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ORIGINAL ARTICLE

MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-κB and TGF-β signaling pathways

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MicroRNAs (miRNAs) as modulators of gene expression have been described to display both tumor-promoting and tumor-suppressive functions. Although their role has been studied in different tumor types, little is known about how they regulate nuclear factor κB (NF-κB) signaling in breast cancer. Here, we performed an unbiased whole genome miRNA (miRome) screen to identify novel modulators of NF-κB pathway in breast cancer. The screen identified 13 miRNA families whose members induced consistent effects on NF-κB activity. Among those, the miR-520/373 family inhibited NF-κB signaling through direct targeting of *RELA* and thus strongly reduced expression and secretion of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8. With a combination of *in vitro* and *in vivo* approaches, we propose a metastasis-suppressive role of miR-520/373 family. miR-520c and miR-373 abrogated both *in vitro* cell invasion and *in vivo* intravasation of highly invasive MDA-MB-231 cells. However, knockdown of *RELA* did not affect their metastatic ability. mRNA profiling of MDA-MB-231 cells on overexpression of miR-520/373 members revealed a strong downregulation of transforming growth factor-β (TGF-β) signaling. Mechanistically, the metastasis-suppressive role of miR-520/373 can be attributed to direct suppression of *TGFBR2*, as the silencing of *TGFBR2* phenocopied the effects of miR-520/373 overexpression on suppression of Smad-dependent expression of the metastasis-promoting genes *parathyroid hormone-related protein*, *plasminogen activator inhibitor-1* and *angiopoietin-like 4* as well as tumor cell invasion, *in vitro* and *in vivo*. A negative correlation between miR-520c and *TGFBR2* expression was observed in estrogen receptor negative (ER^-) breast cancer patients but not in the ER positive (ER^+) subtype. Remarkably, decreased expression of miR-520c correlated with lymph node metastasis specifically in ER^- tumors. Taken together, our findings reveal that miR-520/373 family has a tumor-suppressive role in ER^- breast cancer by acting as a link between the NF-κB and TGF-β pathways and may thus contribute to the interplay of tumor progression, metastasis and inflammation.

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Introduction

Breast cancer is the most frequent type of cancer in women (23% of all cancers) and is still the second leading cause of cancer mortality in women worldwide. Approximately 90% of all cancer-related deaths arise from metastatic spread of primary tumors (Parkin *et al*., 2005; Christofori, 2006). As a disease, breast cancer is heterogeneous, consisting of different subtypes: luminal A, luminal B, basal and ERBB2 positive. Specifically, the basal-like and the ERBB2-positive subtypes are associated with short overall survival, while the presence of estrogen receptor (ER) is correlated with better prognosis (Sorlie, 2007). Alterations in signal transduction pathways such as receptor tyrosine kinase, nuclear factor κB (NF-κB) or transforming growth factor-β (TGF-β) signaling contribute to breast cancer initiation, progression and metastasis (Sovak *et al*., 1997; Spencer *et al*., 2000; Huber *et al*., 2004; Park *et al*., 2007; Padua *et al*., 2008). NF-κB transcription factor has a crucial role in tumor development through transcriptional regulation of genes associated with tumor growth, invasion and metastasis, including cytokines and chemokines, such as interleukin-8 (IL-8) and C-X-C motif chemokine 1 (CXCL1), adhesion molecules, such as the intracellular adhesion molecule-1 (ICAM-1), anti-apoptotic genes, such as *BCL2* or the chemokine receptor CXCR4 (Huang *et al*., 2000; Helbig *et al*., 2003; Zhou *et al*., 2007; Meylan *et al*., 2009). It is thus not surprising that NF-κB signaling has been described

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to have a crucial role in breast cancer progression by stimulating proliferation and preventing apoptosis (Biswas *et al*., 2003). NF-κB has also been found to be constitutively activated in ER^- but not in ER^+ breast tumors, where ERα signaling has been shown to repress RelB NF-κB subunit (Biswas *et al*., 2004; Wang *et al*., 2007). *In vivo* and *in vitro* studies have demonstrated that inhibition of NF-κB signaling in ER^- breast cancer results in reduced tumor growth through induction of apoptosis, suppressed cell invasion and inhibition of osteolytic bone metastasis (Park *et al*., 2007; Singh *et al*., 2007; Wang *et al*., 2007).

Apart from NF-κB signaling, other key mediators of extravasation, microenvironment remodeling and metastasis have been identified. TGF-β is a pleiotropic, anti-inflammatory cytokine and a pivotal regulator of a wide range of cellular processes, including cell cycle, proliferation, differentiation, epithelial-to-mesenchymal transition and metastasis (Yingling *et al*., 2004; Katsuno *et al*., 2008). TGF-β signaling is frequently deregulated in breast cancer and high expression of TGF-β receptors has been correlated with highly reduced overall survival of ER^- breast cancer patients (Buck *et al*., 2004). However, the role of TGF-β in cancer progression appears to be dual and context-, subtype- or stage-dependent. It can display either tumor-suppressing or -promoting effects even within the same tumor type (Yin *et al*., 1999; Cheng *et al*., 2005; Levy and Hill, 2006; Padua *et al*., 2008).

MicroRNAs (miRNAs) are endogenous small non-protein-coding RNAs of ~22 nucleotides in length and constitute a class of negative regulators of gene expression mainly by base pairing with the 3′-untranslated region (3′-UTR) of their target messenger RNA (mRNA). This class of regulators has been described to have an important role in a vast range of biological processes such as proliferation, differentiation and apoptosis (Esquela-Kerscher and Slack, 2006; Gu *et al*., 2009). Recent progress in cancer biology has revealed that miRNAs are frequently deregulated in various human cancers, including breast cancer. For instance, elevated miR-21 expression has been reported in breast tumors (Volinia *et al*., 2006), while miR-335 and miR-200c have been shown to inhibit metastatic cell invasion (Tavazoie *et al*., 2008; Uhlmann *et al*., 2010). These findings demonstrate the ability of miRNAs to modulate the development of malignancies by regulating critical cancer-related genes and signaling pathways. While numerous studies have revealed the role of miRNA–target interactions in single signaling pathways, there is limited information about the interaction of specific miRNAs with components of different signaling pathways, thus potentially leading to multiple and/or enhanced phenotypic effects.

Here, we performed a genome-wide cell-based miRNA screening, to systematically identify and characterize miRNAs that regulate NF-κB signaling pathway activity with an impact on breast cancer. We hereby show that miR-520/373 family targets a network of key tumor-related pathways. In particular, we demonstrate that miR-520/373 inhibits NF-κB and TGF-β signaling pathways in breast cancer cells by direct targeting of *RELA* and *TGFBR2,* respectively. Remarkably, the resulting inhibition of *RELA* expression led to reduced secretion of the pro-inflammatory cytokines IL-6 and IL-8. We further demonstrate that miR-520/373 abrogated *in vitro* cell invasion and *in vivo* intravasation of breast cancer cells, via downregulation of TGFBR2. Finally, a significant downregulation of miR-520c expression in ER^- clinical breast cancer metastasis samples was observed and correlated inversely with *TGFBR2* expression, thus suggesting a tumor-suppressive role of this miRNA family in ER^- breast cancer.

Results

Identification of miRNAs regulating NF-κB signaling using a genome-wide miRNA screen

To identify novel miRNAs regulating NF-κB pathway activity, we performed an unbiased genome-wide miRNA screen with a library of 810 miRIDIAN miRNA-mimics using a luciferase-based reporter assay. For the initial screen, miRNA-mimics were co-transfected with a reporter plasmid containing 3 consensus NF-κB-binding sites followed by a luciferase gene into HEK293FT cells. NF-κB signaling was triggered 48 h later by stimulating the cells with the pro-inflammatory cytokine tumor necrosis factor (TNF)-α. The screen revealed differential effects of the miRNAs on NF-κB activity (Supplementary Table S1).

In a network-based approach, hit-miRNAs were classified according to their seed region (2–7 nucleotide, miRBase v12.0) into miRNA-families, because members of miRNA families sharing the same seed region are expected to have overlapping target profiles. This strategy should further reduce the number of potential false-positive hits. Subsequently, we selected families for which the calculated z-scores in the screen showed a consistent activating or inhibitory effect on NF-κB activity ($P<0.20$). In total, we identified 13 miRNA families whose members coherently modulated the NF-κB pathway (Figure 1a). Two of these families, let-7/98 and miR-181, have recently been reported to negatively and positively regulate NF-κB signaling, respectively (Iliopoulos *et al*., 2009, 2010). These findings support the biological relevance as well as the potential of our screen to identify negative and positive regulators.

To validate miRNAs also in the context of ER^- breast cancer, where NF-κB signaling is frequently constitutively activated (Biswas *et al*., 2004; Singh *et al*., 2007), we applied an independent secondary validation screen based on NF-κB target gene regulation in the breast cancer cell line MDA-MB-231, which is an established model for constitutively activated NF-κB (Park *et al*., 2007). One representative miRNA from each of the 13 selected miRNA families identified in the primary screen was overexpressed in MDA-MB-231 cells and, 2 days later, mRNA lysates were analyzed by quantitative real-time PCR for expression of the NF-κB target genes *IL-6*, *IL-8* and *CXCL1* (Figure 1b). Here, miR-373 showed a strong and coherent inhibitory effect on NF-κB

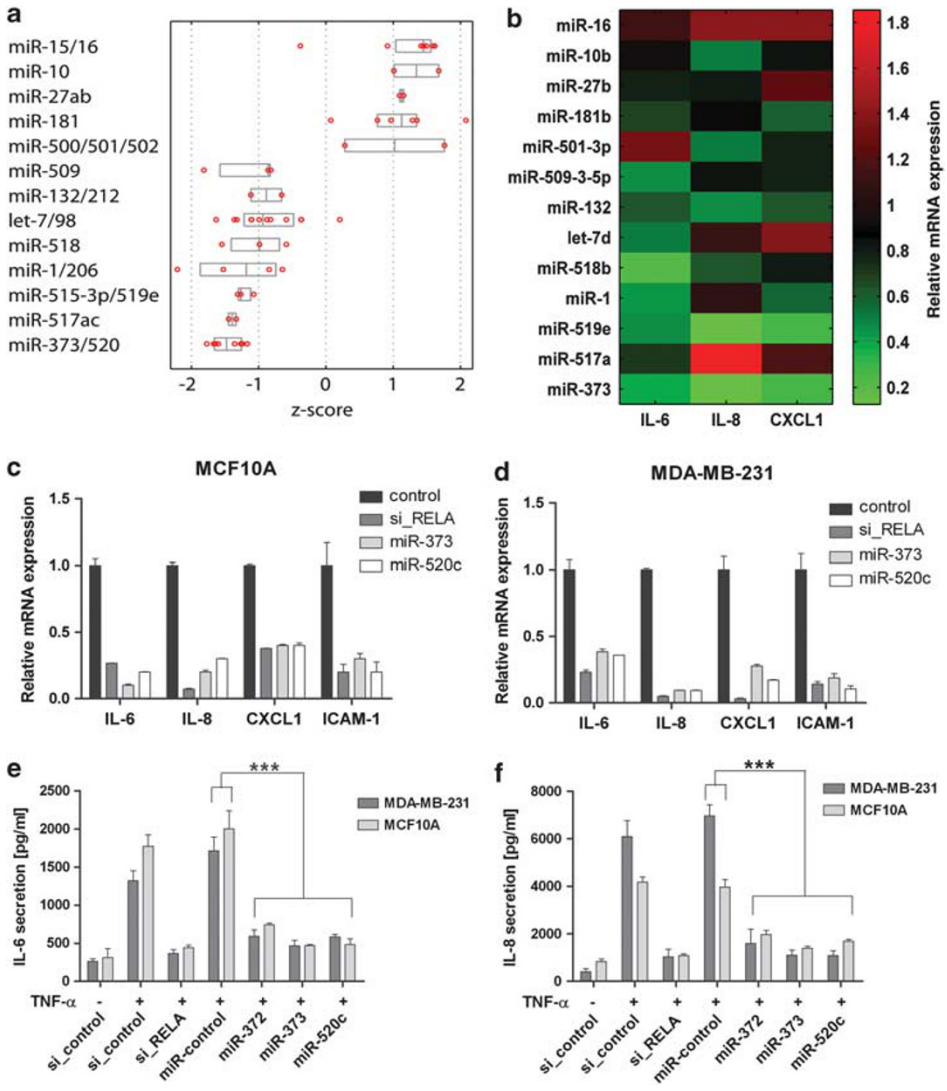

Figure 1 miRNAs modulating NF-κB signaling in breast cancer. (**a**) miRNAs sharing the same seed region were classified into 13 miRNA-families that show a coherent positive or negative modulation of TNF-induced NF-κB activity, as determined by reporter-based luciferase assay. Red dots represent individual miRNAs from each family, vertical line represents the median and the box edges the 25 and 75 percentiles of the z-score. (**b**) Heatmap representation of altered expression of the NF-κB target genes *IL-6, IL-8* and *CXCL1* on overexpression of one representative miRNA from each of the 13 candidate families compared with control-transfected cells. miRNA mimic-transfected cells were treated 2 days after transfection with 20 ng/ml of TNF-α for 5 h and gene expression was determined by qRT–PCR. Expression was normalized to *GAPDH* and *HPRT-1* expression. Reduced expression of NF-κB target genes is shown in green, while increased expression is shown in red. (**c**, **d**) The mRNA levels of the NF-κB-dependent target genes *IL-6, IL-8, CXCL1* and *ICAM-1* after transfection with miR-520c and miR-373 mimics and stimulation with TNF-α for 5 h determined by qRT–PCR in MCF10A (**c**) or MDA-MB-231 breast cancer cells (**d**). Expression was normalized to *GAPDH* and *HPRT-1* expression. (**e**, **f**) IL-6 (**e**) and IL-8 (**f**) secretion (ELISA assay) from TNF-α treated MDA-MB-231 or MCF10A cells transfected with miR-520/373 mimics or negative control (***$P<0.001$). All experiments were performed with four replicates and data are shown as mean ± s.d.

transcriptional activity (Figures 1a and b). This miRNA belongs to the miR-520/373 family, which consists of three different miRNA clusters sharing an identical seed region, miR-302/367, miR-371/372/373 and miR-520. The miR-302/367 cluster is located on chromosome 4, while miR-371/372/373 and miR-520 clusters are located on chromosome 19 (Suh *et al*., 2004). Owing to the strong and consistent inhibitory effect of miR-373 on NF-κB signaling activity, we focused on miR-520/373 family for further analysis.

The miR-520/373 family inhibits NF-κB signaling in normal breast epithelial and cancer cells

To further confirm the impact of miR-520/373 on NF-κB signaling, we examined whether these miRNAs would have an effect on transcriptional regulation of known NF-κB target genes. Therefore, miR-373 and miR-520c mimics were overexpressed in MDA-MB-231 cells and in the immortalized but non-transformed MCF10A breast epithelial cells, in which expression of cytokines such as IL-6 and IL-8 is strongly regulated by

TNF-α-induced NF-κB activity (Supplementary Figure S1). Cells were treated with TNF-α and mRNA expression changes were determined by quantitative reverse transcriptase (qRT)–PCR. In accordance with our aforementioned results, miR-520/373 family strongly inhibited expression of *IL-6*, *IL-8*, *CXCL1* and *ICAM-1*, similarly to the small interfering RNA-induced knockdown of *RELA* in both cell lines (Figures 1c and d). Furthermore, secretion of IL-6 and IL-8 into the supernatant of MDA-MB-231 or MCF10A cells transfected with miR-520/373 mimics was strongly reduced compared with the control cells (Figures 1e and f). This effect seems to be mediated by down-regulation of NF-κB activity as small interfering RNA-induced knockdown of *RELA* also diminished expression and secretion of IL-6 and IL-8 (Figures 1c–f). Together, these findings indicate that expression of the miR-520/373 family negatively modulates NF-κB signaling and NF-κB-mediated physiological effects in both normal breast epithelial and breast cancer cells.

RELA is a direct target of miR-520/373 family

Next, we attempted to identify direct targets of the miR-520/373 family, which could, at least in part, explain the decreased NF-κB transcriptional activity. The bioinformatic prediction tool Miranda (Lewis *et al*., 2005) predicted two target sites for the miR-520/373 family in the *RELA* 3′-UTR (Figure 2c). Thus, we first analyzed the effect of miR-520/373 family on *RELA* expression. Indeed, all analyzed members of the miR-520/373 family strongly reduced *RELA* mRNA level in several human breast cell lines (Figure 2a, Supplementary Figure S2a). Also protein levels of p65/RelA were reduced by enhanced miR-373 and miR-520c expression in MDA-MB-231 and MCF10A cells (Figure 2b). This reduction in p65/RelA expression is also reflected at the activity level, as cells transfected with miRNA-mimics for the miR-520/373 family members showed strongly reduced phosphorylation of p65, which is indicative of reduced p65 activity. However, the expression of p50, the partner of p65 in the classical NF-κB heterodimer, was not affected by overexpression of the miR-520/373 family members (Figure 2b).

To determine whether reduced RELA expression levels were due to direct miRNA targeting of the *RELA* 3′-UTR, we next cloned a reporter construct containing the 3′-UTR region of the *RELA* gene downstream of the Renilla luciferase open reading frame. This construct was co-transfected in MCF-7 cells together with the mimics for the members of miR-520/373 family and compared with control mimic-transfected cells. All analyzed miR-520/373 members strongly reduced the relative luciferase activity by targeting the 3′-UTR of *RELA*. Finally, in order to further validate that the observed effect of miR-520/373 is indeed due to direct binding of its seed region to the seed-matching sequences in the 3′-UTR of *RELA*, we introduced mutations into either one or both predicted target sites by mutating four nucleotides within the seed-matching sequences (Figure 2d). Disruptions of both target sites completely abolished luciferase reduction on miR-520/373 mimic transfection, confirming that *RELA* is a direct target of miR-520/373 family and that both target sites contribute to and are sufficient for the regulation of *RELA* by these miRNAs (Figure 2e). In a different approach, we co-transfected miR-606, which differs in two nucleotides in the seed region compared with the miR-520/373 family, with the WT 3′-UTR of *RELA*. No reduction in luciferase activity was observed, thus further confirming that *RELA* is a specific target of miR-520/373 family (Supplementary Figure S3). Taken together, these results indicate that *RELA* is post-transcriptionally controlled by miR-520/373 family in breast cancer cells.

miR-520/373 family directly targets TGFBR2 and their expression inversely correlates in ER$^-$ primary breast cancer samples

As the pro-inflammatory cytokines IL-6 and IL-8 had previously been reported to modulate cell motility in breast cancer (Wu *et al*., 2008; Walter *et al*., 2009; Camp *et al*., 2011), and NF-κB inhibition in ER$^-$ breast cancer cells had been previously reported to abrogate metastasis formation (Park *et al*., 2007), we hypothesized that miR-373 and miR-520c might have an inhibitory role in cell invasion through deregulation of NF-κB signaling. To investigate this hypothesis, we used the highly invasive and ER$^-$ breast carcinoma cell line MDA-MB-231. Indeed, overexpression of both miRNAs significantly reduced the invasive capacity of MDA-MB-231 cells (Supplementary Figure S4). Interestingly, this effect was not mediated through targeting of *RELA*, as silencing of *RELA* did not phenocopy the observed effect of miR-373 and miR-520c (Supplementary Figure S4). As miRNAs often target several genes, we therefore investigated whether the miR-520/373 family would regulate other signaling molecules apart from NF-κB that might have a regulatory role in breast cancer invasion. To this end, we performed a genome-wide mRNA microarray profiling using MDA-MB-231 cells transfected with either of the four miR-520/373 family members (miR-373, miR-302d, miR-520c and miR-520e) or mimic control (miR-control). This analysis revealed a variety of deregulated genes compared with control. Strikingly, genes of the TGF-β signaling pathway were enriched among the top hits (Figure 3a). Combining the profiling results with the prediction tool TargetScan 5.0, we focused on the *TGFBR2* gene, as it was predicted to contain two highly conserved target sites and showed a significantly lower expression ($P<0.001$) on overexpression of miR-520/373 members compared with control (Figure 3a). Moreover, RNA interference-induced knockdown of *TGFBR2* gene reduced the invasive capacity of MDA-MB-231 cells similarly to miR-520/373 overexpression (Supplementary Figure S4). The reduction of *TGFBR2* mRNA by the miR-520/373 family was further confirmed by qRT–PCR, while inhibition of endogenously expressed miR-520c resulted in increased expression of *TGFBR2* in both MDA-MB-231 and MCF10A cells (Figures 3b and c; Supplementary Figures S2b and S5). Moreover, western blot analysis validated that miR-520c and miR-373

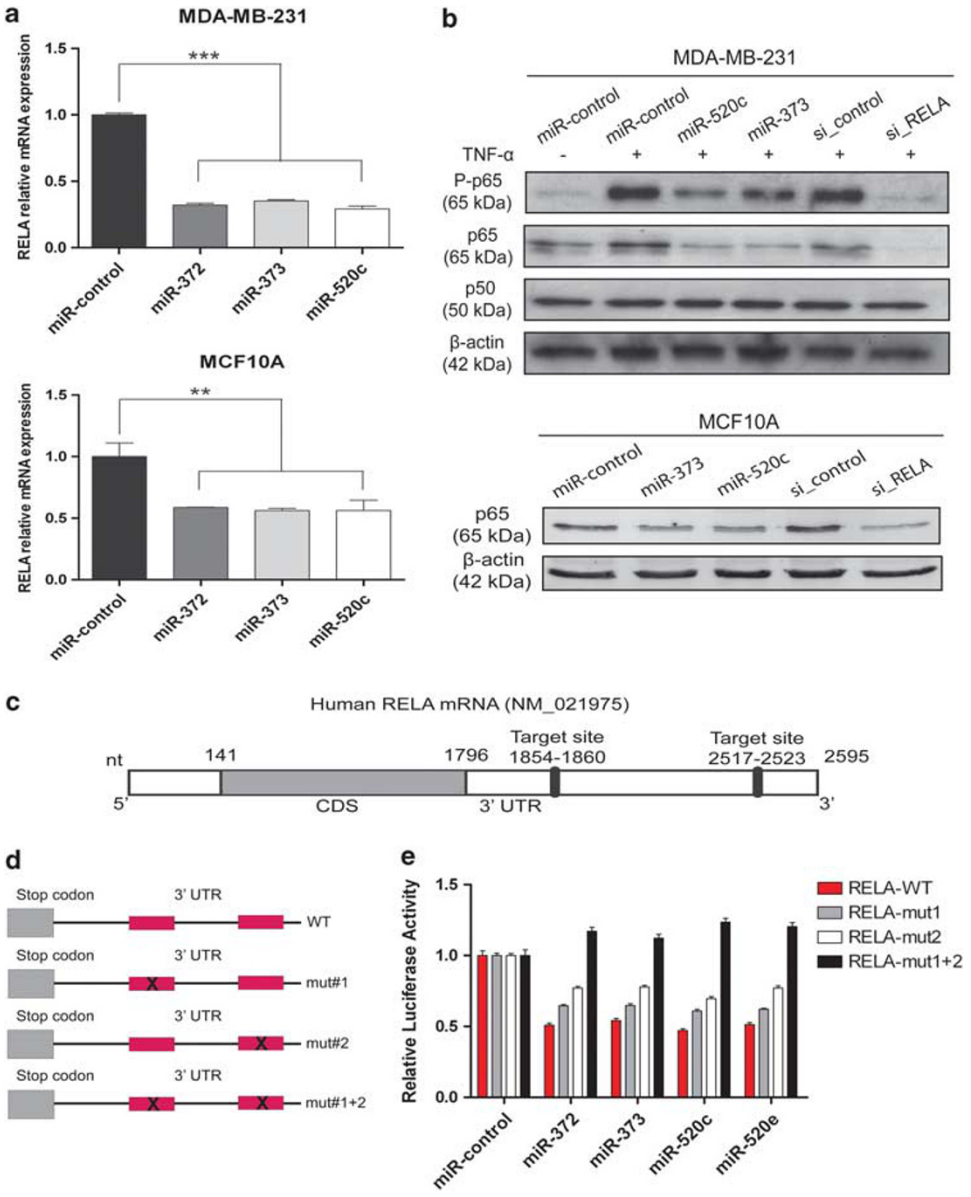

Figure 2 miR-520/373 family directly regulates expression of the RelA/p65 NF-κB subunit in normal breast and cancer cells. (**a**) *RELA* mRNA levels in MDA-MB-231 (***$P<0.001$) or MCF10A (**$P<0.01$) cells transfected with miR-520/373 family mimics compared with control as determined by qRT–PCR. (**b**) RelA/p65 protein levels after transfection of MDA-MB-231 or MCF10A cells with miR-520c, miR-373 mimics, *RELA* knockdown compared with control-transfected cells. After 2 days, MDA-MB-231 cells were treated with 20 ng/ml TNF-α for 10 min and total cell lysates were analyzed by western blot. (**c**) Schematic representation of the gene structure of *RELA* with the predicted target sites for miR-520/373 family members. (**d**) Four different psiCHECK2 reporter vectors were constructed carrying the wild-type, single or double mutated *RELA* 3′UTR, as indicated. (**e**) Luciferase reporter assay of MCF-7 cells transfected with wild-type or mutated 3′UTRs of *RELA* mRNA and miR-520/373 mimics or negative control. Experiments were performed with four replicates and data are shown as mean ± s.d.

negatively regulate expression of *TGFBR2* also at the protein level (Figure 3d).

Next, we analyzed the 3′UTR of *TGFBR2* gene to verify a direct regulation by miR-520/373, as it contains two putative target sites that are highly conserved among different species (Figure 4a). Indeed a strong reduction of the luciferase activity was observed when a reporter construct containing the full-length 3′UTR of the *TGFBR2* gene downstream of the Renilla luciferase open reading frame was transfected in combination with several miR-520/373 mimics. Conversely, introduction of four point mutations in both target sites was sufficient to diminish this effect (Figure 4b). Similarly to *RELA*, no reduction in luciferase activity was observed on co-transfection of miR-606 with the WT 3′UTR of *TGFBR2*, thus further confirming that *TGFBR2* is a specific direct target of miR-520/373 family (Supplementary Figure S3).

To address whether downregulation of *TGFBR2* by miR-520/373 is relevant to breast cancer, we examined

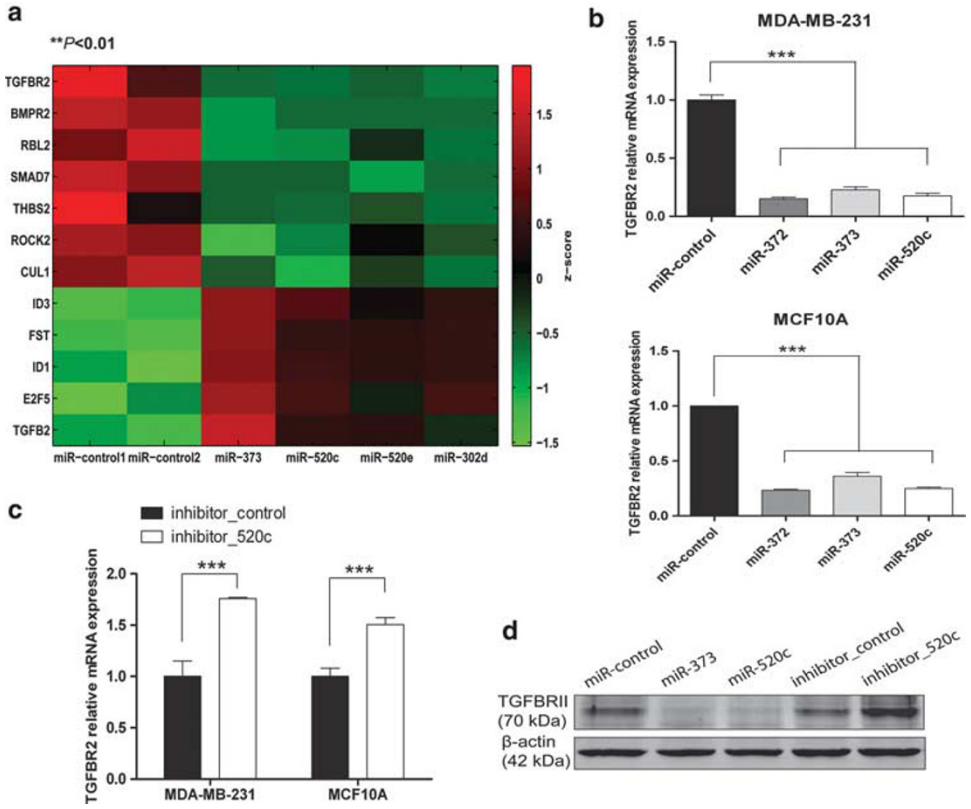

Figure 3 *TGFBR2* is negatively regulated by miR-520/373 family. (**a**) Heatmap representation of the mRNA expression profiling showing selected deregulated genes (**$P<0.01$) within the TGF-β signaling pathway (annotated with KEGG pathway analysis). (**b**, **c**) *TGFBR2* mRNA expression levels in MDA-MB-231 or MCF10A cells 2 days after transfection with mimics (***$P<0.001$) or inhibitor (***$P<0.001$) of miR-520/373 family, as determined by qRT–PCR. (**d**) Regulation of TGFBR2 by miR-520/373 members in protein level was validated by western blot analysis, 2 days after transfection of miR-520/373 mimics or inhibitors in MCF10A cells. Experiments were performed in triplicate and data are shown as mean ± s.d.

the expression of miR-373, miR-520c and TGFBR2 in 76 primary breast tumors by qRT–PCR. No significant reverse correlation was observed between miR-520c and *TGFBR2* expression (data not shown). Strikingly, when tumor specimens were classified according to their ER status in ER^+ ($n=55$) and ER^- ($n=21$), a significant inverse relationship between miR-520c and TGFBR2 was observed in ER^- tumors ($r=-0.43$; $P=0.048$; Figure 4c). In contrast to ER^- tumors, no significant correlation was observed in the subset of ER^+ tumors (Figure 4d). Expression analysis of a possible correlation between miR-373 and TGFBR2 was not feasible because miR-373 expression was below the detection level in both patient groups (data not shown). To conclude, this significant reverse correlation strongly argues for a mechanistic relationship between *TGFBR2* and miR-520c specifically in ER^- breast cancer.

miR-373 and miR-520c overexpression is sufficient to inhibit TGF-β signaling in ER⁻ breast cell lines

For the functional characterization of miR-520/373 on TGF-β signaling, we restricted ourselves to the ER^- cell lines MDA-MB-231 and MCF10A, as an inverse correlation between *TGFBR2* and miR-520c was only observed in ER^- tumors (Figure 4d), while endogenous *TGFBR2* expression was higher in ER^- than in ER^+ cell lines (Supplementary Figure S9). To assess the effect of miR-520/373 family members on the signaling downstream of TGFBR2, we examined whether ectopic expression of miR-373 and miR-520c would impact TGF-β-induced phosphorylation of Smad2 and Smad3 transcription factors, two of the main transducers of extracellular TGF-β signaling from the cellular membrane into the nucleus. Phosphorylation of both Smad2 and Smad3 was indeed significantly reduced on TGF-β stimulation when the two miRNAs were overexpressed in MDA-MB-231 cells, however, the levels of total Smad2 and Smad3 were unchanged (Figure 5a). These alterations in Smad2 phosphorylation were also verified in MCF10A cells (Figure 5b). As phosphorylation is required for activation of Smad proteins and their subsequent nuclear accumulation, we next wanted to verify that miR-520/373 family affects Smad2/3 heterodimer translocation into the nucleus. To this end, we overexpressed miR-520/373 family members in MDA-MB-231 cells treated with TGF-β for 2 h, and then quantified nuclear translocation of Smad2/3 by immunostaining. miR-373 completely abolished TGF-β-induced translocation of Smad2/3 heterodimers into the nucleus, similarly to the small interfering RNA-induced knockdown of *TGFBR2* (Figures 5c and d).

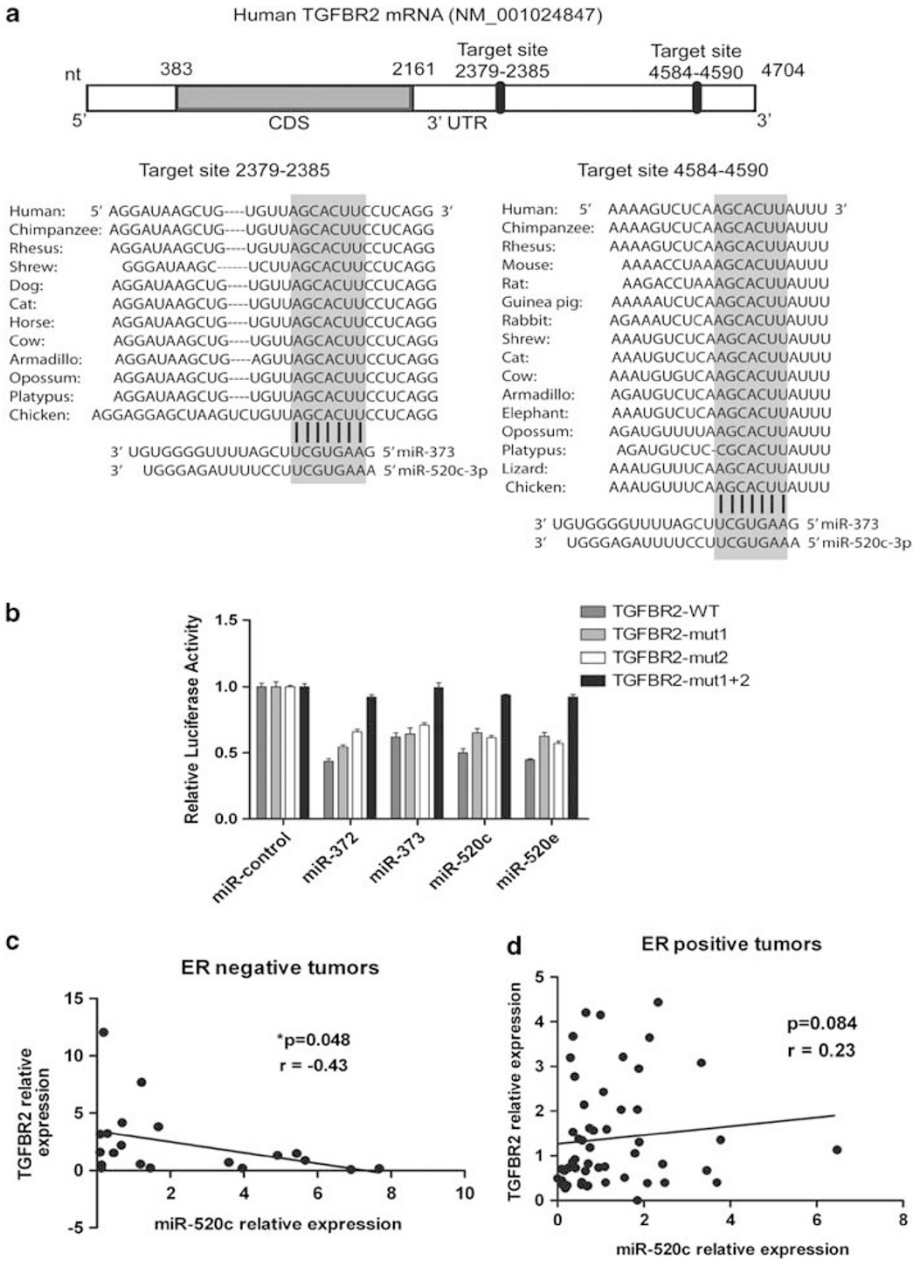

Figure 4 *TGFBR2* is a direct target of miR-520/373 family. (**a**) Schematic representation of the *TGFBR2* mRNA. Two predicted target sites for miR-520/373 family are located within the *TGFBR2* 3′UTR, which are highly conserved among species. The 'seed' sequence of miR-520/373 (2–7 nt) and the target sites on the *TGFBR2* mRNA are indicated in grey. (**b**) Luciferase reporter assay in MCF-7 cells with wild-type or mutated 3′UTR of *TGFBR2* after overexpression of miR-520/373 family members. Experiments were performed with four replicates and data are shown as mean ± s.d. (**c**, **d**) miR-520c and *TGFBR2* mRNA expression levels in 21 ER^- (**c**) or 55 ER^+ (**d**) breast primary tumors, with each data point representing an individual sample and correlation coefficient (*r*) indicated (spearman correlation).

Next, we investigated the physiological effects of impaired TGF-β signal transduction on the transcriptional regulation of critical TGF-β target genes known to have a strong impact on breast cancer progression and metastasis. We, therefore, analyzed expression levels of angiopoietin-like 4 (ANGPTL4), parathyroid hormone-related protein (PTHrP) and plasminogen activator inhibitor-1 (PAI-1), which have been selectively associated with metastasis formation to the lungs, bones and lymph nodes, respectively (Yin *et al*., 1999; Weigelt *et al*., 2005; Padua *et al*., 2008; Ganapathy *et al*., 2010). Expression of all the three TGF-β-dependent genes was diminished in response to enhanced levels of miR-520/373 family members in both MDA-MB-231 and MCF10A cells (Figures 6a and c). Moreover, secretion of ANGPTL4 into the supernatant of MDA-MB-231 or MCF10A cells transfected with miR-520/373 mimics was strongly reduced compared with the control

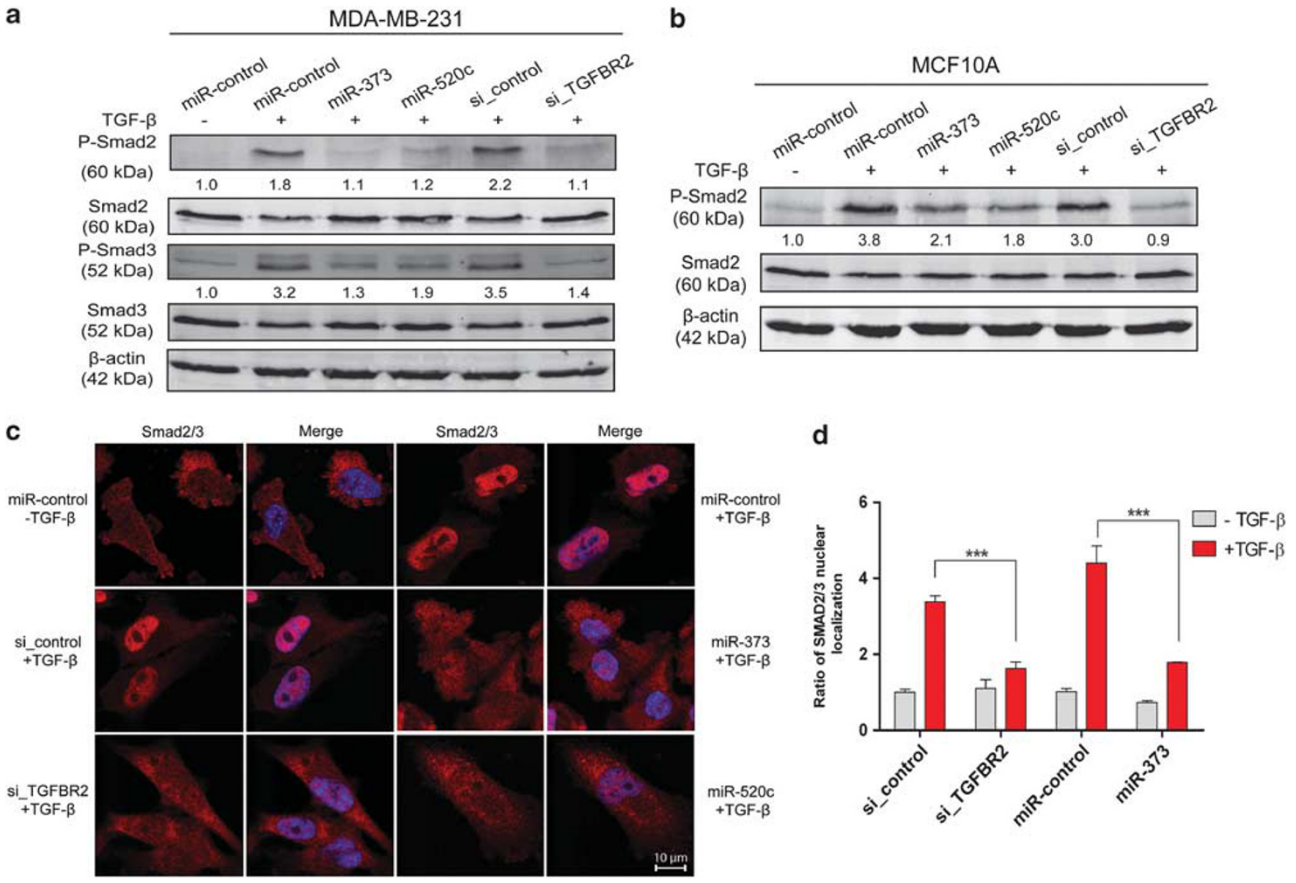

Figure 5 TGF-β signaling is inhibited by miR-520/373. (**a**, **b**) Phosphorylation of Smad2 and Smad3 was examined in MDA-MB-231 (**a**) and phosphorylation of Smad2 was examined in MCF10A cells (**b**) after transfection with miR-373, miR-520c, si_TGFBR2 or respective negative control. Cells were stimulated with TGF-β (10 ng/ml) for 1 h and total cell lysates were analyzed by western blot. Fold change was determined after normalization to actin and is indicated below the blots. (**c**) Decreased Smad2/3 translocation into the nucleus after TGF-β stimulation of MDA-MB-231 transfected with miR-520/373 members or si_TGFBR2 compared with control-transfected cells, as shown by confocal microscopy. Cells were fluorescently stained with anti-Smad2/3 antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). (**d**) Quantitative analysis of Smad2/3 translocation to the nucleus in MDA-MB-231 cells on TGF-β stimulation for 2 h (***$P<0.001$). Images were acquired using Olympus ScanR microscope from approximately 1500 cells for each sample and analyzed by Olympus ScanR analysis software (Olympus, Hamburg, Germany). Cells with a ratio of nuclear/cytoplasmic localization of Smad2/3 higher than 1 were considered as positive for nuclear localization, while cells with a ratio <1 were considered as negative for nuclear localization. Data are shown as fold changes of the ratio of Smad2/3 nuclear localization. Results are shown as mean ± s.d.

cells (Figures 6e and f). These effects seem to be mediated via downregulation of TGFBR2 as silencing of *TGFBR2* also diminished expression of these genes and secretion of ANGPTL4 in both cell lines (Figures 6b and d–f). These data suggest that TGF-β signaling and its downstream mediators are negatively regulated by miR-520/373 family via direct targeting of *TGFBR2*.

miR-520/373 family inhibits TGF-β-induced invasion in vitro *as well as vascular intravasation of ER⁻ breast cancer cells* in vivo

Having shown that the miR-520/373 family suppresses TGF-β signaling, we further studied the functional role of miR-373 and miR-520c in TGF-β-induced cell invasion. Highly invasive, TGF-β responsive and ER⁻ MDA-MB-231 cells were transfected with miR-520/373 family mimics and were then stimulated with TGF-β. Induction of invasion was analyzed in an *in vitro* assay. miR-373 as well as miR-520c overexpression strongly reduced the invasive capacity of MDA-MB-231 cells (Figure 7a). Conversely, when the endogenous levels of miR-520c were reduced using an antisense inhibitor (inhibitor_520c), the number of invaded cells was increased (Figure 7c, Supplementary Figure S5). Furthermore, RNA interference-induced knockdown of *TGFBR2* led to a similar reduction of TGF-β-induced invasion as overexpression of miR-520/373 (Figure 7b). Finally, to examine the importance of *TGFBR2* in the context of cell invasion on overexpression of miR-520/373, *TGFBR2* without its 3′UTR was ectopically expressed. Rescuing TGFBR2 levels significantly increased the number of invaded MDA-MB-231 cells overexpressing miR-373 or miR-520c (Supplementary Figure S6). Together, these findings support the notion that targeting of *TGFBR2* by miR-520/373 family is the major mediator of this phenotype.

To further investigate the ability of miR-520/373 family to suppress the metastatic potential of breast

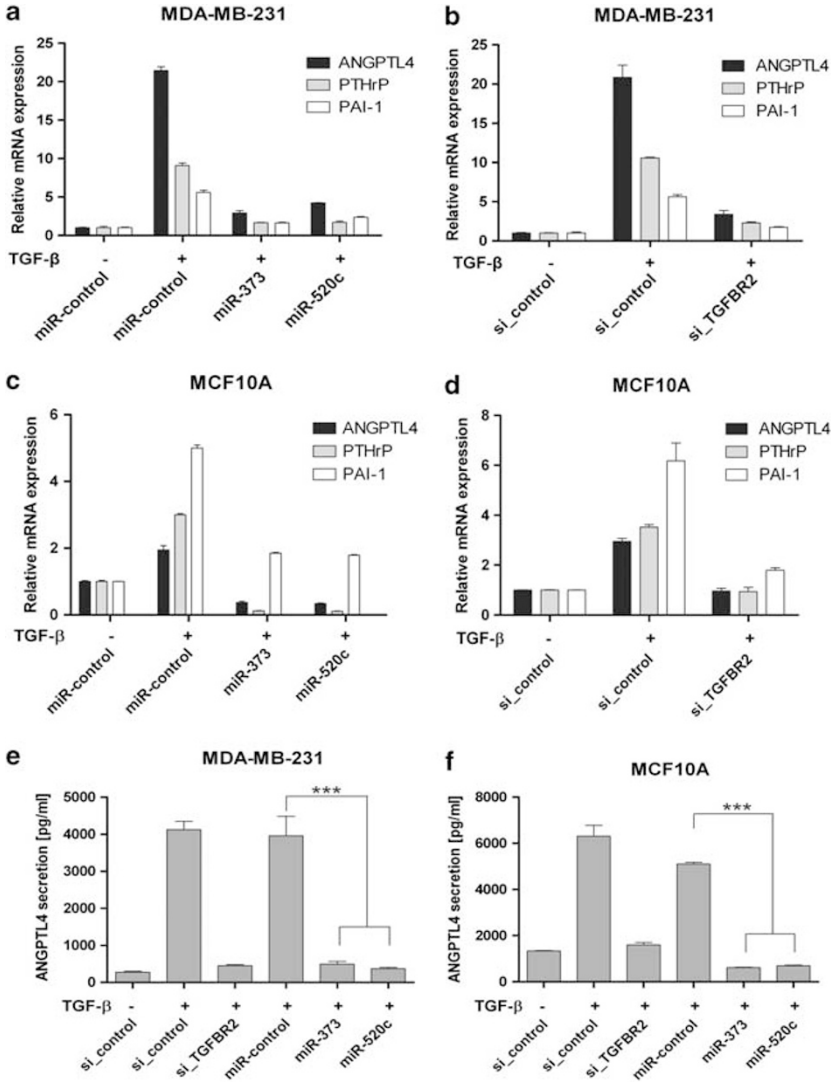

Figure 6 TGF-β target gene expression is inhibited by miR-520/373. (**a**, **b**) Changes in mRNA expression levels of *ANGPTL4, PTHrP* and *PAI-1*. MDA-MB-231 cells transfected with miR-520/373 members, si_TGFBR2 or control were treated with 10 ng/ml TGF-β for 4 h and changes in mRNA level of the Smad-dependent TGF-β target genes were determined by qRT–PCR. (**c**, **d**) Changes in mRNA expression levels of *ANGPTL4, PTHrP* and *PAI-1* in MCF10A-transfected cells with miR-520/373 members, si_TGFBR2 or control were determined by qRT–PCR on treatment with 10 ng/ml TGF-β for 4 h. Expression was normalized to *GAPDH* and *HPRT-1* expression. (**e**, **f**) ANGPTL4 secretion (ELISA assay) from MDA-MB-231 (**e**) or MCF10A (**f**) cells transfected with miR-520/373 mimics or negative control and treated with 10ng/ml TGF-β for 16 h (***$P<0.001$). All experiments were performed in triplicate and data are shown as mean ± s.d.

cancer cells *in vivo*, a chorioallantoic membrane (CAM) assay was performed (Zijlstra *et al*., 2002; van der Horst *et al*., 2004). MDA-MB-231 cells were transfected with miR-520/373 family mimics or with small interfering RNAs targeting *RELA* and *TGFBR2* genes and were inoculated on the upper CAM of 10-day-old chicken embryos. After 5 days, the number of tumor cells that had intravasated into the lower CAM vasculature was significantly reduced when miR-373 or miR-520c were overexpressed as compared with miR-control cells, as quantified by *alu* qRT–PCR (Figure 7d; Supplementary Figure S7). Similarly, RNA interference-induced knockdown of *TGFBR2* resulted in a significant inhibition of lower CAM intravasation as miR-520/373 overexpression. In contrast, downregulation of *RELA* did not affect tumor cell dissemination (Figure 7e). These results are in line with our *in vitro* observations, thus further supporting the role of miR-520/373 family as a metastasis suppressor in ER$^-$ breast cancer, likely via direct downregulation of *TGFBR2*.

miR-520c expression is inversely correlated to nodal metastasis status of ER$^-$ breast tumor samples

To address whether miR-520/373 has clinical relevance in breast cancer metastasis formation, we examined expression levels of miR-373 and miR-520c in 76

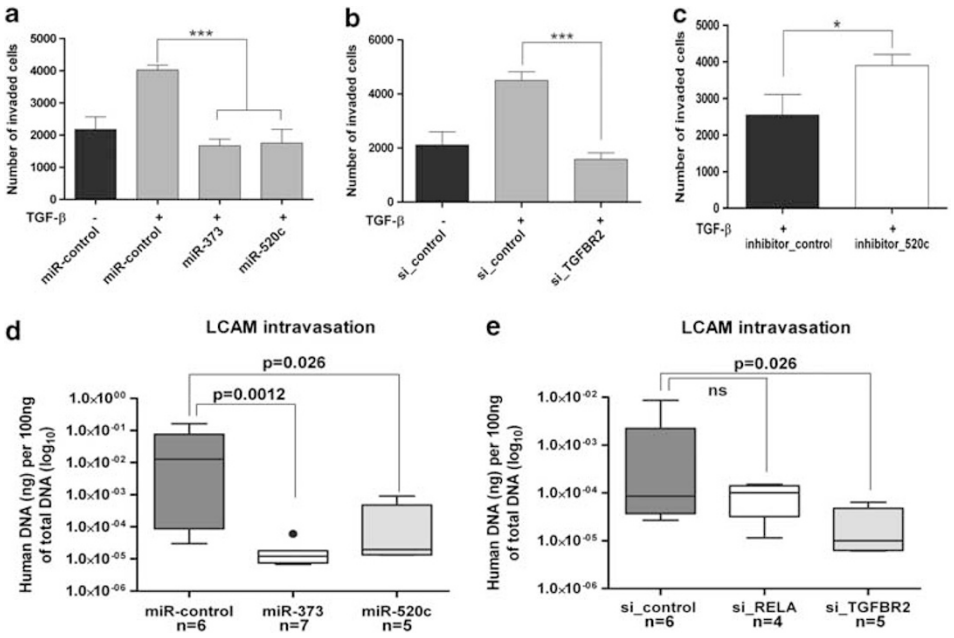

Figure 7 miR-520/373 family inhibits cell invasion *in vitro* and *in vivo* intravasation. (**a**, **b**) Highly invasive MDA-MB-231 cells transfected with miR-373, miR-520c, si_TGFBR2 or negative control were seeded into trans-well inserts in the presence or absence of TGF-β ligand. The number of invaded cells was determined by flow cytometry (***$P<0.001$). (**c**) Number of invaded cells through the trans-well membrane after inhibition of endogenous miR-520c and TGF-β treatment (*$P<0.05$). Experiments were performed in triplicate and data are shown as mean ± s.d. (**d**, **e**) Chicken embryo (CAM) assay was performed using MDA-MB-231 cells transfected with miR-373, miR-520c, si_RELA, si_TGFBR2 or negative control. The number of eggs with primary tumor formation for each group is indicated (*n*). Genomic DNA was analyzed by real-time *alu*-PCR to determine the amount of human DNA corresponding to human tumor cells that had intravasated to the lower CAM (LCAM). Data were analyzed using Mann–Whitney one-tailed *t*-test.

primary human breast cancer tumor samples with known lymph node metastasis status, by qRT–PCR. As a reverse correlation between miR-520c and *TGFBR2* expression was only observed in ER^- and not in ER^+ tumors (Figures 4c and d), and *TGFBR2* expression had previously been reported to correlate with poorer prognosis only in ER^- patients (Buck *et al*., 2004), tumor samples were classified into two groups depending on their ER status. 55 samples were ER^+ and 21 were ER^-. Of the patients with ER^- tumors, 6 showed infiltration to the sentinel lymph nodes (lymph node positive), while 15 patients did not (lymph node negative). miR-520c expression was significantly lower in patients with lymph node metastasis than in patients without (Figure 8a; $P=0.022$). In contrast to miR-520c, miR-373 expression was below the detection level in both patient groups (data not shown). We next investigated whether miR-520c expression was correlated with lymph-node status also in the ER^+ subtype. In contrast to ER^- tumors, no significant difference in miR-520c expression was observed between the lymph node-positive and the lymph node-negative groups in ER^+ patients (Supplementary Figure S8). We next investigated the expression of *TGFBR2* in the ER^- tumor samples by qRT–PCR. Mean expression of *TGFBR2* was significantly increased in the group of patients with lymph node metastasis compared with the group without metastasis formation (Figure 8b; $P=0.0003$). Together, these data suggest that the regulation of *TGFBR2* by miR-520c is clinically relevant specifically for ER^- breast cancer progression. Together with the observed inhibitory role of the miR-520/373 family in *in vitro* and *in vivo* invasion assays, these data indicate that miR-520c might be a key regulator of metastasis formation selectively in the ER^- breast cancer subtype.

Discussion

Several studies have demonstrated the regulation of the NF-κB pathway by miRNAs, such as miR-301a in pancreatic cancer (Lu *et al*., 2011), miR-146a in human monocytes (Taganov *et al*., 2006) and miR-199b in ovarian cancer (Chen *et al*., 2008). However, little is known about the regulation of NF-κB signaling in the context of breast cancer. In this study, we performed an unbiased genome-wide miRNA screen to identify miRNAs modulating NF-κB signaling. This screen revealed 13 miRNA families that significantly up- or downregulated NF-κB activity. Among those, we identified the miR-520/373 family as a strong inhibitor of NF-κB signaling through direct targeting of *RELA*. Consequently, the reduced transcriptional activity of NF-κB also inhibited the production of pro-inflammatory cytokines by breast cancer cells. Recently, numerous studies have demonstrated that inflammation and inflammatory cells associated with the tumor microenvironment promote tumor growth, angiogenesis and

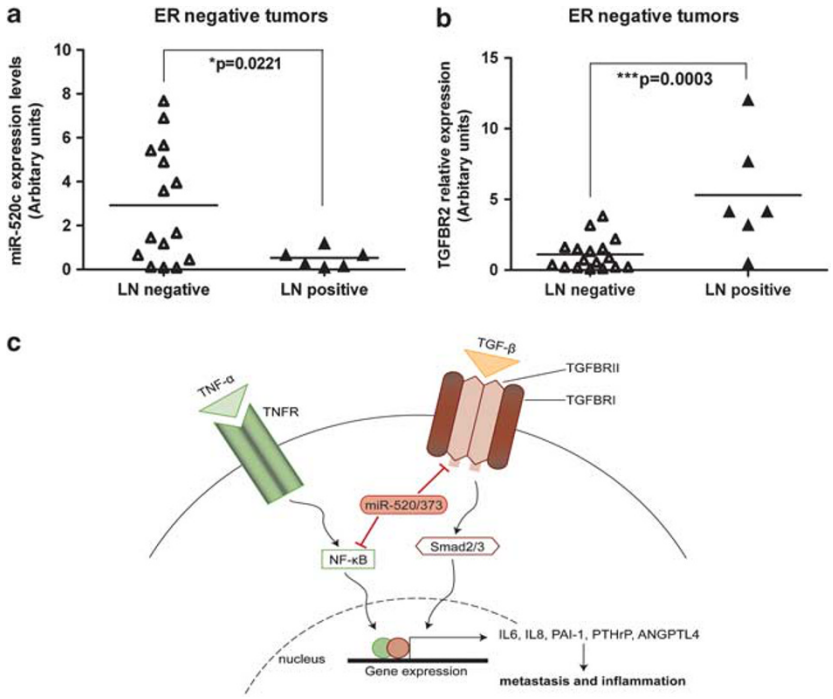

Figure 8 miR-520c is inversely correlated with lymph node metastasis in ER$^-$ breast cancer. (**a**) Expression of miR-520c in ER$^-$ primary tumors with ($n=6$) or without ($n=15$) lymph node metastasis (LN-positive or -negative). Vertical line represents the median. Data were analyzed using one-tailed unpaired *t*-test ($*P=0.0221$). (**b**) The same samples as in (**a**) were analyzed for *TGFBR2* expression levels (one-tailed unpaired *t*-test, $***P=0.0003$). (**c**) Model of the molecular mechanisms by which miR-520/373 regulate breast cancer cell invasion, metastasis and transcriptional regulation of metastasis-promoting genes.

metastasis (Hagemann *et al*., 2004; Cheng *et al*., 2005, 2011). Pro-inflammatory cytokines such as IL-6, IL-8 or CXCL1 enable tumor cells to intravasate, thus enhancing their metastatic potential (Wang *et al*., 2006; Wu *et al*., 2008; Iliopoulos *et al*., 2009; Walter *et al*., 2009; Yu *et al*., 2010). Moreover, co-cultivation of tumor cells with macrophages has been reported to promote invasiveness of breast cancer cells via TNF-α secretion from the macrophages (Hagemann *et al*., 2004). In addition, there is evidence that tumor-associated macrophages are positively associated with angiogenesis and negatively with prognosis in breast cancer (Leek *et al*., 1996). Interestingly, we did not observe any effect of *RELA* knockdown on breast cancer cell invasion (Supplementary Figure S4), although NF-κB inhibition at the IκB Kinase (IKK) level (Park *et al*., 2007) or by expressing the IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) super-repressor (Huber *et al*., 2004) has previously been described to reduce metastasis formation in advanced breast tumors. We speculate that other NF-κB subunits such as RelB, c-Rel, p50 or p52 might compensate for this effect. Nevertheless, the miR-520/373 family might influence the interaction of cancer cells with the tumor microenvironment, including stroma cells or immune cells such as tumor-associated macrophages, via reduced secretion of IL-6 and IL-8. However, further studies are needed to reveal the contribution of miR-520/373 family-induced changes in breast cancer progression via altered NF-κB signaling and cytokine production.

Tumor-cell invasion and metastatic spread are indicative of advanced tumor progression and depend on changes in cell–cell and cell–matrix adhesion. There, TGF-β signaling has been described as one important factor through the regulation of epithelial-to-mesenchymal transition and the transcriptional control of metastasis-related genes (Yin *et al*., 1999; Weigelt *et al*., 2005; Bierie and Moses, 2006; Padua *et al*., 2008; Padua and Massague, 2009; Ganapathy *et al*., 2010). Here, we have shown that TGF-β signaling is inhibited through direct suppression of *TGFBR2* by the miR-520/373 family, thus leading to a reduced expression of the Smad-dependent tumor-promoting genes *PAI-1, PTHrP* and *ANGPTL4*. Therefore, we hypothesized a putative connection of this miRNA family to breast cancer progression and metastasis. Indeed, our findings suggest a tumor-suppressive function for miR-520/373 family specifically in ER$^-$ breast cancer via direct suppression of *TGFBR2*. These results are consistent with previous studies that have reported a tumor-promoting role for TGF-β signaling in human ER$^-$ mammary cancer. There is clinical and molecular evidence that PTHrP and ANGPTL4 are critical mediators of distant breast cancer metastasis formation. In particular, TGF-β in the tumor microenvironment has been shown to prime breast cancer cells for selective lung metastatic seeding via upregulation of ANGPTL4 (Padua *et al*., 2008). Of note, in a murine model of bone metastasis, expression of a dominant-negative mutant of TGFBR2 (TβRIIΔcyt) in MDA-

MB-231 cells resulted in less bone destruction and prolonged survival compared with control, and this effect was mediated by transcriptional regulation of PTHrP (Yin *et al*., 1999).

The miR-520/373 clusters had initially been reported to be specifically expressed in human embryonic stem cells (Suh *et al*., 2004; Laurent, 2008; Ren *et al*., 2009). However, recently they have been associated also with cancer. In particular, the miR-520/373 family seems to have a dual role in tumor progression, depending on the cancer type and context: miR-373 has been described to function as an oncogene in testicular germ tumors (Voorhoeve *et al*., 2006), esophageal cancer (Lee *et al*., 2009) and hepatocellular carcinoma (Wu *et al*., 2011), while in cholangiocarcinoma this miRNA has been shown to act as tumor suppressor (Chen *et al*., 2010). Huang *et al*. (2008) identified miR-373 and miR-520c as promoters of breast cancer metastasis by suppressing the expression of CD44. Overexpression of miR-373 or miR-520c in ER^+ and non-invasive MCF-7 cells enhanced their metastatic potential. These findings appear to be contradictory to our *in vitro* and *in vivo* observations with the ER^- MDA-MB-231 cells, however, could be explained by the fact that breast cancer is a heterogeneous disease, separating into different cancer subtypes. Consequently, the opposing effects of miRNAs could be cancer type and context dependent. This is in line with a previous study where expression profiling of ER^+ and ER^- breast cancer patients revealed distinct miRNA-signatures correlated with tumor aggressiveness. There, miR-7, miR-128a and miR-516-3p were shown to be associated with tumor aggressiveness specifically in the ER^+ subtype (Foekens *et al*., 2008). The clinical data obtained in our study support this hypothesis; a significant downregulation of miR-520c expression in LN-positive breast cancer samples was observed specifically in ER^- tumors but not in ER^+ tumors. In support of our findings, TGF-β signaling had previously been correlated with the formation of distant metastasis in ER^- breast tumors, which was not the case for ER^+ tumors (Padua *et al*., 2008). Moreover, clinical studies in ER^- tumors demonstrate that a high expression of *TGFBR2* is correlated with shorter overall survival compared with patients with low *TGFBR2* expression. However, in ER^+ tumors *TGFBR2* expression did not influence overall survival (Buck *et al*., 2004). These differences might be attributed to different functions signaling pathways, such as TGF-β, might have even within the same cancer type, depending on the cellular context and the time point of activity (Tang *et al*., 2003). Thus, our data support a context-dependent effect of miR-520/373 on patient outcome. In a recent study that aimed at the identification of genes regulating TGF-β signaling in HaCaT keratinocytes, miR-373 was coincidentally identified as a regulator of TGFBR2 (Schultz *et al*., 2011). This finding demonstrates that the regulation of TGF-β signaling via miR-520/373 is not restricted to breast cancer and that further studies are thus needed to better understand this complex regulation.

In summary, we have identified the miR-520/373 family as a negative regulator of *in vitro* cell invasion and *in vivo* intravasation of ER^- breast cancer cells. These *in vitro* and *in vivo* findings were further validated in patient samples, where miR-520c expression was inversely correlated to lymph node metastasis in the ER^- but not in the ER^+ tumors. Mechanistically the tumor-suppressive role of miR-520/373 family can be attributed to inhibition of two different pathways; NF-κB and TGF-β signaling. The first regulation occurs via direct targeting of *RELA,* whereas the latter occurs via direct targeting of *TGFBR2*. To conclude, our results add a mechanistic insight regarding the role of miR-520/373 in the aggressiveness of ER^- breast cancer subtype. These findings do not only reveal the direct targeting of certain cellular components by miR-520/373, but also provide information about the cross-talk between functionally related pathways that cooperate to promote metastasis (Figure 8c) (Christofori, 2006). Hence, miR-520/373 act as a link between the NF-κB and TGF-β pathways and may contribute to the interplay of tumor progression, metastasis and inflammation which, however, might well be specific for certain cancer subtypes. Here, we revealed a tumor-suppressive function of miR-520/373 family in this particular subtype of breast cancer. Therefore, these miRNAs might have important implications for preventing ER^- breast cancer metastasis formation. As ER^- breast cancer presents a therapeutic challenge due to the lack of molecular targets, a therapeutic miRNA approach, where several oncogenic pathways are targeted, might prove more efficient than current strategies that target only one gene within a certain pathway.

Materials and methods

All experimental procedures and reagents used are summarized in supplementary Materials and methods.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)