Authors preprint of Matrix Biology doi: 10.1016/j.matbio.2017.08.002

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

Zoi Piperigkou [1,2], Marco Franchi [3], Martin Götte [2] and Nikos K. Karamanos [1]

1 - Biochemistry, Biochemical Analysis & Matrix Pathobiology Res. Group, Laboratory of Biochemistry, Department of Chemistry, University of Patras, Patras 26110, Greece;
2 - Department of Gynecology and Obstetrics, Münster University Hospital, Münster 48149, Germany;
3 - Department for Life Quality Studies, University of Bologna, Rimini 40126, Italy

Correspondence to: Nikos K. Karamanos, Biochemistry, Biochemical Analysis & Matrix Pathobiology Res. Group, Laboratory of Biochemistry, Department of Chemistry, University of Patras, 26110 Patras, Greece, n.k.karamanos@upatras.gr and Martin Götte, Department of Gynecology and Obstetrics, Münster University Hospital, Münster, Germany, martingotte@uni-muenster.de

Abstract

Even though the role of estrogen receptor alpha (ERα) in the modulation of breast cancer cells' behavior is thoroughly studied, the biological functions of its isoform, ERβ, are less elucidated. The suppression of ERβ in the aggressive ERα-negative MDA-MB-231 breast cancer cells resulted in the inhibition of epithelial to mesenchymal transition (EMT) through 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 suggested the contribution of ERβ in the aggressive phenotype of MDA-MB-231 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 ERβ 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. Treatment 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 that the diverse effect of estradiol on the miRNA expression profile depends on the ER status of breast cancer cells. Transfections of miR-10b precursor and miR-145 inhibitor 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 shERβ MDA-MB-231 cells, thus affecting 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 shERβ 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 ERβ in MDA-MB-231 cells are closely related to certain epigenetic miRNA-induced alterations. Targeting the ERβ-regulated miR-10b and miR-145 is a promising tool for diagnosis and pharmaceutical targeting in breast cancer.

Key words: breast cancer, microRNA, estrogen receptor beta, epithelial-to-mesenchymal transition

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

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 ERα-negative and ERβ-positive, highly invasive MDA-MB-231 breast cancer cells, we have recently established a stable transfected ERβ-suppressed cell line (named shERβ MDA-MB-231) [32]. The suppression of ERβ 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 ERβ 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 noncoding RNA molecules, which have an important role in post-transcriptional regulation 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 ERα and ERβ 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 ERα-positive and low aggressive MCF-7, ERα-negative and ERβ-positive MDA-MB-231 and the shERβ MDA-MB-231 breast cancer cells. The data obtained demonstrate that the knockdown of ERβ is followed by significant changes, similar to ERα-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 shERβ MDA-MB-231 breast cancer cells, highlighting the novel role of ERβ, along with epigenetic alterations of miRNAs in breast cancer cell behavior.

Results

ERβ is associated with distinct morphology and microRNA expression patterns of breast cancer cells

Our group has recently demonstrated that the strong suppression of ERβ in ERβ-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 ERβ gene in shERβ 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, ERα-positive MCF-7 cells exhibit cell-cell junctions (Fig. 1B, panel B3) and are grown by forming cell aggregates, whereas shERβ 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 ERα and ERβ 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 ERβ-dependent modulation. To evaluate the effects of ERβ 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 ERβ 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 ERβ 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. Notable, ERα-positive MCF-7 breast cancer cells exhibited a 99% decrease of miR-10b, a 14- and a 34-fold increase of miR-145 and miR-200b expression levels, 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 treatment of ERα-positive MCF-7 and ERα-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 treated 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 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 estradiol (E2) that signals via ERα 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 of ERβ-positive MDA-MB-231 breast cancer 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 ERβ regulates the expression of miR-10b and miR-145, as its loss resulted in the most profound effects among the miRNAs tested. Therefore, further studies regarding the effects of miRNAs on MDA-MB-231 and shERβ 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 shERβ 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 ERβ resulted in the significant downregulation of miR-10b in shERβ 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 cancer cell properties (cell proliferation, migration and invasion) in this breast cancer model, following treatment with the precursor of miR-10b (pre-miR-10b), which induces miR-10b overexpression. Interestingly, miR-10b overexpression in shERβ MDA-

MB-231 cells (Fig. 3A) was accompanied by significant alterations in the functional properties of these cells (Fig. 3B). Our data depicted significant upregulation of shERβ MDA-MB-231 cell proliferation, as compared to the control miRNA (control miR). Furthermore, shERβ MDA-MB-231 cells seem to significantly attenuate their invasive capacity following the pre-miR-10b treatment (*ca* 85%). Moreover, an increase in the migratory capacity of shERβ MDA-MB-231 cells treated with the pre-miR-10b was also observed.

SEM microscopy showed that the induction of miR-10b expression in shERβ 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, shERβ 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 shERβ MDA-MB-231 cells (Fig. 3D). The F-actin staining for cytoskeleton confirms the mesenchymal-like characteristics that shERβ MDA-MB-231 cells exhibited after pre-miR-10b transfection, in comparison with the characteristic cytoskeleton formation of shERβ MDA-MB-231 cells.

The observed changes in the phenotype of shERβ 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 treatment of shERβ 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 shERβ MDA-MB-231 cells treated 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 ERβ suppression, in shERβ MDA-MB-231 cells. On the other hand, the expression levels of EGFR in shERβ MDA-MB-231 cells were not significantly affected by pre-miR-10b. It is worth noticing that the decreased levels of VEGF in the ERβ-suppressed cells were significantly induced following pre-miR-10b treatment, suggesting a crucial role of miR-10b in the induction of the angiogenic and aggressive potential in shERβ 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 shERβ 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 treatment resulted in the significant upregulation of the phosphorylated Erk1/2 forms in shERβ 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 shERβ MDA-MB-231 cells, thus affecting the ECM composition, proteolytic behaviour and metastatic potential of these cells.

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

As mentioned above, the suppression of ERβ 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 shERβ 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 shERβ MDA-MB-231 cells resulted in the increase of cell proliferation 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 shERβ MDA-MB-231 cells treated with control miRNA and anti-miR-145 clearly depict that the decreased expression of miR-145 in shERβ 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 shERβ 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 demonstrate 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 shERβ 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 shERβ 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, shERβ 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 shERβ 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 shERβ MDA-MB-231 cells resulted in the strong upregulation of the phosphorylated Erk1/2 forms in these cells that is correlated with the high proliferation 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 shERβ MDA-MB-231 cells breast cancer cells.

Discussion

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 [59-61]. 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, 62, 63]. Moreover, its expression levels are elevated in metastatic breast cancers [41, 44]. Importantly, the mRNAs encoding ERα and ERβ 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, 64]. Downregulated expression levels of this miRNA can be found in metastatic and breast cancer stem cell-like cells *in vitro* [65, 66]. Moreover, it is demonstrated that members of the miR-200 family target directly ERα and ERβ [67]. 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 shERβ 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 ERβ in shERβ 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 [68]. The obtained data showed that miR-10b, which is overexpressed in clinical samples of metastatic breast cancer and positively regulates tumor invasion *in vivo* [62], is significantly downregulated in shERβ MDA-MB-231 cells, as compared to MDA-MB-231cells. Consistent with the ERα-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 [65], has been found to be significantly upregulated in MCF-7 and shERβ MDA-MB-231 breast cancer cells. Interestingly, long-term treatment 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 treated 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-ERα 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 [69]. Interestingly, the induction of its expression by transfecting shERβ 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 proliferation and motility [70], suggesting the critical role of miR-10b in this signaling pathway. These data suggest that in ERβ-suppressed shERβ MDA-MB-231 cells, miR-10b presence is 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 shERβ 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 shERβ 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 shERβ 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 ERβ-suppressed shERβ 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. Therefore, miRNAs may serve as prognostic and predictive biomarkers as well as therapeutic agents for breast cancer. The data from this study pinpointed that ERβ 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 ERβ 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, and 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, ERα-negative, ERβ-positive) and MCF-7 (low metastatic potential, ERα-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) CO_2 and 95% air. shERβ MDA-MB-231 (ERβ-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) Na_2EDTA. 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 mirVanaTM 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 $2^{-\Delta\Delta Ct}$ 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 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% OsO_4 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 [71]. 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 [71], using 30–60 μg of protein/lane.

Statistical analysis

Reported values are expressed as mean ± 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.

Funding information

This work was supported by the EU Horizon 2020 project RISE-2014, action No. 645756 "GLYCANC – Matrix glycans as multifunctional pathogenesis factors and therapeutic targets in cancer". Z.P. was supported by the DAAD agency, grant No. 91607321.

Author Contributions

Z.P. performed the main experimental part and prepared the manuscript draft and the figures, M.F. performed SEM image analysis and contributed to manuscript writting. 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.

Acknowledgements

We wish to thank Dr. Achilleas Theocharis (University of Patras), Dr. Maurizio Onisto (University of Padova), Prof. Burkhard Greve (University of Münster) and Dr. Franz Josef Wischmann (University of Münster) for suggestions, useful discussions and technical assistance during this work.

References

[1] A. Jemal, F. Bray, M.M. Center, J. Ferlay, E. Ward, D. Forman, Global cancer statistics, CA Cancer J Clin 61(2) (2011) 69-90.

[2] M. Gottlicher, S. Heck, P. Herrlich, Transcriptional cross-talk, the second mode of steroid hormone receptor action, J Mol Med (Berl) 76(7) (1998) 480-9.

[3] R. Losel, M. Wehling, Nongenomic actions of steroid hormones, Nat Rev Mol Cell Biol 4(1) (2003) 46-56.

[4] E.R. Levin, S.R. Hammes, Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors, Nat Rev Mol Cell Biol (2016).

[5] J.K. Mouw, G. Ou, V.M. Weaver, Extracellular matrix assembly: a multiscale deconstruction, Nat Rev Mol Cell Biol 15(12) (2014) 771-85.

[6] A.D. Theocharis, S.S. Skandalis, C. Gialeli, N.K. Karamanos, Extracellular matrix structure, Adv Drug Deliv Rev 97 (2016) 4-27.

[7] N.A. Afratis, K. Karamanou, Z. Piperigkou, D.H. Vynios, A.D. Theocharis, The role of heparins and nano-heparins as therapeutic tool in breast cancer, Glycoconj J (2016).

[8] E. Karousou, S. Misra, S. Ghatak, K. Dobra, M. Gotte, D. Vigetti, A. Passi, N.K. Karamanos, S.S. Skandalis, Roles and targeting of the HAS/hyaluronan/CD44 molecular system in cancer, Matrix Biol (2016).

[9] R.V. Iozzo, L. Schaefer, Proteoglycan form and function: A comprehensive nomenclature of proteoglycans, Matrix Biol 42 (2015) 11-55.

[10] N.A. Afratis, D. Nikitovic, H.A. Multhaupt, A.D. Theocharis, J.R. Couchman, N.K. Karamanos, Syndecans: key regulators of cell signaling and biological functions, FEBS J (2016).

[11] L. Schaefer, C. Tredup, M.A. Gubbiotti, R.V. Iozzo, Proteoglycan neofunctions: regulation of inflammation and autophagy in cancer biology, FEBS J 284(1) (2017) 10-26.

[12] D. Nikitovic, A. Papoutsidakis, N.K. Karamanos, G.N. Tzanakakis, Lumican affects tumor cell functions, tumor-ECM interactions, angiogenesis and inflammatory response, Matrix Biol 35 (2014) 206-14.

[13] C. Gialeli, A.D. Theocharis, N.K. Karamanos, Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting, FEBS J 278(1) (2011) 16-27.

[14] A.D. Theocharis, C. Gialeli, P. Bouris, E. Giannopoulou, S.S. Skandalis, A.J. Aletras, R.V. Iozzo, N.K. Karamanos, Cell-matrix interactions: focus on proteoglycan-proteinase interplay and pharmacological targeting in cancer, FEBS J 281(22) (2014) 5023-42.

[15] Z. Piperigkou, B. Mohr, N. Karamanos, M. Gotte, Shed proteoglycans in tumor stroma, Cell Tissue Res (2016).
[16] T. Neill, L. Schaefer, R.V. Iozzo, Decoding the Matrix: Instructive Roles of Proteoglycan Receptors, Biochemistry 54(30) (2015) 4583-98.
[17] K. Kessenbrock, C.Y. Wang, Z. Werb, Matrix metalloproteinases in stem cell regulation and cancer, Matrix Biol 44-46 (2015) 184-90.
[18] C.S. Curran, P.J. Keely, Breast tumor and stromal cell responses to TGF-beta and hypoxia in matrix deposition, Matrix Biol 32(2) (2013) 95-105.
[19] B. Cieply, C. Koontz, S.M. Frisch, CD44S-hyaluronan interactions protect cells resulting from EMT against anoikis, Matrix Biol 48 (2015) 55-65.
[20] A.D. Theocharis, S.S. Skandalis, T. Neill, H.A. Multhaupt, M. Hubo, H. Frey, S. Gopal, A. Gomes, N. Afratis, H.C. Lim, J.R. Couchman, J. Filmus, R.D. Sanderson, L. Schaefer, R.V. Iozzo, N.K. Karamanos, Insights into the key roles of proteoglycans in breast cancer biology and translational medicine, Biochim Biophys Acta 1855(2) (2015) 276-300.
[21] A.D. Theocharis, S.S. Skandalis, G.N. Tzanakakis, N.K. Karamanos, Proteoglycans in health and disease: novel roles for proteoglycans in malignancy and their pharmacological targeting, FEBS J 277(19) (2010) 3904-23.
[22] S. Kumar, A. Kapoor, S. Desai, M.M. Inamdar, S. Sen, Proteolytic and non-proteolytic regulation of collective cell invasion: tuning by ECM density and organization, Sci Rep 6 (2016) 19905.
[23] R.D. Sanderson, M. Elkin, A.C. Rapraeger, N. Ilan, I. Vlodavsky, Heparanase regulation of cancer, autophagy and inflammation: New mechanisms and targets for therapy, FEBS J (2016).
[24] J.R. Couchman, H. Multhaupt, R.D. Sanderson, Recent Insights into Cell Surface Heparan Sulphate Proteoglycans and Cancer, F1000Res 5 (2016).
[25] V.C. Ramani, I. Vlodavsky, M. Ng, Y. Zhang, P. Barbieri, A. Noseda, R.D. Sanderson, Chemotherapy induces expression and release of heparanase leading to changes associated with an aggressive tumor phenotype, Matrix Biol 55 (2016) 22-34.
[26] I. Kovalszky, A. Hjerpe, K. Dobra, Nuclear translocation of heparan sulfate proteoglycans and their functional significance, Biochim Biophys Acta 1840(8) (2014) 2491-7.
[27] G. Heidari-Hamedani, R.R. Vives, A. Seffouh, N.A. Afratis, A. Oosterhof, T.H. van Kuppevelt, N.K. Karamanos, M. Metintas, A. Hjerpe, K. Dobra, T. Szatmari, Syndecan-1 alters heparan sulfate composition and signaling pathways in malignant mesothelioma, Cell Signal 27(10) (2015) 2054-67.

[28] A.I. Tsonis, N. Afratis, C. Gialeli, M.I. Ellina, Z. Piperigkou, S.S. Skandalis, A.D. Theocharis, G.N. Tzanakakis, N.K. Karamanos, Evaluation of the coordinated actions of estrogen receptors with epidermal growth factor receptor and insulin-like growth factor receptor in the expression of cell surface heparan sulfate proteoglycans and cell motility in breast cancer cells, FEBS J 280(10) (2013) 2248-59.

[29] O. Kousidou, A. Berdiaki, D. Kletsas, A. Zafiropoulos, A.D. Theocharis, G.N. Tzanakakis, N.K. Karamanos, Estradiol-estrogen receptor: a key interplay of the expression of syndecan-2 and metalloproteinase-9 in breast cancer cells, Mol Oncol 2(3) (2008) 223-32.

[30] K. Voudouri, D. Nikitovic, A. Berdiaki, D. Kletsas, N.K. Karamanos, G.N. Tzanakakis, IGF-I/EGF and E2 signaling crosstalk through IGF-IR conduit point affects breast cancer cell adhesion, Matrix Biol (2016).

[31] Z. Piperigkou, K. Karamanou, N.A. Afratis, P. Bouris, C. Gialeli, C.L. Belmiro, M.S. Pavao, D.H. Vynios, A.M. Tsatsakis, Biochemical and toxicological evaluation of nano-heparins in cell functional properties, proteasome activation and expression of key matrix molecules, Toxicol Lett 240(1) (2016) 32-42.

[32] Z. Piperigkou, P. Bouris, M. Onisto, M. Franchi, D. Kletsas, A.D. Theocharis, N.K. Karamanos, Estrogen receptor beta modulates breast cancer cells functional properties, signaling and expression of matrix molecules, Matrix Biol (2016).

[33] V. Koistinen, K. Harkonen, R. Karna, U.T. Arasu, S. Oikari, K. Rilla, EMT induced by EGF and wounding activates hyaluronan synthesis machinery and EV shedding in rat primary mesothelial cells, Matrix Biol (2016).

[34] L.A. Griggs, N.T. Hassan, R.S. Malik, B.P. Griffin, B.A. Martinez, L.W. Elmore, C.A. Lemmon, Fibronectin fibrils regulate TGF-beta1-induced Epithelial-Mesenchymal Transition, Matrix Biol 60-61 (2017) 157-175.

[35] D. Vigetti, M. Viola, E. Karousou, S. Deleonibus, K. Karamanou, G. De Luca, A. Passi, Epigenetics in extracellular matrix remodeling and hyaluronan metabolism, FEBS J 281(22) (2014) 4980-92.

[36] Z.J. Rutnam, T.N. Wight, B.B. Yang, miRNAs regulate expression and function of extracellular matrix molecules, Matrix Biol 32(2) (2013) 74-85.

[37] J. Winter, S. Jung, S. Keller, R.I. Gregory, S. Diederichs, Many roads to maturity: microRNA biogenesis pathways and their regulation, Nat Cell Biol 11(3) (2009) 228-34.

[38] A. Marsico, M.R. Huska, J. Lasserre, H. Hu, D. Vucicevic, A. Musahl, U. Orom, M. Vingron, PROmiRNA: a new miRNA promoter recognition method uncovers the complex regulation of intronic miRNAs, Genome Biol 14(8) (2013) R84.

[39] P.A. Gregory, A.G. Bert, E.L. Paterson, S.C. Barry, A. Tsykin, G. Farshid, M.A. Vadas, Y. Khew-Goodall, G.J. Goodall, The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1, Nat Cell Biol 10(5) (2008) 593-601.

[40] J.X. Bai, B. Yan, Z.N. Zhao, X. Xiao, W.W. Qin, R. Zhang, L.T. Jia, Y.L. Meng, B.Q. Jin, D.M. Fan, T. Wang, A.G. Yang, Tamoxifen represses miR-200 microRNAs and promotes epithelial-to-mesenchymal transition by up-regulating c-Myc in endometrial carcinoma cell lines, Endocrinology 154(2) (2013) 635-45.

[41] L. Ma, J. Teruya-Feldstein, R.A. Weinberg, Tumour invasion and metastasis initiated by microRNA-10b in breast cancer, Nature 449(7163) (2007) 682-8.

[42] B.N. Hannafon, P. Sebastiani, A. de las Morenas, J. Lu, C.L. Rosenberg, Expression of microRNA and their gene targets are dysregulated in preinvasive breast cancer, Breast Cancer Res 13(2) (2011) R24.

[43] M. Gotte, C. Kersting, M. Ruggiero, J. Tio, A.H. Tulusan, L. Kiesel, P. Wulfing, Predictive value of syndecan-1 expression for the response to neoadjuvant chemotherapy of primary breast cancer, Anticancer Res 26(1B) (2006) 621-7.

[44] S.A. Ibrahim, G.W. Yip, C. Stock, J.W. Pan, C. Neubauer, M. Poeter, D. Pupjalis, C.Y. Koo, R. Kelsch, R. Schule, U. Rescher, L. Kiesel, M. Gotte, Targeting of syndecan-1 by microRNA miR-10b promotes breast cancer cell motility and invasiveness via a Rho-GTPase- and E-cadherin-dependent mechanism, Int J Cancer 131(6) (2012) E884-96.

[45] Y.N. Liu, J.J. Yin, W. Abou-Kheir, P.G. Hynes, O.M. Casey, L. Fang, M. Yi, R.M. Stephens, V. Seng, H. Sheppard-Tillman, P. Martin, K. Kelly, MiR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms, Oncogene 32(3) (2013) 296-306.

[46] F. Xu, H. He, W. Huang, Y. Lin, S. Luo, Q. Du, R. Duan, Decreased expression of MicroRNA-200 family in human breast cancer is associated with lymph node metastasis, Clin Transl Oncol 18(3) (2016) 283-8.

[47] M. Gotte, C. Mohr, C.Y. Koo, C. Stock, A.K. Vaske, M. Viola, S.A. Ibrahim, S. Peddibhotla, Y.H. Teng, J.Y. Low, K. Ebnet, L. Kiesel, G.W. Yip, miR-145-dependent targeting of junctional adhesion molecule A and modulation of fascin expression are associated with reduced breast cancer cell motility and invasiveness, Oncogene 29(50) (2010) 6569-80.

[48] J. Zhang, H. Guo, H. Zhang, H. Wang, G. Qian, X. Fan, A.R. Hoffman, J.F. Hu, S. Ge, Putative tumor suppressor miR-145 inhibits colon cancer cell growth by targeting oncogene Friend leukemia virus integration 1 gene, Cancer 117(1) (2011) 86-95.

[49] M. Sachdeva, S. Zhu, F. Wu, H. Wu, V. Walia, S. Kumar, R. Elble, K. Watabe, Y.Y. Mo, p53 represses c-Myc through induction of the tumor suppressor miR-145, Proc Natl Acad Sci U S A 106(9) (2009) 3207-12.

[50] J.H. Noh, Y.G. Chang, M.G. Kim, K.H. Jung, J.K. Kim, H.J. Bae, J.W. Eun, Q. Shen, S.J. Kim, S.H. Kwon, W.S. Park, J.Y. Lee, S.W. Nam, MiR-145 functions as a tumor suppressor by directly targeting histone deacetylase 2 in liver cancer, Cancer Lett 335(2) (2013) 455-62.

[51] S.A. Ibrahim, H. Hassan, M. Gotte, MicroRNA-dependent targeting of the extracellular matrix as a mechanism of regulating cell behavior, Biochim Biophys Acta 1840(8) (2014) 2609-20.

[52] R. Baffa, M. Fassan, S. Volinia, B. O'Hara, C.G. Liu, J.P. Palazzo, M. Gardiman, M. Rugge, L.G. Gomella, C.M. Croce, A. Rosenberg, MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets, J Pathol 219(2) (2009) 214-21.

[53] L. Ma, F. Reinhardt, E. Pan, J. Soutschek, B. Bhat, E.G. Marcusson, J. Teruya-Feldstein, G.W. Bell, R.A. Weinberg, Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model, Nat Biotechnol 28(4) (2010) 341-7.

[54] P. Bouris, S.S. Skandalis, Z. Piperigkou, N. Afratis, K. Karamanou, A.J. Aletras, A. Moustakas, A.D. Theocharis, N.K. Karamanos, Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells, Matrix Biol 43 (2015) 42-60.

[55] S.S. Skandalis, N. Afratis, G. Smirlaki, D. Nikitovic, A.D. Theocharis, G.N. Tzanakakis, N.K. Karamanos, Cross-talk between estradiol receptor and EGFR/IGF-IR signaling pathways in estrogen-responsive breast cancers: focus on the role and impact of proteoglycans, Matrix Biol 35 (2014) 182-93.

[56] D. Barbouri, N. Afratis, C. Gialeli, D.H. Vynios, A.D. Theocharis, N.K. Karamanos, Syndecans as modulators and potential pharmacological targets in cancer progression, Front Oncol 4 (2014) 4.

[57] M. Zheng, X. Sun, Y. Li, W. Zuo, MicroRNA-145 inhibits growth and migration of breast cancer cells through targeting oncoprotein ROCK1, Tumour Biol 37(6) (2016) 8189-96.

[58] H. Zhao, X. Kang, X. Xia, L. Wo, X. Gu, Y. Hu, X. Xie, H. Chang, L. Lou, X. Shen, miR-145 suppresses breast cancer cell migration by targeting FSCN-1 and inhibiting epithelial-mesenchymal transition, Am J Transl Res 8(7) (2016) 3106-14.

[59] F. Du, P. Yuan, Z.T. Zhao, Z. Yang, T. Wang, J.D. Zhao, Y. Luo, F. Ma, J.Y. Wang, Y. Fan, R.G. Cai, P. Zhang, Q. Li, Y.M. Song, B.H. Xu, A miRNA-based signature predicts development

of disease recurrence in HER2 positive breast cancer after adjuvant trastuzumab-based treatment, Sci Rep 6 (2016) 33825.

[60] A. Mathe, M. Wong-Brown, B. Morten, J.F. Forbes, S.G. Braye, K.A. Avery-Kiejda, R.J. Scott, Novel genes associated with lymph node metastasis in triple negative breast cancer, Sci Rep 5 (2015) 15832.

[61] S.A. Ibrahim, H. Hassan, M. Gotte, MicroRNA regulation of proteoglycan function in cancer, FEBS J 281(22) (2014) 5009-22.

[62] L. Ma, Role of miR-10b in breast cancer metastasis, Breast Cancer Res 12(5) (2010) 210.

[63] Y. Liu, J. Zhang, X. Sun, M. Li, EMMPRIN Down-regulating miR-106a/b Modifies Breast Cancer Stem-like Cell Properties via Interaction with Fibroblasts Through STAT3 and HIF-1alpha, Sci Rep 6 (2016) 28329.

[64] V.P. Tryndyak, F.A. Beland, I.P. Pogribny, E-cadherin transcriptional down-regulation by epigenetic and microRNA-200 family alterations is related to mesenchymal and drug-resistant phenotypes in human breast cancer cells, Int J Cancer 126(11) (2010) 2575-83.

[65] J.C. Eggers, V. Martino, R. Reinbold, S.D. Schafer, L. Kiesel, A. Starzinski-Powitz, A.N. Schuring, B. Kemper, B. Greve, M. Gotte, microRNA miR-200b affects proliferation, invasiveness and stemness of endometriotic cells by targeting ZEB1, ZEB2 and KLF4, Reprod Biomed Online 32(4) (2016) 434-45.

[66] Y.Y. Lim, J.A. Wright, J.L. Attema, P.A. Gregory, A.G. Bert, E. Smith, D. Thomas, A.F. Lopez, P.A. Drew, Y. Khew-Goodall, G.J. Goodall, Epigenetic modulation of the miR-200 family is associated with transition to a breast cancer stem-cell-like state, J Cell Sci 126(Pt 10) (2013) 2256-66.

[67] D.W. Thomson, C.P. Bracken, J.M. Szubert, G.J. Goodall, On measuring miRNAs after transient transfection of mimics or antisense inhibitors, PLoS One 8(1) (2013) e55214.

[68] H. Mlcochova, T. Machackova, A. Rabien, L. Radova, P. Fabian, R. Iliev, K. Slaba, A. Poprach, E. Kilic, M. Stanik, M. Redova-Lojova, M. Svoboda, J. Dolezel, R. Vyzula, K. Jung, O. Slaby, Epithelial-mesenchymal transition-associated microRNA/mRNA signature is linked to metastasis and prognosis in clear-cell renal cell carcinoma, Sci Rep 6 (2016) 31852.

[69] R. Singh, R. Pochampally, K. Watabe, Z. Lu, Y.Y. Mo, Exosome-mediated transfer of miR-10b promotes cell invasion in breast cancer, Mol Cancer 13 (2014) 256.

[70] Q. Shao, X. Luo, D. Yang, C. Wang, Q. Cheng, T. Xiang, G. Ren, Phospholipase Cdelta1 suppresses cell migration and invasion of breast cancer cells by modulating KIF3A-mediated ERK1/2/beta- catenin/MMP7 signalling, Oncotarget 8(17) (2017) 29056-29066.

[71] V. Nikolova, C.Y. Koo, S.A. Ibrahim, Z. Wang, D. Spillmann, R. Dreier, R. Kelsch, J. Fischgrabe, M. Smollich, L.H. Rossi, W. Sibrowski, P. Wulfing, L. Kiesel, G.W. Yip, M. Gotte, Differential roles for membrane-bound and soluble syndecan-1 (CD138) in breast cancer progression, Carcinogenesis 30(3) (2009) 397-407.

Figure Legends

Fig. 1. ERβ has a key role in the morphology and miRNAs expression of breast cancer cells. (A) Immunofluorescence analysis of ERβ protein in MDA-MB-231 and shERβ MDA-MB-231 breast cancer cells. (B) Scanning electron micrograph (SEM) of MDA-MB-231 (1), MDA-MB-231 cells cultured on a Millipore filter coated with Matrigel on the upper surface (2), MCF-7 (3) and shERβ 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 μm. (C) ERβ suppression alters miRNA expression in shERβ 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 shERβ 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 treatment with estrogen-free medium induces miR-10b expression and downregulates miR-200b and miR-145 expression levels in MCF-7 breast cancer cells (ERα-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 treatment with estrogen-free medium reduces miR-10b expression and upregulates miR-200b and miR-145 expression levels in MDA-MB-231 breast cancer cells (ERβ-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 shERβ MDA-MB-231 breast cancer cells. The experiments were performed following transfections with pre-miR-10b. (A) MDA-MB-231 and shERβ 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 shERβ MDA-MB-231 breast cancer cells. The induction of miR-10b enhances proliferation, migration and invasion of shERβ MDA-MB-231 breast cancer cells. (C) Cell morphology was monitored through SEM microscopy. shERβ MDA-MB-231 cells following pre-miR-10b stimulation exhibit more cytoplasmic protrusions (arrows), whereas the morphology of MDA-MB-231 was not affected following 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 shERβ MDA-MB-231 cells with or without pre-miR-10b (bar, 10 μm). Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 4. The induction of miR-10b expression results in EMT reprogramming and significant alterations in the expression of ECM components. (A, B) Immunofluorescence (bar, 10 μm) and real-time PCR analyses of EMT markers revealed that transfection of shERβ 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 induction in MDA-MB-231 and shERβ 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 shERβ 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 shERβ MDA-MB-231 breast cancer cells. The experiments were performed following transfections with miR-145 inhibitor (anti-miR-145). (A) The inhibition of miR-145 gene in MDA-MB-231 and shERβ 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 shERβ MDA-MB-231 breast cancer cells. miR-145 inhibition increases proliferation, migration and invasion of

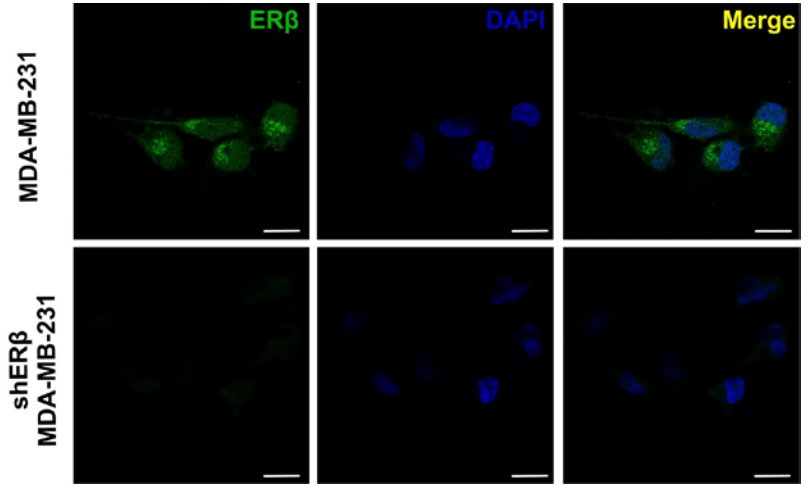
shERβ MDA-MB-231 breast cancer cells. (C) Cell morphology was monitored through SEM microscopy. shERβ MDA-MB-231 cells treated with anti-miR-145 appeared more cytoplasmic protrusions (arrows), whereas the morphology of MDA-MB-231 was not affected following treatment with anti-miR-145. (D) Evaluation 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 shERβ MDA-MB-231 cells with or without anti-miR-145 (bar, 10 μm). Asterisks (*), (**) indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Fig. 6. The inhibition of miR-145 expression in shERβ 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 shERβ MDA-MB-231 cells revealed that anti-miR-145 decreases E-cadherin and upregulates vimentin levels in shERβ MDA-MB-231 cells (bar, 10 μm). (B) Real-time PCR analysis of EMT markers, E-cadherin, vimentin, fibronectin and Snail2/Slug in MDA-MB-231 and shERβ MDA-MB-231 cells following transfections with anti-miR-145. (C) miR-145 inhibition in shERβ 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 shERβ 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).

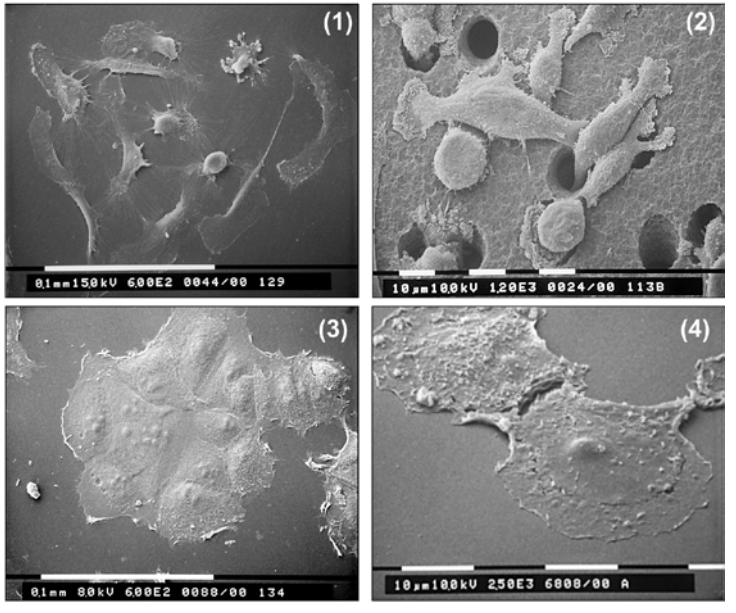
Fig. 7. ERβ is a key modulator of breast cancer cells' aggressiveness through miRNA regulation. (A) Long-term treatment of MCF-7 cells (ERα-positive) with estrogen-free medium increases their aggressiveness following the upregulation of miR-10b and downregulation of miR-200b and miR-145 expression levels, compared to expression in normal medium. MDA-MB-231 (ERβ-positive) breast cancer cells long-term treated 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) ERβ inversely regulates the expression of miR-10b and miR-145. ERβ suppression in shERβ 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 shERβ 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 inhibition

of miR-145 expression increases the metastatic potential of shERβ 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.

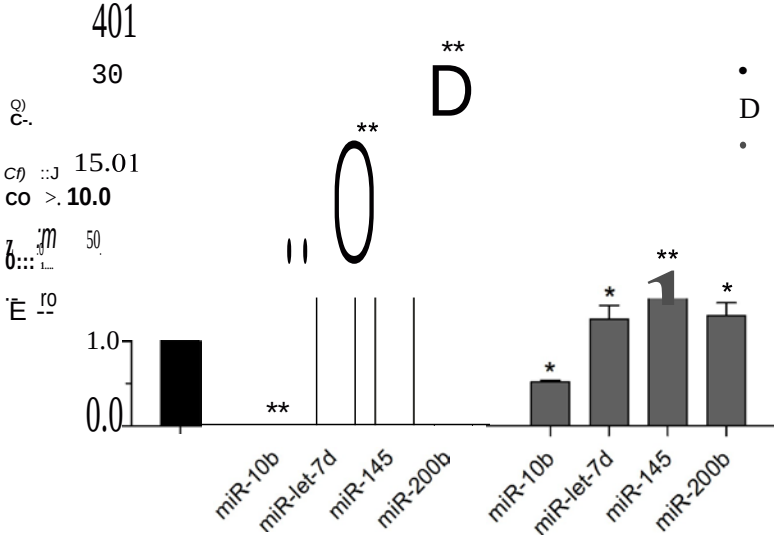
A



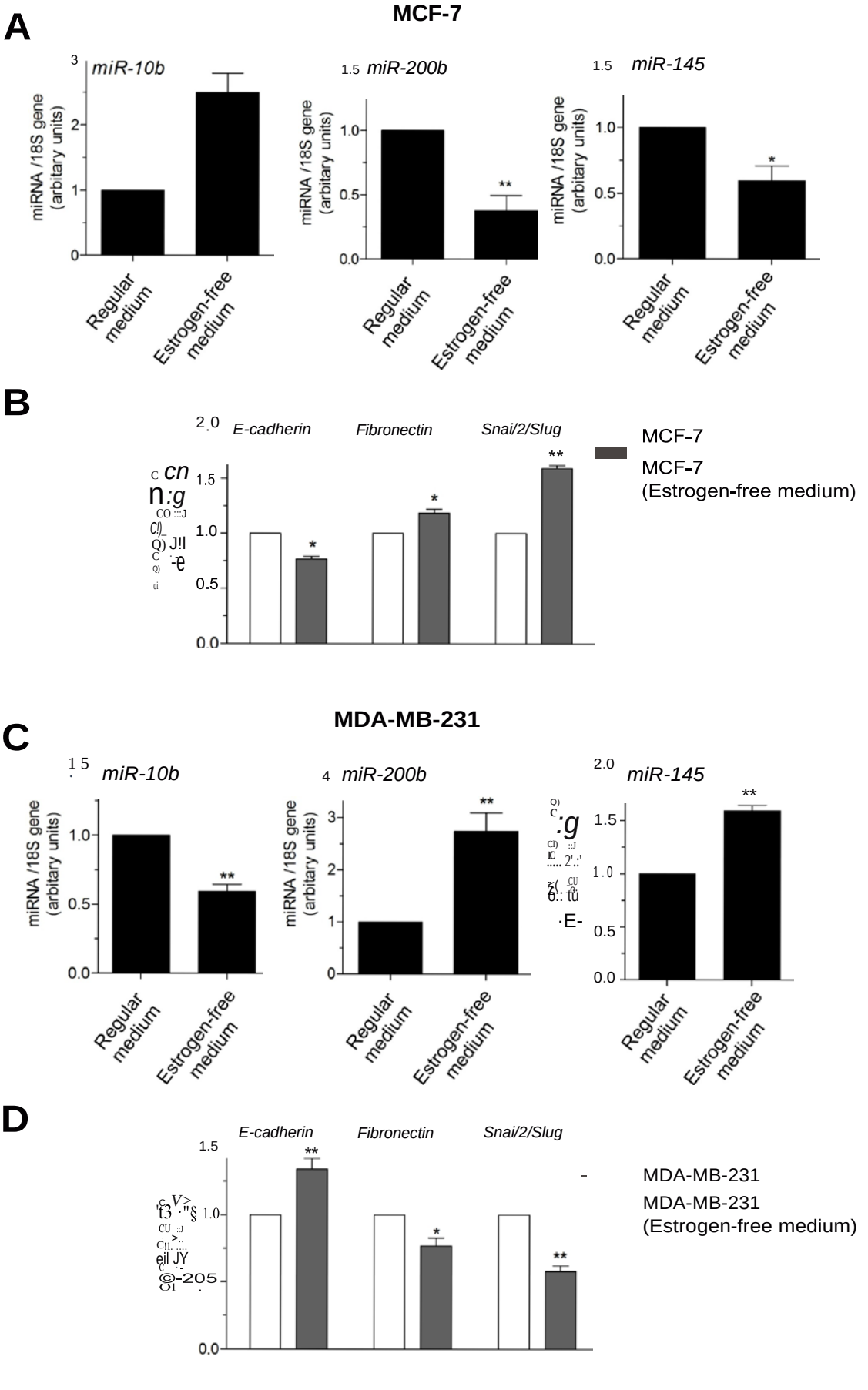
B

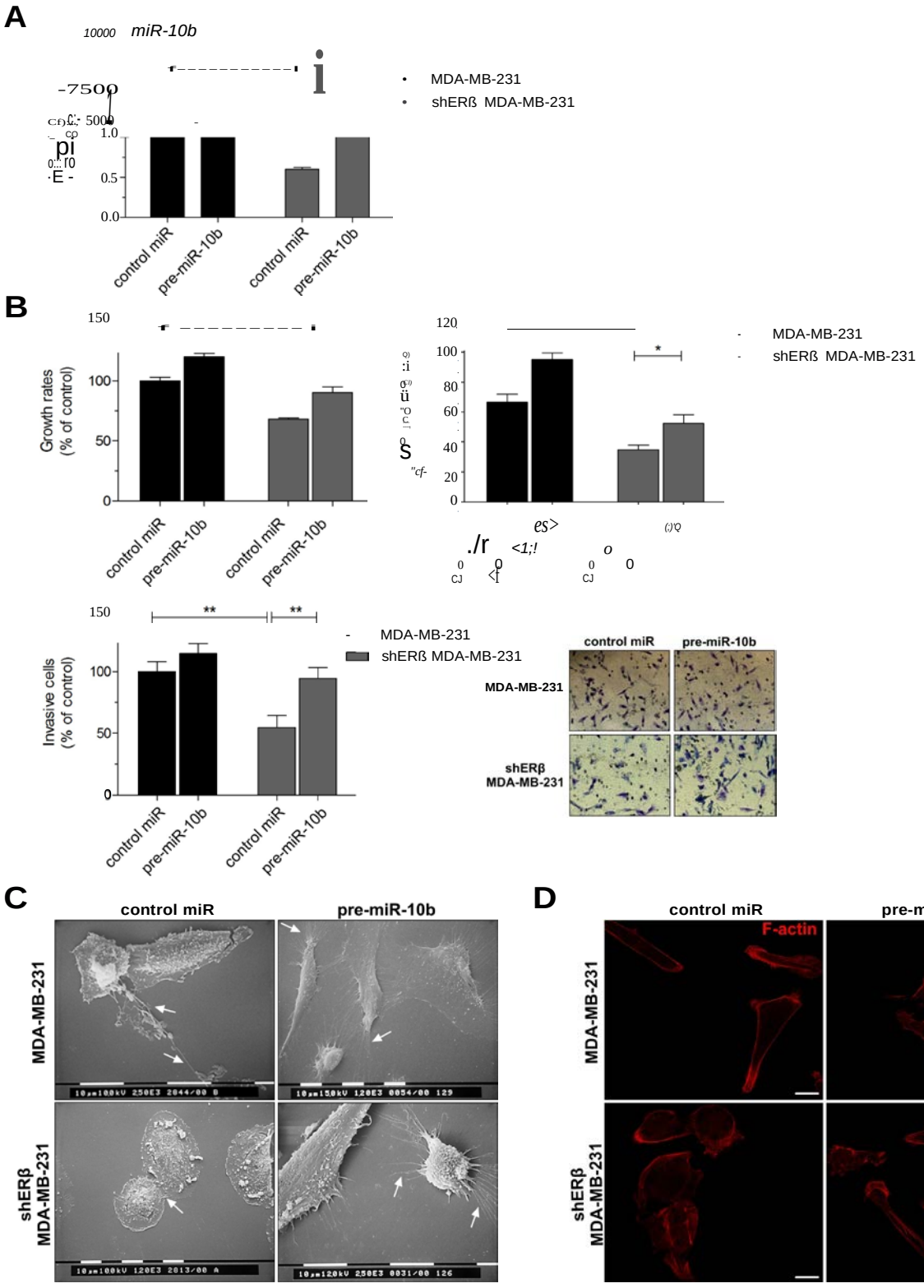


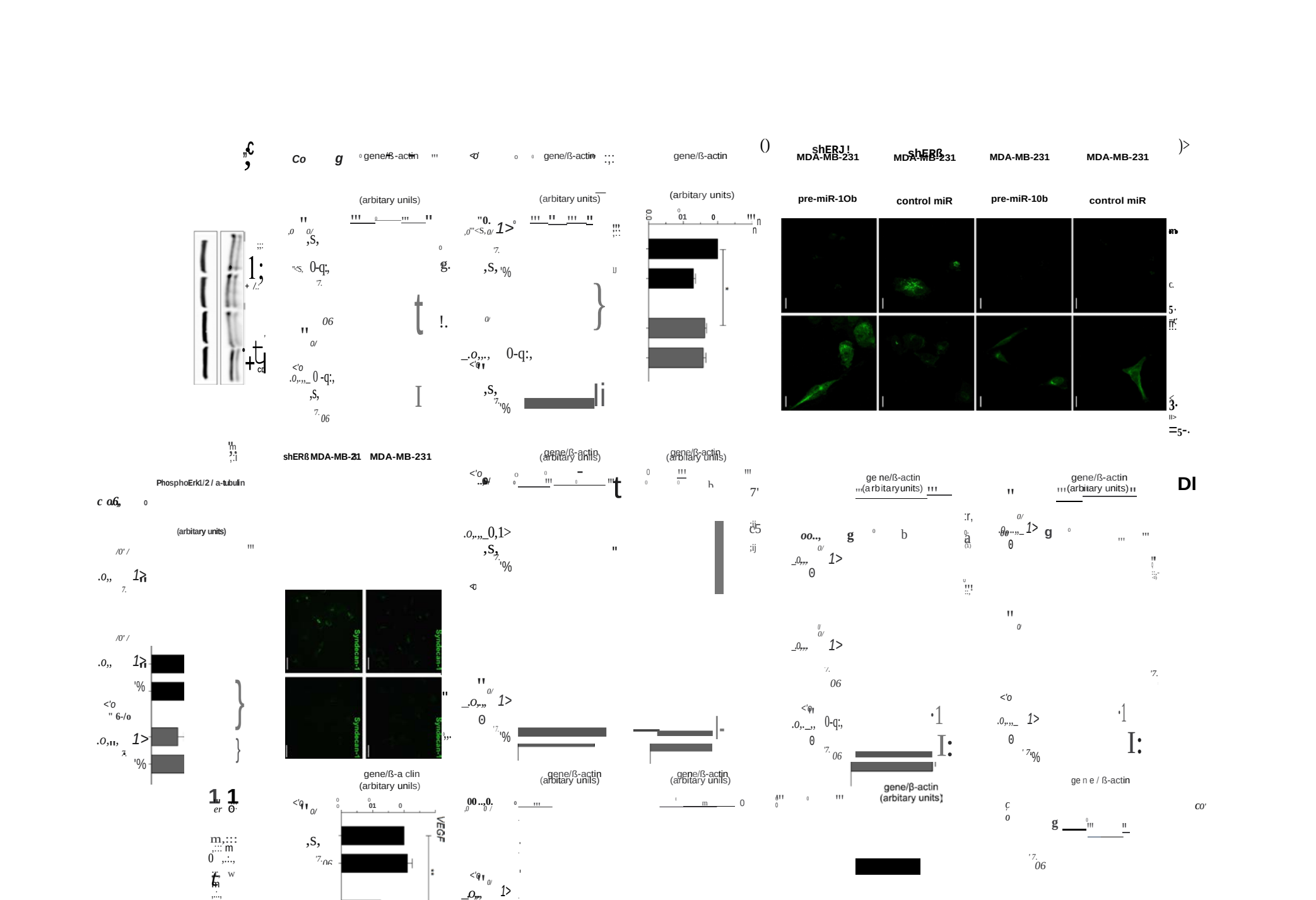
C

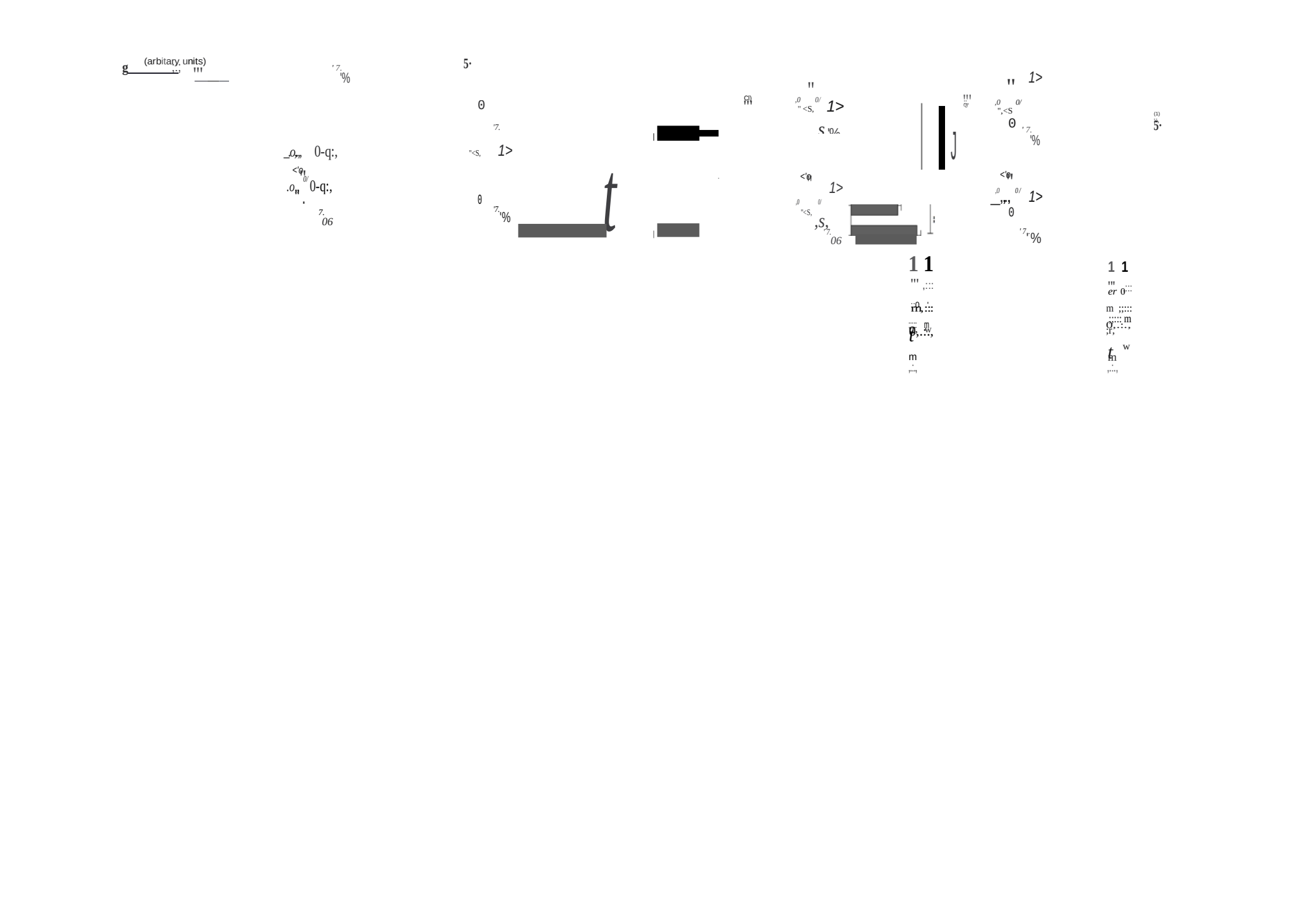


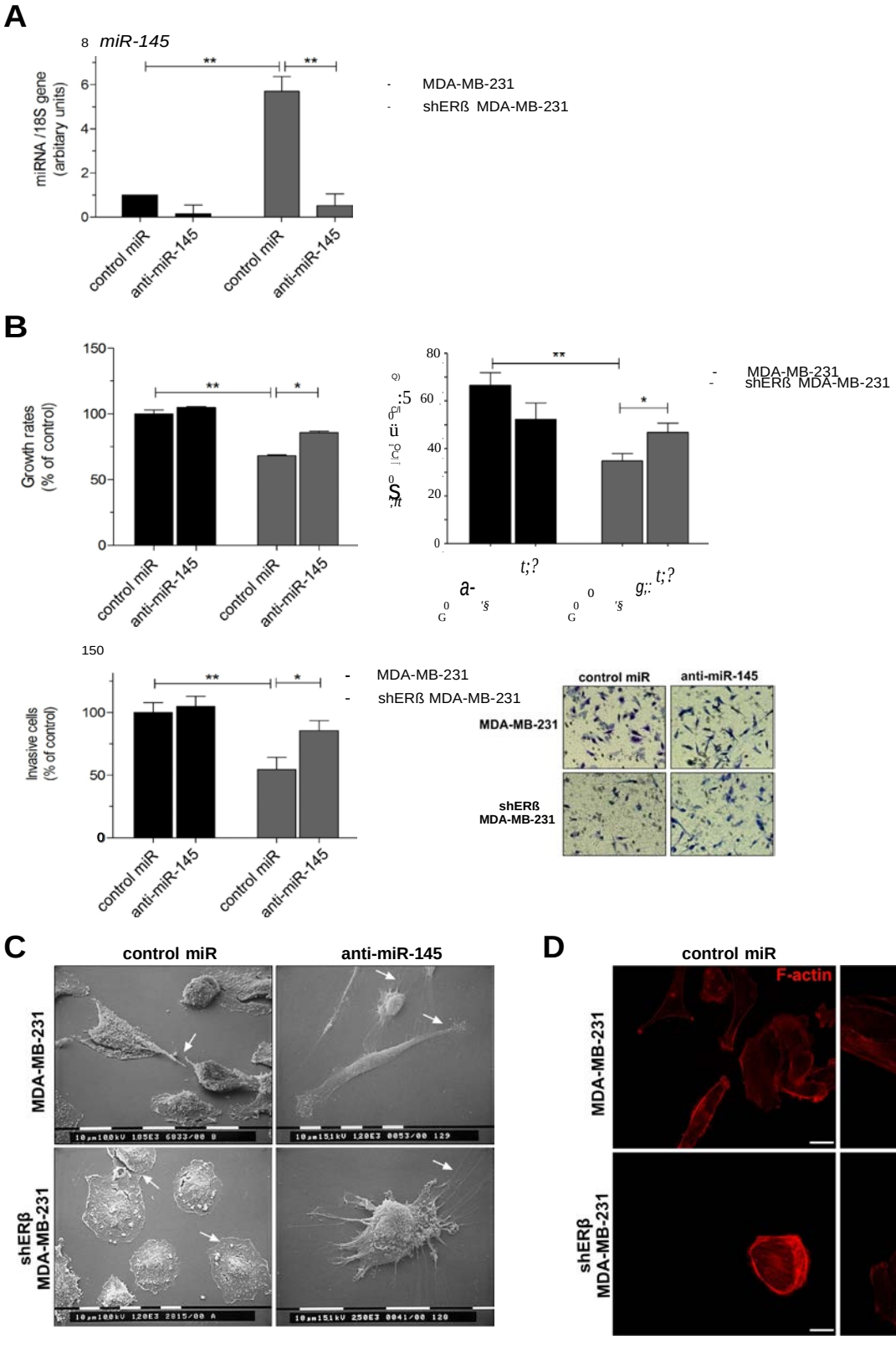
- MDA-MB-231
- MCF-7
- shERß MDA-MB-231

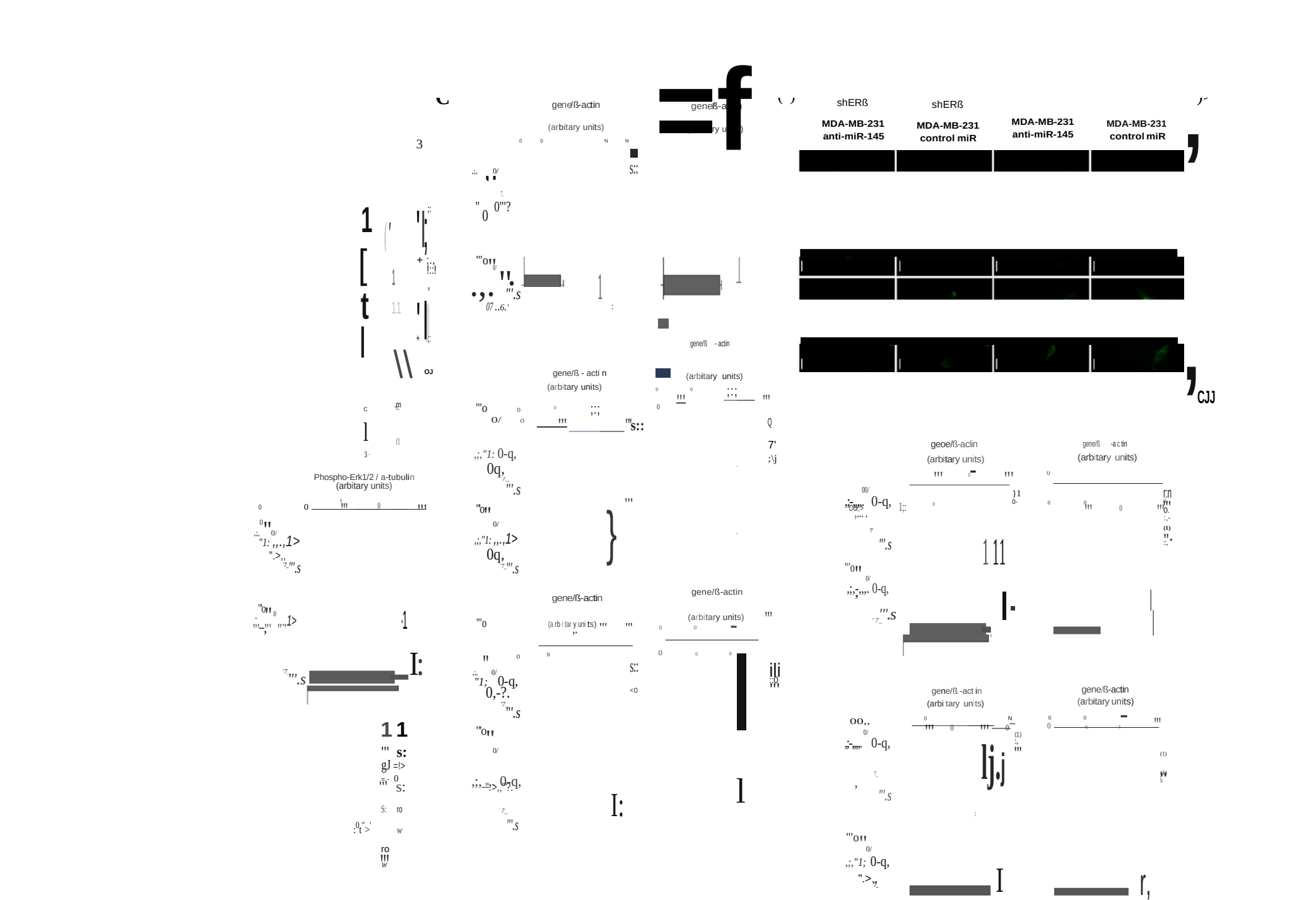

A
MCF-7
miR-10b
miR-200b
miR-145
miRNA /18S gene (arbitary units)
Regular medium
Estrogen-free medium
B
E-cadherin
Fibronectin
Snai/2/Slug
MCF-7
MCF-7 (Estrogen-free medium)
C
MDA-MB-231
miR-10b
miR-200b
miR-145
D
E-cadherin
Fibronectin
Snai/2/Slug
MDA-MB-231
MDA-MB-231 (Estrogen-free medium)


A
miR-10b
MDA-MB-231
shERß MDA-MB-231
control miR
pre-miR-10b
B
Growth rates (% of control)
Invasive cells (% of control)
MDA-MB-231
shERß MDA-MB-231
control miR
pre-miR-10b
C
control miR
pre-miR-10b
MDA-MB-231
shERβ MDA-MB-231
D
control miR
pre-miR-1Ob
F-actin
MDA-MB-231
shERβ MDA-MB-231


shERJ!
MDA-MB-231
shERß
MDA-MB-231
MDA-MB-231
MDA-MB-231
pre-miR-1Ob
control miR
pre-miR-10b
control miR
(arbitary units)
gene/ß-actin
shERß MDA-MB-231
MDA-MB-231
PhosphoErkl/2 / a-tubulin
Syndecan-1
gene/ß-a clin
VEGF
DI

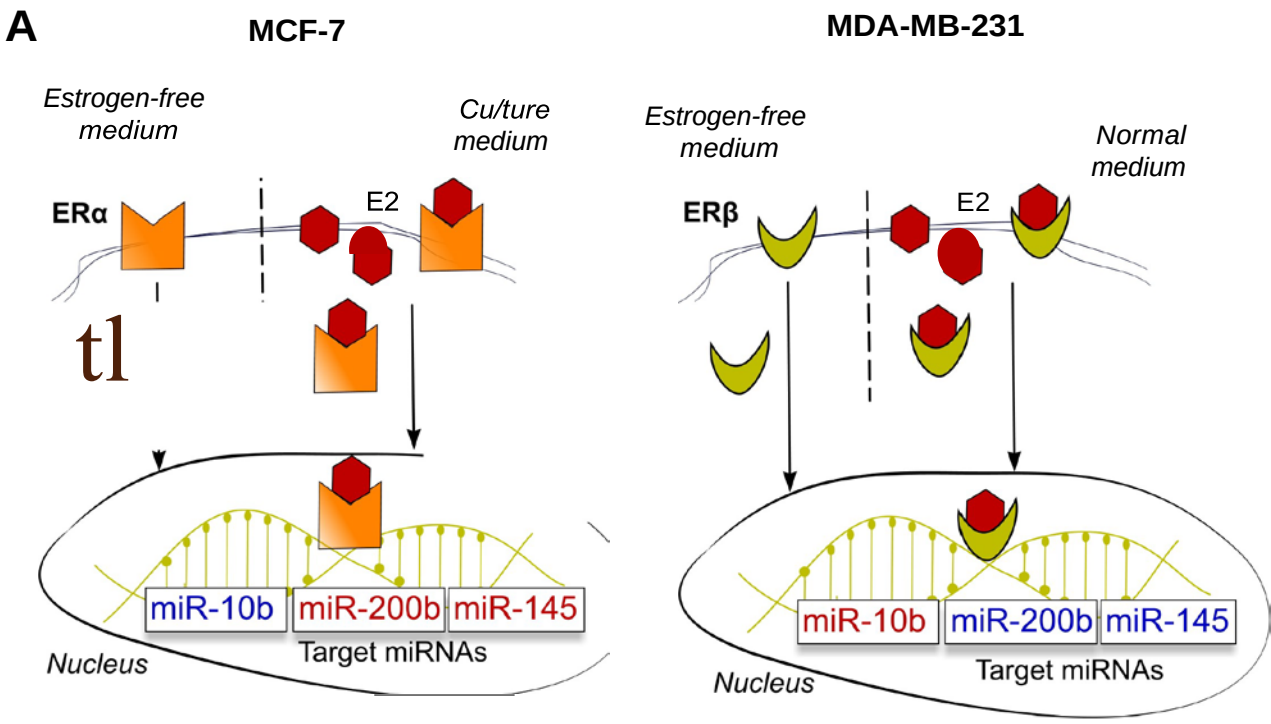

(arbitary, units)

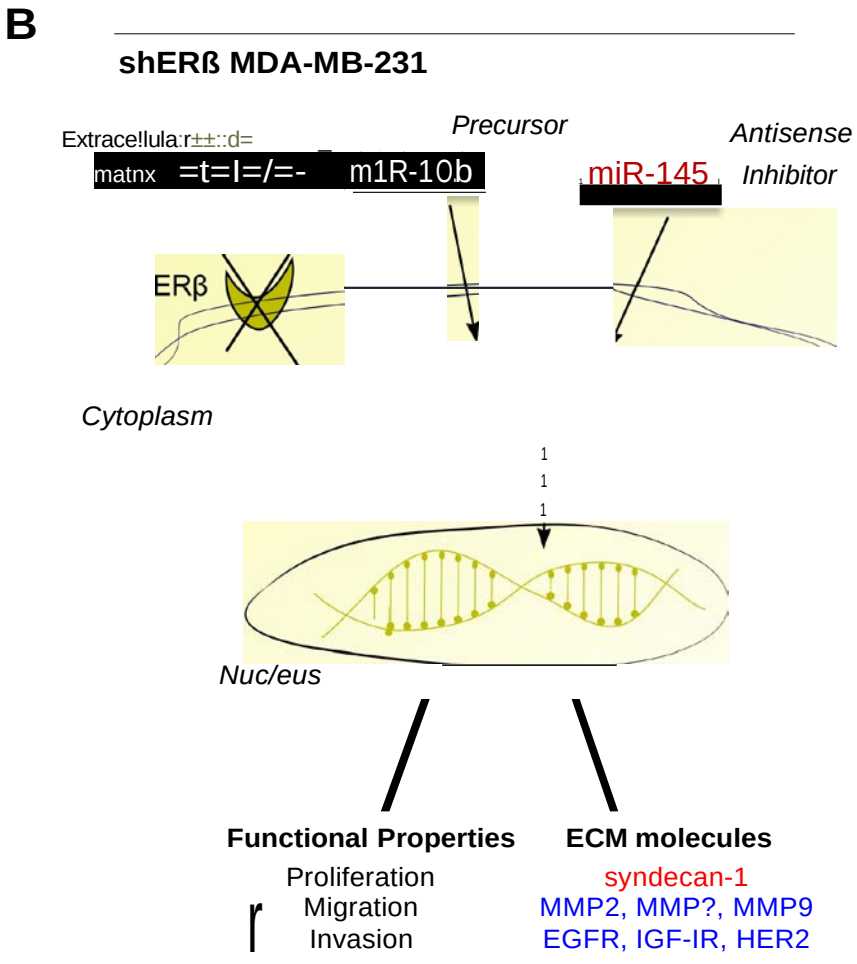

A
miR-145
miRNA /18S gene (arbitary units)
control miR
anti-miR-145
MDA-MB-231
shERß MDA-MB-231
B
Growth rates (% of control)
Invasive cells (% of control)
control miR
anti-miR-145
MDA-MB-231
shERß MDA-MB-231
C
control miR
anti-miR-145
MDA-MB-231
shERβ MDA-MB-231
D
control miR
anti-miR-145
F-actin
MDA-MB-231
shERβ MDA-MB-231


shERß
shERß
MDA-MB-231 anti-miR-145
MDA-MB-231 control miR
MDA-MB-231 anti-miR-145
MDA-MB-231 control miR
gene/ß-actin
(arbitary units)
gene/ß-actin
(arbitary units)
gene/ß - actin
(arbitary units)
gene/ß - actin
(arbitary units)
Phospho-Erk1/2 / a-tubulin
(arbitary units)
gene/ß-actin
(arbitary units)
gene/ß-actin
(arbitary units)
geoe/ß-aclin
(arbitary units)
gene/ß -actin
(arbitary units)
gene/ß -act in
(arbitary units)
gene/ß-actin
(arbitary units)

11

11


A
MCF-7
MDA-MB-231
Estrogen-free medium
Cu/ture medium
Estrogen-free medium
Normal medium
ERα
E2
ERβ
E2
tl
miR-10b
miR-200b
miR-145
Target miRNAs
Nucleus
miR-10b
miR-200b
miR-145
Target miRNAs
Nucleus


B
shERß MDA-MB-231
Precursor
Antisense
Inhibitor
miR-145
ERβ
Cytoplasm
Nuc/eus
Functional Properties
Proliferation
Migration
Invasion
ECM molecules
syndecan-1
MMP2, MMP?, MMP9
EGFR, IGF-IR, HER2

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

Zoi Piperigkou, Marco Franchi, Martin Götte and Nikos K. Karamanos

Supplementary material

Supplementary Table 1. List of antibodies used in this study.

Antibody	**Manufacturer**
Alexa-Fluor 488 anti-rabbit IgG, Goat	Invitrogen Corporation, Carlsbad, USA
Alexa-Fluor 488 anti-mouse IgG, Goat	Invitrogen Corporation, Carlsbad, USA
Alexa-Fluor 568- labeled phalloidin	Invitrogen Corporation, Carlsbad, USA
anti-human E-Cadherin, clone 36, monoclonal, Mouse	BD, Becton & Dickinson Biosciences, Heidelberg, Germany
anti-human Vimentin, clone 13.2, monoclonal, Mouse	Sigma-Aldrich, Steinheim, Germany
anti-human-phospho -p44/42 MAPK (Erk1/2) (Thr202/Tyr204**)**, polyclonal, Rabbit	Cell Signaling Technology, Inc., Danvers, USA
anti-human-Syndecan-1 (DL-101), monoclonal Mouse	Santa Cruz Biotechnology Inc., Santa Cruz, USA
anti-human-α-Tubulin, clone B-5-1-2, Mouse	Sigma-Aldrich, Steinheim, Germany
goat anti-rabbit IgG, Peroxidase Conjugated	Calbiochem, USA
goat anti-mouse IgG, Peroxidase Conjugated	Calbiochem, USA

Supplementary Table 2. List of miRNA expression qPCR assays used in validation phase of the study.

miRBase ID	**miRBase Accession No.**	**Mature miRNA sequence**
hsa-miR-10b	MIMAT0000254	UACCCUGUAGAACCGAAUUUGUG
hsa-miR-145	MIMAT0000437	GUCCAGUUUUCCCAGGAAUCCCU

Supplementary Table 3. List of PCR primers used in this study.

Gene	Primer code / sequence	Primer type
18S rRNA	Hs99999901_s1	ABI TaqMan assay
Actin gamma-2	Hs01123712_m1	ABI TaqMan assay
E-cadherin (CDH1)	Hs00170423_m1	ABI TaqMan assay
Vimentin	Fwd: 5'-TCAGCATCACGATGACCTTGAA-3' Rev: 5'-CTGCAGAAAGGCACTTGAAAGC-3'	conventional PCR primer
Fibronectin	Fwd: 5'-CCAAGCATCACCCTGGGAGT-3' Rev: 5'-CGAAGCAGAACAGGCAATGTG-3'	conventional PCR primer
Snail2/Slug	Fwd: 5'-ATCTGCCAGACGCGAACTCA-3' Rev: 5'-GGCAACCAGACAACCGACAT-3'	conventional PCR primer
EGFR	Fwd: 5'-CAATTGGAAGATTGGAAGATTCAGC-3' Rev: 5'-CCAGTCAGGTTACAGGGCACA-3'	conventional PCR primer
IGF-IR	Hs00541255_21	ABI TaqMan assay
HER2	Fwd: 5'-AAGCGGCCCTAAGGGAGTGT-3' Rev: 5'-CATTGCTGTTCCTTCCTCATGG-3'	conventional PCR primer
VEGF	Fwd: 5'-TCCAATCTCTCTCTCCCTGATCG-3' Rev: 5'-GGGCAGAGCTGAGTGTTAGCAA-3'	conventional PCR primer
MMP-2	Hs00234422_m1	ABI TaqMan assay
MMP-7	Fwd: 5'-GCTGGCTCATGCCTTTGC-3' Rev: 5'-TCCTCATCGAAGTGAGCATCTC-3'	conventional PCR primer
MMP-9	Hs00234579_m1	ABI TaqMan assay
Syndecan-1	Hs00174579_m1	ABI TaqMan assay