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~~The ER β inversely regulated miR-10b and miR-145 are key targets for aggressiveness, epithelial-to-mesenchymal transition and matrix expression of breast cancer cells~~

Estrogen receptor beta as epigenetic mediator of miR-10b and miR-145 in mammary cancer

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Abbreviations: ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal-transition; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; IGF-IR, insulin-like growth factor receptor type I; miR, microRNA; MMP, matrix metalloproteinase

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Abstract

Even though the role of estrogen receptor alpha (ER α) in the modulation of breast cancer cells' behavior is thoroughly studied, the biological functions of its isoform, ER β , are less elucidated. The suppression of ER β in the aggressive ER α -negative MDA-MB-231 breast cancer cells resulted in the inhibition of epithelial to mesenchymal transition (EMT) ~~through~~ and major changes in the basic functional properties expression levels of certain matrix components of breast cancer cells. This arrest in metastatic potential of breast cancer cells suggests the contribution of ER β in the induction of a more aggressive phenotype in MDA-MB-231 breast cancer cells. The epigenetic alterations are responsible for the ability of the tumor cells to metastasize. Here, we report for the first time that the suppression of ER β in MDA-MB-231 breast cancer cells leads to significant changes in the expression profiles of specific microRNAs, including miR-10b, miR-200b and miR-145. Growth of MCF-7 and MDA-MB-231 cells with estrogen-free medium has a diverse impact on miRNA expression and the behaviour of these cells suggesting the diverse effect of estradiol on the miRNA expression profile depending on the ER status of breast cancer cells. ~~Transfections of miR-10b precursor and miR-145 inhibitor clearly revealed~~ Enhanced miR-10b expression or silencing of miR-145 clearly revealed that these microRNAs can regulate the functional properties, EMT program and the expression of major matrix components known to be implicated in breast cancer aggressiveness. Our data revealed that miR-10b is strongly implicated in the regulation of functional properties, EMT program and Erk1/2 signaling in shER β MDA-MB-231 cells, thus affecting the extracellular matrix (ECM) composition, including syndecan-1, proteolytic behaviour, especially MMP2, MMP7 and MMP9 expression and subsequently the aggressiveness of these cells. Accordingly, the inhibition of miR-145 expression significantly increased the aggressiveness of shER β MDA-MB-231 cells and induced EMT. Moreover, miR-145 inhibition resulted in important changes in the gene and protein levels of ECM mediators, such as HER2 and several MMPs, whereas it significantly increased the phosphorylated levels of Erk1/2 kinases in these cells, suggesting the crucial role of miR-145 in this signaling pathway. These novel results suggest that the alterations in cell behaviour and in ECM composition caused by the suppression of ER β in MDA-MB-231 cells are closely related to certain epigenetic miRNA-induced alterations. Targeting the ER β -regulated miR-10b and miR-145 is a promising tool for diagnosis and pharmaceutical targeting in breast cancer.

Introduction

Breast cancer is the most prevalent type of cancer among women, worldwide with the hormone receptor-positive tumor being present in most breast cancer patients [1]. Estrogen receptors (ERs) are among the principal signaling molecules that regulate several cell functions, acting either through the genomic or through the non-genomic pathway [2-4]. Cell signaling via the estradiol (E2)-ER axis affects also the cell behavior and the expression of the extracellular matrix (ECM) macromolecules, thus altering the tumor microenvironment. ECM is a dynamic scaffold, consisting of a variety of functional components, such as proteoglycans, glycoproteins and proteinases [5-12]. This network provides structural stability but could also mediate tumor development and progression through interactions between its macromolecules [13-19]. Alterations in the expression of matrix components result in the remodeling of ECM, thus affecting its ability to regulate several important functions of cancer cells, such as proliferation, migration, adhesion and invasion [10, 20-27]. The expression of major matrix macromolecules is affected by ERs suggesting a critical role of endocrine regulation for the tumor microenvironment and thus the functional properties of breast cancer cells [28-31]. Using the ER α -negative and ER β -positive, highly invasive MDA-MB-231 breast cancer cells, we have recently established a stable transfected ER β -suppressed cell line (named shER β MDA-MB-231) [32]. The suppression of ER β in MDA-MB-231 breast cancer cells evoked striking changes in the gene and protein expression levels of significant ECM components, such as proteoglycans, matrix degrading enzymes, cell surface receptors and signalling molecules, which in turn mediated the behaviour of breast cancer cells. In addition, the suppression of ER β in these cells inhibited epithelial-to-mesenchymal transition (EMT), a critical process for the initiation of metastasis [33, 34], leading to a less aggressive phenotype with decreased cell proliferation, migration and invasion potential [32].

Of particular interest in the understanding of cancer cell behavior is the field of microRNAs (miRNAs). They are endogenous non-coding RNA molecules, which have an important roles in post-transcriptional regulation of a broad set of a wide range of numerous cellular processes [35, 36]. By binding of their 'seed' region to the 3'-untranslated region of target mRNAs, miRNAs are capable of either inducing mRNA degradation via the RNA-induced silencing complex, or of inhibiting mRNA translation [37]. During the last decade, several reports associated the altered expression of various

miRNAs with cell proliferation, resistance to apoptosis, differentiation, immunity and cancer initiation, progression and metastasis. MiRNAs can directly modulate the EMT program and it is well established that ER α and ER β genes are among the direct targets of several miRNAs [38-41]. ~~Moreover, miRNAs are responsible for the regulation of ECM components and of their cellular receptors.~~ In breast cancer, overexpression of the transmembrane heparan sulfate proteoglycan (HSPG) syndecan-1, a predicted target of the pro-metastatic miR-10b, correlates with poor clinical outcome [42, 43]. Recent study has proven that the onco-miR-10b targets specifically syndecan-1 in breast cancer, highlighting the functional relationship or miRNAs and proteoglycans as well as indicating new evidence for the development of new therapeutic approaches for breast cancer [44]. On the other hand, miR-200b is a negative regulator of the EMT process and cancer metastasis. Several reports demonstrated that decreased expression levels of miR-200b are correlated with metastatic breast cancer and poor clinical outcome [45, 46]. There is contradicting evidence regarding the role of miR-145 in breast cancer progression. However, several reports reveal that miR-145 is responsible for the increased apoptosis in cancer cells as well as the decreased cell proliferation, proposing it as a putative tumor suppressor [47-50]. MicroRNA-dependent modulation of the ECM and its cellular receptors has emerged as a novel mechanism of regulating numerous matrix-dependent processes, including cell proliferation and apoptosis, cell adhesion and migration and cell differentiation and have been proposed as a promising target for the development of new therapeutic approaches for breast cancer [51].

In response to the above, we evaluated whether the different ER status could have an impact on the expression profiles of certain miRNAs and on the other hand to investigate the effect of ER-related miRNAs on the basic functional properties, EMT program and matrix composition of breast cancer cells. For these purposes, we utilized the ER α -positive and low aggressive MCF-7, ER α -negative and ER β -positive MDA-MB-231 and the shER β MDA-MB-231 breast cancer cells. The data obtained demonstrate that the knockdown of ER β is followed by significant changes, similar to ER α -positive MCF-7 cells, in the expression profiles of various miRNAs that have been correlated with breast cancer progression, with miR-10b and miR-145 to be major players that are regulated inversely. Moreover, to evaluate the possibility of targeting breast cancer at the level of these two miRNAs, transfections with the respective antisense (inhibiting expression) and precursor (enhancing expression) of the inversely regulated miRNAs were performed. The data demonstrated that miR-10b and miR-145 regulate the functional

properties of shER β MDA-MB-231 breast cancer cells, highlighting the novel role of ER β , along with epigenetic alterations of miRNAs in breast cancer cell behavior.

Results

ER β is associated with distinct morphology and microRNA expression patterns in breast cancer cells

Our group has recently demonstrated that the strong suppression of ER β in ER β -positive MDA-MB-231 breast cancer cells reduced their aggressiveness and lead to striking changes in EMT process as well as in the expression and activity profiles of various ECM components [32]. Interestingly, immunofluorescence analysis revealed that the suppression of ER β gene in shER β MDA-MB-231 cells resulted in the substantial decrease in the protein levels of this ER compared to MDA-MB-231 breast cancer cells (Fig. 1A). As shown in Fig. 1B (panel B1), scanning electron microscopy (SEM) showed that MDA-MB-231 cells have a globular and spindle-like shape and appear distant to each other. Notably, when MDA-MB-231 breast cancer cells were seeded onto a matrix-coated millipore filter, they presented an elongated shape with numerous cytoplasmic protrusions (lamellipodia/ invadopodia) (Fig. 1B, panel B2). On the other hand, ER α -positive MCF-7 cells exhibit cell-cell junctions (Fig. 1B, panel B3) and are grown by forming cell aggregates, whereas shER β MDA-MB-231 breast cancer cells are more round with less cytoplasmic protrusions and have the tendency to form cell aggregates (Fig. 1B, panel B4). The above data confirm the importance of ER α and ER β in the morphology, growth and invasive properties of breast cancer cells.

It is well established that miRNAs regulate several breast cancer functional properties as well as the expression of matrix components, however little is known about their ER β -dependent modulation. To evaluate the effects of ER β in miRNA expression, several miRNAs that are reported to be important regulators of breast cancer progression were screened by qPCR (Fig. 1C). Our data pinpointed that the suppression of ER β resulted in a statistically significant downregulation of the pro-metastatic miR-10b, whereas it significantly induced the expression levels of the metastatic miR-let-7d, miR-200b and the tumor-suppressor miR-145, as compared to MDA-MB-231 breast cancer cells. Importantly, the ER β suppression resulted in a 45% decrease of miR-10b and 11-fold increase in miR-145 expression levels, as compared to MDA-MB-231 cells. Notably, concerning the basal miRNA expression levels of ER α -positive MCF-7 breast cancer

cells, a 99% decrease of miR-10b, a 14- and a 34-fold increase of miR-145 and miR-200b expression levels was observed, respectively, compared to MDA-MB-231 breast cancer cells.

Estrogen-free medium induces alterations in miRNA expression and EMT markers in MCF-7 and MDA-MB-231 breast cancer cells

In order to evaluate the effects of estrogens and the different ER status on the miRNA profile, we performed long-term growth of ER α -positive MCF-7 and ER α -negative MDA-MB-231 breast cancer cells with estrogen-free medium, followed by an evaluation of the expression levels of miR-10b, miR-200b and miR-145, related with breast cancer progression. Notably, when MCF-7 breast cancer cells were cultured with estrogen-free medium, the expression levels of miR-10b were significantly upregulated, whereas miR-200b and miR-145 expression levels were strongly downregulated, as compared to the control cells (Fig. 2A). These alterations were followed by the downregulation of the epithelial marker E-cadherin and the significant upregulation of the mesenchymal markers fibronectin and snail2/slug (Fig. 2B). This result supports the view that E2 that signals via ER α in MCF-7 breast cancer cells is the critical factor for the maintenance of the low expression levels of miR-10b in these cells. On the other hand, ~~treatment~~ growth in estrogen-free medium of ER β -positive MDA-MB-231 cells resulted in the reduced miR-10b expression levels, followed by the strong induction of miR-200b and miR-145 levels. Moreover, estrogen-free medium reduced the expression of fibronectin, vimentin and snail2/slug and the induction of E-cadherin, compared to normal medium (Fig. 2D). These data pinpointed the distinct role of E2 in the regulation of EMT and miRNA expression, depending on the ER status. Taking under consideration the above data, it becomes clear that ER β regulates the expression of miR-10b and miR-145, as its suppression resulted in the most profound effects among the miRNAs tested. Therefore, further studies regarding the effects of miRNAs on MDA-MB-231 and shER β MDA-MB-231 breast cancer cell properties and EMT program have been focused on miR-10b and miR-145.

miR-10b modulates the functional properties and EMT program of shER β MDA-MB-231 cells

It has been previously demonstrated that miR-10b expression is upregulated in clinical samples of metastatic breast cancer [52]. Moreover, miR-10b silencing inhibits breast

cancer metastasis *in vivo* in a mouse mammary tumor model [53]. These observations prompted us to further investigate the role of miR-10b in the regulation of breast cancer cell behavior. The suppression of ER β resulted in the significant downregulation of miR-10b in shER β MDA-MB-231 breast cancer cells, as compared to MDA-MB-231 cells (Fig. 3A). As a next step, we evaluated the role of miR-10b in the regulation of basic cancer cell properties (cell-viability, migration and invasion) in this breast cancer model, following transfections ~~treatment~~ with the precursor miR-10b (pre-miR-10b), which results in ~~induces~~ miR-10b overexpression. Interestingly, miR-10b overexpression in shER β MDA-MB-231 cells (Fig. 3A) was accompanied by significant alterations in the functional properties of these cells (Fig. 3B). Our data depicted that miR-10b overexpression resulted in significant upregulation of shER β MDA-MB-231 cell growth, as compared to the control miRNA (control miR) shER β MDA-MB-231 cells. Furthermore, ~~shER β MDA-MB-231 cells~~ miR-10b overexpression in shER β MDA-MB-231 cells seems to significantly boost ~~attenuate~~ their invasive capacity following the ~~pre-miR-10b treatment~~ (ca 85% upregulation compared to the control miR shER β MDA-MB-231). Moreover, an important increase in the migratory capacity of shER β MDA-MB-231 cells overexpressing ~~treated with the pre-miR-10b~~ was also observed, as compared to the control miR shER β MDA-MB-231.

SEM microscopy showed that the induction of miR-10b expression in shER β MDA-MB-231 breast cancer cells resulted in important morphological changes, whereas the mesenchymal phenotype of MDA-MB-231 breast cancer cells was not substantially affected (Fig. 3C). Notably, shER β MDA-MB-231 cells transfected with pre-miR-10b gain the features of highly mobile cells, as the formation of pseudopodia (arm-like) and filopodia (thread-like) was more prominent compared to controls. These observations are followed by clear changes in cell cytoskeleton (F-actin staining) of shER β MDA-MB-231 cells (Fig. 3D). The F-actin staining for cytoskeleton confirms the mesenchymal-like characteristics that shER β MDA-MB-231 cells exhibited after pre-miR-10b transfection, in comparison with the characteristic cytoskeleton formation of shER β MDA-MB-231 cells.

The observed changes in the phenotype of shER β MDA-MB-231 breast cancer cells as well as in their functional properties generated the question whether miR-10b is also capable of modulating the EMT program. Confocal microscopy showed that pre-miR-10b transfection of shER β MDA-MB-231 cells resulted in increased protein levels of the mesenchymal marker vimentin and the downregulation of the epithelial marker E-

cadherin (Fig. 4A). Moreover, the overexpression of miR-10b significantly affected the expression levels of important EMT markers (Fig. 4B). Specifically, in shER β MDA-MB-231 cells transfected with pre-miR-10b the expression levels of the epithelial marker E-cadherin were decreased, whereas the mesenchymal markers vimentin, fibronectin and Snail2/Slug were significantly upregulated compared to the control miR. It is well established that the receptor tyrosine kinases, IGF-IR and EGFR are important players in the cross-talk with ERs and the functional properties of breast cancer cells [28, 30, 32, 54, 55]. As shown in Fig. 4C, pre-miR-10b significantly increased the low expression levels of IGF-IR and HER2, inhibited by ER β suppression, in shER β MDA-MB-231 cells. On the other hand, the expression levels of EGFR in shER β MDA-MB-231 cells were not significantly affected by pre-miR-10b. It is worth noticing that the decreased levels of VEGF in the ER β -suppressed cells were significantly induced following pre-miR-10b transfection, suggesting a crucial role of miR-10b in the induction of the angiogenic and aggressive potential in shER β MDA-MB-231 cells.

ECM molecules such as syndecans and matrix metalloproteinases play critical roles in the cell-matrix interactions, cell signaling and adhesion/migration of breast cancer cells [13, 14, 56]. Regarding the effect of miR-10b on cell membrane proteoglycans, pre-miR-10b resulted in the strong downregulation of syndecan-1 mRNA and protein levels in shER β MDA-MB-231 cells. As far as the regulation of proteolytic enzymes is concerned, the overexpression of miR-10b resulted in the strong induction of the expression levels of MMP2, MMP7 and MMP9 (Fig. 4C). As it is depicted in Fig. 4D, pre-miR-10b transfection resulted in the significant upregulation of the phosphorylated Erk1/2 forms in shER β MDA-MB-231 cells (ca 285%). Taken together, these results clearly demonstrate that miR-10b is strongly implicated in the regulation of functional properties, EMT program and signaling in shER β MDA-MB-231 cells, thus affecting the ECM composition, proteolytic behavior and metastatic potential of these cells.

miR-145 inhibition induces significant changes in the functional properties and EMT markers of shER β MDA-MB-231 cells

As mentioned above, the suppression of ER β resulted in the strong increase of the expression levels of miR-145, as compared to MDA-MB-231 cells (Fig. 1C). Therefore, it becomes plausible to investigate the role of this miRNA in our breast cancer model. For this reason, MDA-MB-231 and shER β MDA-MB-231 breast cancer cells were transfected with the antisense oligonucleotide that inhibits the expression of miR-145

(Fig. 5A). The inhibition of miR-145 (anti-miR-145) in shER β MDA-MB-231 cells resulted in the increase of cell growth as well as in the profound upregulation of their migratory and invasive capacity, suggesting its protective role in the initiation of a more aggressive profile in these cells (Fig. 5B). Notably, the inhibition of miR-145 does not affect the functional properties of MDA-MB-231 cells. In Fig. 5C, the SEM images of MDA-MB-231 and shER β MDA-MB-231 cells transfected with control miRNA and anti-miR-145 clearly depict that the decreased expression of miR-145 in shER β MDA-MB-231 cells affects their morphology. This is evident due to the increase in their cytoplasmic protrusions and the alterations of their shape to an elongated one, whereas MDA-MB-231 cells were not affected by such treatment. Moreover, confocal microscopy revealed that the cytoskeleton of shER β MDA-MB-231 cells was clearly affected by the miR-145 decreased expression, as a disrupted F-actin network with elongated filaments can be observed (Fig. 5D).

Previous studies demonstrated that miR-145 suppresses growth and migration in breast cancer clinical samples relative to normal breast tissue and that the overexpression of miR-145 strongly inhibited EMT [57, 58]. To this end, using confocal microscopy we observed that the inhibition of miR-145 in shER β MDA-MB-231 breast cancer cells evokes the decrease of E-cadherin and the increase of vimentin protein levels (Fig. 6A). Moreover, anti-miR-145 results in the strong downregulation of E-cadherin and the significant increase of vimentin, fibronectin and Snail2/Slug expression levels in shER β MDA-MB-231 cells (Fig. 6B). Notably, in control MDA-MB-231 cells anti-miR-145 has no effect on the expression profiles of E-cadherin and vimentin, but it increased the expression levels of fibronectin and Snail2/Slug, enhancing their aggressive phenotype. Furthermore, the effects of miR-145 inhibition on major matrix components in this breast cancer cell model were evaluated. Interestingly, as shown in Fig. 6C, shER β MDA-MB-231 cells transfected with anti-miR-145 are characterized by elevated levels of HER2 and followed by the unaltered levels of EGFR and IGF-IR. The inhibition of miR-145 expression decreased the expression of EGFR, IGF-IR and HER2 in MDA-MB-231 cells. Regarding the effects on the components of proteolytic cascade, the transfection of shER β MDA-MB-231 cells with anti-miR-145 resulted in the significant increase of MMP2, MMP7 and MMP9 expression, whereas MDA-MB-231 cells remain unaffected. Notably, the inhibition of miR-145 in shER β MDA-MB-231 cells resulted in the strong upregulation of the phosphorylated Erk1/2 forms in these cells that is correlated with the high growth levels induced by anti-miR-145 (Fig. 6D). These data suggest that miR-145

serves as a protective partner regarding the low aggressiveness of breast cancer cells, as its inhibition results in the induction of EMT program by increasing their aggressive behavior through important changes in their functional properties and the expression of major matrix components of shER β MDA-MB-231 cells breast cancer cells.

Discussion

It is well established that miRNAs are powerful mediators of the development, metastasis and progression of breast cancer, therefore the investigation of their functions is of great clinical relevance and may provide novel targets for therapeutic approaches in breast cancer [36, 59]. ~~The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs [37].~~ Numerous miRNAs have been identified as regulators of breast cancer cell properties [60-62]. While some miRNAs act as tumor suppressors, others act as oncogenes, depending on their respective molecular targets. MiR-10b has been demonstrated as a pro-metastatic miRNA as it positively regulates cell migration and invasion *in vitro* and it initiates tumor invasion *in vivo* [41, 63, 64]. Moreover, its expression levels are elevated in metastatic breast cancers [41, 44]. Importantly, the mRNAs encoding ER α and ER β are targets of this miRNA [38]. In contrast to the onco-miR-10b, miR-200b is linked to the mesenchymal phenotype of breast cancer cells, as it is one of the negative regulators of the EMT process, invasion and metastasis [39, 65]. Downregulated expression levels of this miRNA can be found in metastatic and breast cancer stem cell-like cells *in vitro* [66, 67]. Moreover, it is demonstrated that members of the miR-200 family target directly ER α and ER β [68]. There is contradicting evidence regarding the role of miR-145 in breast cancer. However, several reports reveal that miR-145 is responsible for the increased apoptosis in cancer cells as well as the decreased cell proliferation, proposing it as a putative tumor suppressor [47-50].

In this study, we evaluated the miRNA regulation in the EMT process and the functional properties of shER β MDA-MB-231 breast cancer cells. We pinpointed that miR-10b and miR-145 were among the most significantly deregulated miRNAs after the suppression of ER β in shER β MDA-MB-231 cells. These miRNAs are closely linked to the EMT program and could contribute to cell-cell and/or cell-ECM interactions, affecting crucial breast cancer cells properties [69]. The obtained data showed that miR-10b, which is overexpressed in clinical samples of metastatic breast cancer and positively regulates

tumor invasion *in vivo* [63], is significantly downregulated in shER β MDA-MB-231 cells, as compared to MDA-MB-231 cells.

Consistent with the ER α -dependent expression, E2 has a positive role in this regulation, as its absence from the culture medium significantly increased miR-10b expression levels in MCF-7 cells. Accordingly, miR-200b, which is negative modulator of tumor invasion and metastasis [66], has been found to be significantly upregulated in MCF-7 and shER β MDA-MB-231 breast cancer cells. Interestingly, ~~long-term treatment~~ growth in estrogen-free medium of MCF-7 cells resulted in decreased expression of miR-200b and miR-145 and significant increase in the expression levels of mesenchymal markers. On the other hand, when MDA-MB-231 cells were long-term cultured with estrogen-free medium, exhibited upregulated expression levels of miR-200b and miR-145, as well as decreased aggressiveness. These data suggest that miR-200b, together with the E2-ER α axis, are beneficial for the conservation of an epithelial-like phenotype for breast cancer cells.

It has been previously described that miR-10b promotes the invasiveness of breast cancer cells [70]. Interestingly, the induction of its expression by transfecting shER β MDA-MB-231 cells with pre-miR-10b caused a significant increase in proliferation, migration and invasion rates of these cells. Moreover, the induction of miR-10b expression resulted in important changes in cell morphology, EMT markers, as well as expression of cell surface receptors, ECM components, including syndecan-1, which is a target of miR-10b [44], and proteolytic enzymes such as MMP2, MMP7 and MMP9. These alterations were accompanied by the strong increase in the phosphorylated levels of Erk1/2 kinase, which has been correlated with breast cancer cell growth and motility [71], suggesting the critical role of miR-10b in this signaling pathway. These data suggest that the low expression levels of miR-10b following the ER β suppression - ~~suppressed~~ in shER β MDA-MB-231 cells, ~~miR-10b presence is~~ are beneficial for the conservation of an epithelial-like phenotype for breast cancer cells.

Functional analysis revealed that miR-145 overexpression results in the strong induction of the aggressiveness of shER β MDA-MB-231 cells, inducing striking changes in their morphology, affecting basal functional properties and expression profiles of several ECM modulators, such as MMPs. Moreover, we highlighted the role of miR-10b in the initiation of EMT in shER β MDA-MB-231 cells, as anti-miR-145 significantly boosted this process by downregulating the epithelial marker E-cadherin and increasing several mesenchymal markers, in mRNA and protein levels. Importantly, anti-miR-145 significantly induced the

phosphorylated levels of Erk1/2 signaling pathway, confirming the aggressive phenotype of shER β MDA-MB-231 cells induced by the inhibition of anti-miR-145. Overall, these data indicate that estrogen-dependent regulation of miR-145 expression is mechanistically related to the strong phenotypic changes observed in ER β -suppressed shER β MDA-MB-231 cells.

In conclusion, ~~miRNA represents an epigenetic code involved in gene regulation and it is clearly responsible for the effect of the cell microenvironment on cell behavior~~ epigenetic modifications of miRNA represent a code involved in gene regulation. Altered gene expression does not only affect the tumor cell itself, it also influences cell behavior by altering the ECM in the tumor microenvironment. Therefore, miRNAs may serve as prognostic and predictive biomarkers as well as therapeutic agents for breast cancer. The data from this study pinpointed that ER β together with specific miRNAs, including miR-10b and miR-145, are key players for the initiation of breast cancer cell aggressiveness. Here we report for the first time that ER β inversely regulates miR-10b and miR-145, which are critical modulators of functional properties, EMT and ECM composition of breast cancer cells. The studies on the epigenetic regulation of gene expression open a new research field that may shed light on molecular mechanisms that are critical in various physiological and pathological conditions. This new knowledge, summarized in Fig. 7, could serve as a groundwork for the future development of new drug targets to inhibit oncogenic signaling and breast cancer progression.

Experimental Procedures

Chemicals reagents and antibodies

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin were all obtained from Gibco BRL (Karlsruhe, Germany). All other chemicals used were of the best commercially available grade. Antibodies used are listed in Supplementary Table 1.

Cell culture

MDA-MB-231 (high metastatic potential, ER α -negative, ER β -positive) and MCF-7 (low metastatic potential, ER α -positive) breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and routinely cultured as monolayers at 37°C in a humidified atmosphere of 5% (v/v) CO₂ and 95% air. shER β MDA-MB-231 (ER β -suppressed cells) were previously described [32]. Breast cancer cells were cultured in

DMEM supplemented with 10% (v/v) FBS, 1% L-glutamine and 1% penicillin/streptomycin. Puromycin (0.8 µg/mL) was included in the cultures of shERβ MDA-MB-231 cells. Cells were harvested by trypsinization with 0.05% (w/v) trypsin in PBS containing 0.02% (w/v) Na₂EDTA. All experiments were conducted in serum-free conditions. The transfection reagent for the performed experiments was the DharmaFECT (Dharmacon, GE Healthcare, UK), which was used according to the manufacturer's instructions.

microRNA transfection

Transfections of MDA-MB-231 empty vector and shERβ MDA-MB-231 cells with the miRNA precursor pre-miR-10b, or the miRNA inhibitor anti-miR-145 (10nM, ABI, Darmstadt, Germany), or miR negative control (10nM, ABI) were performed according to the manufacturer's instructions and as described previously [47].

MTT cell proliferation assay

Breast cancer cells were seeded in 96-well plates (5,000 cells/well) 48h after the miRNA transfections and cultured for additional 48h. MTT assay was performed as described previously [47].

***In vitro* invasion assay**

In total, 25,000 breast cancer cells in 0.5 mL DMEM/10% FCS were added in triplicates to the upper compartments of Matrigel Invasion Chambers (BD Biosciences, Heidelberg, Germany) 48h after miRNA transfections. After 24h, the medium in the upper chamber was replaced by serum-free DMEM. After 48h, cells on the lower surface were fixed and stained with DiffQuik (Medion, Duedingen, Switzerland). Relative invasiveness was expressed as percentage of cells on compound-treated compared to control inserts (n > 3).

***In vitro* wound healing assay**

Transfected breast cancer cells were seeded in 12-well cell culture plates at a density of 25,000 cells/well. Breast cancer confluent cell layers were serum starved for 16 h and then wounded by scratching with a sterile 100 µL pipette tip. Detached cells were removed by washing twice with PBS and fresh culture medium, in the absence of serum, was added. The wound closure was monitored at 0, 16 and 24 h using a digital camera

connected to a microscope. Wound surface area was quantified by image analysis (Image J software).

RNA isolation and real-time PCR analysis

Total cellular RNA was isolated using rna-OLS (OMNI Life Science, Hamburg, Germany) and reverse transcribed (Advantage First strand cDNA synthesis kit; Fermentas, St. Leon-Rot, Germany). For miRNA isolation and analysis, the mirVana™ miRNA Isolation Kit (ABI) and the TaqManVR MicroRNA RT kit (ABI) were employed. Quantitative real-time PCR (qPCR) and melting curve analysis were performed using Qiagen QuantiTect SYBR Green PCR kit in a LightCycler (Roche, IN). Expression of miRNAs and additional mRNAs was analyzed using TaqMan probes on an ABI PRISM 7300 Sequence Detection System as described previously [47]. The $2^{-\Delta\Delta C_t}$ method was used to determine relative gene transcript levels after normalization to 18S rRNA. Primer sequences, QuantiTect assay and TaqMan probe IDs are listed in Supplementary Tables 2 and 3.

Confocal immunofluorescence microscopy

Transfected breast cancer cells were seeded onto coverslips in 24-well plate and incubated in DMEM containing 10% FCS for 24 h, fixed in 4% paraformaldehyde in PBS and permeabilized with PBS/0.1% TritonX-100. Nonspecific binding was blocked with PBS/1% Aurion BSA-c (DAKO, Glostrup, Denmark). Coverslips were subsequently incubated with the primary antibodies at 4°C overnight and incubated with Alexa Fluor-conjugated antibodies for 30 minutes at room temperature. Primary antibody omission served as a negative control. Slides were analyzed with the LSM 510 META confocal microscope equipped with the oil immersion objective Plan-Apochromat 63x/1.40 (Carl Zeiss, Jena, Germany). Representative images were analyzed by image analysis (ZEN Software).

SEM imaging

Breast cancer cells on a Matrigel Millipore filter or cells seeded in culture flasks 48h after miRNA transfections, were firstly rinsed with a phosphate buffer solution to prevent cells detachment and then fixed in a Karnovsky's solution for 20 min. Flasks with adhering cells were again rinsed three times with 0.1% cacodylate buffer, post-fixed in 1% OsO₄

in cacodylate buffer for 20 min, dehydrated with increasing concentrations of ethanol, and finally dehydrated with hexamethyldisilazane (Sigma-Aldrich Inc) for 15 min. The specimens were mounted on appropriate stubs, coated with a 5nm palladium gold film (Emitech 550 sputter-coater) to be observed under a SEM (Philips 515, Eindhoven, The Netherlands) operating in secondary-electron mode.

Western blot analysis

Cell lysates of breast cancer cells were prepared 72h after transfection with control or miRNAs as described previously [72]. Cell lysates were separated on 10% SDS-PAGE, the proteins were electrophoretically transferred to PVDF membranes (Bio-Rad, USA) and blotted with the indicated antibodies as described previously [72], using 30–60 µg of protein/lane.

Statistical analysis

Reported values are expressed as mean $\pm$ standard deviation (SD) of experiments in triplicate. Statistically significant differences were evaluated using the analysis of variance (ANOVA) test and were considered statistically significant at the level of at least $p \leq 0.05$. Statistical analysis and graphs were made using GraphPad Prism 6 (GraphPad Software).

Declarations of interest

The authors declare no competing interests.

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Author Contributions

Z.P. performed the main experimental part and prepared the manuscript draft and the figures, M.F. performed SEM image analysis and interpretation. M.G. and N.K.K. had the supervision of the experiments, demonstration of the data and contributed to manuscript

writing and editing. N.K.K. had also the overall supervision and submitted the manuscript. All authors reviewed the manuscript.

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Figure Legends

Fig. 1. ER β has a key role in the morphology and miRNAs expression of breast cancer cells. (A) Immunofluorescence analysis of ER β protein in MDA-MB-231 and shER β MDA-MB-231 breast cancer cells. (B) Scanning electron micrograph (SEM) of MDA-MB-231 (1), MDA-MB-231 cells on the upper surface of a Millipore filter coated with Matrigel (not shown), trying to pass through the pores (2), MCF-7 (3) and shER β MDA-MB-231 breast cancer cells. The lower surface of the Millipore filter presents cells moving through the holes of the Millipore filter after passing the Matrigel (metastasing cells). These cells show a rounded/globular aspect but also an elongated shape with lamellipodia/invadopodia. At the cell surface, cytoplasmic blebs (vesicles) and filopodia, characteristic of highly moving cells, are also detectable. The Millipore filter and the holes can be observed in the background. Bar, 10 μ m. (C) ER β suppression alters miRNA expression in shER β MDA-MB-231 breast cancer cells. The mRNA levels of miR10b, miR-let-7d, miR-145 and miR-200b were assessed by real-time PCR analysis in MDA-MB-231, MCF-7 and shER β MDA-MB-231 breast cancer cells and were normalized to the respective miRNA expression levels in MDA-MB-231 breast cancer cells. Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 2. The absence of E2 from the culture medium of MCF-7 and MDA-MB-231 respectively has a different impact, depending on the ER status, on the aggressiveness of these cells. (A) Long-term growth with estrogen-free medium induces miR-10b expression and downregulates miR-200b and miR-145 expression levels in MCF-7 breast cancer cells (ER α -positive). (B) Real-time PCR analysis of EMT markers (E-cadherin, fibronectin, Snail2/Slug), in MCF-7 cells cultured in regular or estrogen-free medium. The mRNA levels were evaluated using β -actin as reference gene. (C) Long-term culture with estrogen-free medium reduces miR-10b expression and upregulates miR-200b and miR-145 expression levels in MDA-MB-231 breast cancer cells (ER β -positive). (D) Real-time PCR analysis of EMT markers (E-cadherin, fibronectin, Snail2/Slug), in MDA-MB-231 cells cultured in regular or estrogen-free medium. The mRNA levels were evaluated using β -actin as reference gene. The mRNA levels of miR10b, miR-145 and miR-200b were assessed by real-time PCR and were normalized to the respective miRNA expression levels in breast cancer cells cultured in

regular/normal medium. Expression was normalized to 18S rRNA expression. Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 3. Evaluation of miR-10b effects on MDA-MB-231 and shER β MDA-MB-231 breast cancer cells. The experiments were performed following transfections with pre-miR-10b. (A) MDA-MB-231 and shER β MDA-MB-231 cells were transfected with a control miRNA (control miR) or a miR-10b precursor (pre-miR-10b) and the overexpression of miR-10b gene was monitored with real-time PCR analysis. Expression was normalized to 18S rRNA expression. (B) The effect of pre-miR-10b on functional properties of MDA-MB-231 and shER β MDA-MB-231 breast cancer cells. The induction overexpression of miR-10b enhances cell growth, migration (after 24h) and invasion of shER β MDA-MB-231 breast cancer cells. (C) Cell morphology was monitored through SEM microscopy. shER β MDA-MB-231 cells following pre-miR-10b expression stimulation exhibit more cytoplasmic protrusions (arrows), whereas the morphology of MDA-MB-231 was not affected following expression of treatment with pre-miR-10b. (D) Evaluation of cytoskeleton formation of breast cancer cells after pre-miR-10b transfection. Immunofluorescence analysis of F-actin staining and confocal microscopy in MDA-MB-231 and shER β MDA-MB-231 cells with or without pre-miR-10b (bar, 10 μ m). Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 4. The overexpression induction of miR-10b expression results in EMT reprogramming and significant alterations in the expression of ECM components. (A, B) Immunofluorescence (bar, 10 μ m) and real-time PCR analyses of EMT markers revealed that transfection of shER β MDA-MB-231 cells with pre-miR-10b attenuates E-cadherin and upregulates EMT markers vimentin, fibronectin, ZEB2 and Snail2/Slug. (C) Screening of major cell surface receptors (EGFR, IGF-IR and HER2), the angiogenic factor (VEGF), the cell membrane proteoglycans (syndecan-1) and major matrix metalloproteinases (MMP2, MMP7 and MMP9) following miR-10b overexpression induction in MDA-MB-231 and shER β MDA-MB-231 cells with real-time PCR analysis and immunofluorescence microscopy. The mRNA levels were evaluated using β -actin as reference gene. (D) Immunoblots of phospho-Erk1/2 and α -tubulin in MDA-MB-231 and shER β MDA-MB-231 cells in the presence or absence of pre-miR-10b. Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 5. miR-145 inhibition increases the aggressiveness of shER β MDA-MB-231 breast cancer cells. The experiments were performed following transfections with miR-145

inhibitor (anti-miR-145). (A) The silencing inhibition of miR-145 gene in MDA-MB-231 and shER β MDA-MB-231 cells was monitored with real-time PCR analysis. Expression was normalized to 18S rRNA expression. (B) The effects of anti-miR-145 on functional properties of MDA-MB-231 and shER β MDA-MB-231 breast cancer cells. miR-145 inhibition increases cell growth, migration (after 24h) and invasion of shER β MDA-MB-231 breast cancer cells. (C) Cell morphology was monitored through SEM microscopy. shER β MDA-MB-231 cells transfected treated with anti-miR-145 appeared more cytoplasmic protrusions (arrows), whereas the morphology of MDA-MB-231 was not affected following the silencing of treatment with anti-miR-145. (D) Evaluation of cytoskeleton formation of breast cancer cells after anti-miR-145 transfections. Immunofluorescence analysis of F-actin staining and confocal microscopy in MDA-MB-231 and shER β MDA-MB-231 cells with or without anti-miR-145 (bar, 10 μ m). Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 6. The silencing inhibition of miR-145 expression in shER β MDA-MB-231 cells results in striking changes in EMT markers and significant alterations in the expression of ECM components. (A) Confocal microscopy for immunostaining of E-cadherin and vimentin in MDA-MB-231 and shER β MDA-MB-231 cells revealed that anti-miR-145 decreases E-cadherin and upregulates vimentin levels in shER β MDA-MB-231 cells (bar, 10 μ m). (B) Real-time PCR analysis of EMT markers, E-cadherin, vimentin, fibronectin and Snail2/Slug in MDA-MB-231 and shER β MDA-MB-231 cells following transfections with anti-miR-145. (C) miR-145 silencing inhibition in shER β MDA-MB-231 cells affects major ECM mediators, including HER2, MMP2, MMP7 and MMP9. The mRNA levels were evaluated with real-time PCR analysis. Expression was normalized to β -actin expression. (D) Immunoblots of phospho-Erk1/2 and α -tubulin in MDA-MB-231 and shER β MDA-MB-231 cells in the presence or absence of anti-miR-145. Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 7. ER β is a key modulator of breast cancer cells' aggressiveness through miRNA regulation. (A) Long-term growth of MCF-7 cells (ER α -positive) with estrogen-free medium increases their aggressiveness following the upregulation of miR-10b and downregulation of miR-200b and miR-145 expression levels, compared to expression in normal medium. MDA-MB-231 (ER β -positive) breast cancer cells long-term cultured in estrogen-free conditions exhibited attenuated expression levels of miR-10b and increased levels of miR-200b and miR-145, which strongly decreased these cells'

aggressiveness, compared to the ones cultured in normal medium. (B) ER β inversely regulates the expression of miR-10b and miR-145. ER β suppression in shER β MDA-MB-231 breast cancer cells strongly upregulates the expression levels of miR-145 and eliminates miR-10b expression compared to MDA-MB-231 cells. Overexpression of miR-10b in shER β MDA-MB-231 cells increases their aggressiveness through striking changes in key players of EMT program as well as the expression of major signaling (Erk1/2) and matrix macromolecules (EGFR, IGF-IR, HER2, VEGF, syndecan-1, MMP2, MMP7, MMP9). Accordingly, the silencing inhibition of miR-145 expression increases the metastatic potential of shER β MDA-MB-231 cells and critically affects EMT program, their functional properties, the expression of specific matrix components (HER2, MMP2, MMP7, MMP9) and signaling molecules (Erk1/2). Downregulated genes are depicted with red and upregulated genes are depicted with blue.

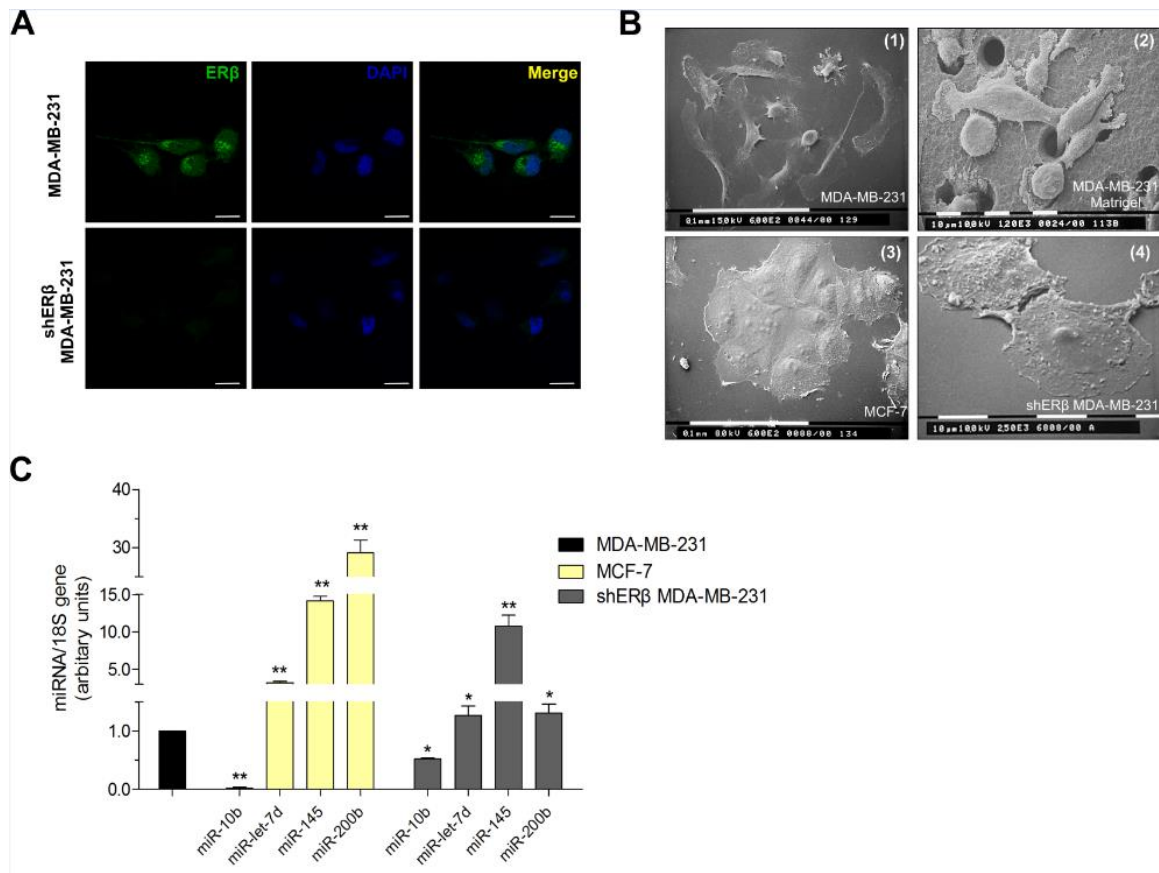


Figure 1

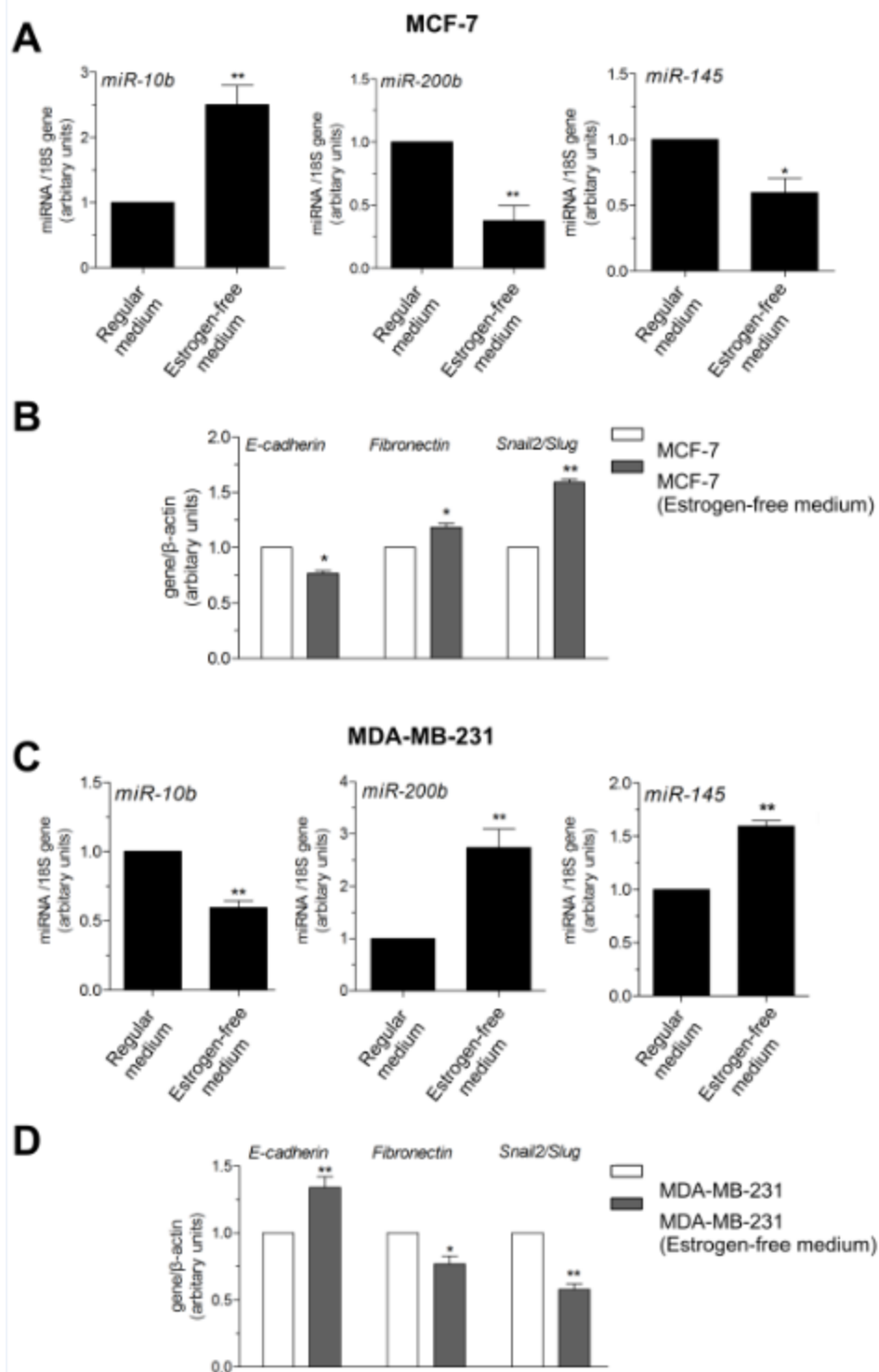


Figure 2

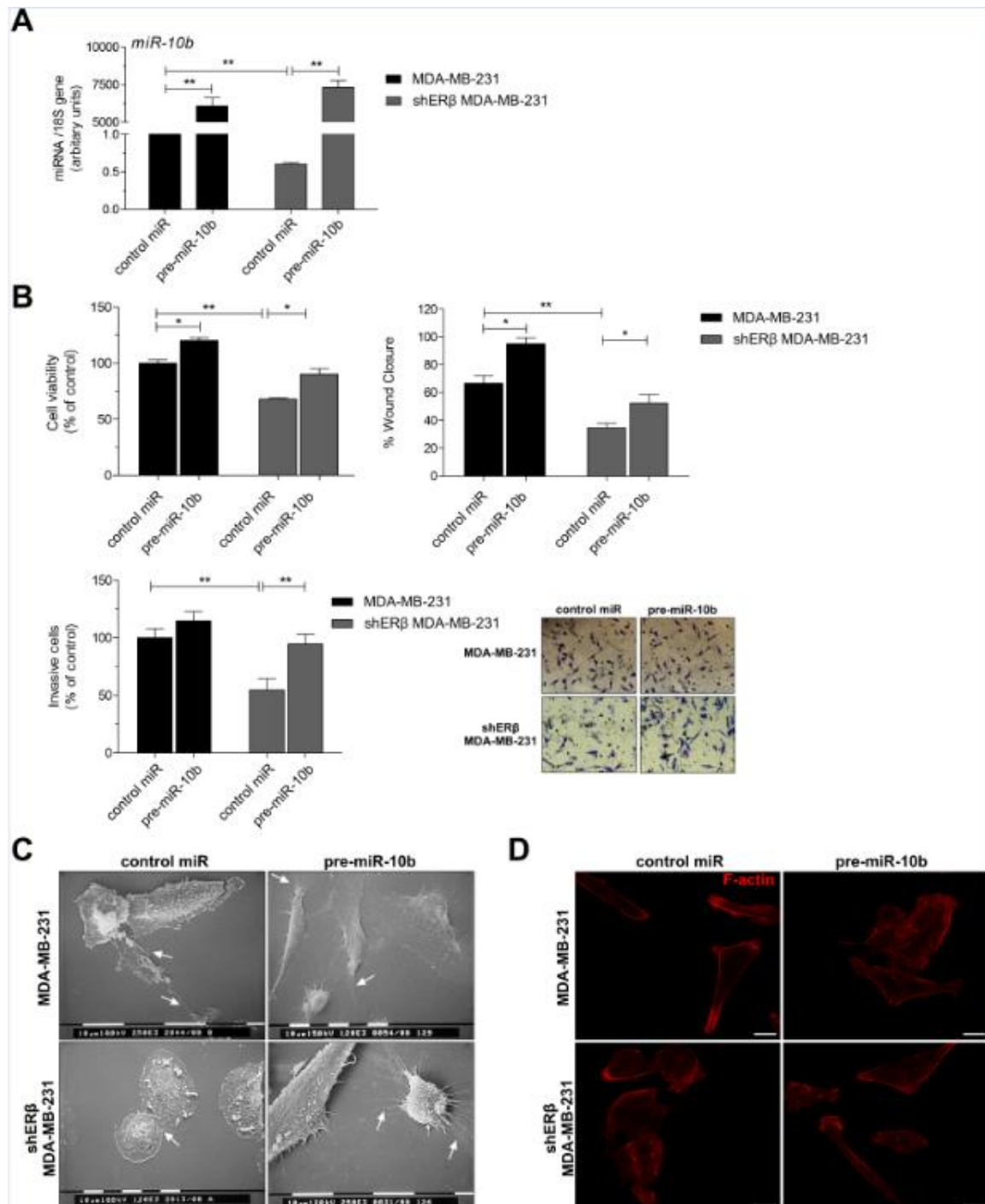


Figure 3

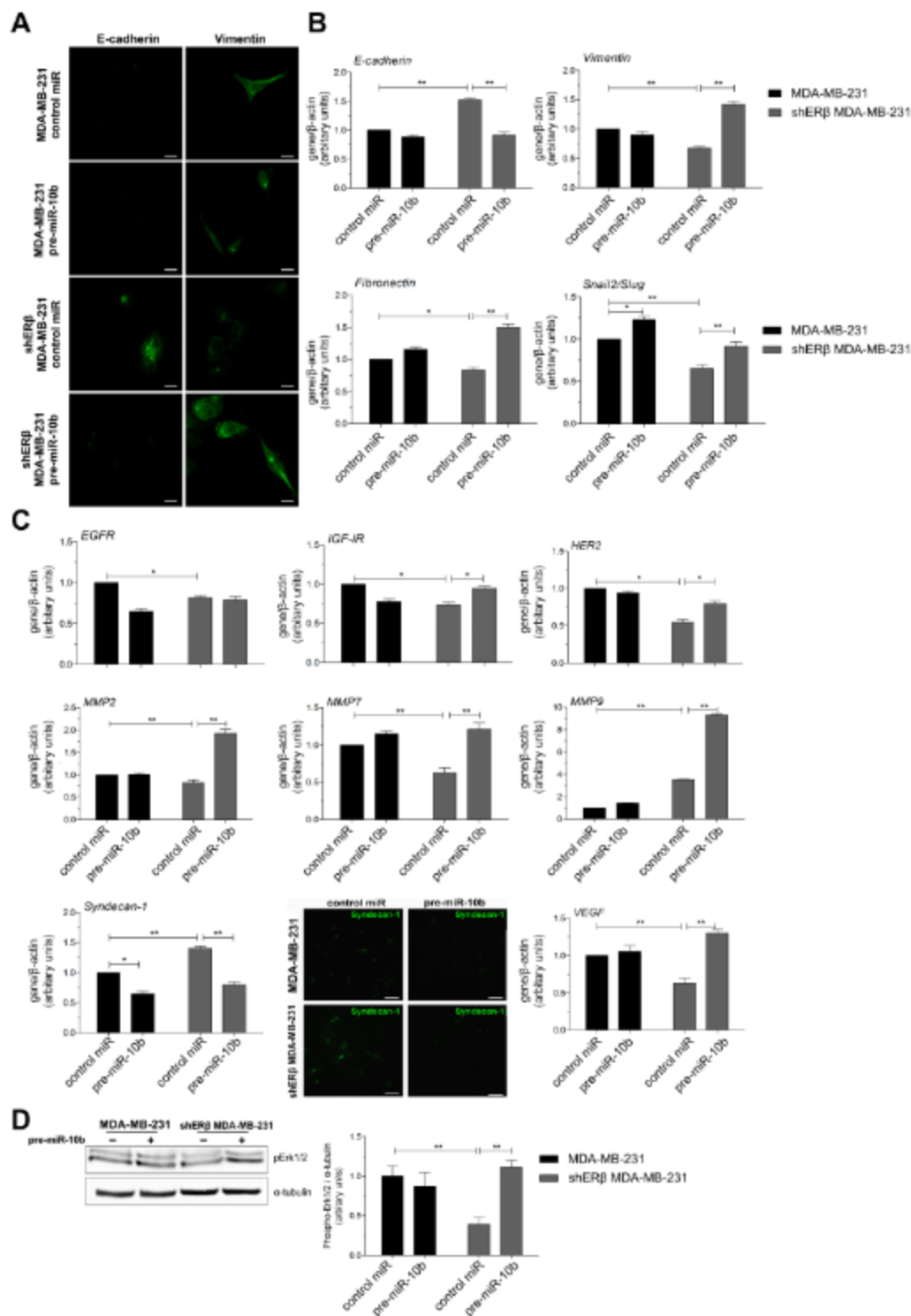


Figure 4

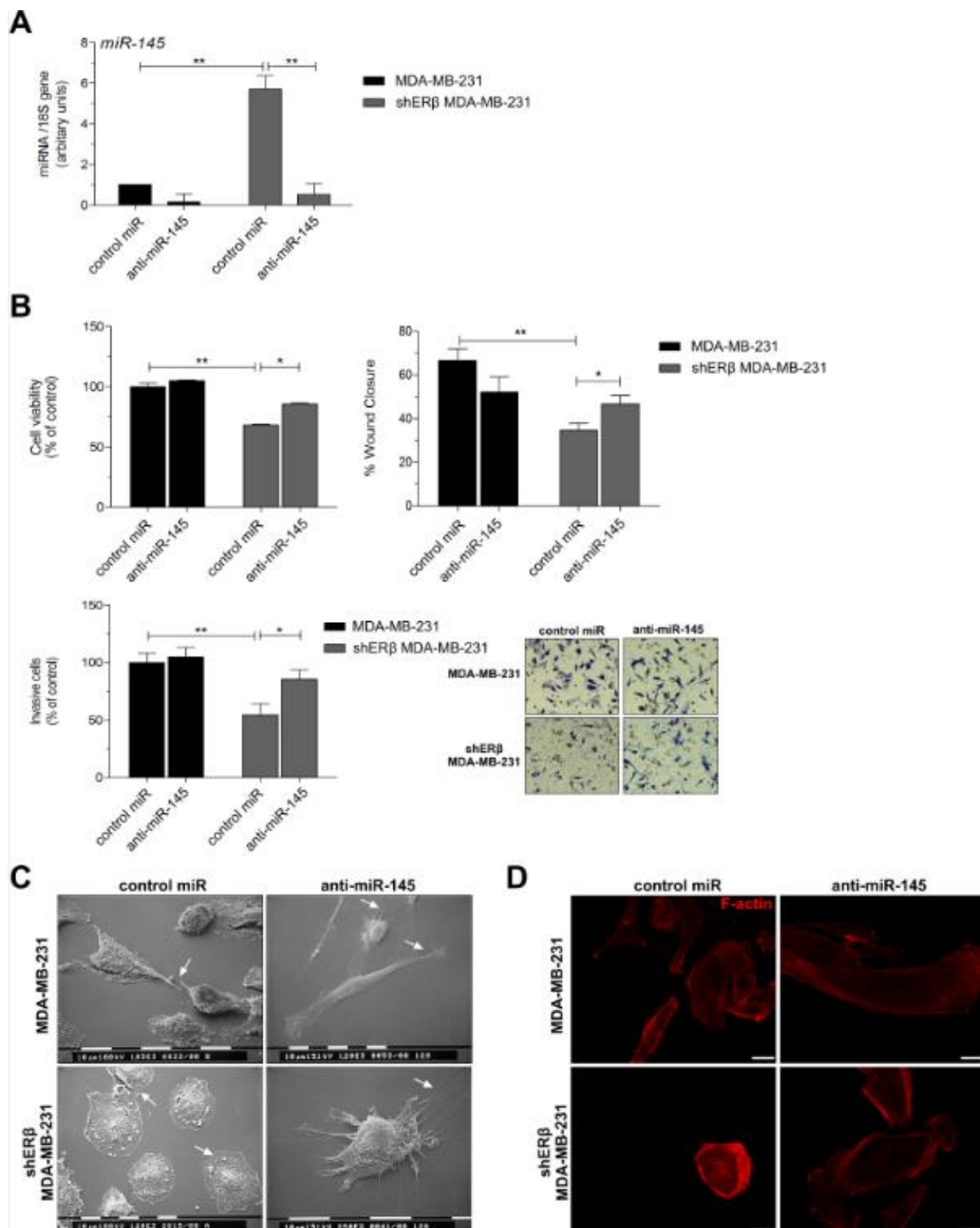
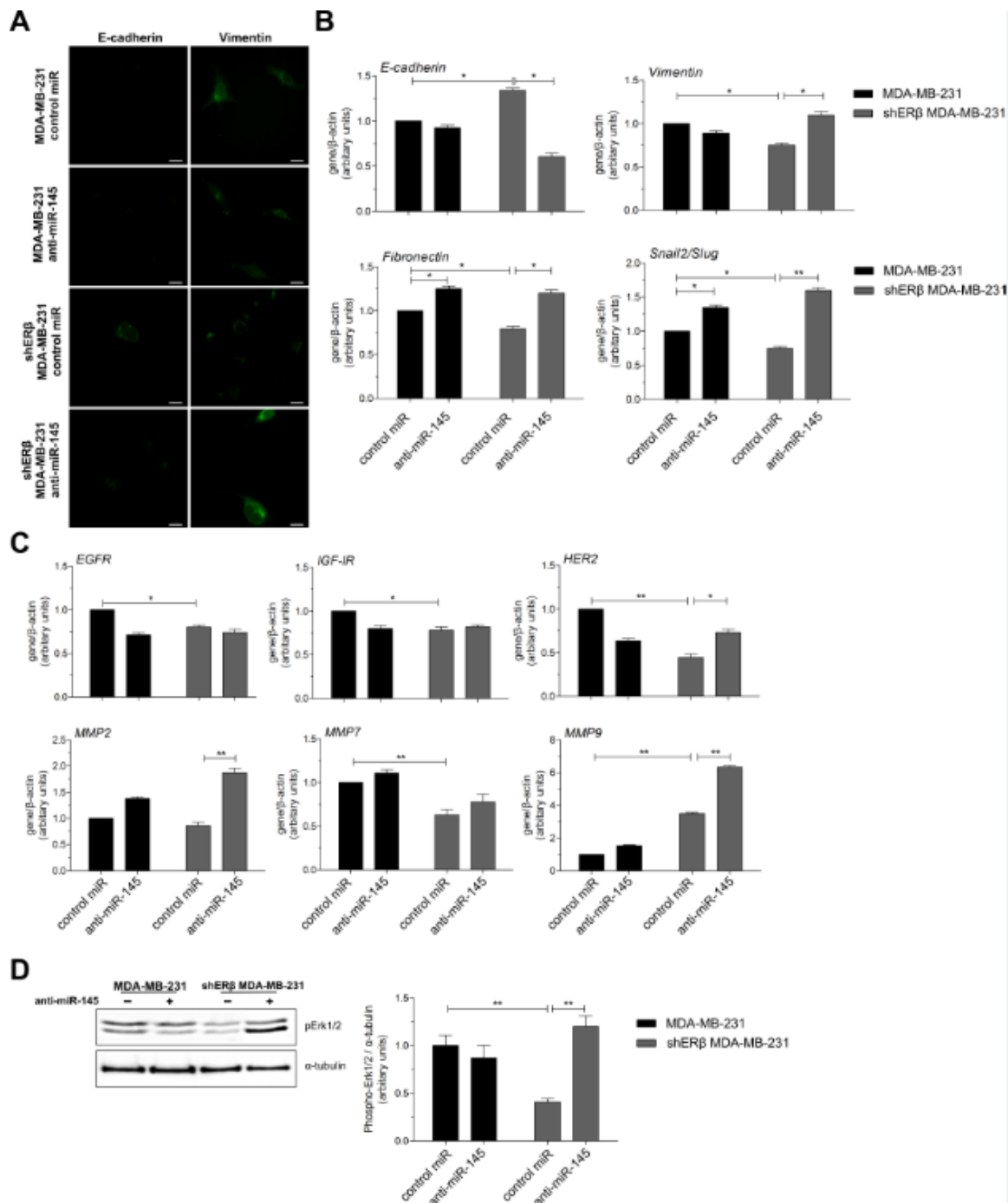


Figure 5



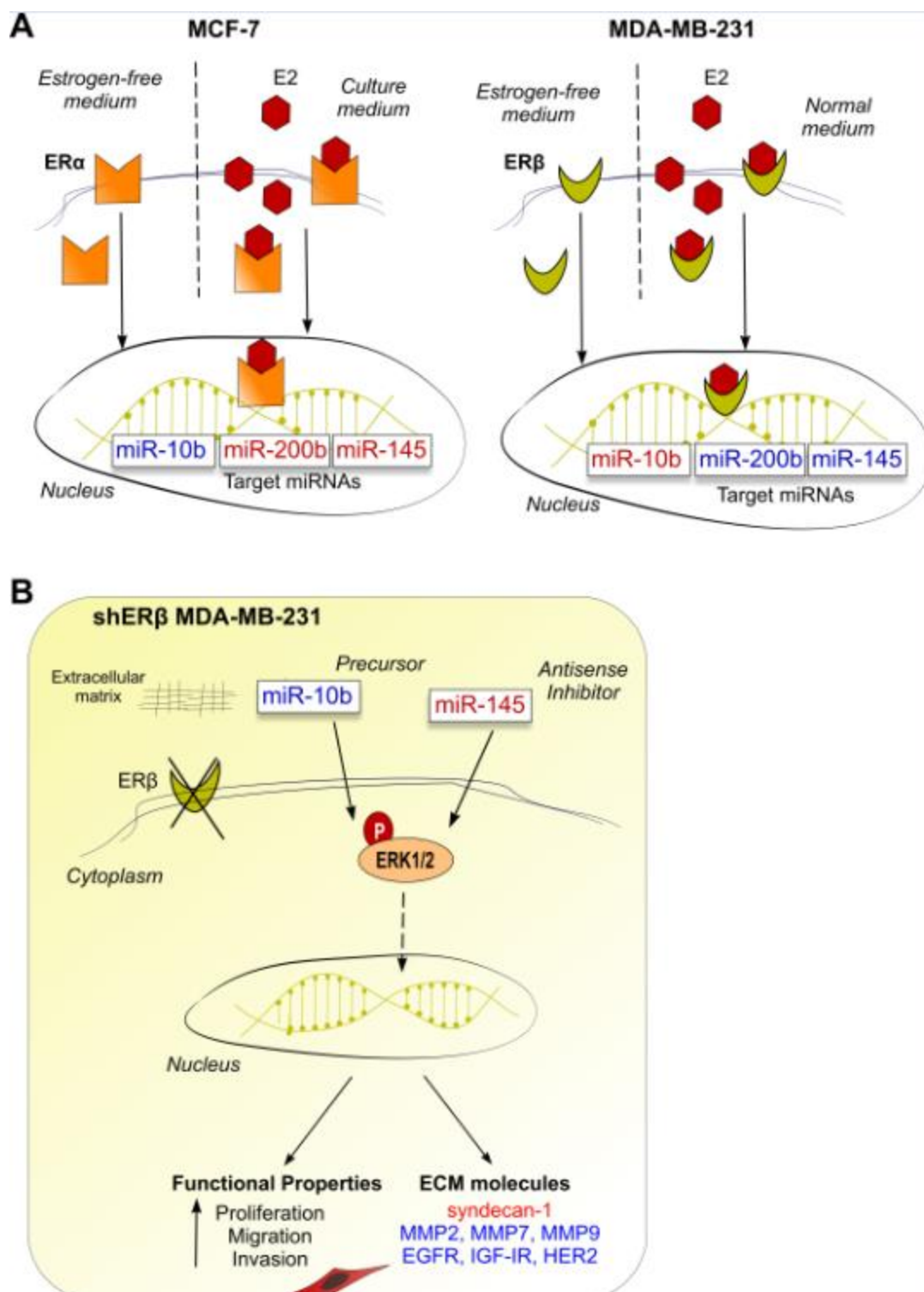


Figure 7

Highlights

- MicroRNA expression profile depends on the ER status of breast cancer cells.
- Suppression of ER β in MDA-MB-231 cells leads to significant changes in the expression profiles of specific microRNAs.
- Transfections with miR-10b precursor and miR-145 inhibitor clearly revealed that these microRNAs regulate the functional properties, EMT program, proteolytic behavior, the expression of major matrix components and Erk1/2 signaling.
- Targeting the ER β -regulated miR-10b and miR-145 is a promising tool for diagnosis and pharmaceutical targeting in breast cancer.