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TWISTing Stemness, Inflammation, and Proliferation of Epithelial Ovarian Cancer Cells through MIR199A2/214

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Abstract

Cancer stem cells are responsible for sustaining the tumor and giving rise to proliferating and progressively differentiating cells. However, the molecular mechanisms regulating the process of cancer stem cell differentiation is not clearly understood. Recently, we reported the isolation of the epithelial ovarian cancer (EOC) stem cells (Type I/CD44+). In this study we show that Type I/CD44+ cells are characterized by low levels of both miR-199a and miR-214, whereas mature EOC cells (Type II/CD44-) have higher levels of miR-199a and miR-214. Moreover, these two miRNAs are regulated as a cluster on pri-miR-199a2 within the human Dnm3os gene (GenBank FJ623959). This study identify Twist1 as a regulator of this unique miRNA cluster responsible for the regulation of the IKKβ/NFκB and PTEN/AKT pathways and its association of ovarian cancer stem cell differentiation. Our data suggest that Twist1 may be an important regulator of "stemness" in EOC cells. The regulation of *MIR199A2/214* expression may be used as a potential therapeutic approach in EOC patients.

Keywords

ovarian cancer stem cells; Twist1; miR-199a/214 cluster; IKKβ

Introduction

Epithelial ovarian cancer (EOC) is the fourth leading cause of cancer-related deaths in women in the United States and the leading cause of gynecologic cancer deaths. Although 80%-90% of patients initially respond to first-line chemotherapy agents carboplatin and paclitaxel, less than 10–15% remain in complete remission and most patients eventually recur(Alison et al 2008) with chemoresistant tumors (Espey et al 2007, Jemal et al 2006). Treatment advances have led to an improved five-year survival of about 45%, but not in

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overall survival. Thus, recurrence and chemoresistance are major hurdles in the management of patients with ovarian cancer (Schwartz 2002)(Clarke-Pearson 2009, Woolas et al 1993).

Recently it has been postulated that cancer stem cells (CSCs) are not only the potential origin of the tumor, but also the source of recurrence and chemoresistance (Clarke et al 2006, Clarke and Fuller 2006). Similar to normal stem cells, CSCs are characterized by a high degree of plasticity and capacity for repair and differentiation (Ahmed et al 2007). In the ovary, normal stem cells are thought to contribute to healing the ovarian surface after ovulation (Salamanca et al 2004). This capacity of normal stem cells to repair may be shared by the CSCs. As such, chemoresistant CSCs that persist after surgery and chemotherapy may “heal” the cancer and lead to recurrence(Alvero et al 2009a, Alvero et al 2009b).

Recently, we reported the identification of two populations of epithelial ovarian cancer (EOC) cells, Type I and Type II (Chen et al 2007, Chen et al 2008). Type I/CD44+ EOC cells have many characteristics associated with CSCs. They are: (1) tumorigenic and can recapitulate the heterogeneity of the original tumor; (2) can form self-renewing spheroids; (3) have high levels of stem cell markers β-catenin, Oct-4, and SSEA-4; (4) have constitutively active IKKβ/NF-κB; (5) constitutively secrete IL-6, IL-8, MCP-1, and GRO-α; and (6) are chemo-resistant (Alvero et al 2009a). Type II/CD44- EOC cells represent a differentiated population and are sensitive to chemotherapy (Chen et al 2008). Furthermore, a pure population of Type I/CD44+ EOC cells differentiate *in vitro* into Type II/CD44- EOC cells. This differentiation is accompanied by the loss of stem cell markers and reversion of chemoresistance. The demonstration of this differentiation event is the first step in understanding the molecular regulation of CSC differentiation and may allow the identification of specific signals that regulate this process. It should be emphasized that this differentiation process may yield a more chemosensitive cancer cell population, which may lack the capacity for self-renewal and repair. Moreover, this process may involve molecular events that can change the regulation of apoptosis, cell division, angiogenesis and inflammation. Epigenetic factors have been suggested as the regulatory source promoting the transition from cancer stem-like cells into mature/differentiated cancer cells; including the expression and function of microRNAs.

MicroRNAs (miRNAs) are ∼23 nucleotide noncoding RNAs, which negatively regulate gene expression in a sequence specific manner. Numerous studies suggest that miRNAs are key regulators of several fundamental biological processes, including neoplasia and tumor formation (Taylor and Gercel-Taylor 2008, Valencia-Sanchez et al 2006, Yang et al 2008); (Liu et al 2005). Recently, we observed a distinct miRNA profile between Type I/CD44+ and Type II/CD44- EOC cells. In addition, we identified hsa-miR-199a, which is highly expressed in Type I/CD44+ EOC cells, as a major regulator of the IKKβ/NF-κB pathway (Chen et al 2008).

Twist1 is a highly conserved transcription factor that belongs to the family of basic helix-loop-helix (bHLH) proteins (Bialek et al 2004, Cheng et al 2008c). Twist1 has been implicated in the differentiation of multiple cell lineages including muscle, cartilage and osteogenic cells (Bialek et al 2004, Lee et al 1999, Lee et al 2000, Ota et al 2004). In mice, Twist1 was shown to be required for proper development of the head mesenchyme, somites,

and limb buds (Lee et al 2009). Mice lacking *Twist1* die at E10.5 confirming its important role in development and differentiation (Baylies and Bate 1996). Recently, Lee et al reported the regulation of miR-199/214 in the mouse suggesting a role for Twist1 and these miRNAs in the development of mice neural cell population (Lee et al 2009). In addition, Twist1 has been shown to be important in the regulation of inflammation and programmed cell death (Cheng et al 2008b, Cheng et al 2008c). However, the mechanisms regulating Twist1 expression and function and how Twist1 regulates inflammation, differentiation, and programmed cell death has not been described.

In the present study, we show that in EOC, Twist1 is associated with the transition of stem-like Type I/CD44+ cells to Type II/CD44- cells, through the regulation of two major pathways: IKKβ/NF-κB and PTEN/AKT pathways. Furthermore, we demonstrate that the regulation of these two pathways by Twist1 is through the expression and function of the miRNA cluster *MIR199A2/214*. Specifically, we show that Twist1, through its regulation of *MIR199A2/214*, can control the processes of inflammation, differentiation, and programmed cell death.

Results

hsa-miR-199a in EOC cells corresponds to *MIR199A2* gene on Chromosome 1

Type I/CD44+ cells possess a functional and responsive TLR-MyD88-NFκB pathway, while Type II/CD44- EOC cells do not. We reported previously that this specific characteristic of the Type I/CD44+ cells is regulated by hsa-miR-199a, which controls the expression of IKKβ (Chen et al 2008). To determine how hsa-miR-199a is regulated, we determined its location in the human genome. Using the NIH-gene database, we identified two genes that potentially encode pri-miR-199a, the primary precursor of hsa-miR-199a. The first gene is *MIR199A1* on Chromosome 19 (NCBI GeneID 406976) and the second is *MIR199A2* on Chromosome 1 (NCBI GeneID 406977). These two sites were predicted from two distinct pre-miR-199a sequences(pre-miR-199a-1 and -2) (Sanger Institute miRBase Stem-loop Sequence ID MI0000242 and MI0000281, respectively). In order to determine which of these sequences correspond to hsa-miR-199a expressed in EOC cells, we evaluated the transcription of pri-miR-199a-1 (from *MIRN199A1*) and pri-miR-199a-2 (from *MIRN199A2*) in EOC cells by RT-PCR. Our results showed that only pri-miR-199a-2 was expressed in EOC cells, with higher expression levels in Type II/CD44- than in Type I/CD44+ EOC cells (Fig. 1). This suggests that the expression of hsa-miR-199a in EOC cells might be due to the differential regulation at the transcriptional level of the *MIR199A2* gene.

Characterization of full length pri-miR-199a-2 transcript reveals a novel gene cluster

Having determined that *MIR199A2* was the gene responsible for hsa-miR-199a expression in EOC cells, we then sought to further characterize this gene and its regulation. We first used 5′- and 3′-RACE to determine the 5′- and 3′-ends of this transcript, thus defining the full length of pri-miR-199a-2. Using cDNA reverse transcribed from pri-miR-199a-2, RACE results showed that the full transcript is 6253 bp in length (Supplementary Fig. 1A and 1B). Interestingly, at the 3′-end of the pri-miR-199a-2 transcript, we found the precursor sequence for another miRNA pair – hsa-miR-214 and hsa-miR-214*. Thus, we renamed the

gene encoding this transcript as *MIR199A2/214*. This gene encodes a minimum of 4 miRNA molecules: hsa-miR-199a-5p (or miR-199a), hsa-miR-199a-3p (or miR-199a*), hsa-miR-214, and hsa-miR-214* (GenBank FJ623959). We then evaluated whether the expression of hsa-miR-214 in EOC cells follows the same pattern as that observed with hsa-miR-199a. Using quantitative RT-PCR we found that similar to hsa-miR-199a, hsa-miR-214 is highly expressed in Type II/CD44- cells and is absent in Type I/CD44+ cells (Figs. 2 A&B).

By BLASTing the obtained sequence in the NCBI database, a murine pri-miR-199a-2/214 transcript was identified as Dnm3os (NCBI GeneID 474332) (*2*). The identification of Dnm3os as the mouse homolog of pri-miR-199a-2/214 (and *Dnm3os* as mouse *MiR199a2/214*) was further supported by the sequence and predicted genome context information in the Sanger database (Sanger Institute miRBase Stem-loop Sequence ID MI0000713 and MI0000698). Comparison of the 5′- and 3′-ends of human *MIR199A2/214* with the mouse homolog showed 88% and 89% homology, respectively, with the pre-miR-199a-2 region scoring 99% homology, and the pre-miR-214 region 100% homology (Supplementary Fig. 1A and 1B). The high sequence conservation of the *MIR199A2/214* gene at the pre-miR-199a-2 and the pre-miR-214 regions in human and mouse indicates important roles of hsa-miR-199a and hsa-miR-214 across species.

Twist1 regulates the hsa-miR-199a/hsa-miR-214 cluster in EOC cells

After we confirmed the differential expression of *MIR199A2/214* in EOC cells, our next goal was to understand its regulation. Twist1 is a bHLH transcription factor known to regulate the expression of Dmn3os in mouse (Lee et al 2009). Therefore, we evaluated the expression of *Twist1*, the human homolog of murine *Twist1*, and determined its role in regulating *MIR199A2/214* expression in EOC cells. Western blot analysis and qRT-PCR showed that, similar to MIR199A2/214, Twist1 is highly expressed in Type II/CD44- cells but not in Type I/CD44+ EOC cells (Fig. 3A and B). To determine if Twist1 has a regulatory role over this microRNA cluster in human EOC cells, we used *Twist1*-specific siRNA to knock down Twist1 expression. Transfection of Type II/CD44- cells with *Twist1* siRNA is able to significantly decrease *Twist1* expression (Fig. 4A). More importantly, analysis of hsa-miR-199a and hsa-miR-214 expression levels after *Twist1* knockdown showed a parallel decrease on both miRNAs (Fig. 4A). These results suggest that Twist1 positively regulates the expression of hsa-miR-199a/hsa-miR-214 cluster in EOC cells.

Twist1 inhibits IKKβ expression by regulating hsa-miR-199a

Recently we demonstrated that hsa-miR-199a regulates IKKβ expression by inhibiting its translation (Chen et al 2008). The demonstration that Twist1 regulates hsa-miR-199a expression suggests that Twist1 can regulate IKKβ. Therefore, we knocked-down Twist1 in Type II/CD44- cells (which have high endogenous Twist1 and low IKKβ) and monitored the effect on IKKβ expression at both the message and protein levels. qRT-PCR (Fig. 4B) and Western blot results (Fig. 4C) revealed significant increase in IKKβ expression upon *Twist1* knockdown. To further confirm that the inhibition of IKKβ by Twist1 is through the regulation of hsa-miR-199a, we then sought to reverse the effect of *Twist1* knockdown on IKKβ by adding exogenous pre-miR-199a. As shown in Figure 5, the addition of exogenous

pre-miR-199a is able to reverse the effect of *Twist1* knockdown on the levels of IKKβ (Fig. 5). This suggests that Twist1 affects IKKβ expression through its regulation of hsa-miR-199a. The ability of Twist1 to reduce IKKβ expression suggests that Twist1 has an inhibitory role in the NF-κB signaling pathway, and may serve as a regulator of inflammation as well as cancer development and progression.

Twist1 negatively regulates NF-κB-dependent cytokine production through IKKβ

Activation of NF-κB pathway can increase Twist1 expression, and Twist1 plays an antagonistic role in NF-κB-dependent cytokine expression (Sosic et al 2003). However, the specific mechanism by which Twist1 negatively regulates NF-κB is not known. Because Twist1, through hsa-miR-199a, can down regulate IKKβ, we hypothesize that Twist1 is able to negatively regulate NF-κB-dependent cytokine expression by controlling the levels of IKKβ.

In contrast to Type I/CD44+ cells, Type II/CD44- EOC cells do not constitutively express cytokines, have high levels of Twist1, and have low levels of IKKβ. To show that Twist1 can negatively regulate NF-κB dependent cytokine expression, we knocked-down *Twist1* in Type II/CD44- cells and determined the levels of Rantes, which is known to be regulated by NF-κB. Type II/CD44- EOC cells do not express Rantes (Fig 6). Knockdown of *Twist1* in these cells however, did not result in an increase in Rantes although IKKβ levels were elevated. This suggests that an additional stimulus may be required to induce Rantes production. Thus, we treated the cells with TNFα after Twist1 knockdown. We observed a significant increase in Rantes expression after TNFα treatment in cells where *Twist1* was knocked down compared to cells transfected with non-specific siRNA (Fig 6). These results suggest that inhibition of Twist1 can activate the NF-κB-dependent Rantes production induced by TNFα treatment in Type II/CD44- EOC cells.

Differential expression of hsa-miR-214 in Type I/CD44+ and Type II/CD44- EOC cells corrrelatesw with affects the AKT survival pathway

As shown above, knockdown of *Twist1* affects both hsa-miR-199a and hsa-miR-214 levels. To determine whether hsa-miR-199a and hsa-miR-214 have parallel expression in EOC cells, we evaluated the expression of hsa-miR-214 in primary cultures of Type I/CD44+ and II/CD44- EOC cells by RT-PCR. As shown in Figure 2A, hsa-miR-214 follows a similar expression pattern as hsa-miR-199a: it is highly expressed in Type II/CD44- EOC cells and mostly undetectable in Type I/CD44+ cells (Fig. 2B). Taken together with the data described above, these results further supports that hsa-miR-199a and hsa-miR-214 are regulated as a cluster.

Because Twist1 is able to regulate the MIR199A2/214 cluster and affect the levels of the miR-199a target, IKKβ, our next objective is to determine the effect of Twist1 on the biological function of hsa-miR-214. hsa-miR-214, has been shown to directly inhibit PTEN expression at the post-transcriptional level (Cheng et al 2008b). PTEN is a critical inhibitor in the AKT survival pathway, an important pathway involved in cancer development as well as chemoresistance (Cheng et al 2008a). Therefore, we next examined whether there is a correlation between the levels of hsa-miR-214, PTEN, and AKT phosphorylation in EOC

cells. As expected, we observed an inverse correlation between hsa-miR-214 and PTEN expression in the two types of EOC cells. Type I/CD44+ cells are characterized by low levels of hsa-miR-214 and high levels of PTEN expression. In contrast, Type II/CD44- cells expressed high levels of hsa-miR-214 and low levels of PTEN (Fig. 7A). Furthermore, this differential phenotype correlates with AKT activity (Fig. 7A) as determined by its phosphorylation status. Type II/CD44- EOC cells have a higher level of phospho-AKT, compared to Type I/CD44+ EOC cells (Fig. 7A). Levels of total AKT was however similar in the two cell types (Fig. 7A). These results suggest a regulatory role for the Twist1/miR-214/PTEN axis in the control of AKT activity.

To further determine the specificity of each miRNA we evaluated the levels of IKKβ and PTEN expression in Type I/CD44+ cells transfected with hsa-miR-199. As shown in supplemetary Figure 2, expression of hsa-miR-199 inhibits IKKβ expression but does not have any effect on levels of PTEN (Supp Fig. 2).

In vitro differentiation of Type I/CD44+ EOC cells into Type II/CD44- cells is associated with increase in Twist1 expression

Twist1 has been shown to be involved in the process of differentiation (Lee et al 1999). Furthermore, we see differential expression of Twist1 in the two sub-populations of EOC cells. We previously demonstrated that pure cultures of Type I/CD44+ EOC cells can differentiate *in vitro* into Type II/CD44- cells (Alvero et al 2009a). Therefore, we determined if Twist1 is associated with this differentiation process. Thus, Type I/CD44+ EOC cells were induced to differentiate as previously described by frequent passing and plating in low confluence (Alvero et al 2009a). When the cells showed morphologic characteristics of Type II/CD44- EOC cells, such as low nuclear/cytoplasm ratio and fast cell division rate, cells were collected and protein expression determined by Western blot. As shown in Figure 7B, Type I/CD44+ cells do not express Twist1. However, following differentiation, we observed a significant increase in Twist1 expression. The acquisition of Twist1 is accompanied by a decrease in IKKβ, MyD88, and PTEN and an increase in the phosphorylation of AKT (Threonine 308 and Serine 473).

Recently we reported that Type I/CD44+ EOC cells have stem-like properties and that it has the capacity to differentiate into endothelial cells. This property is not observed in Type II/CD44- cells (Alvero et al 2009b). Therefore, we determine whether inhibition of Twist1 expression could restore some “stemness” potential in the Type II/CD44- cells. As shown in Supplementary Figure 3a, Type I/CD44+ cells can form tube-like structures when cultured in Matrigel. In contrast, Type II/CD44- cells form cell clusters in Matrigel (Supp. Fig. 3b). Inhibition of Twist1 expression in Type II/CD44- cells partially restore the capacity of these cells to form tubes (Supp. Fig. 3c); this was not observed in Type II/CD44- cells transfected with a non-specific siRNA (Supp. Fig. 3d).

Discussion

In this study, we demonstrate that Twist1 may regulate the differentiation of Type I/CD44+ ovarian cancer stem cells through the expression and function of a newly identified miRNA cluster, the hsa-miR-199a/hsa-miR-214 (*MIR199A2/214.)* The differentiation of CSCs into

mature cancer cells involves significant changes in their cellular characteristics, particularly in growth rate and capacity for repair and inflammation (Alison et al 2008). Twist1 has been implicated in the differentiation of multiple cell lineages, including cancer cells. In this study we provide evidence that Twist1 through the expression of hsa-miR-199a/hsa-miR-214, can negatively regulate the pro-inflammatory NF-κB pathway and can positively regulate the AKT survival pathway. In addition, we showed the association betweenTwist1 expression and the activation status of these two pathways during the process of EOC differentiation.

Growing evidence suggest that miRNAs play prominent roles in numerous cellular processes including growth, proliferatio, and apoptosis. These suggest that miRNAs are involved in development, genome organization, and also human disease, including neurodegenerative, metabolic, inflammatory diseases, and a variety of cancers (Alvarez-Garcia and Miska 2005, Bartel 2004, Miska 2005, Taylor and Gercel-Taylor 2008, Valencia-Sanchez et al 2006). In addition, new evidence suggests that miRNAs may also be involved in the process of differentiation, including stem cell differentiation (Papagiannakopoulos and Kosik 2008, Papagiannakopoulos et al 2008). Therefore, it is plausible that miRNAs may also play a role in the differentiation process in CSCs.

Ovarian cancer stem cells are characterized by slow cell division, low levels of pAKT, and constitutive NF-κB activity. Upon differentiation into fast dividing mature ovarian cancer cells, pAKT levels increase and the cells lose the constitutive NF-κB activity. Strikingly, we found that these two processes are regulated by Twist1. Furthermore, we found that the mechanism by which Twist1 promotes these changes is through the expression of a miRNA cluster located in the Dnm3 gene.

Recently, we showed that miR-199a can decrease IKKβ expression and therefore negatively regulate NF-κB activity (Chen et al 2008). In the current study, we identified the full length of pri-miR-199a2 gene, and found hsa-miR-214 located in its 3′ terminal with hsa-miR-199a in the 5′-terminal. Interestingly, we found the location of pri-miR-199a2 has overlap with human Dnm3os (opposite strand of intron of Dnm3).

Dnm3os was identified by Loebel et al. (Loebel et al 2002) while evaluating Twist-regulated genes in Twist$^{-/-}$ mutant mice embryos. The gene was named Dnm3os since it is encoded by the opposite strand of Dnm3. Both Dnm3os and Dnm3 are down regulated in Twist$^{-/-}$ mutants (Loebel et al 2002). Interestingly, we found that the reported Dnm3os gene sequence corresponds to the hsa-miR-199a/hsa-miR-214 cluster and its expression is regulated by Twist1. This was confirmed in the knockdown experiments showing that inhibition of Twist1 by siRNA follows a significant decrease on the expression of both miR-199a and miR-214. Furthermore, we found an active E-box (a promoter element known to be bound by Twist1) in the region of the miR-199/214 cluster (-640-0 bp go DNM3os). This is similar as reported in the mouse by Lee et al (Lee et al 2009). These data suggest that Twist1 role as a transcriptional regulatory factor can be mediated through the expression of miRNAs.

In the mouse, Lee et al (Lee et al 2009) demonstrated that Twist1 drives the expression of mi199a/214 and is associated with embryonic development; suggesting that Twist1, through micro-RNAs, may regulate processes of differentiation. Our study demonstrate that similar regulation is present in humans, but more specific in human ovarian cancer cells.

Twist1 is temporally expressed during normal development, but it has been reported to be constitutively expressed in some forms of cancer (Entz-Werle et al 2005, Kwok et al 2005, Mironchik et al 2005, Valsesia-Wittmann et al 2004, Yang et al 2004). Similarly, miR-199/214 might be important for development, as described by Lee et al (Lee et al 2009) and may also play a role in cancer progression. Indeed, we found that the expression of hsa-miR-199a, hsa-miR-214, and *Twist1* are higher in mature ovarian cancer cells compared to the ovarian cancer stem cells. Similarly, another group reported that miR-214 is differentially expressed in ovarian cancer cells and that its expression correlates with high levels of pAKT (Yang et al 2008).

An important observation is the differential expression of Twist1, hsa-miR-199a, and hsa-miR-214 in the ovarian cancer stem cells and the mature ovarian cancer cells. This differential expression correlates with the levels of IKKβ and PTEN, respectively. More importantly, this correlation was maintained during the differentiation process from Type I/CD44+ EOC stem cells to Type II/CD44- mature cancer cells. The increase in Twist1 levels during the *in vitro* differentiation process is accompanied by an increase in miR-199a and miR-214, decrease in IKKβand PTEN, and increase in AKT activity. It should be emphasized that the *in vitro* differentiation process is associated with the loss of stem cell phenotype and acquisition of characteristics of a mature cancer cell such as a more rapid cell proliferation. Therefore, we propose that Twist1 activation is an essential factor in the process of transition from ovarian cancer stem cells (Type I/CD44+) into mature fast dividing ovarian cancer cells (Type II/CD44-) (Fig. 8a,b).

In summary, we report the characterization of Twist1 as a regulator of a unique miRNA cluster responsible for the regulation of the IKKβ/NFκB and PTEN/AKT pathways and its association of ovarian cancer stem cell differentiation. Our data suggest that Twist1 may be an important regulator of “stemness” in EOC cells. The regulation of *MIR199A2/214* expression may be used as a potential therapeutic approach in EOC patients.

Materials and Methods

Cell cultures and culture conditions

Cells used in these studies were isolated from either ovarian ascites or ovarian cancer tissues and grown as previously described (Kamsteeg et al 2003). All patients signed consent forms, and the use of patient samples was approved under Yale University's Human Investigations Committee (HIC no. 10425).

Reagents

Control siRNA (no. AM4611), control pre-microRNA (no. AM17110), siRNA of Twist1 (no. s14523), pre-miR-199a-5p (no. PM10893), and siPORT NeoFX™ Transfection Agent (AM4510) were purchased from Ambion (Austin, TX, USA). Rabbit anti-human β-actin

antibody was purchased from Sigma (St Louis, MO, USA). Rabbit anti-human MyD88 antibody was purchased from eBioscience (San Diego, CA, USA). Mouse anti-human Twist1 (sc-81417) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human IKKβ (no. 2684), Rabbit anti-human PTEN (no. 9552), rabbit anti-human Akt (no.9272), rabbit anti-human phospho-AKT(Ser473, no.9271), and rabbit anti-human phospho-AKT(Thr308, no.9275) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Rapid Amplification of cDNA Ends (RACE)

5′ and 3′ RACE were performed with the 5′ RACE System for Rapid Amplification of cDNA Ends Kit and the 3′ RACE System for Rapid Amplification of cDNA Ends Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Primers used: for 5′ RACE, 5GSP1: 5′-ttctccttggaaacagccc-3′, 5GSP2: 5′-aatcctctcccggtcctca-3′, and 5GSP3: 5′-ctgcgctgactgagacacact-3′; for 3′ RACE, 3GSP1: 5′- gggctgtttccaaggagaa-3′ and 3GSP2: 5′-caggctgattgtatctgtctatgagc-3′. The determined full-length transcript was deposited to GenBank with the Accession Number FJ623959.1.

Protein preparation

Protein extraction was done as previously described (Kamsteeg et al 2003). Briefly, cell pellets were lysed on ice in 1×phosphate-buffered saline with 1% NP40, 0.1% SDS and freshly added 20 ml/ml protease inhibitor cocktail (Sigma Chemical, St Louis, MO, USA) and 2mM phenylmethylsulfonyl fluoride (Sigma Chemical). Protein concentration was determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA), and proteins were stored at -80°C until further use.

SDS–PAGE and western blots

A quantity of 20 μg of each protein sample was denatured in sample buffer and subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE) as previously described (Kamsteeg et al., 2003). The following antibody dilutions were used: rabbit anti-human MyD88 (1:1,000), rabbit anti-human IKKβ (1:2,000), mouse anti-human Twist1 (1:500), rabbit anti-human phospho-Akt (Ser473, 1:1000), rabbit anti-human phospho-Akt (Thr308, 1:1000), rabbit anti-human PTEN (1:1000) and rabbit anti-human β-actin (1:10,000). Specific protein bands were visualized using enhanced chemiluminescence (Pierce Biotechnology).

Cytokine profiling

Levels of Rantes were measured from cell-free supernatants using the Bioplex Pro Cytokine Assay (Biorad, Hercules, CA). Data were acquired using the Bioplex system (Biorad) and analysis was performed using the Bioplex software as previously described (Koga et al 2009).

Twist1 knockdown and pre-miR-199a transfection

5×10^5 cells were seeded in 6-cm dish plates and transfected with either 10 nM of control siRNA/microRNA (Ambion) or with the siRNA of Twist1/pre-miR-199a-5p, then incubated

in 5% CO_2 incubator for different time point. Transfections were carried out using the siPORT transfection kit (Ambion) in accordance with the manufacturer's protocol.

RNA isolation and reverse transcription–PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed on 2 mg of total RNA using the First Strand cDNA Synthesis kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. The primers for pri-miR-199a2: forward, 5′- ttctccttggaaacagccc-3′; reverse, 5′- tgaggaccgggagaggatt-3′. The primers for Twist1: forward, 5′- gctacgccttctcggtct-3′; reverse, 5′- agctccagagtctctagac-3′. The primers for ACTB: forward, 5′-tgacggggtcacccacactgtgcccatcta-3′; reverse, 5′-ctagaagcatttgcggtggacgatggaggg-3′. Thirty cycles of PCR were performed at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s.

mRNA and microRNA qRT-PCR

Total RNA was prepared from Type I and Type II cell lines using the TRIZOL reagent (Invitrogen). Total RNA isolated from each samples was then used as a template for cDNA synthesis, prepared with a first-strand cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, UK) for mRNA, and prepared with a Ncode miRNA first-strand cDNA synthesis kit (Invitrogen) for microRNA. The expression of various transcripts was assessed by real-time polymerase chain reaction (PCR) amplification [50 °C/2 minutes; 95°C/10 minutes; (95°C/15 seconds; 60°C/1 minute; 40 cycles); 95°C/15 seconds] with Real-Time SYBR Green/Rox PCR supermix (Invitrogen), using the ABI 7500 Real Time Standard Cycler (Applied Biosystems, Foster City, CA, USA). Primer sets used in this study: GAPDH, (Fwd 5′- ctctgctcctcctgttcgac -3′; Rev 5′- acgaccaaatccgttgactc -3′); Twist-1, (Fwd 5′- ggcatcactatggactttctctatt -3′, Rev 5′- ggccagtttgatcccagtatt -3′); IKKβ (Fwd 5′- agccagccaagaagagtgaa -3′, Rev 5′- tctagcagggtgcagaggtt -3′); NRU6B-F, (5′- acgcaaattcgtgaagcgttcca -3′); hsa-miR-199a-F, (5′- cccagtgttcagactacctgttc -3′); hsa-miR-214-F, (5′- acagcaggcacagacaggcagt -3′). All PCR reactions were performed in triplicate and validated by the presence of a single peak in the melt curve analysis. Changes in gene expression were calculated relative to GAPDH using the $2^{-\Delta Ct}$ method, and changes in microRNA expression were calculated relative to NRU6B using the standard Curve method.

Statistical analysis

Data were presented as mean±s.d. (O'Dwyer et al 1994). Statistical significance ($P<0.05$) was determined using one-way ANOVA with the Bonferonni correction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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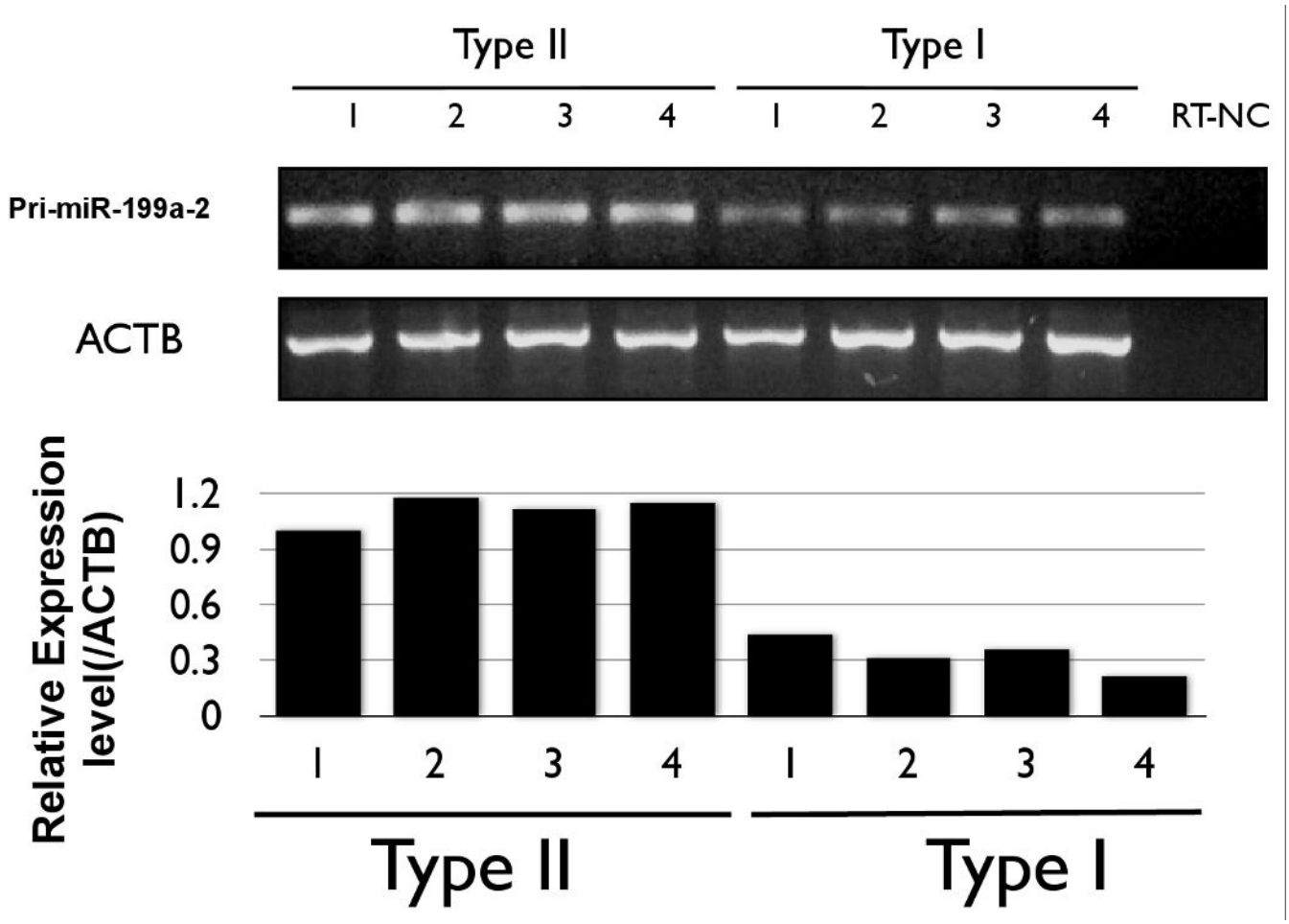


Figure 1.

Differential expression of pri-miR-199a-2 (from *MIRN199A2*) in EOC cells. Levels of pri-miR-199a-2 was evaluated in a panel of Type I/CD44+ and Type II/CD44- EOC cells using RT-PCR. ACTB: β-actin house keeping gene. RT-NC: Negative control for RT reaction.

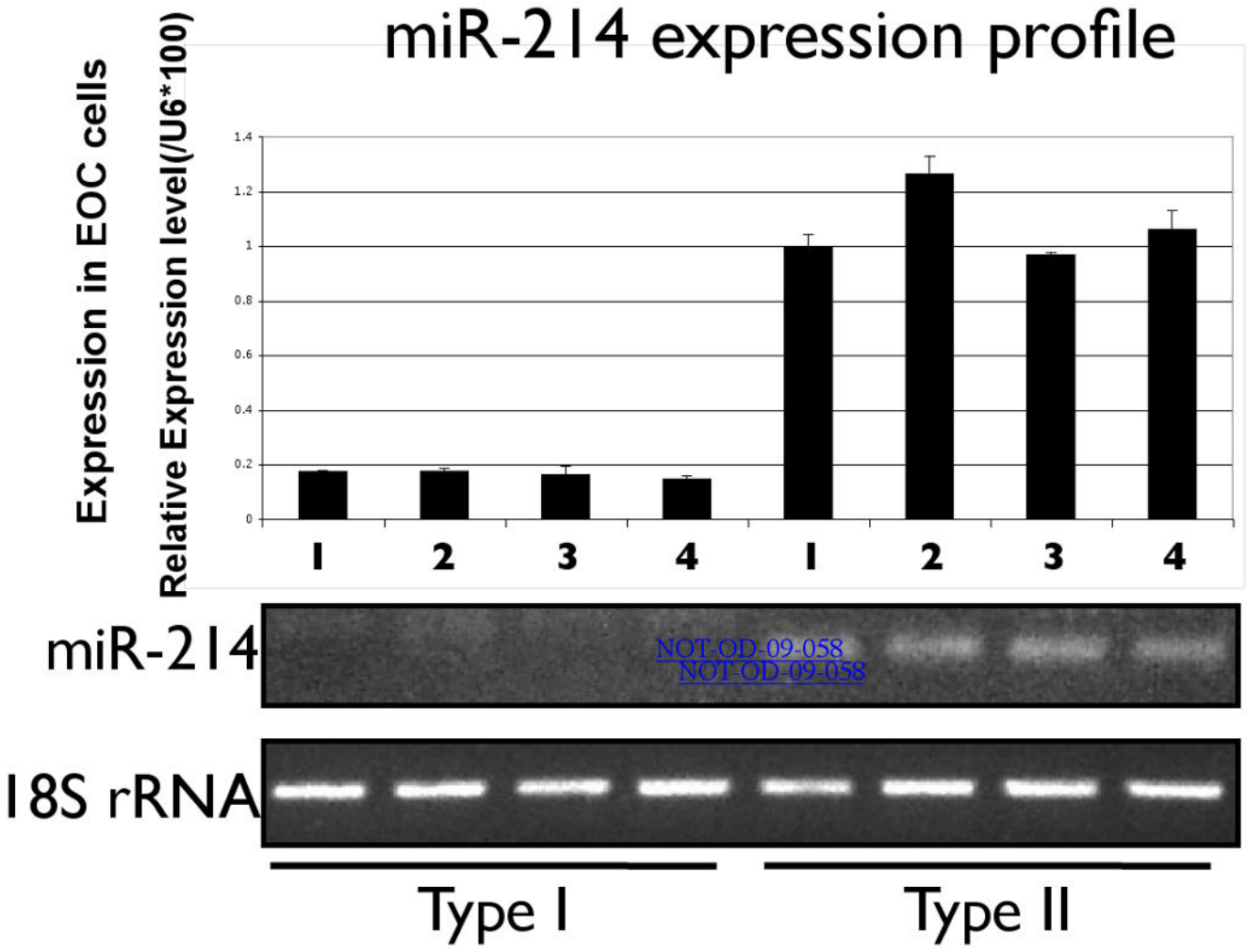


Figure 2B

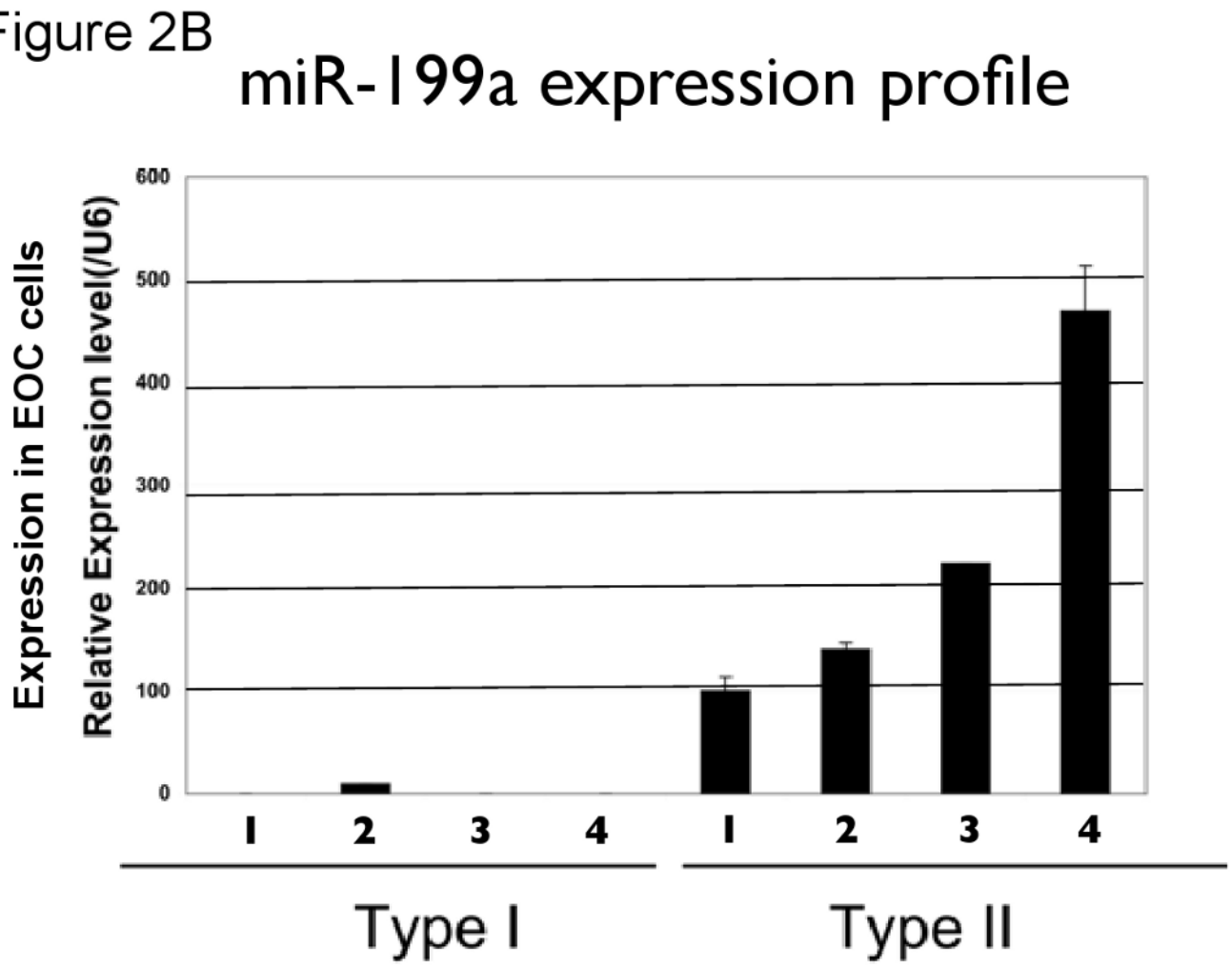


Figure 2.

Differential expression of miR-214 (a) and miR-199a (b) in EOC cells. Expression of the two miRNAs was determined in a panel of EOC cells by RT-PCR.

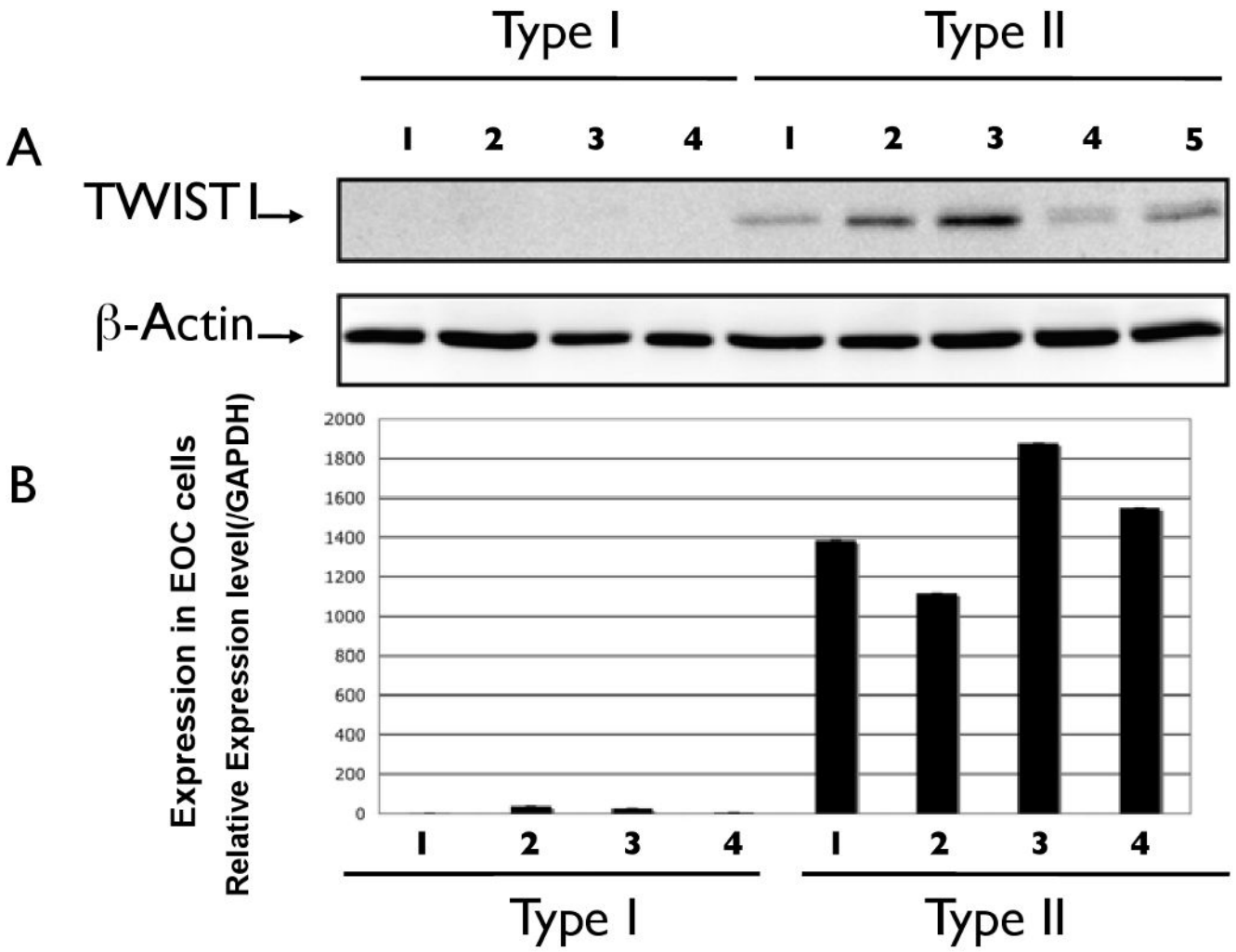
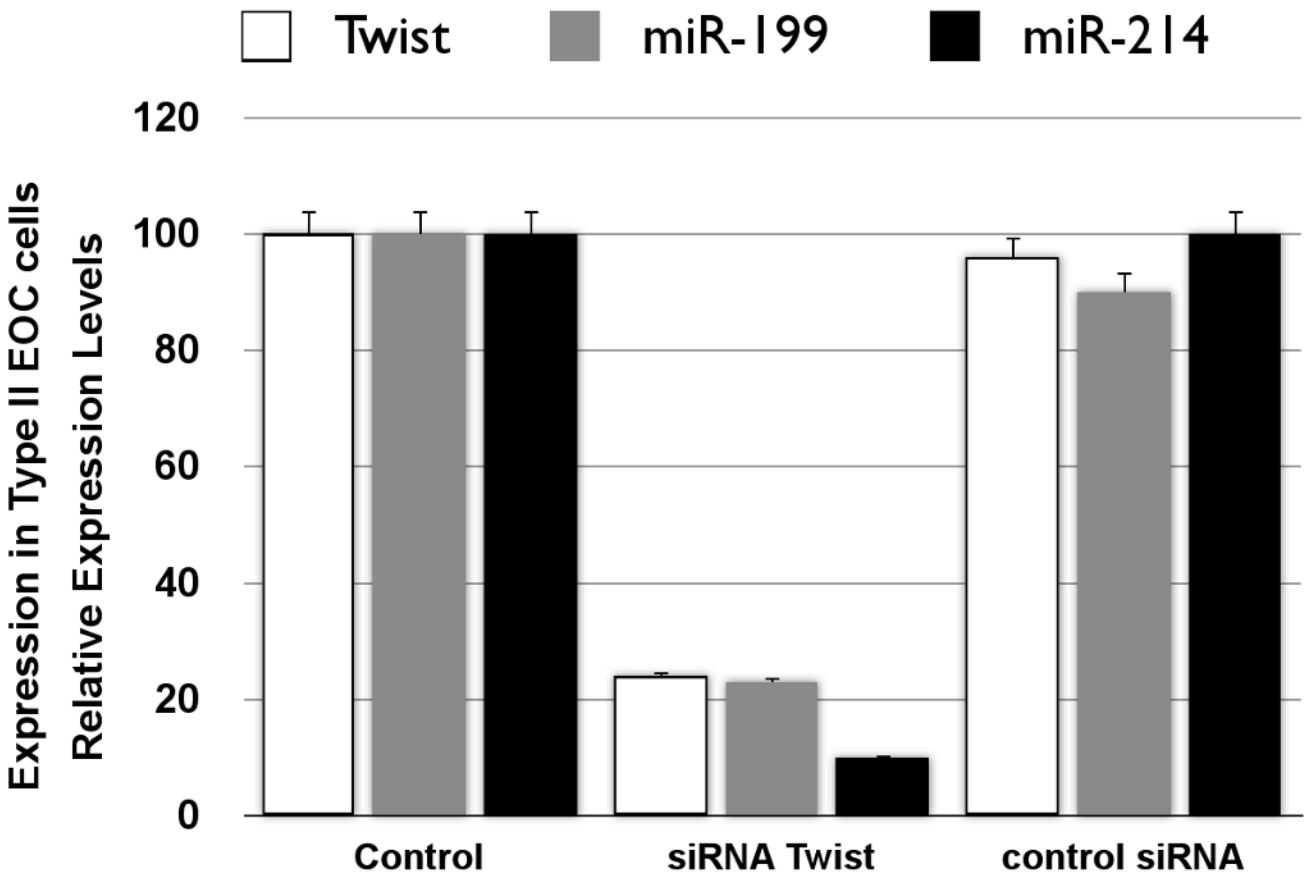


Figure 3.

Differential expression of Twist 1 in EOC cells. (a) Levels of Twist1 at the protein level was evaluated using Western blot analysis. (b) *Twist1* mRNA levels were evaluated using RT-PCR.

Figure 4A



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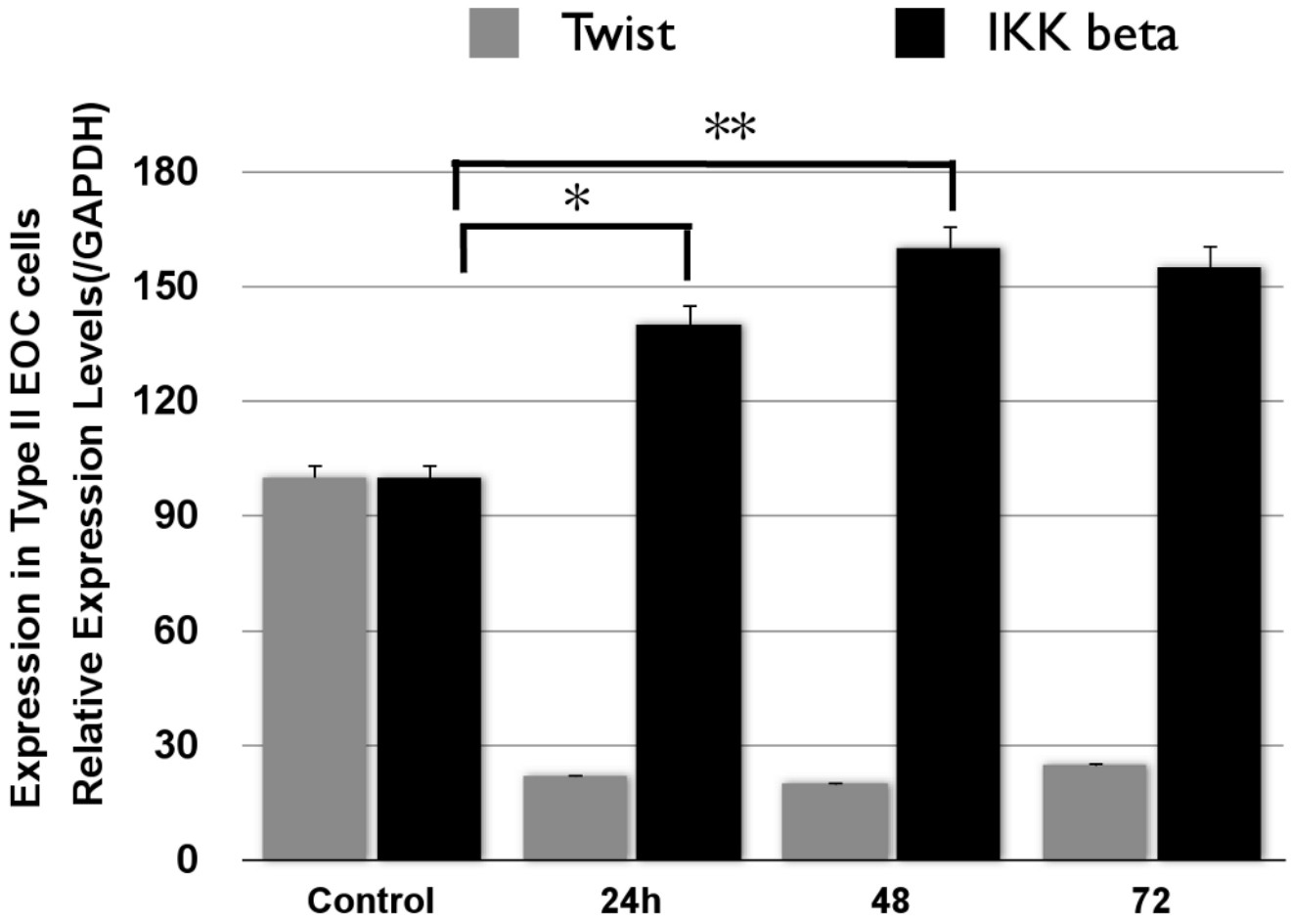

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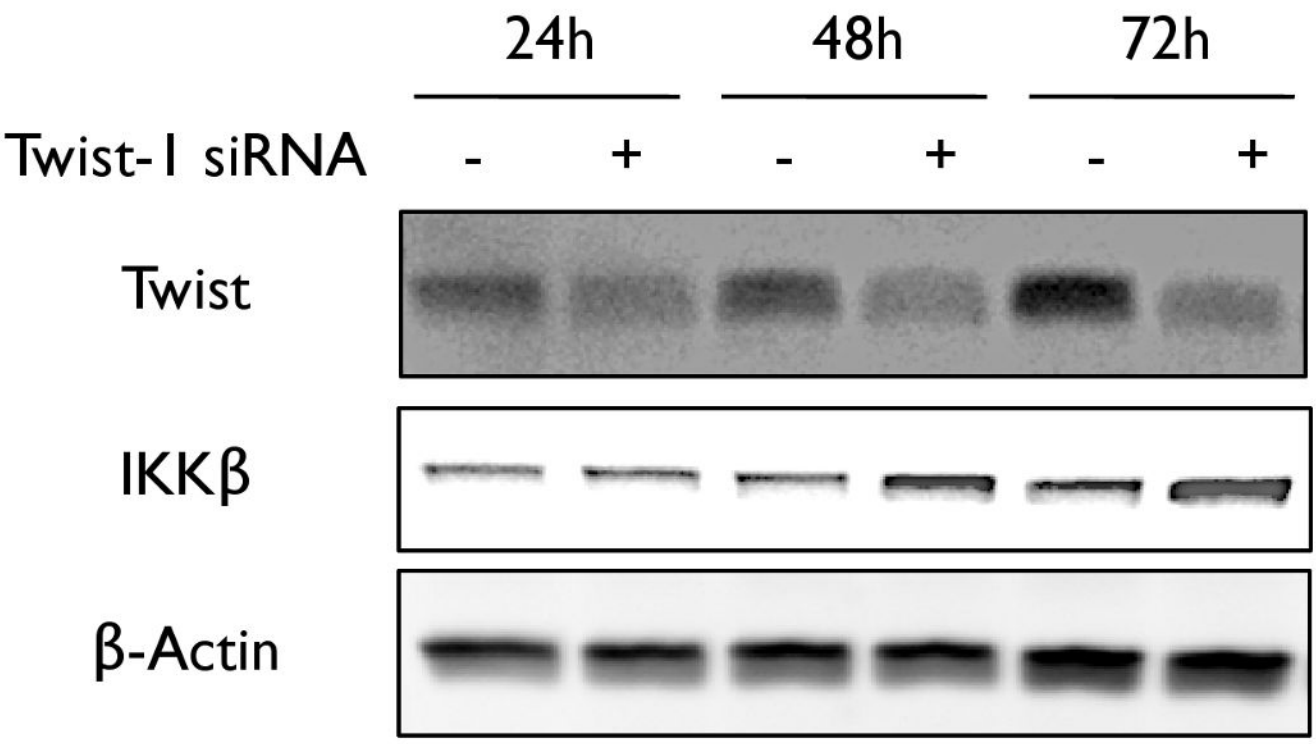


Figure 4D

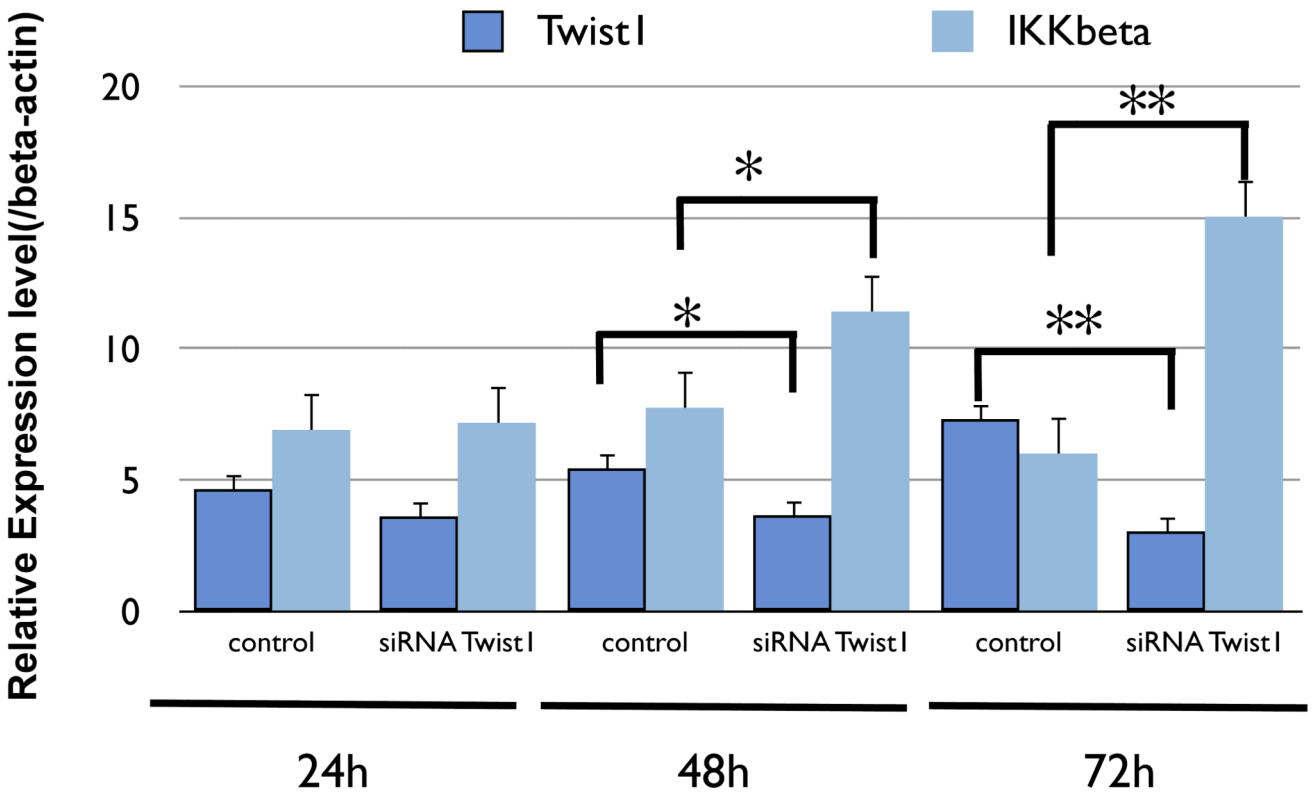


Figure 4.

Effect of Twist1 knockdown on levels of miR-199a, miR-214, and IKKβ. (a)Type II/CD44- EOC cells were transfected with Twist1 siRNA. Levels of Twist 1, miR-199a, and miR-214 were determined by RT-PCR. Note the significant decrease on miR-199a and miR-214 following Twist1 knockdown. No changes were observed in cells treated with control siRNA. (b) Type II/CD44- EOC cells were transfected with Twist1 siRNA and levels of IKKβ mRNA determined by RT-PCR in a time dependent manner. (c and d) Type II/CD44- EOC cells were transfected with Twist1 siRNA and levels of IKKβ determined by western blot analysis. Note that inhibition of Twist1 expression is associated with increase in IKKβ expression in a time dependent manner. *= p>0.05; **= p>0.001

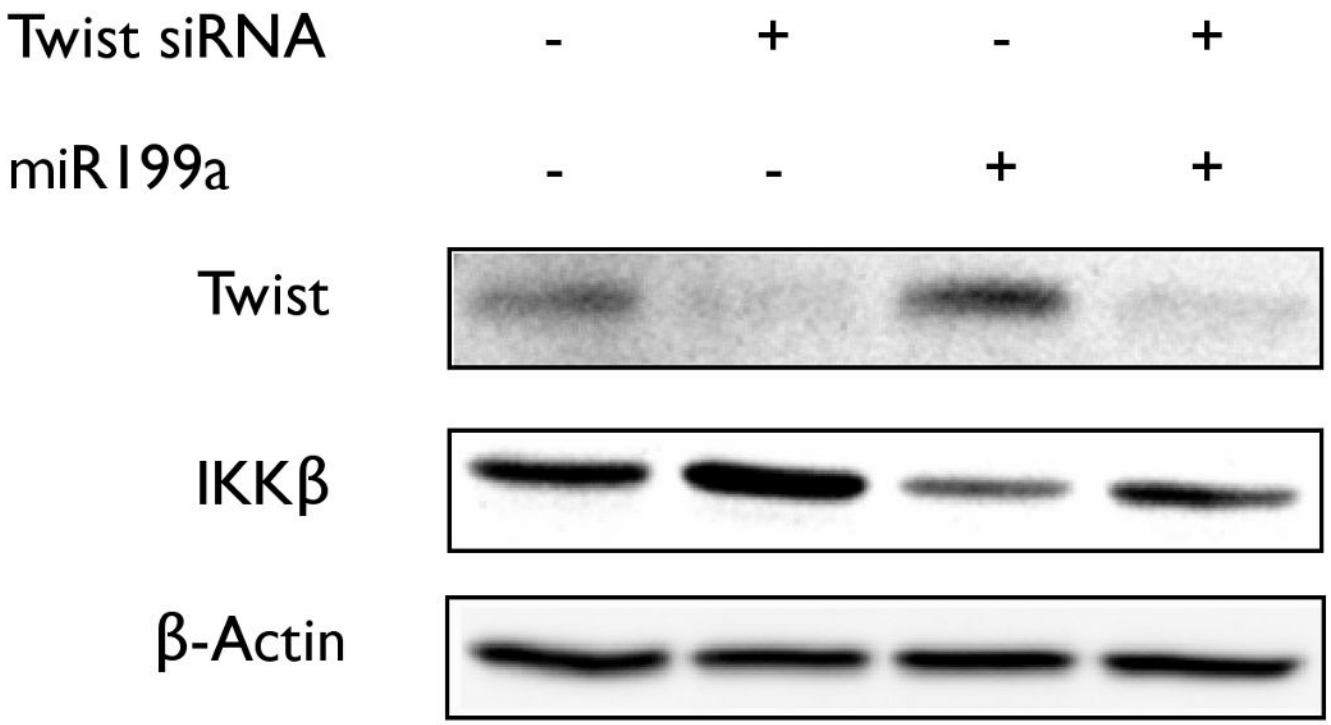


Figure 5.

Exogenous pre-miR-199a is able to reverse the effect of *Twist1* knockdown on the levels of IKKβ. Type II/CD44- EOC cells were transfected with siRNA for Twist1. Afterwards, cells were incubated in the presence or absence of pre-miR-199a for additional 24 h and IKKβ expression was determined by Western blot analysis. Note that the presence of miR199a is able to reverse the effect of Twist1 inhibition.

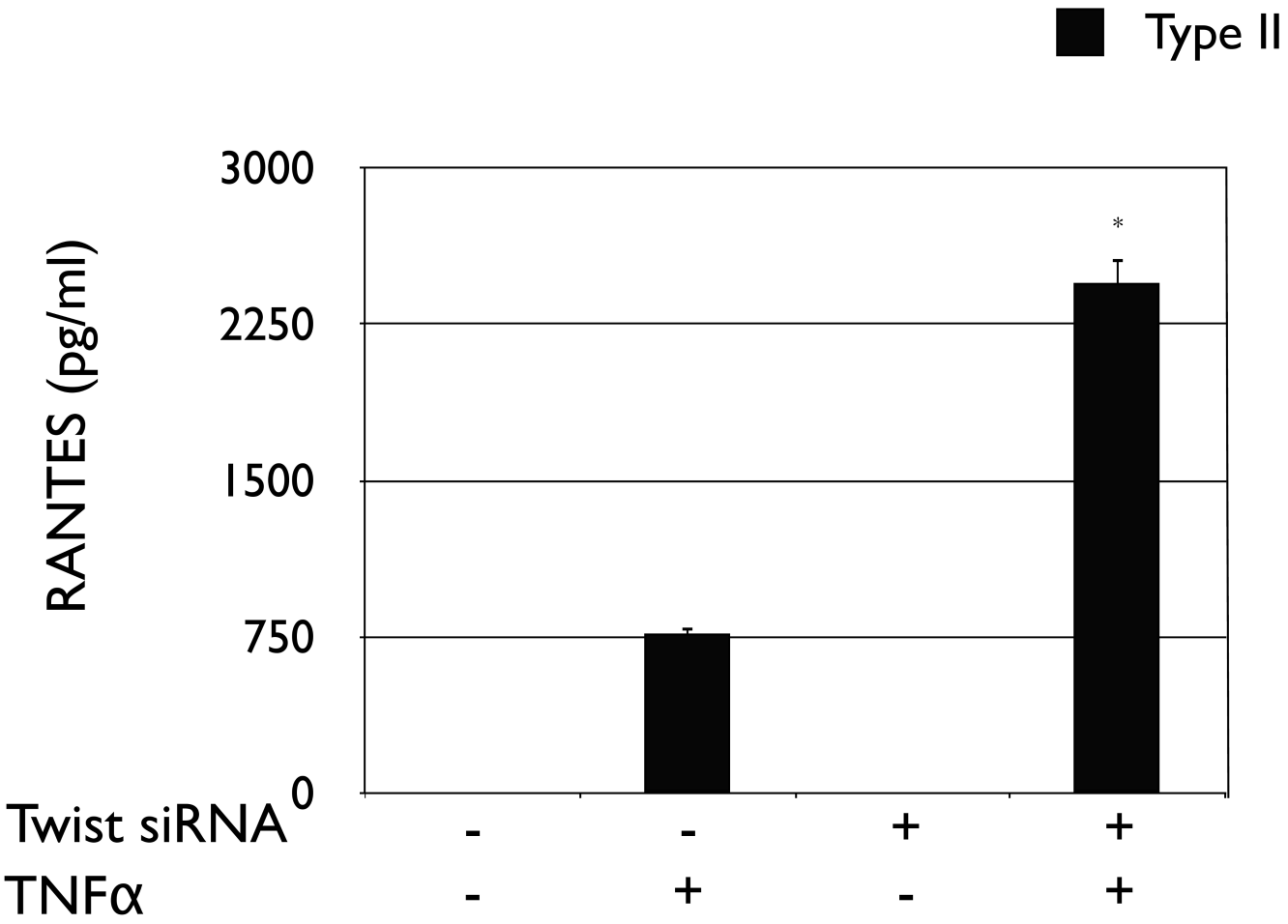
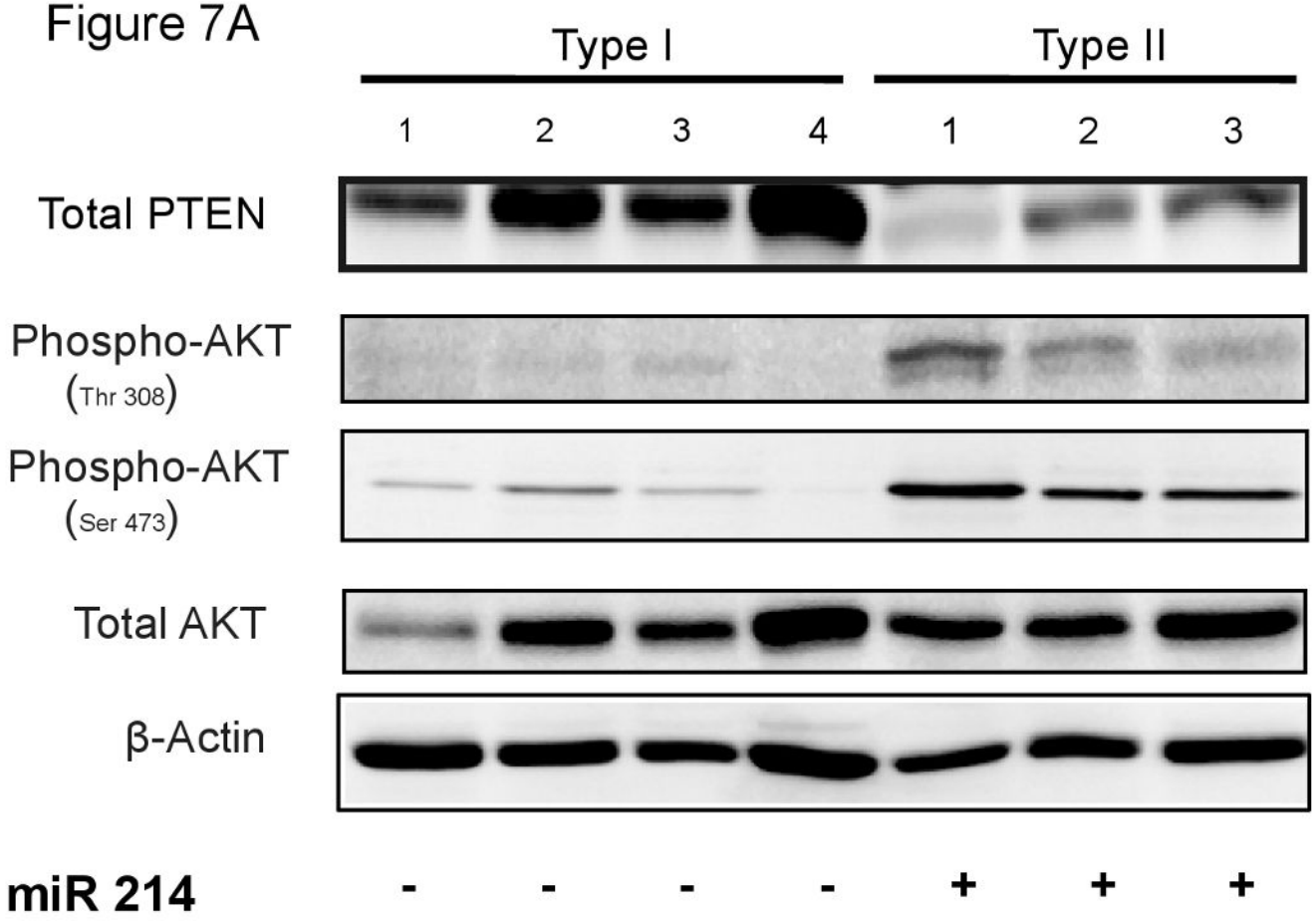


Figure 6.

Inhibition of Twist1 in Type II/CD44- EOC cells can reactivate the NF-κB pathway. Type II/CD44- EOC cells were transfected with siRNA for Twist1. Afterwards, cells were incubated in the presence or absence of TNFα (10 nM) for additional 24 hours. Levels of RANTES was determined in the supernatant. Note the significant increase in RANTES secretion in Type II/CD44- cells following Twist1 inhibition. Graph shown is representative of three independent experiments done in triplicate. * p=0.001

Figure 7A



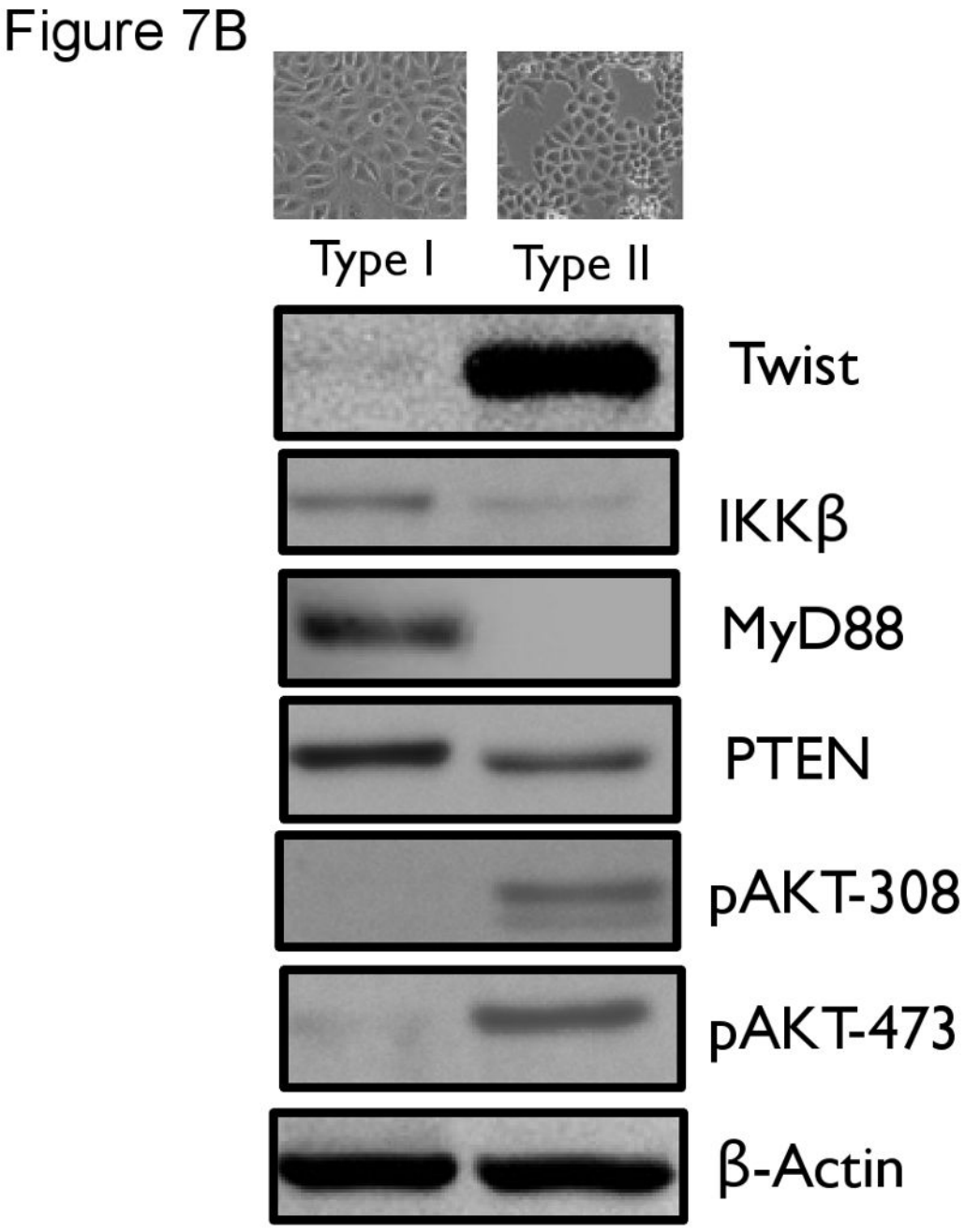
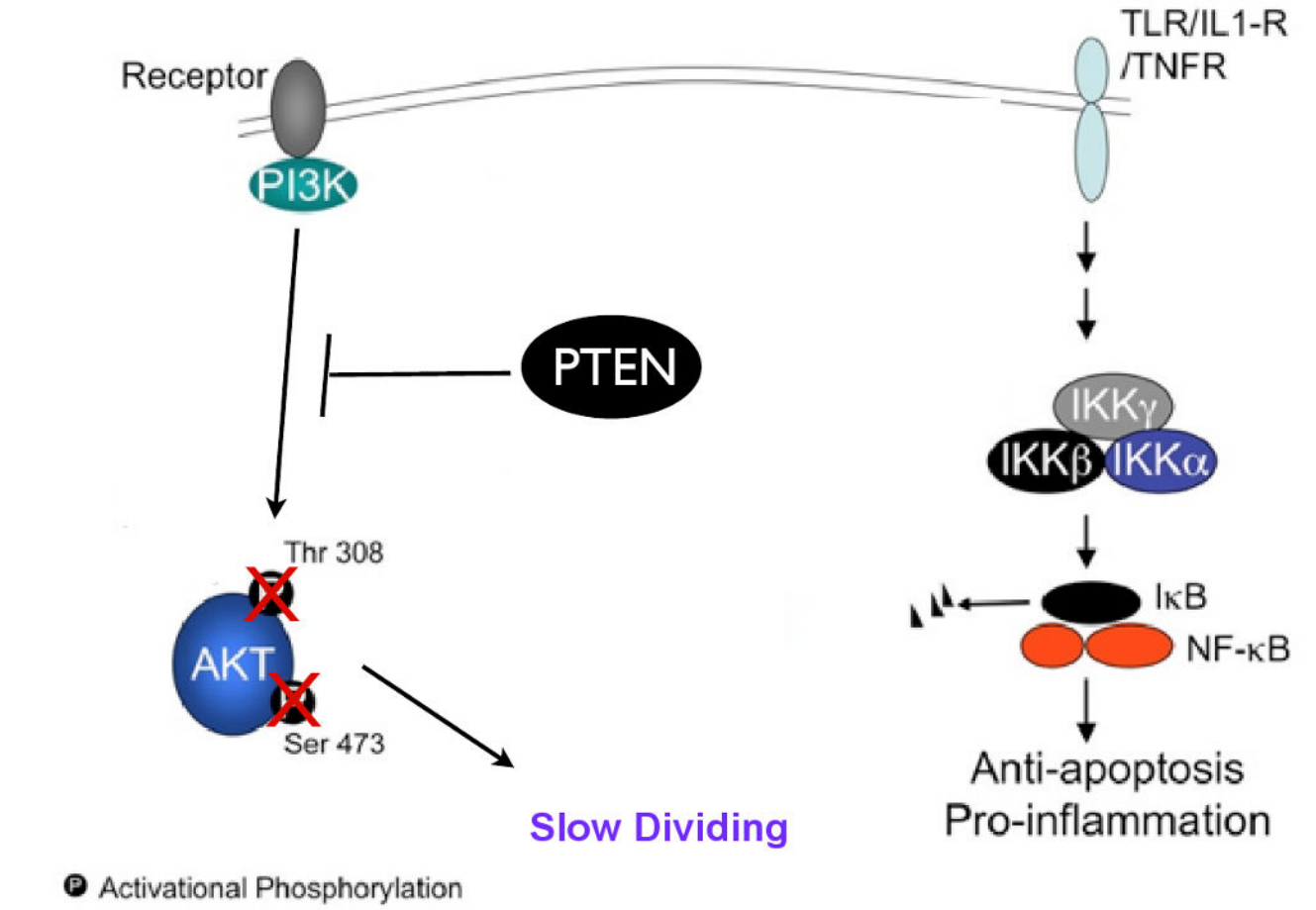


Figure 7.

Expression of miR-214 correlates with the expression of PTEN and AKT phosphorylation status.(a) Levels of PTEN and two AKT phosphorylation sites were evaluated in a panel of Type I/CD44+ and Type II/CD44- EOC cells by western blot. Note the correlation between levels of PTEN, AKT phosphorylation and miR-214 expression. (b) Type I/CD44+ EOC cells were induced to differentiate *in vitro* into Type II/CD44- EOC cells and levels of Twist1, IKKβ, MyD88, PTEN, and pAKT was determined by western blot.

Figure 8A



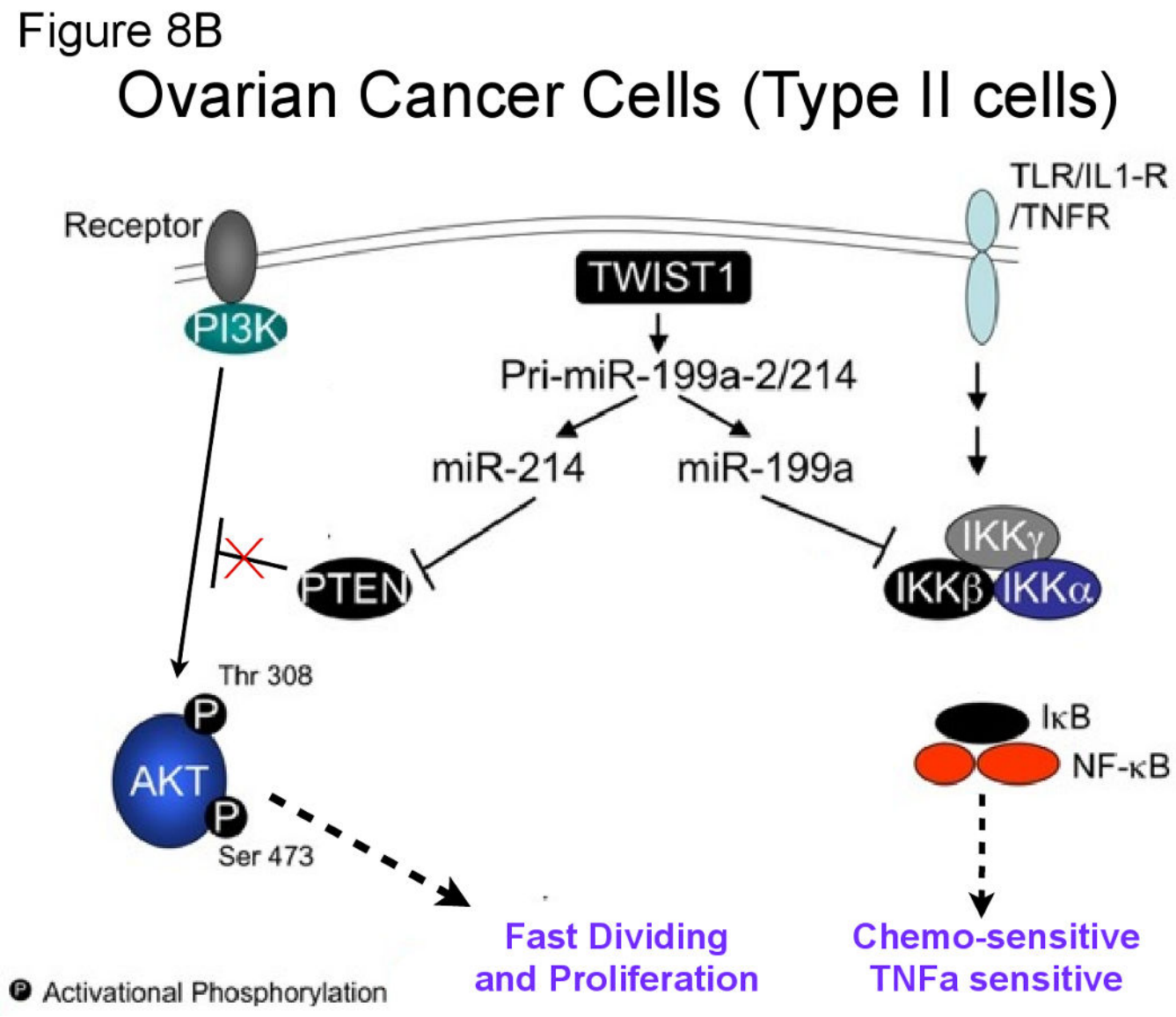


Figure 8.

Proposed model for the role of Twist1 in the ovarian cancer stem cells. (a) In the ovarian cancer stem cells, undetectable levels of Twist1 results in undetectable expression of hsa-miR-199a and hsa-miR-214, the inhibitors of IKKβ and PTEN, respectively. This leads to high expression of both IKKβ and PTEN. As a result, the ovarian cancer stem cells have constitutive NF-kB activity, which creates a pro-inflammatory and anti-apoptotic environment, and an inactive Akt pathway, leading to slow proliferation. (b) In the mature ovarian cancer cells, high levels of Twist1 leads to the expression of hsa-miR-199a and hsa-miR-214, which downregulates IKKβ and PTEN, respectively. As a result, mature/differentiated ovarian cancer cells do not have a constitutive NF-kB pathway, but instead have a highly active Akt pathway. These cells do not have a pro-inflammatory environment, are chemosensitive, and proliferate faster.