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MiR-27b targets *PPARγ* to inhibit growth, tumor progression, and the inflammatory response in neuroblastoma cells

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Abstract

The *PPARγ* nuclear receptor pathway is involved in cancer, but it appears to have both tumor suppressor and oncogenic functions. In neuroblastoma cells, miR-27b targets the 3′UTR of *PPARγ* and inhibits its mRNA and protein expression. miR-27b overexpression or *PPARγ* inhibition blocks cell growth *in vitro* and tumor growth in mouse xenografts. *PPARγ* activates expression of the pH regulator *NHE1*, which is associated with tumor progression. Lastly, miR-27b through *PPARγ* regulates NF-κB activity and transcription of inflammatory target genes. Thus, in neuroblastoma, miR-27b functions as a tumor suppressor by inhibiting the tumor-promoting function of *PPARγ*, which triggers an increased inflammatory response. In contrast, in breast cancer cells, *PPARγ* inhibits *NHE1* expression and the inflammatory response, and it functions as a tumor suppressor. We suggest that the ability of *PPARγ* to promote or suppress tumor formation is linked to cell-type specific differences in regulation of *NHE1* and other target genes.

Keywords

miR-27b; *PPARγ*; *NHE1*; NF-κβ; inflammation; neuroblastomas

INTRODUCTION

Peroxisome proliferators-activated receptors (PPAR) are members of the nuclear receptor superfamily of ligand-activated transcription factors. Three isoforms, PPARα, PPARβ/δ and PPARγ, are encoded by three genes that respond to diverse, but distinct, sets of ligands (Michalik et al., 2004). PPARγ has emerged as an attractive target for cancer therapy due to its association with many human cancers such as colon, thyroid, breast and prostate (Michalik et al., 2004). PPARγ is abundant in adipose tissues and is also expressed at a lower level in skeletal muscles, liver, heart, intestine, vascular smooth muscle, lung, breast,

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colon and prostate. Interestingly abundant PPARγ expression has been detected in different tumors such as transformed human B lymphocyte and myeloid cells lines, astrocytomas (Chattopadhyay et al., 2000), glioblastoma (Nwankwo & Robbins, 2001; Morosetti et al., 2004) and neuroblastoma (Han et al., 2001).

The role of PPARγ in tumor development is controversial. It has been suggested that PPARγ is a tumor suppressor, because ligands that activate PPARγ promote growth inhibition and apoptosis in cancers of breast (Mueller et al., 1998; Mehta et al., 2000; Kim et al., 2006), colon (Sarraf et al., 1998), liposarcoma (Tontonoz et al., 1997), and neuroblastoma (Cellai et al., 2006; Cellai et al., 2010). However, it has been suggested the anti-tumor effect induced by such PPARγ ligands occurs via a PPARγ-independent pathway without the presence of the PPARγ receptors (Abe et al., 2002; Lecomte et al., 2008). Alternatively, several lines of evidence suggest that activated PPARγ is not a tumor suppressor, but rather functions as an oncogene. First, expression of PPARγ is higher in human prostate cancer cells than in normal prostate tissues (Han & Roman, 2007). Second, PPARγ exhibits a pro-tumor effect in mice bearing a mutation in the *APC* tumor suppressor gene, because PPARγ agonists increase the frequency and size of colon tumors (Lefebvre et al., 1998; Saez et al., 1998). Third, PPARγ antagonists have anticancer effects in other cell lines and mouse models (Cui et al., 2002; Burton et al., 2008).

MicroRNAs play critical roles in many biological processes including cancer by directly interacting with specific mRNAs through base pairing and then inhibiting expression of the target genes through a variety of molecular mechanisms (Bartel, 2009; Croce, 2009; Ventura & Jacks, 2009). The miR-27 family (miR-27a and miR-27b) directly targets PPARγ, and it inhibits adipocyte differentiation (Karbiener et al., 2009; Kim et al., 2010) and is induced upon inflammation in macrophages (Jennewein et al., 2010). Here, we show that miR-27b also targets *PPARγ* in neuroblastoma cells. miR-27b overexpression or *PPARγ* inhibition blocks neuroblastoma growth *in vitro* and *in vivo*, and this growth inhibition is associated with decreased expression of *NHE1*, a PPARγ target gene, and a reduced inflammatory response. In contrast, PPARγ inhibits *NHE1* expression, the inflammatory response, and growth of a breast cancer cell line. These results suggest that miR-27b functions as a tumor suppressor, that *PPARγ* promotes tumor formation in neuroblastomas, and that cell-type-specific regulation of *NHE1* by PPARγ underlies the difference between the oncogenic and tumor suppressing functions of PPARγ in different cell types.

RESULTS

miR-27b inhibits PPARγ expression via its 3′UTR in neuroblastoma

As the miR-27 family (miR-27a and miR-27b) directly targets PPARγ in adipocytes and macrophages (Karbiener et al., 2009; Jennewein et al., 2010; Kim et al., 2010), we examined whether PPARγ is a direct target of miR-27b in a cancer context. Luciferase reporter plasmids containing the wild-type (WT) 3′UTR sequence of PPARγ or a deletion mutant (lacking the 8-bp seed sequence) were transfected into the SK-N-AS neuroblastoma cancer cell line with miR-27b or an anti-sense RNA against miR-27b (as-miR-27b). PPARγ luciferase activity of the wild-type reporter is reduced 5-fold upon miR-27b overexpression, whereas it is increased by 60% upon miR-27b inhibition (Figure 1a). In contrast, no changes

in PPARγ luciferase activity are observed in the mutant reporter plasmid upon overexpression of miR-27b or as-miR-27b. As expected, antisense-mediated inhibition of either miR-27a or miR-27b results in increased levels of PPARγ mRNA (Figure 1b). In addition, PPARγ protein levels are decreased upon overexpression of miR-27b and increased upon addition of antisense against miR-27b (Figure 1c). Lastly, in 10-day old tumors generated by injection of SK-N-AS cells in nude mice, PPARγ mRNA expression is reduced 3-fold in tumors injected intra-tumorally with miR-27b, but not with the control miRNA (Figure 1d). Thus, miR-27b inhibits PPARγ expression in neuroblastomas cells.

miR-27b inhibits neuroblastoma cell growth *in vitro* and tumor growth in mouse xenografts

We investigated the role of miR-27b in neuroblastoma cell growth by overexpressing either miR-27b or its antisense RNA. Overexpression of miR-27b or miR-27a inhibits cell growth, whereas overexpression of as-miR-27b or as-miR-27a increases cell growth (Figures 2a). More importantly, in mouse xenografts involving the neuroblastoma cell line, administration of four cycles of miR-27b, but not a control miRNA, strongly reduces tumor growth, whereas tumor growth is enhanced by treatment with as-miR-27b (Figure 2b). These observations are indicative of a tumor suppressive role for miR-27b in neuroblastomas, and they are in accord with studies in other types of cancer. Specifically, miR-27b acts as a tumor suppressor gene in breast cancer, and it is highly expressed in human normal breast tissues (Lu et al., 2005) but less expressed in breast cancer tissues (Tsuchiya et al., 2006). In addition, miR-27b expression is suppressed in anaplastic thyroid cancer (Braun et al., 2010).

miR-27b levels are reduced in neuroblastoma tissues

To examine whether the tumor-suppressor effects of miR-27b in neuroblastoma cell lines are relevant to the human disease, we measured miR-27b RNA levels in tissue samples from human patients. In all 9 cases tested, miR-27b levels in neuroblastoma tissue were 2–3 fold lower than in the adjacent non-cancer tissue (Figure 2c). Thus, reduced levels of miR-27b are associated with neuroblastoma.

PPARγ plays a tumor-promoting role in neuroblastoma

The functional role of PPARγ activation during cancer development remains controversial, in part because the experiments have been performed with PPARγ agonists or antagonists that may mediate their effects through non-PPARγ mechanisms (see Introduction). To avoid this problem, we inhibited expression of the PPARγ gene by an siRNA and found that this resulted in reduced cell viability (Figure 2a). In accord with these experiments, treatment of these neuroblastoma cells with the PPARγ antagonist GW9662 inhibits cell growth *in vitro* (Figure 2d) and in mouse xenografts (Figure 2e). In addition GW9662 inhibit growth of a different neuroblastoma cell line (SK-N-SH; Supplementary Figure 1). Lastly, as mentioned above, miR-27b acts as a tumor suppressor, providing an independent line of evidence that reduction of PPARγ levels is associated with reduced cancer cell growth. Collectively these observations strongly suggest that PPARγ has a growth-stimulating and tumor-promoting role in neuroblastoma cells.

PPARγ activates NHE1 in neuroblastoma cells

Activation of the pH regulator *NHE1* causes tumors to become more acidic extracellularly and more alkaline intracellularly even during the early stages of neoplastic progression, and hence *NHE1* activation is tumor-promoting (Hagag et al., 1987; Ober & Pardee, 1987; Siczkowski et al., 1994; Reshkin et al., 2000). Indeed, si-RNA-mediated inhibition of *NHE1* expression results in reduced growth of SK-N-AS neuroblastoma cells (Figure 2a). *NHE1* expression is directly regulated by binding of PPARγ to target sites (PPREs) in the *NHE1* promoter, and activated PPARγ inhibits *NHE1* expression in breast cancer cell lines (Kumar et al., 2009; Venkatachalam et al., 2009). These observations are consistent with a number of studies concluding that PPARγ has anti-tumor effects in breast cancer (Mueller et al., 1998; Mehta et al., 2000; Girnun et al., 2002; Kim et al., 2006; Kumar et al., 2009).

We independently confirmed the anti-tumor effects of PPARγ in breast cancer cells using an isogenic model of cellular transformation involving non-transformed mammary epithelial cells (MCF-10A)(Soule et al., 1990) containing ER-Src, a derivative of the Src kinase oncoprotein (v-Src) that is fused to the ligand-binding domain of the estrogen receptor (Aziz et al., 1999). Treatment of such cells with tamoxifen rapidly induces Src, and morphological transformation is observed within 24–36 hours (Hirsch et al., 2009; Iliopoulos et al., 2009), thereby making it possible to kinetically follow the transition between non-transformed and transformed cells. In this isogenic model, siRNA-mediated inhibition of PPARγ or exogenous expression of miR-27b results in increased tumorigenicity (colonies growing in soft agar; Figure 3a) and invasive growth (MATRIGEL assay; Figure 3b). Furthermore, tumors derived from these transformed ER-Src cells in mouse xenograft grow more quickly upon injection of siRNA against PPARγ (Figure 3c). Similar effects of miR-27b on reducing *PPARγ* expression (Figure 3d) and increasing invasive growth (Figure 3e) are observed in two other breast cancer cells lines (MDA-MB-231 and MDA-MB-468).

In contrast to the results in breast cancer cells, several lines of evidence indicate that PPARγ activates *NHE1* expression in neuroblastomas cells. First, expression of as-miR-27b causes increased *NHE1* expression (Figure 4a) along with increased *PPARγ* expression (Figure 1b, c) in cell culture. Conversely, expression of miR-27b in mouse xenografts reduces *NHE1* (Figure 4b) and *PPARγ* expression (Figure 1d). Second, treatment of neuroblastoma cells with siRNA against PPARγ causes a 4-fold decrease in *NHE1* expression levels (Figure 4c). Third, the PPARγ antagonist GW9662 inhibits both *PPARγ* and *NHE1* expression in cell culture (Figure 4d) and in mouse xenografts (Figure 4e). Taken together, these observations suggest that *PPARγ* can activate or inhibit *NHE1* expression in a cell-type-specific manner, and that the differential regulation of *NHE1* expression accounts for the opposing tumor-promoting or tumor-inhibiting effects in these different cell types.

miR-27b and PPARγ regulate the inflammatory response in neuroblastoma cells

The inflammatory transcription factor NF-κB physically interacts with PPARγ (Chung et al., 2000), and there is a great deal of evidence linking NF-κB and inflammation to cancer (Balkwill & Mantovani, 2001; Karin, 2006; Naugler & Karin, 2008; Iliopoulos et al., 2009). We therefore examined the effect of miR-27 and PPARγ on the inflammatory response. Inhibition of miR-27b in SK-N-AS neuroblastoma cells increases mRNA levels of four

inflammatory factors (IL-1A, JAK2, IL-6 and IL-1B), whereas expression of miR-27b results in decreased expression (Figure 5a). In addition, mRNA levels of these inflammatory factors are strongly reduced upon siRNA-mediated (Figure 5a) or pharmacological inhibition (GW9662) of PPARγ (Figure 5b). Importantly, the increased expression of inflammatory factors upon reduction of miR-27b is blocked by simultaneous inhibition of PPARγ (Figure 5a), suggesting that the effects of miR-27b are mediated through PPARγ. In accord with these observations, tumors harvested from the mice either treated with GW9662 or with miR-27b show significantly lower NF-κB activity and reduced IL-6 mRNA expression relative to control groups (Figure 5c). Lastly, neuroblastoma cell growth is inhibited upon treatment with an NF-κB inhibitor (BAY-117082; Figure 5d) at concentrations that do not affect the growth of non-transformed cells (Supplementary Figure 2). Thus, miR-27b and PPARγ regulate the inflammatory response in neuroblastoma cells.

DISCUSSION

Our study identifies a molecular pathway important for growth and tumor progression of neuroblastomas cells (Figure 6). Specifically, miR-27b functions as a tumor suppressor by directly inhibiting the expression of PPARγ. Inhibition of PPARγ by miR-27b, si-RNA, or a pharmacological antagonist reduces expression of *NHE1* (presumably by direct binding to the promoter region) and the inflammatory response (by an unknown mechanism). Furthermore, inhibition of PPARγ results in reduced cell growth *in vitro* and tumor growth in mouse xenografts, indicating that PPARγ functions as a tumor-promoting factor in neuroblastomas. In accord with this tumor-promoting function, PPARγ stimulates *NHE1* expression and inflammation, both of which are linked to tumor progression in multiple cell types. Our results do not exclude additional cancer-related functions for miR-27b or for PPARγ in neuroblastoma, and indeed these are likely.

Our study also provides new insights on how a transcription factor can act either as an oncogene or tumor suppressor depending on the cell type. PPARγ activates *NHE1* expression in neuroblastomas, but it inhibits *NHE1* expression in breast cancer cells, and this discordant regulation of *NHE1*, an oncogenic factor, is linked to tumor suppression in breast cells and tumor promotion in neuroblastomas (Figure 6). There are many examples in which a DNA-binding transcription factor can directly activate or repress genes in a given cell type, or directly activate or repress a given gene in different cell types. We therefore suggest that PPARγ has oncogenic or tumor suppressor functions in different cell types by virtue of cell-type-specific regulation of *NHE1* and perhaps other target genes.

MATERIALS AND METHODS

Cell lines

The neuroblastoma cell line SK-N-AS (American Type Culture Collection) was maintained in DMEM media (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals), and penicillin/streptomycin (Invitrogen) at 37°C with 5% CO_2. The breast epithelial cell line MCF-10A cells containing the ER-Src fusion protein was grown in DMEM/F12 medium supplemented with 5% donor HS, 20 ng/ml epidermal growth factor (EGF), 10 mg/ml insulin, 100 mg/ml hydrocortisone, 1 ng/ml cholera toxin, and 50 units/ml pen/strep, with

the addition of puromycin (Hirsch et al., 2009; Iliopoulos et al., 2009). To induce transformation, the Src oncogene was activated by the addition of 1 mM tamoxifen (Sigma, St. Louis) for 36 hours.

Luciferase assays

The firefly luciferase reporter plasmids contained the entire wild-type 3′UTR of *PPARγ* (Genecopeia Inc.) or a mutated derivative deleted for the 8 bp seed sequence deleted generated by inverse-PCR (Supplementary Table 1). The *Renilla* plasmids (0.8 μg) were co-transfected into SK-N-AS cells either with 33 nM of as-miR-27b (AM10750, Ambion), miR-27b (C-300589-05, Dharmacon) or non-targeting control (NC) (PM11440, Ambion) using Lipofectamine™ 2000 (Invitrogen) to the cells. The PPARγ luciferase activity of the luciferase vector construct only (UT) was normalized to one and the other transfection combinations were compared with UT. Cells were harvested 48 h after transfection and assayed using the Dual Luciferase Reporter Assay System (Promega).

RNA analysis

RNA was purified by the Trizol method (Invitrogen, Carlsbad, CA), treated with RNase-free DNase (Ambion), and reverse transcribed with using SuperScript III RT (Invitrogen) to generate cDNA. RNA levels were determined by SYBR Green-based real-time PCR of the cDNA, with the level of β-actin used as a loading control. Each sample was run in triplicate, and the data represent the mean ± SD of three independent experiments. PCR primers used for these analyses are shown in Supplementary Table 1.

Western blotting

Total protein (50 μg) from neuroblastoma cells was isolated by standard methods in RIPA buffer (25 mM Tris.HCl pH7.6, 150 mM NaCl, 1% NP-40, 1% sodium, deoxycholate, 0.1% SDS), electrophoretically separated, and transferred to nitrocellulose filters. The filters were incubated overnight at 4°C with anti-PPARγ (1:200; ab27649, Abcam Inc.) and anti-α-tubulin (1:3000; Clone DM1A, Sigma). The density of the bands was quantified and normalized by the loading control, γ-tubulin.

Genetic and pharmacological analysis of cell growth

For genetic analysis, SK-N-AS cells seeded in 6- or 12-well plates were transfected with 100 nM miRNAs, antisense (as)-miRNAs or siRNAs using the siPORT NeoFX transfection agent (Ambion) and incubated for 24 hours. The number of viable cells was measured at various times after this initial incubation period. For pharmacological analysis, cells were seeded in 24-well plates for an initial 20 hour incubation period, after which time they were treated with medium containing 15 μM GW9662 (PPARγ antagonist; Cayman Chemical), a 5 μM BAY-11-72 (NF-kB inhibitor; Sigma), or DMSO (vehicle). Medium containing these inhibitors was changed every 24 hours.

Soft agar colony and invasion assays

The soft agar colony and MATRIGEL invasion assays for MCF-10A-ER-Src cells were performed as described previously (Iliopoulos et al., 2009; Hirsch et al., 2010).

Xenograft experiments

SK-N-AS cells (5×10^6) were injected into the right flank of nu/nu mice (Charles River Laboratories), all of which developed tumors in 10 days with size of ~60 mm^3. The mice were randomly distributed into groups (typically 4 mice per group) and treated with miR-27b (100 nM), miRNA negative control (miR-NC; 100 nM), GW9662 (2.5 mg/kg), or DMSO (0.1 ml/10 g body weight). All treatments were given by intraperitoneal (i.p) injection every five days starting on day 10 to day 25 for 4 cycles. Tumor volumes were monitored every five days. Tumors were harvested on day 35 for mRNA analysis of *PPARγ* and *NHE1* and for measurements of NF-kB activity (ActivELISA kit IMK-503 from Imgenex). All mouse experiments were performed according to the Institutional Animal Care and Use Committee procedures and guidelines of Tufts University.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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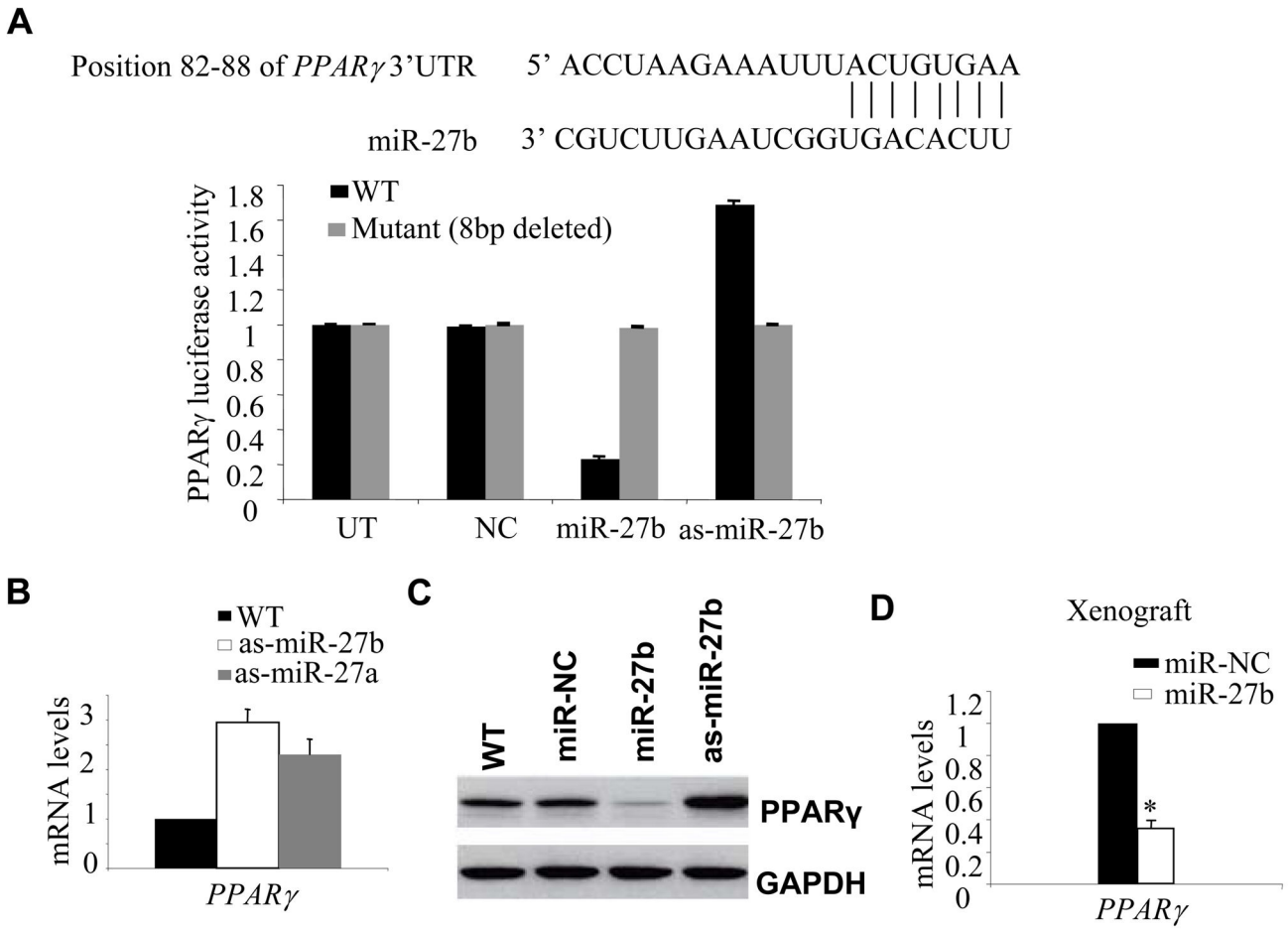

Figure 1. miR-27b targets the 3′UTR of *PPARγ*

(**a**) Sequence complementarity (vertical lines showing the seed sequence between positions 82–88) between miR-27b and the *PPARγ*. Luciferase activity of reporters containing the wild-type or 8-bp deleted 3′UTR of *PPARγ* 24h after transfection with miR-27b, antisense (as) against miR-27b or miR negative control or non-transfected cells (UT). (**b**) *PPARγ* mRNA levels in SK-N-AS cells transfected with as-miR-27a (gray bar) or as-miR-27b (white bar). (**c**) Western blot showing *PPARγ* protein levels in cells transfected with the indicated RNAs; levels of GAPDH serve as a loading control. (**d**) *PPARγ* mRNA levels in mouse xenografts (SK-N-AS cells) that are or are not injected with miR-27b.

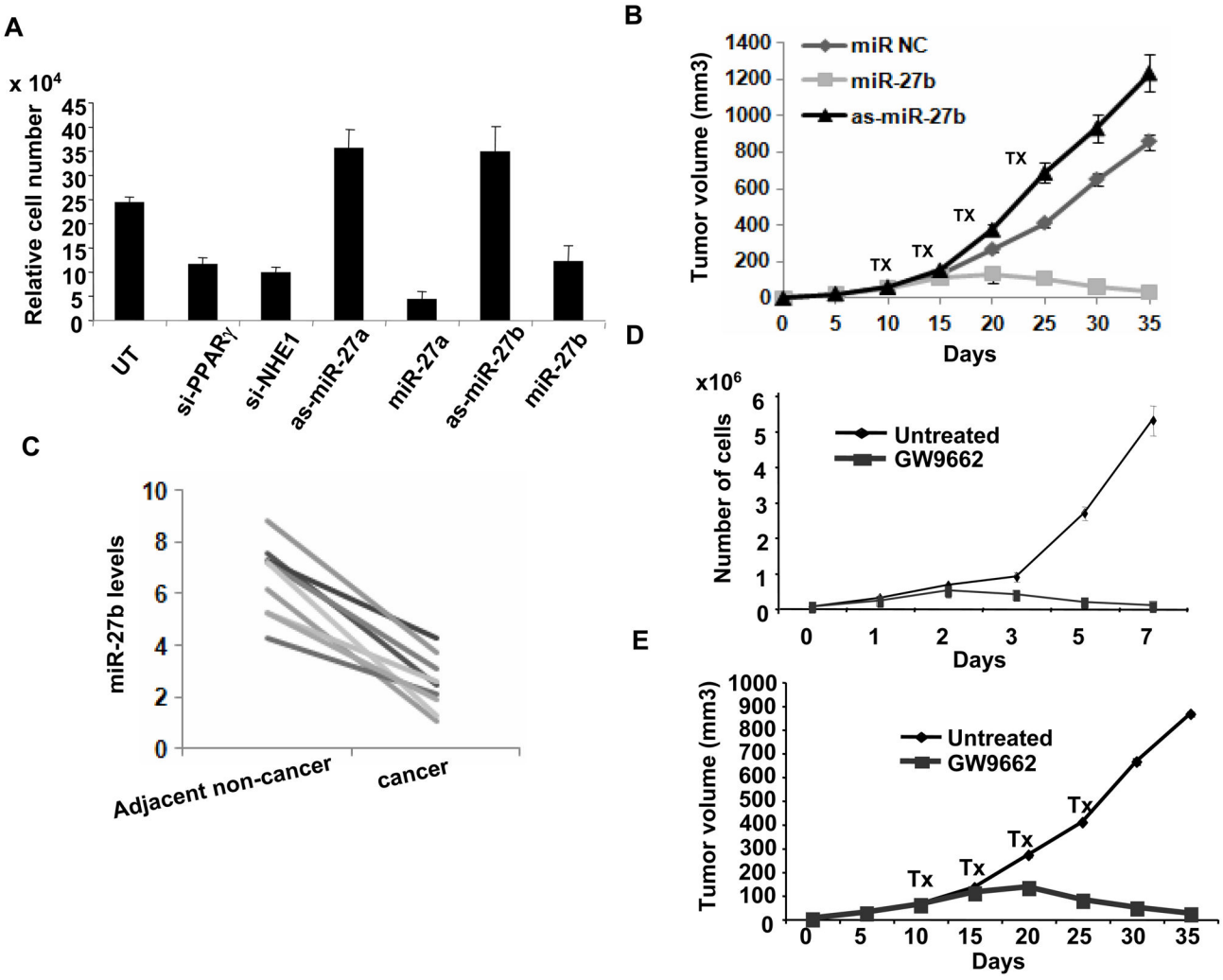

Figure 2. miR-27b through *PPARγ*affecting the cell growth in neuroblastoma cancer*in vitro* and *in vivo*

(**a**) Relative umber of viable SK-N-AS cells that were transfected with the indicated RNAs for 24 hours and then allowed to grow for an additional 24 hours. UT indictes untreated (i.e. no siRNA). (**b**) Tumor growth (mean ± SD) of mouse xenografts containing neuroblastoma (SK-N-AS) cells after intraperitoneal treatment with miR-27b, as-miR-27b or control miRNA on the indicated number of days after the initial injection of cancer cells. (**c**) miR-27b RNA levels in neuroblastoma and adjacent non-cancer tissues from 9 patients, with each line representing an individual patient. (**d**) Growth of SK-N-AS cells in the presence or absence of GW9962 for the indicated number of days. (**e**) Tumor growth (mean ± SD) of mouse xenografts containing neuroblastoma (SK-N-AS) cells after intraperitoneal treatment with GW9662 (or no treatment) on the indicated number of days after the initial injection of cancer cells.

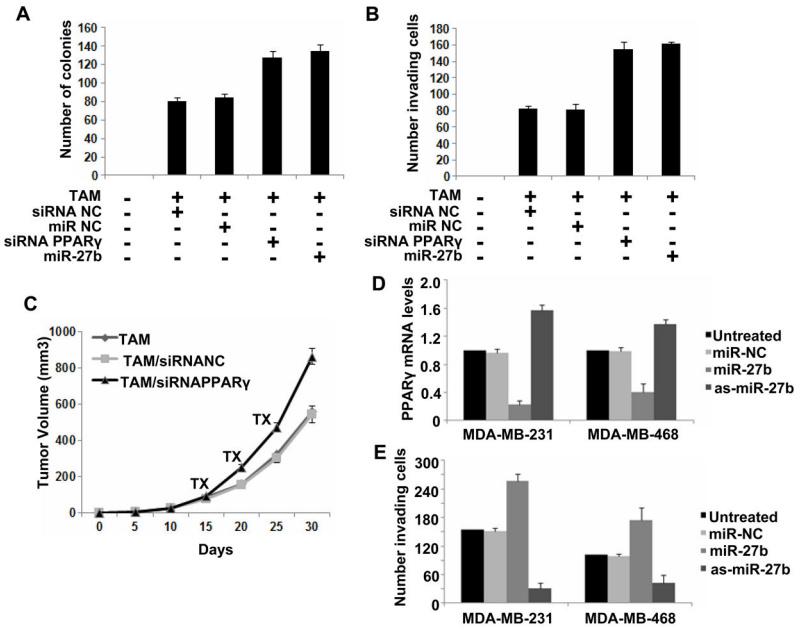

Figure 3. *PPARγ* functions as a tumor suppressor in a isogenic model of transformation in breast cells

(**a**) Colony formation in soft agar of the ER-Src cells that were or were not treated with tamoxifen (TAM) and/or transfected with miR-27b, siRNA against *PPARγ*, or control siRNA and miRNA. (**b**) Invasive growth (invading cell/field after wounding) of the cells described in panel **a**. (**c**) Tumor growth (mean ± SD) of mouse xenografts containing transformed ER-Src cells after intraperitoneal treatment with siRNA against *PPARγ* or control siRNA on the indicated number of days after the initial injection of cancer cells. (**d**) *PPARγ* RNA levels in the indicated breast cancer cell lines treated with miR-27b, as-miR-27b, or control miRNA. (**e**) Invasive growth in the indicated breast cancer cell lines treated with miR-27b, as-miR-27b, or control miRNA.

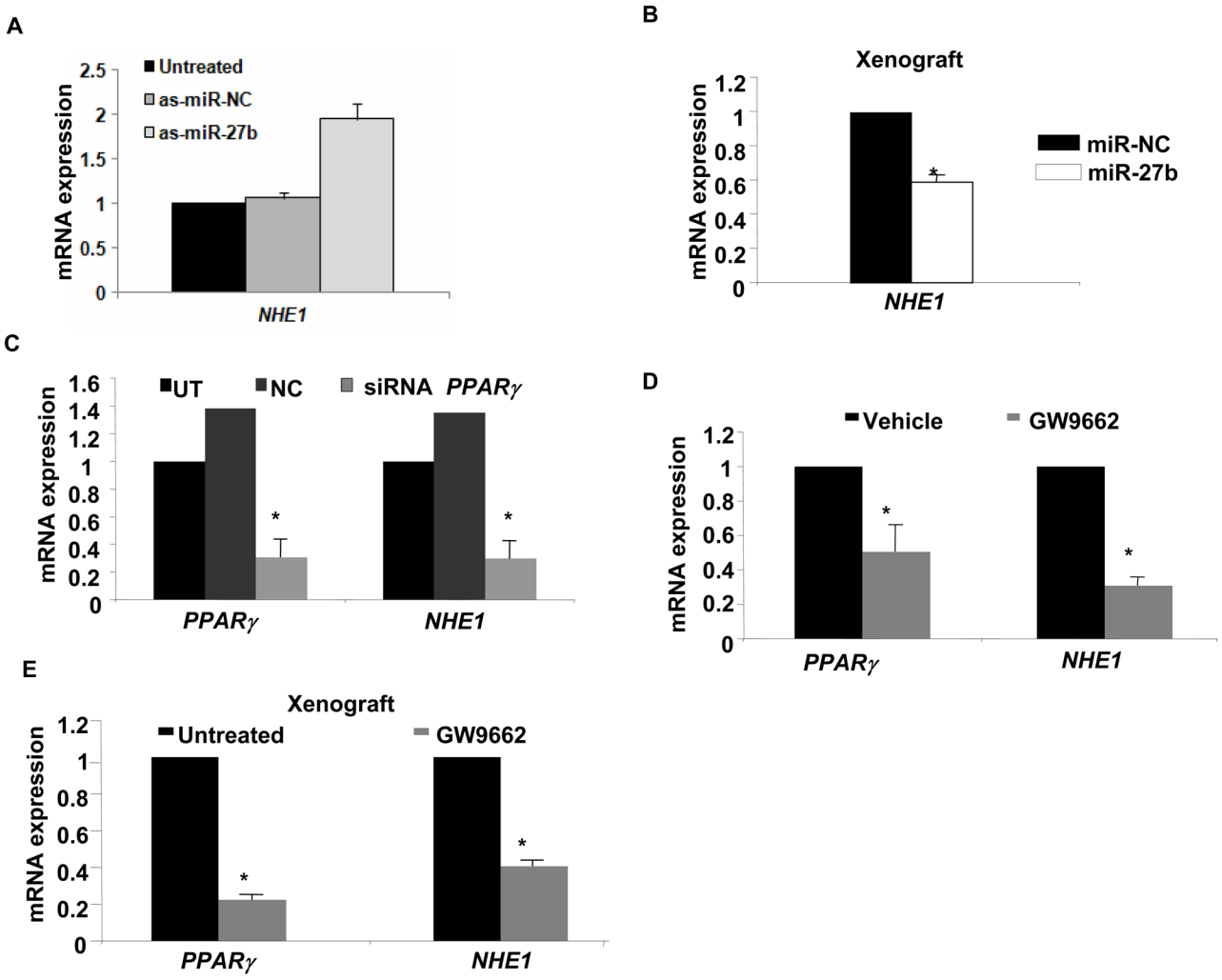

Figure 4. PPARγ promotes cell-growth *in vitro* and *in vivo*

(**a**) *NHE1* RNA levels in SK-N-AS cells that were or were not treated with as-miR-27b RNA or control miRNA. (**b**) *NHE1* RNA levels in mouse xenografts (SK-N-AS cells) that are or are not injected with miR-27b. (**c**) *PPARγ* and *NHE1* RNA levels in SK-N-AS cells treated with siRNA against *PPARγ* or control siRNA. (**d**) *PPARγ* and *NHE1* RNA levels in SK-N-AS cells that were or were not treated with GW9662. (**e**) *PPARγ* and *NHE1* RNA levels in mouse xenografts containing SK-N-AS cells that were or were not treated with GW9662.

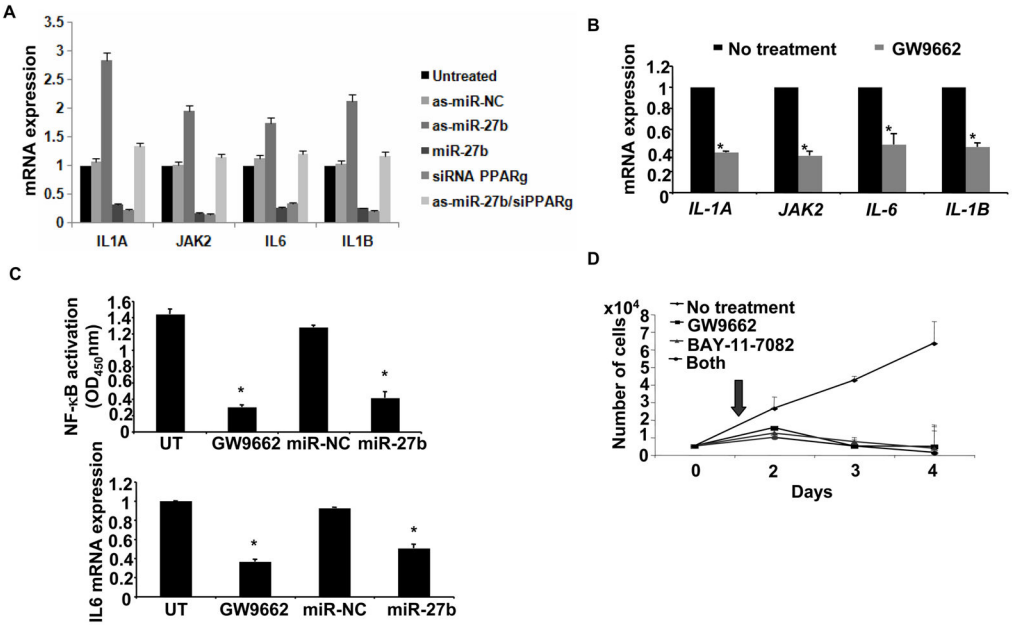

Figure 5. miR-27b through *PPARγ* regulates the NF-κB pathway in neuroblastoma cells and tumors

(**a**) RNA levels of the indicated inflammatory genes in SK-N-AS cells treated with the indicated RNAs. (**b**) RNA levels of the indicated inflammatory genes in SK-N-AS cells that were or were not treated with GW9662. (**c**) NF-κB activity or IL6 RNA levels in tumors from mouse xenografts (SK-N-AS cells) that are treated with miR-27b or control miRNA or GW9662. (**d**) Number of SK-N-AS cells after treatment with the indicated inhibitors.

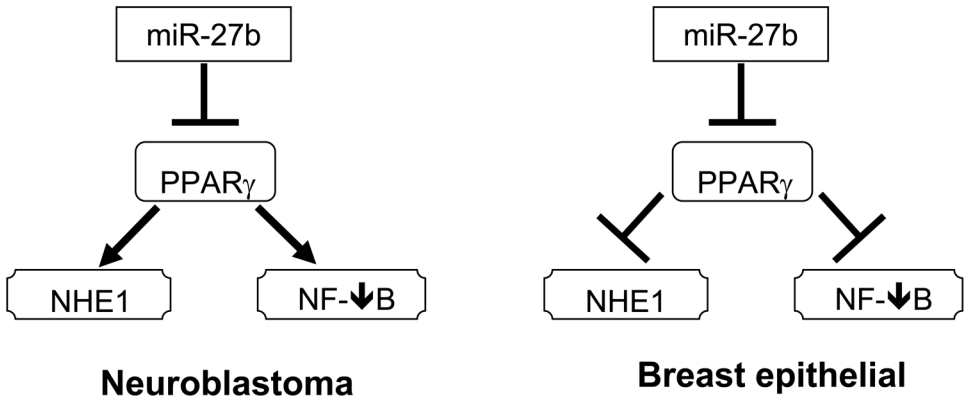

Figure 6. Model

In neuroblastoma, miR-27b inhibits *PPARγ*, which functions as an oncogene that activates downstream targets *NHE1* and NF-κβ in tumor development. In breast cancer cells, *PPARγ* functions as a tumor suppressor that inhibits *NHE1* expression.