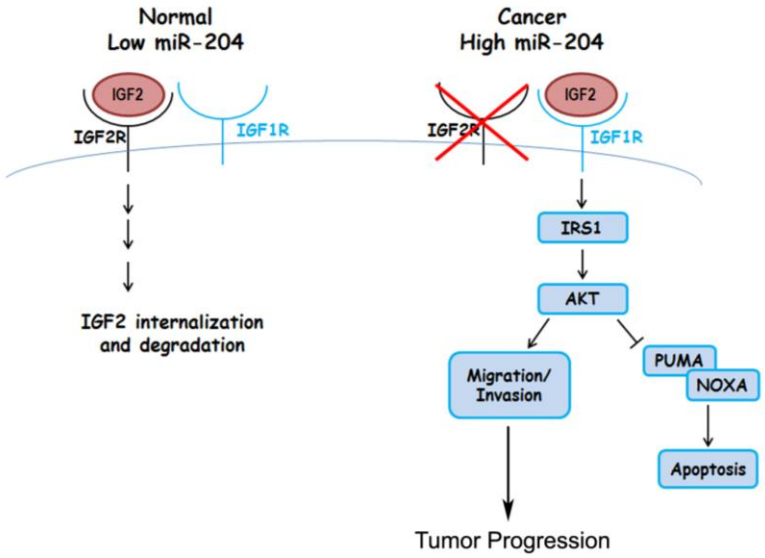

Figure 6: Schematic model of our proposed mechanism for miR-204 mediated tumor progression. In normal (low miR-204) cells, IGF2 preferentially binds to the IGF2R where it gets internalized and degraded by the lysosomes. Therefore, IGF1R signaling is kept to a minimum. However, in cancer (high miR-204) cells, we propose that miR-204 mediated inhibition of IGF2R frees IGF2 to bind the IGF1R leading to hyperactivation of this pathway resulting in increased proliferation, migration and invasion, processes required for tumor progression.

Reduced IGF2R expression correlates with poor patient prognosis in BC patients (Chappell et al., 1997; Hankins et al., 1996), and a recent study showed significantly higher levels of IGF2R in Caucasian Americans (CA) compared to African American (AA) tumor samples, suggesting that decreased IGF2R expression may contribute to BC disparity (Kalla Singh et al., 2010). We observed elevated miR-204 levels in AA compared to CA women; specifically in the low-grade samples. Interestingly, loss of IGF2R is an early transformational event in breast cancer, occurring in the initiation rather than the progression stage of carcinogenesis. Therefore, we postulate that overexpression of miR-204 may be an early initiating event in AA women, leading to the loss of IGF2R and more aggressive disease (Figure 6).

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Conflict of interest

The authors declare that no competing or conflict of interests exist. The funders had no role in study design, writing of the manuscript, or decision to publish.

Authors' contributions

LMN and JDF performed the luciferase and western blot assays. LMN and LB performed the qPCR assays. LMN and DPT performed the functional and rescue experiments. LMN and CEB performed the in vivo experiments. KLH performed the histological analysis of the tumors. EGM analyzed and performed statistical analysis on the *in vivo* data. VJF conceived of the study and participated in its design and coordination, and with DPT and DKW drafted the manuscript. All authors read and approved the final manuscript.

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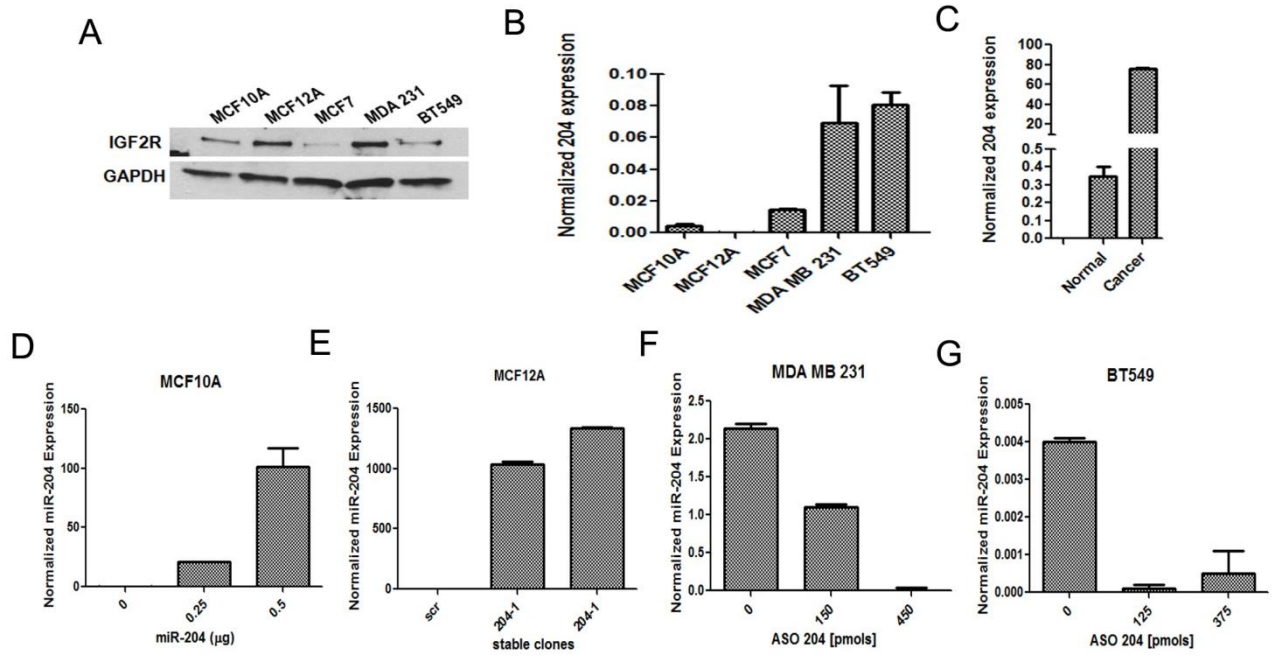
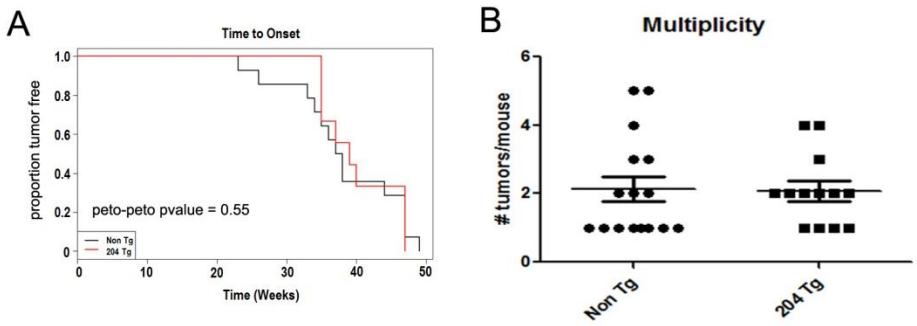
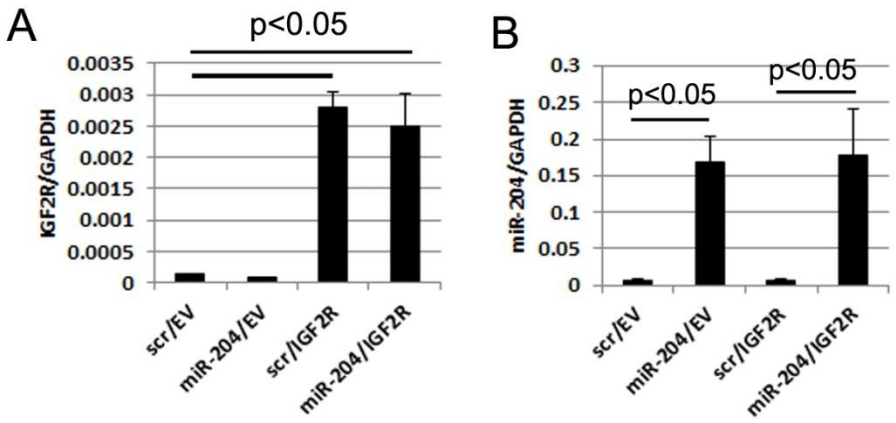
Supplementary Data:



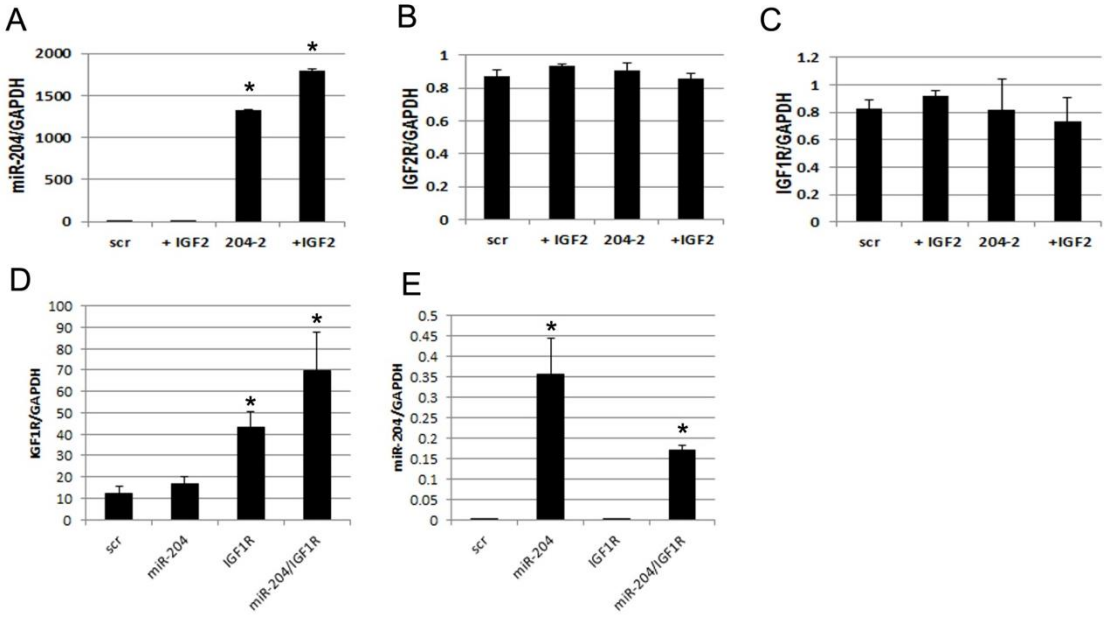
Supplemental Figure 1: Western blot of IGF2R (A) and qPCR analysis of mir-204 expression levels in various human breast cell lines (B) and normal and breast cancer mouse tissue (C). qPCR analysis of miR-204 expression levels in cell lines after either miR-204 overexpression in MCF10A(D) and MCF12A(E) cells or miR-204 inhibition in MDA-MB-231 (F) and BT549(G) cells. *p < 0.05



Supplemental Figure 2: Time to tumor onset (A) and tumor multiplicity (B) in non Tg (black lines) and miR-204 Tg (red lines) mice.



Supplemental Figure 3: qPCR analysis of (A) IGF2R and (B) mir-204 expression levels in MCF10A cells transiently transfected with IGF2R or empty vector (EV) control.



Supplemental Figure 4: qPCR analysis of (A) miR-204, (B) IGF2R and (C) IGF1R expression levels in MCF12A cells stably transfected with miR-204 and either untreated or treated with 50nM IGF2 for 5 minutes. qPCR analysis of (D) IGF1R and (E) mir-204 expression levels in MCF10A cells stably expressing IGF1R or control cells and then transiently transfected with miR-204 or scr control.