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miR-221/222 control tumor progression of luminal breast cancers by regulating different targets

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ABSTRACT

The α6β4 integrin is an adhesion molecule for laminin receptors involved in tumor progression. We searched for a link between β4 integrin expression and miR-221/222 in the most prevalent mammary tumors in humans, the luminal invasive carcinomas (LICs). Using human primary tumors, displaying different levels of β4 integrin expression and grade, we showed that miR-221/222 expression inversely correlates with the tumor proliferating index, Ki67. Interestingly, the majority of high grade tumors express β4 integrin and low miR-221/222 levels. Thus, to investigate the possibility that miR-221/222 could regulate β4 expression, we ectopically transfected miR-221/222 in human-derived mammary tumor cell-line, that recapitulates the luminal subtype. We demonstrated that over-expression of miR-221/222 resulted in a down-regulation of β4 expression and was associated with inhibition of breast cancer cell proliferation and invasion. The role of miR-221/222 in driving β4 integrin expression was also confirmed by mutating the miR-221/222 seed sequence for the β4 integrin 3'UTR. Furthermore, we showed that these two miRNAs were also key regulators of both breast cancer cell proliferation and invasion through the post-transcriptional regulation of the signal transducer and activator of transcription 5A (STAT5A) and of a disintegrin and metalloprotease-17 (ADAM-17), respectively. By silencing ADAM-17 or using a dominant-negative or an activated form of STAT5A we further confirmed these data. miR-221/222-driven β4 integrin, STAT5A and ADAM-17 did not occur in the MCF-10A cells, denoted as "normal" breast epithelial cells, indicating that such a mechanism is specific of cancer cells.

These results for the first time provide evidence of a post-transcriptional mechanism that regulates β4 integrin, STAT5A and ADAM-17 expression to control proliferation and invasion of breast cancer cells. This suggests that exploiting pre-miR-221/222 in aggressive luminal subtype might represent a novel and powerful therapeutic anti-cancer strategy.

INTRODUCTION

Integrins are a family of heterodimeric transmembrane receptors, which mediate cell–extracellular matrix (ECM) and cell–cell interactions (1). The β4 integrin subunit was initially identified in cancer as a tumor-related antigen associated with tumor progression (2). Human studies demonstrated that β4 expression correlates with size and grade of breast cancer and with a poor patient prognosis in a variety of tumors of epithelial origin (3-4). The β4 integrin and its heterodimeric subunit α6 are the receptor for laminins (5-7). In physiological conditions β4 anchors the cytoskeleton to laminins in the basement membrane via hemidesmosomes located on the basal surface of epithelial cells (8). In several tumors of epithelial origin, which lack hemidesmosome anchorage, β4 integrin becomes diffusely distributed over the entire cell surface resulting in an increase of expression and signaling, and as consequence tumor invasion (9-10). Indeed, recently it has been demonstrated that β4 integrin depletion inhibits tumor cell expansion and invasion, mainly by impairing the activity of the phosphatidylinositol 3-kinase (PI3K) pathway (11).

Alternatively, cancer dissemination occurs via degradation of the extacellular matrix (ECM). In recent years, the members of *a d*isintegrin *a*nd *m*etalloproteinase family (ADAM) have attracted interest because of their adhesive and proteolytic properties (12). In addition, ADAMs regulates signaling events relevant for cell proliferation (12). ADAM-17, a multi-domain protein (13-14), is the most extensively investigated member of this family (15). ADAM-17 is widely distributed and its expression changes not only during embryonic development but also in adult life (16). Moreover, ADAM-17 protein is up-regulated in several diseases and tumors (17). In particular, increased expression of ADAM-17 correlates with mammary cancer development and adverse outcomes in breast cancer patients (18).

MicroRNAs (miRNAs), the endogenous small non-coding single-stranded RNAs, are key players in gene regulation (19). Their imperfect pairing with the 3'untranslated region (3'UTR) of target mRNAs generally results in mRNA degradation or translational inhibition (19). Deregulation of miRNA expression is considered a hallmark of cancer (20-21). Although altered expression of miRNAs has been reported in breast cancers (22-23), the functional consequences of specific miRNA aberrant expression in tumor progression and invasion remain to be elucidated. Recently, miR-221/222 over-expression has been shown in several advanced malignancies, including mammary tumors (24-25). Among miR-221/222 target genes the signal transducers and activator of transcription (STAT) 5A is included (26). In the context of breast cancer, active STAT5 can promote tumorigenesis in rodents (27), whereas in human breast cancer, STAT5 has been shown to positively correlate with the differentiation status of tumors (28) or with mammary epithelial cell differentiation and responsiveness to endocrine therapy (29-30). Collectively, these studies suggest a dual role of STAT5 in the mammary gland, as an initiator of tumor formation and a promoter of established tumor differentiation. However, the observations that inhibition of STAT5 in breast cancers impairs the proliferative rate suggest that the debate on the role of STAT5 in breast cancer is still open to debate (31-32).

Self-governing molecular mechanisms in breast cancer development and progression have been widely explored. Likewise, the role of β4 integrin as signaling receptor in mammary tumors has been extensively documented (3-4). However, more recently, a new scenario in which β4 integrin expression modulates miRNA pattern has been proposed for basal-like breast cancers (33). In this study we investigated, *in vitro* and *in vivo*, the functional relationship between miR-221/222

and β4 integrin, ADAM-17, and STAT5 in the progression of the luminal subtype breast cancers, which would benefit from a further prognostic stratification.

RESULTS

β4 integrin and miR-221/222 expression in human luminal invasive carcinomas (Lum-ICs). It has been shown that β4 expression modulates miR-221/222 expression in basal-like carcinomas (33). We evaluated the expression of miR-221/222 and β4 integrin in oestrogen receptor (ER)-positive (luminal) Lum-ICs using the gene expression profiling dataset generated by the TCGA consortium (The Cancer Genome Atlas Network) (34). We found that both miR-221/222 and β4 are expressed in a significant fraction of luminal breast cancers (Fig. 1A). Consistent with this observation, we demonstrated that β4 and miR-221/222 are expressed in the human-derived luminal breast cancer cell lines MCF-7, MDA-MB361 and T47D (Fig. 1B-C) (35). However, when β4 protein expression was evaluated on 15 primary human breast carcinoma samples (luminal subtypes), β4 was only found in the 50% of the cases that were preferentially high grade and highly proliferating tumors (Fig. 1D). By contrast, all tumor samples expressed miR-221/222 (Fig. 1E) even though at different levels. Significantly, low levels of miR-221/222 were observed in samples expressing β4 integrin (Fig. 1D-E), while high level of miR-221/222 was observed in low-grade tumors. Consistently, miR221/222 expression inversely correlated (miR221 p=0.0013; miR222 p=0.037) with the proliferating index, evaluated by Ki67 nuclear expression (Fig. 1F). All features of primary samples are reported in Table I.

β4 integrin expression is post-transcriptionally regulated by miR-221/222 in breast cancer cells. Based on the above results we hypothesized that miR-221/222 could control β4 integrin expression in luminal breast cancer subtype. To validate this possibility gain-of-function experiments were performed in MCF-7 wild type (wt) cell line (Fig. S1A). Data reported in Fig 2A show that over-expression of miR-221/222 led to a down-regulation of β4 integrin, suggesting that miR-221/222 could post-transcriptionally regulate β4 integrin expression. Recently, non-canonical interactions of miRNAs to their putative target genes have been reported (36). Thus the full-length 3'UTR nucleotide sequence of β4 integrin was analyzed for miR-221/222 blasting sequence and several base pairing were found (259-281bp 3'UTR β4 integrin) (Fig. 2B). The luciferase reporter vector containing the full-length 3'UTR of β4 integrin was then transfected in MCF-7 over-expressing miR-221/222. As control MCF-7 cells transfected with the luciferase reporter empty vector was used. As expected, the luciferase activity was not detectable in MCF-7 cells over-expressing miR-221/222 (Fig. 2C). These results were further confirmed by using a luciferase reporter vector containing a point mutation in the seed sequence for miR-221/222 of the β4 integrin 3'UTR (Fig. 2D). The finding that miR-221/222 control β4 integrin expression and not vice-versa is sustained by the results reported in Fig. 2E-F. Indeed, a transient silencing of β4 integrin did not modify miR-221/222 expression in MCF-7 cells.

Finally, to evaluate whether miR-221/222-driven β4 expression specifically regulates cancer cell fate, the expression of both β4 integrin and miR-221/222 was evaluated in MCF-10A cells. As shown in Fig. S2 these cells express low levels of β4 integrin and high levels of miR-221/222. Moreover, when loss-of function experiments was performed (Fig S1C) we were not able to detect changes in β4 expression (Fig S2A). This suggests that miR-221/222-driven β4 integrin expression is peculiar of tumor cells.

miR-221/222-driven β4 integrin down-regulation is crucial for MCF-7 proliferation and invasion. To investigate the biological relevance of miR-221/222-induced post-transcriptional regulation of β4 integrin, functional studies were performed in MCF-7 wt cells over-expressing miR-221/222. Over-expression of miR-221/222, resulting in β4 integrin down-regulation, was associated with an impaired ability of cells to pass across the extracellular matrix (Fig. 3E) and with an impaired cell proliferation, as sustained by cell counting, by expression of DNA-polymerase-δ auxiliary protein (e.g. Proliferating-Cell Nuclear Antigen, PCNA), by cyclin D1 expression and by the content of phosphorylated Akt (Fig.-3A-C), and impaired ability of cells to pass across the extracellular matrix (Fig. 3E). FACS analysis demonstrates that over-expression of miR-221/222 led to G0/G1 cell-cycle arrest (Fig. 3D). The biological relevance of miR-221/222 over-expression in MCF-7 cells was also confirmed by a clonogenic assay (Fig. 3F). As expected we were unable to detect a significant increase of MCF-10A cell proliferation when miR-221/222 antagomir were used (data not shown).

These data were further confirmed by using a stable clone, lacking the β4 integrin subunit (MCF-7 β4i) generated from the MCF-7 cell line (11). As shown in Fig. 4A the level of β4 integrin subunit in the MCF-7 scr-shRNA clone (MCF-7c) was similar to that of the parental cells and these cells were used as internal control. As expected, functional studies performed in these two clones demonstrated a 50% reduction of proliferation rate and invasiveness of cells depleted of β4 integrin (Fig. 4B-D). Unexpectedly MCF-7 β4i cells displayed a 10-fold increase of miR-221/222 expression (Fig. 4E) compared to the MCF-7c clone. This suggests that, as in-vivo, the level of miR-221/222 might control the proliferative and invasive capability of this tumor subtype. To evaluate this possibility loss-of function experiments were performed in MCF-7 β4i cells (Fig. S1B). As shown in Fig. 5 antagomir expression promoted MCF-7 β4i cell proliferation (Fig. 5A-B), cyclin D1 expression, Akt phosphorylation (Fig. 5C) and invasion (Fig. 5D).

miR-221/222 target STAT5A to regulate breast cancer cell proliferation. The above results suggest that even in the case of β4 integrin silencing, miR-221/222 still control breast cancer cell behavior by regulating additional targets. To investigate this hypothesis other known target genes were explored. As shown in Fig. 6A p27^{Kip1}and p57^{Kip2} level of expression was unchanged in MCF-7 β4i or in MCF-7c cells. Similar results were obtained in MCF-7 wt over-expressing miR-221/222 (data not shown). By contrast, the expression of STAT5A inversely correlated with that of miR-221/222 in the two different clones (Fig. 6A). Similar results were obtained when gain-and loss-of-function experiments were performed (Fig. 6A and Fig. S1A-B). The post-transcriptional regulation of STAT5A by miR-221/222 in our experimental models was further confirmed by the luciferase reporter assay performed in both clones, using the full-length 3'UTR of STAT5A gene or the point mutated 3'UTR seed sequence of STAT5A (Fig. 6B-C). Functional studies were then performed in both MCF-7 clones by over-expressing a dominant-negative STAT5A (ΔSTAT5A) or a constitutively activated STAT5 construct (1*6 STAT5A) (37). As reported in Fig. 6D, inactivation of STAT5A in MCF-7c or induction of STAT5A in MCF-7 β4i cells led to inhibition or induction of cell proliferation, respectively. On the contrary, we were unable to detect changes in cell invasiveness either by inactivating or activating STAT5A (Fig. 6E). These data along with the finding that high level of STAT5 expression was found in highly proliferative MDA-MB361 and T47D cell lines (Fig.S3), suggest that miR-221/222-driven STAT5A expression strictly controls

breast cancer cell proliferation. Consistent with this hypothesis STAT5A expression was not modulated by antagomir expression in MCF-10A cells (Fig. S2A).

miR-221/222 target ADAM-17 to drive cell invasion. Finally, the possibility that proteins involved in cell invasion could be also post-transcriptionally regulated by miR-221/222 was evaluated. To this end a number of prediction software (miRBase-MicroCosm version v5, miRanda, TargetScan and RNAhybrid) was interrogated. Particular attention has been paid to ADAM-17, that we found expressed in cells obtained from the MCF7c, MDA-MB361 and T47D cell lines, but not from the MCF-7 β4i clone (Fig. 7B and S2). Indeed, we found that miR-222 matched with the 3'UTR of the ADAM-17 gene (Fig. 7A). The inverse correlation between ADAM-17 and miR-221/222 in the two clones (MCF-7c and MCF-7 β4i) (Fig. 7B-C) was further confirmed by loss- and gain-of-function experiments (Fig. S1A-B). Thus, the luciferase reporter vector, containing the full-length 3'UTR of ADAM-17 was transfected in MCF-7c and MCF-7 β4i cells and the luciferase activity was evaluated. As expected, the luciferase activity was detected only in the MCF-7c cells (Fig. 7D). These results were confirmed by over-expressing or down-regulating miR-221/222 in MCF7c and MCF7b4i cells, respectively (Fig. 7D). The specificity of miR-221/222-mediated post-transcriptional regulation of ADAM-17 was further confirmed by using a luciferase reporter vector containing a point mutation in the seed sequence of the ADAM-17 3'UTR (Fig. 7E).

To evaluate the biological relevance of ADAM-17 in our models, siRNA technology was applied (Fig. 8A). As shown in Fig. 8B-C, MCF-7c cells depleted of ADAM-17 were still able to proliferate, as sustained by the cell counting, cyclin D1 expression and the phosphorylated Akt content. On the contrary, ADAM-17 depletion impairs cell invasive capability (Fig. 8E) without affecting β4 integrin expression (Fig 8D). Once again antagomir expression in MCF-10A cells had no effect on ADAM-17 expression (Fig. S2A)

Finally, the presence of a disintegrin domain in the ADAM-17 sequence led us to evaluate whether β4 integrin and ADAM-17 could act in cooperation to mediate cell invasion. Indeed, we found that β4 integrin is physically associated to ADAM-17 in our experimental condition (Fig. 8F).

DISCUSSION

In this study we demonstrate that miR-221/222 play a role in regulating tumor growth and invasion of breast carcinomas of the luminal subtype by regulating different genes. miRNAs are abnormally expressed in a variety of cancer types including breast cancers and they can act as *oncomiRs* or *oncosuppressor-miRs* depending on the cellular contest (38). This is particularly true for miR-221/222 that may act as oncomiRs in tumors of epithelial origin (39) or as oncosuppressor in hemopoietic malignancies (40). In the context of breast cancer a correlation between miR-221/222 and the response of breast cancer cells to tamoxifen has been reported (41). Moreover, the miRNA microarray screening of luminal- and basal-like subtypes revealed that basal-like tumors express high level of miR-221/222 (42). More recently these miRs (33) were found inversely correlated with β4 integrin expression. By analyzing the gene expression profiling dataset generated by the TCGA consortium (36) we found that miR-221/222 and β4 integrin are expressed in luminal carcinomas as well. However, we failed to detect an inverse correlation between the expression of miR-221/222 and that of β4 integrin. Since the TCGA is a genomic database and did not consider tumor grade, a possible correlation between miR-221/222 and β4 integrin protein expression in human carcinoma samples of luminal subtype, displaying distinct grade of differentiation and proliferation activity was investigated. Consistent with the role of β4 integrin in tumor progression, we found that the expression of β4 was associated with poor differentiation of primary luminal LIC (G3 tumors) and with low expression of miR-221/222. On the other hand, we found an inverse correlation between the expression of both miRs and the tumor proliferating index, Ki67.

Expression, localization, and cytoskeletal interactions of β4 integrin are crucial driver of proliferation and invasion of cancer cells (43). The increase of β4 expression in cancers of epithelial origin has suggested that β4 expression might be regulated, at least in part, at the transcriptional level (44). In the present study we first demonstrate that in the MCF-7 cell line, a known breast carcinoma cell line of luminal subtype (45), β4 integrin expression is under the control of miR-221/222. The concept that miR-221/222 by regulating β4 expression dictates tumor aggressiveness was further validated by functional studies. The cells over-expressing miR-221/222 displayed a low proliferative rate and invasiveness as well as an almost undetectable PI3K/Akt activation (46). The finding that miR-221/222 down regulation in MCF-10A cells did not affect β4 integrin expression further sustains that such a mechanism controls specifically cancer cell fate.

The role of the PI3K signaling cascade in mediating breast tumor progression (47) has spurred the development of different classes of PI3K, Akt, and mTOR inhibitors that at present are used in clinic (47-48). However, the appearance of resistance to such approaches highlighted the need for novel anti-cancer strategies. A combined targeting of the PI3K/mTOR and the JAK2/STAT5 pathways in breast cancers has provided new therapeutic opportunity (49). Indeed the STAT pathway is activated in response to different stimuli (50-52) and a cross talk between STAT5 and integrins has been also reported (53-55). We herein first demonstrate that miR-221/222 strictly control STAT5A expression in breast cancer cells. Moreover, loss-of-function experiments in cells depleted of β4 integrin indicate that such post-transcriptional event is independent of β4 integrin expression while depends on miR-221/222 deregulation. Although the role of STAT5 in breast cancers is still debated (28), agents that down-regulate STAT5 reduce the growth of breast cancer cell lines derived from different breast cancer sub-types (31-32). Consistently, we demonstrated that low STAT5 expression was associated with a low proliferation rate. The biological relevance of

STAT5 in mediating breast cancer cell proliferation is further sustained by ectopic expression of the constitutively active form or the dominant negative STAT5 construct. STAT5 is crucial for biological functions as cell proliferation and survival, but also for cell migration (53,56). In our models we failed to detect a relevant role of STAT5 in cell invasion. This suggests that in carcinomas of the luminal subtype, STAT5 primarily controls cell proliferation.

Cell invasion in tumors lacking hemidesmosome anchorage is facilitated by β4 integrin switching into a more active signaling receptor (9-10). However, extracellular proteases, as ADAMs, also actively participate in invasion by acting as a control device of cell-ECM interactions (57). ADAMs have a complex multi-domain structure having proteolytic potential and adhesive and signaling properties (56). Data from pre-clinical cancer models indicate the 'shedding' activities of ADAMs, cleaving or solubilizing the ectodomain of cytokines, growth factors, receptors and adhesion molecules may regulate different activities that include cell migration and proliferation (57). Indeed, a correlation between the expression of ADAM-17 and high-grade invasive breast tumors has been reported in humans (18). In keeping with clinical data, we demonstrate that human derived cell lines, displaying high proliferative rate and invasive capability, express ADAM-17. Possibly due to the presence of a disintegrin domain, it is able to bind and to regulate the activity of integrins (12,16), ADAM-17 physically interacts with the β4 integrin and controls β4 integrin-mediated cell invasion, but not cell proliferation. This is particularly true as knock down of ADAM-17 was associated with an impaired β4 integrin-mediated invasion, while it had no effect on cell proliferation. Finally, in the present study we first demonstrate that ADAM-17 expression is strictly controlled by post-transcriptional mechanisms involving miR-221/222 only in tumor cells.

In cancer cells β4 over-expression supports cellular events involved in tumor progression (9-10). Although the precise mechanisms involved still remain unclear, we identify a post-transcriptional mechanism driven by miR-221/222 that modulates β4 integrin, STAT5A and ADAM-17 expression in luminal carcinomas displaying a more aggressive behavior. This suggests that the levels of expression of miR221/222 in combination with that of β4 integrin, ADAM17, and STAT5 would confer additional benefits to the prognostic stratification of the luminal breast cancer subtypes. Moreover, the pleiotropic effects of miR-221/222, occurring in cancer cells only, indicates that efforts should now be made to develop successful *in vivo* delivery systems to investigate the therapeutic potential of pre-miR-221/222 in reverting cancer cell behavior.

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MATERIALS AND METHODS

Reagents and antibodies are presented in online supplements.

Laminin 5 preparation. A laminin 5–enriched matrix was prepared from 804G cells as previously described (58). Briefly, confluent 804G cells in either 100-mm dishes or 96-well plates were washed in sterile PBS and detached from the underlying laminin 5–enriched matrix by treatment for 10 min in 20 mmol/L NH_4OH at 4°C and subsequent washing twice with sterile PBS. Poly-L-lysine (Calbiochem, Milan, Italy) was used as control matrix at a concentration of 10 mg/mL.

Cell lines. The human breast carcinoma cell lines MCF-7, MDA-MB361 and T47D were obtained from the American Type Culture Collection (Manassas, VA), maintained in RPMI medium supplemented with 10% FCS (Invitrogen, Milan, Italy) and cultured on laminin. Generation of MCF-7 scrambled short hairpin RNA (scr-shRNA), defined MCF7c and the MCF-7 β4 shRNA cell subclones, defined MCF7 β4i, expressing or not α6β4 integrin, respectively, were obtained as previously described (22). To produce matrigel the rat bladder epithelial cell line 804G, to produce matrigel, was kindly provided by Dr. G. Meneguzzi (Faculty of Medicine, Institut National de la Sante´ et de la Recherche Me´dicale U634, Nice, France). The human mammary cell line MCF-10A, used as control (59), was kindly provided by Prof. D. Taverna (Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy).

Cell proliferation. The proliferative activity was assayed by direct cell count, by three individual operators in triplicate, as previously described (number ± SEM of cells per field, 10X magnification) (52). Proliferation was also evaluated as expression of DNA-polymerase-δ auxiliary protein (e.g. Proliferating-Cell Nuclear Antigen, PCNA) or by flow cytometer (FACScan, Becton Dickinson Immunocytometry Systems, San Jose, CA) analysis. Cell-cycle phases were also analyzed: breast cancer cells, treated as indicated, were fixed with 70% ethanol. After digestion with RNase, DNA was stained with propidium iodide and analyzed with FACScan (53).

Cell invasion. Cell invasion was assessed using a 48-well modified Boyden's chamber (NeuroProbe, Pleasanton, CA) and 8-μm pore polyvinyl pyrrolidone–free polycarbonate Nucleopore filters (Costar, New York, NY). The filters were coated with 3 mg/mL Cultrex (Trevigen, Gaithersburg, MD). The lower compartment of the chamber was filled for 24h with conditioned serum free medium produced from NIH3T3 fibroblasts. MCF-7 wt, MCF-7c and MCF-7 β4i cells (5×10^4cells/ml), treated as indicated, were harvested and placed in the upper compartment of the Boyden's chamber. After 8h of incubation at 37°C, the cells migrated on the lower surface of the filters were fixed and stained with DiffQuick (Merz-Dade, Dudingen, Switzerland). The migrated cells in 12 high power fields were counted. Each assay was carried out in quadruplicate and repeated at least three times. The ability of the cells to adhere to the filters was verified by staining the upper side of the filter for each cell line.

Clonogenic assay To investigate the ability of tumor cells to form colonies, 1×10^5 cells, over-expressing or not miR-221/222, were re-suspended in 0.3% agarose (low gelling temperature agarose) and seeded on 6 well plates pre-plating with 0.6% regular agarose. Colonies were counted

after 3 weeks by three individual operators in triplicate (number ± SEM of cells per field, 10X magnification)

Analysis of TCGA data. Gene and microRNA expression profiling data of primary breast tumors were obtained from the website (https://tcga-data.nci.nih.gov/docs/publications/brca_2012/) associated to Ref. (PMID 2300897), and selected based on the clinical and molecular parameters reported in the same website.

Human carcinoma samples (luminal subtype, Lum-ICs). A series of 15 ER positive invasive carcinomas of no special type (60) were retrieved from the archives of the Pathology Unit at our Institution. The study was approved by the ethic institutional review board for "Biobanking and use of human tissue for experimental studies" of the Pathology Services of the Azienda Ospedaliera Città della Salute e della Scienza di Torino. Written informed consent was obtained from all patients to authorize their tissue to be used in research.
The cohort comprised 6 low grade (G1) carcinomas and 9 high grade (G3) carcinomas (61-62). ER positivity was scored according to the ASCO/CAP guidelines (62) and proliferation index was evaluated as a continuous variable (percentage of stained cells) (63).

Immunohistochemistry (IHC). IHC was performed on 3 μm thick sections of formalin fixed paraffin embedded tissues (FFPE) using the Ventana BenchMark® XT automated immunostainer (Ventana Medical Systems, Tucson,AZ, USA). Slides were incubated with anti-β4-integrin antibody (diluition 1:200) for 32 minutes at room temperature, after protease 1 (Ventana Medical Systems, Tucson, AZ, USA) pretreatment (4 minutes). Positive and negative controls (omission of the primary antibody and IgG-matched serum) were included for each immunohistochemical run.

RNA isolation and quantitative real-time PCR (qRT-PCR) for miRNAs. Total RNA was isolated using mirVana extraction kit (Ambion) from MCF-7 wt and cell clones, from MDA-MB361, T47D and MCF-10A cells. RNA was also extracted from fresh frozen blocks. The number of sections needed to obtain a nucleic acid yield adequate for molecular analysis depended on the type of tissue samples (fibrosis, cellularity) and sample dimension. Sections were collected in a 1.5 ml sterile Eppendorf tube. RNA extraction from fresh frozen sections was performed with 1 ml of Trizol reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNA was then reverse-transcribed using a TaqMan microRNA RT kit specific for miR-221 and miR-222.
Reverse miRNAs were subjected to quantitative real-time PCR using TaqMan microRNA assay kit and the ABI PRISM 7700 sequence detection system (Applied Biosystems). Expression of miRNAs was normalised to small nuclear RNA, RNU6B. Gain- and loss-of-function experiments were performed in MCF-7 wt, MCF-7c, MCF-7 β4i or MCF-10A cells as previously described (26)

ADAM-17 silencing by small interfering RNAs (siRNA). To obtain ADAM-17 inactivation, MCF-7c cells were transiently transfected with MISSION siRNA for ADAM-17 or with duplex siRNAs purchased by Sigma-Aldrich. Transfection was performed according to the vendor's instructions. 48h later whole cell extracts were processed. Cell viability was evaluated at the end of each experiment.

Dominant negative STAT5A (ΔSTAT5A) and STAT1*6 vector transfection. MCF-7c and MCF-7 β4i cells were transiently transfected with the ΔSTAT5A construct or with STAT1*6 plasmid vector, as previously described (37). The empty vector pCNeo was used as control.

Luciferase miRNA target reporter assay. The luciferase reporter assay was performed using a construct generated by sub-cloning into restriction site XbaI of the luciferase reporter vector pGL3 Basic Vector (Promega, Madison, WI, USA) the PCR products amplified from full-length 3'UTR of ADAM-17, STAT5A and β4 integrin DNA. The PCR products were obtained using the following primers:
ADAM-17: sense, 5'TCTAGATTTAGTTCTCAGCTCTTCTGAC3'
       antisense, 5'TCTAGAGTCTCACTCTGTCACCCA3':

STAT5A: sense, 5'AAGAGCTCATGTTTGAATCCCACGCT3'
       antisense,5'TTGAGCTCACACAAATGTGTGGTCTT3':

β4 integrin: sense, 5'TCTAGATGACCGCACCCTGCCCCACC3'
       antisense, 5'TCTAGAAGCAGTAGCAAAACCATTAT3'
A site-directed mutagenesis of the 3'UTR ADAM-17 and of the 3'UTR β4 integrin amplified PCR product was performed to obtain the mutated miR-222 or miR-221 binding site, respectively. The sequence was generated using the Quik-Change SiteDirect Mutagenesis kit (Stratagene, La Jolla, CA, USA). The oligonucleotides used were: sense, 5'CTAGTTATTACC*TATAT*TTTTTATGTAGC3' for ADAM-17 and sense, 5'TGTAACCAAAGA*TA*TG*TA*AGCAGCACAAG3' for β4 integrin, containing the desired mutation, was designed according to the manufacturer's instructions (the mutated nucleotides are underlined and italicized). The mutated 3'UTR STAT5A luciferase vector was obtained as previously described (26).
The insert identities were verified by sequencing. The pGL3, pGL3-3'UTR ADAM-17, pGL3-3'UTR STAT5A and pGL3-3'UTR β4 full length or mutated reporter vectors were transiently co-transfected in MCF-7c or MCF-7 β4i cells, treated as indicated, at 30:1 molar ratio with the pRL vector, coding for the *Renilla* luciferase, used as internal control of luciferase assay as previously described (64). Luciferase activities were analyzed 48 h after transfection by Dual-Luciferase Report Assay System (Promega) as previously described (26,64).

Statistical analysis. Comparison and significance of differences between two groups was performed with t test. Comparison between three or more groups was performed with one-way ANOVA and significance of differences were evaluated with Newman-Keuls multicomparison post test. p values *<0.05, **<0.01 and ***<0.001 were considered significant and were indicated with different symbols, as detailed in each figure legend. All statistical analyses were carried out with Graph Pad Prism version 5.04 software (Graph Pad Software, Inc, USA). Densitometric analysis was used to calculate the differences in the fold induction of protein levels and normalized to β actin or to Akt content and reported as "Relative amount" in figures.

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Table I. Histopathological and immunophenotypical features of primary human tumor samples of luminal subtype (Lum-ICs). All cases showed high expression (range: 75%-100%) of estrogen receptor (ER) with variable expression of progesterone receptor (PR) and a proliferation index (Ki-67) ranging between 5% and 60%. ER, PR and Ki-67 are expressed as percentage of positive cells. Histological type is categorized according to the latest WHO classification (IC-NST: invasive carcinoma of no special type; ILC: invasive lobular carcinoma; MPC: micropapillary carcinoma: TUB: tubular carcinomas)

Sample	Histological Type	Grade	% ER	%Ki-67	% PR
1	IC-NST+ILC	1	98	12	90
2	IC-NST	1	100	5	95
3	IC-NST	1	100	20	70
4	IC-NST+ TUB	1	95	16	90
5	IC-NST	1	95	6	95
6	IC-NST	1	85	15	80
7	IC-NST	3	98	47	25
8	IC-NST + MPC	3	95	35	20
9	IC-NST	3	98	23	95
10	IC-NST	3	95	60	75
11	IC-NST + MPC	3	75	39	30
12	IC-NST	3	98	46	0
13	IC-NST	3	100	31	30
14	IC-NST	3	100	39	70
15	IC-NST	3	90	30	<1

FIGURE LEGENDS

Figure 1. β4 integrin and miR-221/222 expression in Lum-IC samples. (A) β4 integrin and miR-221/222 distribution in basal- and luminal-like carcinomas from dataset of TCGA consortium. **(B)** Cell extracts from luminal-derived MCF-7, MDA-MB361 and T47D cell lines were analysed by western blot for β4 integrin content by densitometry (Relative amount). Protein levels were normalised to β actin content. The results are representative of four different experiments performed in triplicate (n=4). **(C)** MCF-7, MDA-MB361 and T47D cell lines were analysed by qRT-PCR to evaluate miR-221 and miR-222 expression. The reported data were normalised to RNU6B and are representative of five different experiments performed in triplicate (n=5). **(D)** Representative immunohistochemical staining for β4 integrin expression on human Lum-IC sections, including grade 1 and grade 3 samples. Scale bar: 80 μm (×40 magnification). **(E)** To evaluate miR-221 and miR-222 expression qRT-PCR was performed in human Lum-IC samples (n=6, grade 1; n=9, grade 3). The reported data are normalized to RNU6B and are representative of all samples, performed in triplicate. MCF-7 cell line was used as control for miRNA expression. (*** *p<0.001* grade 3 vs grade 1 for miR-221; * *p<0.05* grade 3 vs grade 1 for miR-222). **(F)** Correlation between the proliferating index Ki67 and miR-221 or miR-222 expression in the luminal carcinoma samples. r correlation coefficient and p values are reported.

Figure 2. miR-221/222 post-transcriptionally regulate β4 integrin expression. (A) MCF-7 wild type (wt) cells were transfected with pre-miR negative control (neg c) or with pre-miR-221 or pre-miR-222. 48h later cell extracts were subjected to western blot analysis to evaluate β4 integrin and β actin content (*** *p<0.001* MCF-7 transfected with pre-miR-221 and pre-miR-222 vs pre-miR neg c). The results are representative of three different experiments performed in triplicate (n=3). **(B)** Blast analysis of human miR-221 sequence and 3'UTR full length of β4 integrin shows several base pairing from bp 259 to 281 of the β4 integrin 3'UTR. **(C)** pGL3 empty vector or pGL3-3'UTR β4 integrin luciferase constructs were transfected into MCF-7 wild type cells, previously transfected with pre-miR neg c or with pre-miR-221 or pre-miR-222. The relative luciferase activity is reported (*** *p<0.001* pre-miR neg c in presence of pGL3-3'UTR β4 vs pGL3; *** *p<0.001* pre-miR-221 or pre-miR-222 vs pre-miR neg c in pGL3-3'UTR β4-transfected cells) (n=4). **(D)** Luciferase activity was evaluated in MCF-7 cells co-transfected with pre-miR neg c or pre-miR-221 or pre-miR-222 precursors and pGL3 or pGL3-3'UTR β4 integrin mutated into the seed sequence (*mut*). Relative luciferase activity is reported (*** *p<0.001* pre-miR neg c in presence of pGL3-3'UTR*mut* β4 vs pGL3) (n=3). **(E)** MCF-7 wild type cells were transfected for 48h with scramble or short hairpin (sh)-β4 integrin and evaluated for β4 integrin and β actin content. (*** *p<0.001* sh-β4 integrin vs scramble for β4 integrin content). The results are representative of four different experiments performed in triplicate (n=4). **(F)** qRT-PCR was performed to evaluate miR-221 and miR-222 expression in MCF-7 wild type cells transfected with scramble or sh-β4 integrin. The reported data were normalised to RNU6B and are representative of five different experiments performed in triplicate (n=5).

Figure 3. miR-221/222 over-expression impairs MCF-7 cell proliferation and invasion. (A) Cell proliferation assay was performed for the indicated time intervals on MCF-7 wild type cells transfected with pre-miR neg c, pre-miR-221 or pre-miR-222 (*** *p<0.001* pre-miR neg c vs pre-miR-221 and pre-miR-222 transfected MCF-7 cells) (n=4). **(B)** Representative FACS analysis to

evaluate PCNA expression in MCF-7 cells, transfected as above. The PCNA staining (48h after transfection) is reported. Percentage ± SEM of reduced proliferation: pre-miR-221, 60±5.6; pre-miR-222, 40±4.4, ***$p<0.001$. **(C)** Cyclin D1 and phospho (p)Akt content in pre-miR neg c-, pre-miR-221- and pre-miR-222-transfected MCF-7 cells was evaluated by western blot analysis. Protein levels were normalised to β actin or Akt content, respectively. The results are representative of four different experiments performed in triplicate (n=4) (*** $p<0.001$ pre-miR-221 and pre-miR-222 vs pre-miR neg c transfected MCF-7 cells). **(D)** Cell proliferation assay was performed by FACS analysis to evaluate the percentage of cells in each cell-cycle phase. The results are representative of three different experiments performed in triplicate (data are expressed as mean, n=3). **(E)** Invasion assay was performed on MCF-7 transfected as above. Percentage of invading cells is reported (*** $p<0.001$ pre-miR-221 and pre-miR-222 vs pre-miR neg c transfected MCF-7 cells) (n=4). **(F)** Clonogenic assay was performed on MCF-7 cells treated as above. The number of formed clones per field (20X magnification) is reported in the histogram (*** $p<0.001$ pre-miR-221 and pre-miR-222 vs pre-miR neg c transfected MCF-7 cells) (n=3).

Figure 4. β4 integrin depletion led to cell proliferation and cell invasion inhibition in breast cancer cells. (A) Cell extracts from MCF-7 wt cells, MCF-7 scr-shRNA clone (MCF7c) or MCF-7 β4-shRNA clone (MCF-7 β4i) were subjected to SDS-PAGE to evaluate β4 integrin and β actin content (*** $p<0.001$ MCF-7 β4i vs MCF-7 wt and MCF-7c cells). The results are representative of three different experiments performed in triplicate (n=3). **(B)** Cell proliferation assay was performed for indicated time intervals on MCF-7c and MCF-7 β4i clones (*** $p<0.001$ MCF-7 β4i vs MCF-7c) (n=3). **(C)** PCNA staining in MCF-7 cell clones analysed by FACS. Percentage ± SEM of reduced proliferation: MCF-7 β4i 50±5.7, ***$p<0.001$. **(D)** Invasion assay was performed on MCF-7c and MCF-7 β4i cells. Percentage of invading cells is reported (*** $p<0.001$ MCF-7 β4i vs MCF-7c) (n=4). **(E)** miR-221 and miR-222 expression was analysed by qRT-PCR on MCF-7c and MCF-7 β4i cells. The reported data were normalised to RNU6B and are representative of four different experiments performed in triplicate (n=4) (*** $p<0.001$ miR-221 and miR-222 expression in MCF-7 β4i vs MCF-7c).

Figure 5. miR-221/222 loss-of-function in the MCF-7 β4i clone rescues proliferation and invasiveness. (A) Cell proliferation assay was performed for indicated time intervals on MCF-7 β4i cells transfected with anti-miR neg c, anti-miR-221 or anti-miR-222 (*** $p<0.001$, anti-miR-221 or anti-miR-222 vs anti-miR neg c transfected MCF-7 β4i cells) (n=3). **(B)** PCNA staining in MCF-7 β4i cells transfected as above. Percentage ± SEM of increased proliferation: anti-miR-221, 60±3.7; anti-miR-222, 50±4.2 ***$p<0.001$. **(C)** MCF-7 β4i cells treated as above were analysed for cyclin D1, pAkt and β4 integrin content. Protein levels were normalised to β actin or Akt content. The results are representative of three different experiments performed in triplicate (n=3) (*** $p<0.001$ anti-miR-221 and anti-miR-222 vs anti-miR neg c for cyclin D1 and pAkt content). **(D)** Invasion assay was performed on MCF-7 β4i cells treated as indicated. Percentage of invading cells is reported (*** $p<0.001$ anti-miR-221 and anti-miR-222 vs anti-miR neg c) (n=4).

Figure 6. miR-221/222 drive mammary tumor cell proliferation by regulating STAT5A expression. (A) p27[Kip1], p57[Kip2], STAT5A and β actin content were analysed by western blot in MCF7-7c cells transfected with pre-miR neg c, pre-miR-221 or pre-miR-222 precursors or in the MCF-7 β4i cells transfected with anti-miR neg c, anti-miR-221 or anti-miR-222 oligonucleotides.

The results are representative of three different experiments performed in triplicate (n=3) (*** *p<0.001* gain- and loss-of-function values vs control values for STAT5A content). **(B)** Luciferase activity was evaluated in MCF-7 clones treated as indicated. Relative luciferase activity is reported (*** *p<0.001* pre-miR neg c-transfected MCF-7c in presence of pGL3-3'UTR STAT5A vs pGL3; *** *p<0.001* pre-miR-221 or pre-miR-222 vs pre-miR neg c transfected MCF-7c; *** *p<0.001* anti-miR-221 or anti-miR-222 vs anti-miR neg c transfected MCF-7 β4i) (n=4). **(C)** pGL3 empty vector or the pGL3-3'UTR STAT5A mutated seed sequence (*mut*) were co-transfected with pre-miR neg c or pre-miR-221 or pre-miR-222 precursors in MCF-7c. Relative luciferase activity is reported (*** *p<0.001* pre-miR neg c transfected MCF-7c in presence of pGL3-3'UTR*mut* STAT5A vs pGL3) (n=3). **(D)** Cell proliferation assay was performed for indicated times in MCF-7c cells, transfected with pCNeo empty vector or pCNeo-ΔN STAT5A construct, and in MCF-7 β4i cells, transfected with pCNeo empty vector or pCNeo-1-6*STAT5A construct (*** *p<0.001* pCNeo-ΔN STAT5A and pCNeo-1-6 *STAT5A constructs vs pCNeo empty vector) (n=3). **(E)** Invasion assay was performed on MCF-7 clones treated as above. Percentage of invading cells is reported (n=4).

Figure 7. miR-221/222 post-transcriptionally regulate ADAM-17 expression. (A) Alignment of miR-222 with its potential binding site in the 3'UTR of ADAM-17 mRNA (miRANDA web site). **(B)** ADAM-17 and β actin content in MCF-7c and MCF-7 β4i clones (*** *p<0.001* MCF-7 β4i vs MCF-7c). The results are representative of three different experiments performed in triplicate (n=3). **(C)** Cell extracts from MCF-7c and MCF-7 β4i cells subjected to gain- and loss-of-function experiments respectively were analysed by western blot for ADAM-17 content. Protein levels were normalised to β actin content (*** *p<0.001* gain- and loss-of-function values vs control values for ADAM-17 content). The results are representative of four different experiments performed in triplicate (n=4). **(D)** Luciferase activity was evaluated in MCF-7 clones treated as indicated. Relative luciferase activity is reported (*** *p<0.001* pre-miR neg c-transfected MCF-7c in presence of pGL3-3'UTR ADAM-17 vs pGL3; *** *p<0.001* pre-miR-221 or pre-miR-222 vs pre-miR neg c transfected MCF-7c; *** *p<0.001* anti-miR-221 or anti-miR-222 vs anti-miR neg c transfected MCF-7 β4i) (n=4). **(E)** pGL3 empty vector or pGL3-3'UTR ADAM-17 mutated seed sequence (*mut*) were co-transfected with pre-miR neg c or pre-miR-221 or pre-miR-222 precursors in MCF-7c. Relative luciferase activity is reported (*** *p<0.001* pre-miR neg c transfected MCF-7c in presence of pGL3-3'UTR*mut* ADAM-17 vs pGL3) (n=3).

Figure 8. miR-221/222-regulated ADAM-17 expression drives mammary tumor cell invasion. (A) ADAM-17, cyclin D1 and β actin content were analysed by western blot in MCF-7c cells transfected for 48h with scramble or ADAM-17 siRNA (*** *p<0.001* siRNA ADAM-17 vs scramble for ADAM-17 content).. The results are representative of four different experiments performed in triplicate (n=4). **(B)** Western blot analysis for pAkt and Akt content in MCF-7c silenced or not for ADAM-17. The results are representative of three different experiments performed in triplicate (n=3). **(C)** Cell proliferation assay for indicated time intervals on MCF-7c cells treated as above (n=4). **(D)** Cell extracts from MCF-7c cells silenced or not for ADAM-17 were analysed by western blot to evaluate β4 integrin and β actin content. The results are representative of three different experiments performed in triplicate (n=3). **(E)** Invasion assay was performed on MCF-7c cells treated as above. Percentage of invading cells is reported (*** *p<0.001* siRNA ADAM-17 vs scramble) (n=4). **(F)** Co-immunoprecipitation experiments were performed in

MCF-7 wt cells using ADAM-17 and β4 integrin antibodies. β4 integrin and ADAM-17 content was evaluated. The results are representative of three different experiments performed in triplicate (n=3).

Figure S1. Gain and loss-of-function experiments. (A-C). Following transfection of MCF-7 wt and MCF-7c cells with pre-miR neg c or pre-miR-221 or pre-miR-222 **(A)**, or transfection of MCF-7 β4i and MCF-10A cells with anti-miR neg c or anti-miR-221 or anti-miR-222 **(B-C)**, qRT-PCR was performed to evaluate miR-221 and miR-222 expression. The reported data are normalized to RNU6B and are representative of five experiments (n=5) (*** *p<0.001* miR-221 and miR-222 expression in MCF-7 wt and MCF-7c cells transfected with pre-miR-221/222 vs cells transfected with pre-miR neg c, A; ** *p<0.01* miR-221 and miR-222 expression in MCF-7 β4i and MCF-10A cells transfected with anti-miR-221/222 vs cells transfected with anti-miR neg c, B-C).

Figure S2. miR-221/222 expression in MCF-10A cells do not affect β4 integrin, STAT5A or ADAM-17 expression. (A) MCF-10A cells transfected with anti-miR neg c or anti-miR-221 or anti-miR-222 were analysed for β4 integrin, STAT5A and ADAM-17 content. Protein levels were normalised to β actin content. The results are representative of three different experiments performed in triplicate (n=3). **(B)** qRT-PCR was performed to evaluate miR-221 and miR-222 expression in MCF-7 wt and MCF-10A cells. The reported data are normalized to RNU6B and are representative of five experiments (n=5) (*** *p<0.001* miR-221 and miR-222 expression in MCF-10A vs MCF-7 wt cells).

Figure S3. STAT5A, ADAM-17 and pAkt content in MCF-7, MDA-MB361 and T47D cell lines. (A) Cell extracts from MCF-7, MDA-MB361 and T47D cell lines were analysed by western blot for STAT5A, ADAM-17 and pAkt content by densitometry (Relative amount). Protein levels were normalised to β actin (STAT5A and ADAM-17) or to Akt (pAkt) content. The results are representative of four different experiments performed in triplicate (n=4). **(B)** Cell proliferation assay was performed for the indicated time intervals on luminal cell lines (n=4). **(C)** Invasion assay was performed on MCF-7, MDA-MB361 and T47D cells. Percentage of invading cells is reported (n=4).
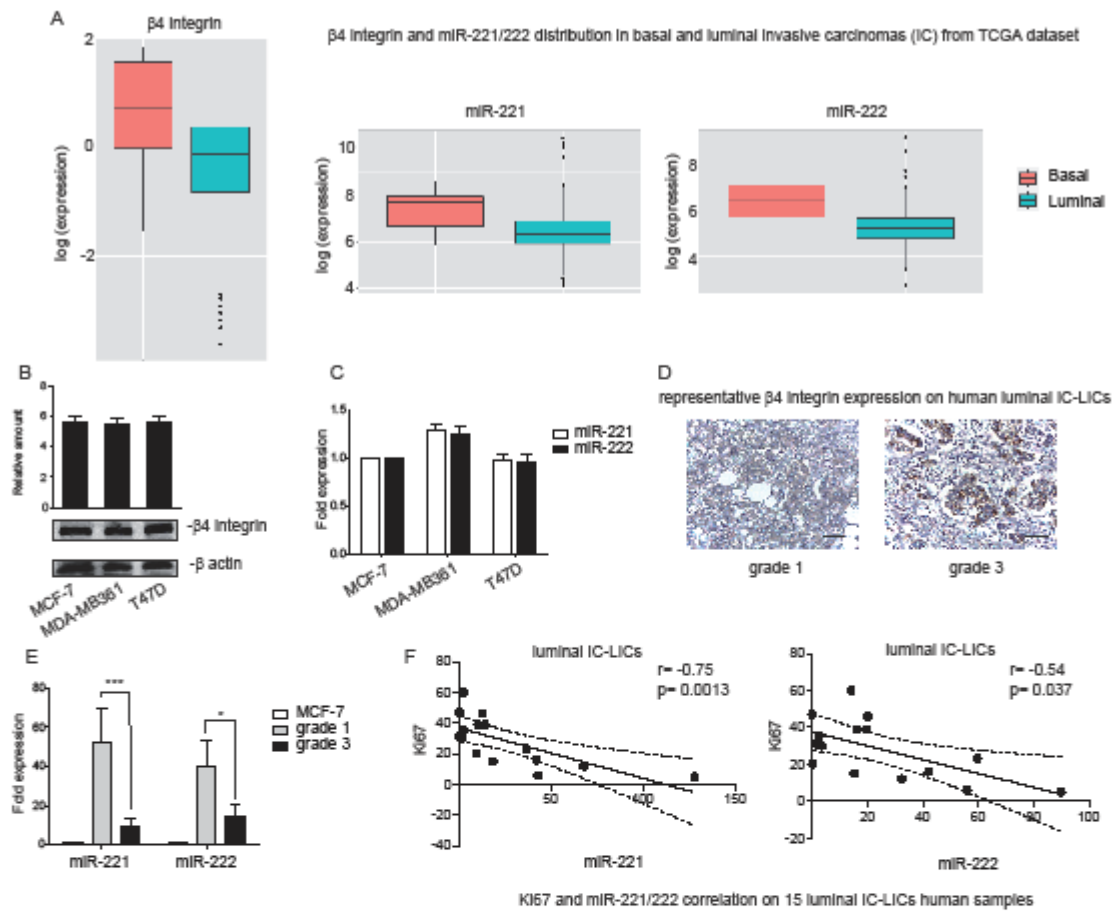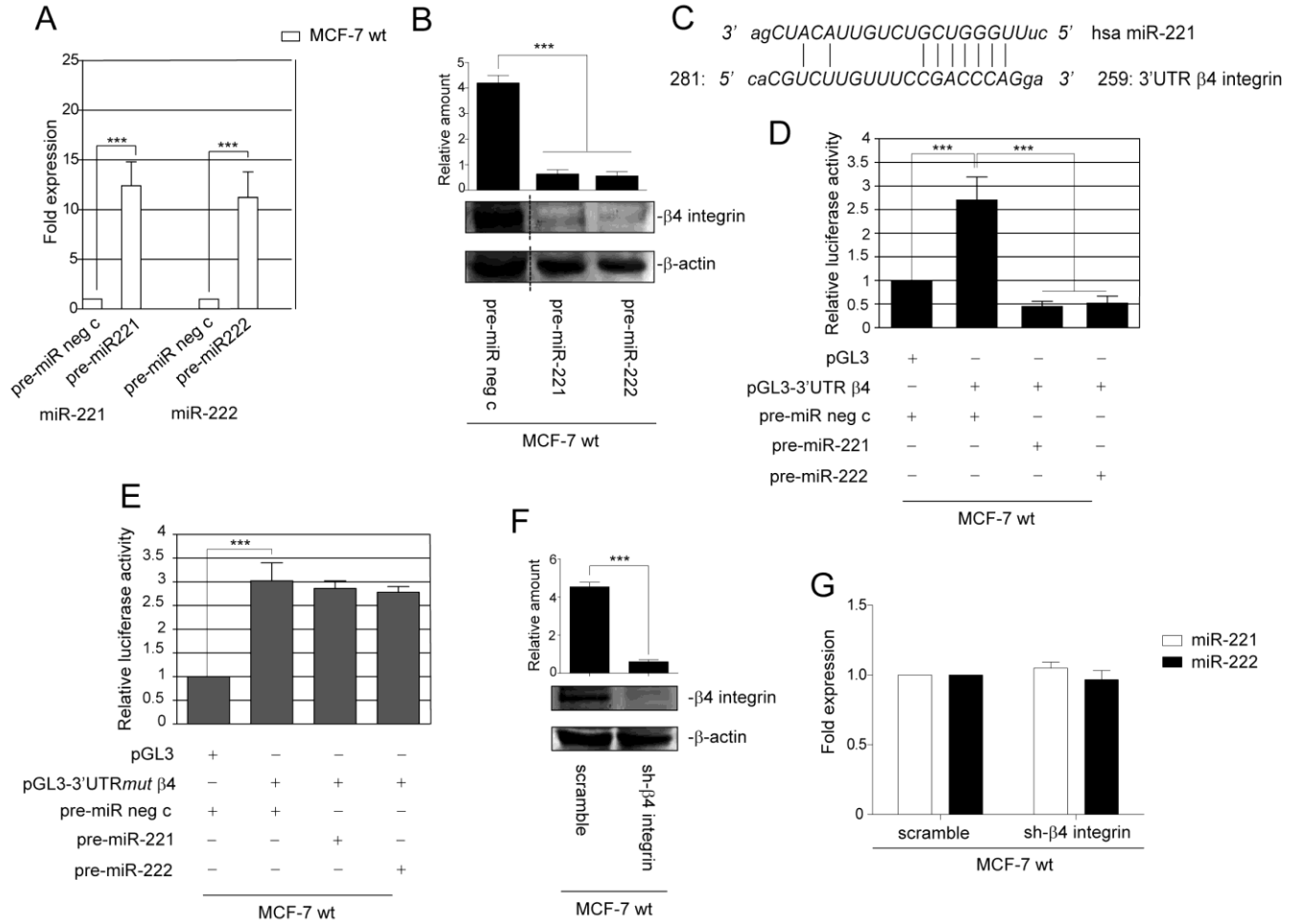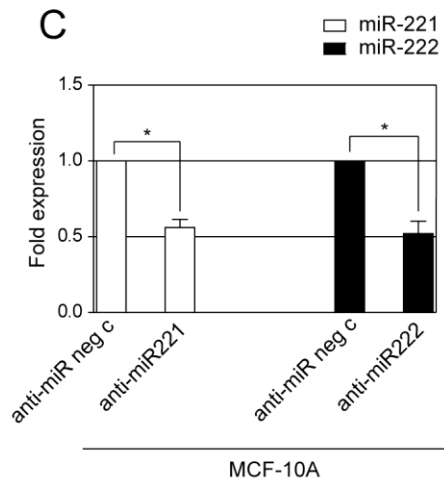
Figure 1

A

β4 integrin
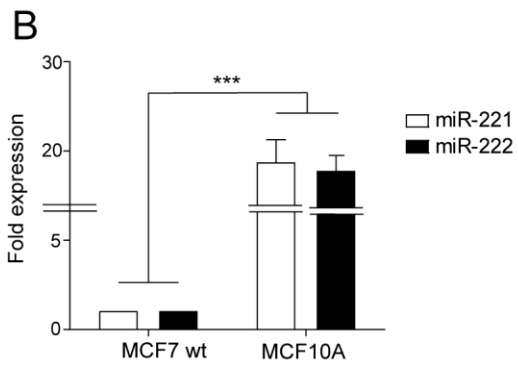
β4 integrin and miR-221/222 distribution in basal and luminal invasive carcinomas (IC) from TCGA dataset

miR-221

miR-222

Basal
Luminal

B

Relative amount

-β4 integrin

-β actin

MCF-7   MDA-MB361   T47D

C

Fold expression

miR-221
miR-222

MCF-7   MDA-MB361   T47D

D

representative β4 integrin expression on human luminal IC-LICs

grade 1   grade 3

E

Fold expression

MCF-7
grade 1
grade 3

***   *

miR-221   miR-222

F

luminal IC-LICs   r= -0.75
                        p= 0.0013

KI67

miR-221

luminal IC-LICs   r= -0.54
                        p= 0.037

KI67

miR-222

KI67 and miR-221/222 correlation on 15 luminal IC-LICs human samples

Figure 2

A

☐ MCF-7 wt

Fold expression

*** (pre-miR neg c / pre-miR221)
*** (pre-miR neg c / pre-miR222)

pre-miR neg c — pre-miR221 — miR-221
pre-miR neg c — pre-miR222 — miR-222

B

Relative amount

***

-β4 integrin
-β-actin

pre-miR neg c   pre-miR-221   pre-miR-222

MCF-7 wt

C

3' *agCUACAUUGUCUGCUGGGUUuc* 5'   hsa miR-221

281: 5' *caCGUCUUGUUUCCGACCCAGga* 3'   259: 3'UTR β4 integrin

D

Relative luciferase activity

***   ***

pGL3	+	−	−	−
pGL3-3'UTR β4	−	+	+	+
pre-miR neg c	+	+	−	−
pre-miR-221	−	−	+	−
pre-miR-222	−	−	−	+

MCF-7 wt

E

Relative luciferase activity

***

pGL3	+	−	−	−
pGL3-3'UTR*mut* β4	−	+	+	+
pre-miR neg c	+	+	−	−
pre-miR-221	−	−	+	−
pre-miR-222	−	−	−	+

MCF-7 wt

F

Relative amount

***

-β4 integrin
-β-actin

scramble   sh-β4 integrin

MCF-7 wt

G

Fold expression

☐ miR-221   ■ miR-222

scramble   sh-β4 integrin

MCF-7 wt

Figure 3

Figure 4

A

B

PCNA staining

C

-cyclin D1
-β-actin

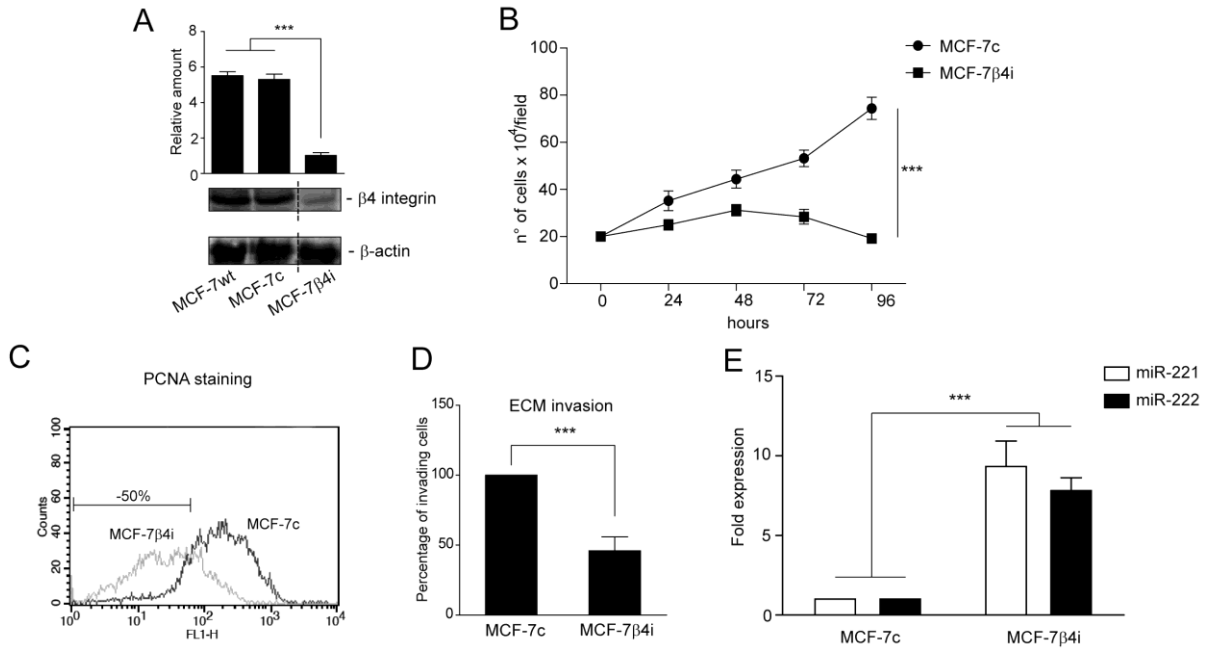-pAkt
-Akt

D

ECM invasion

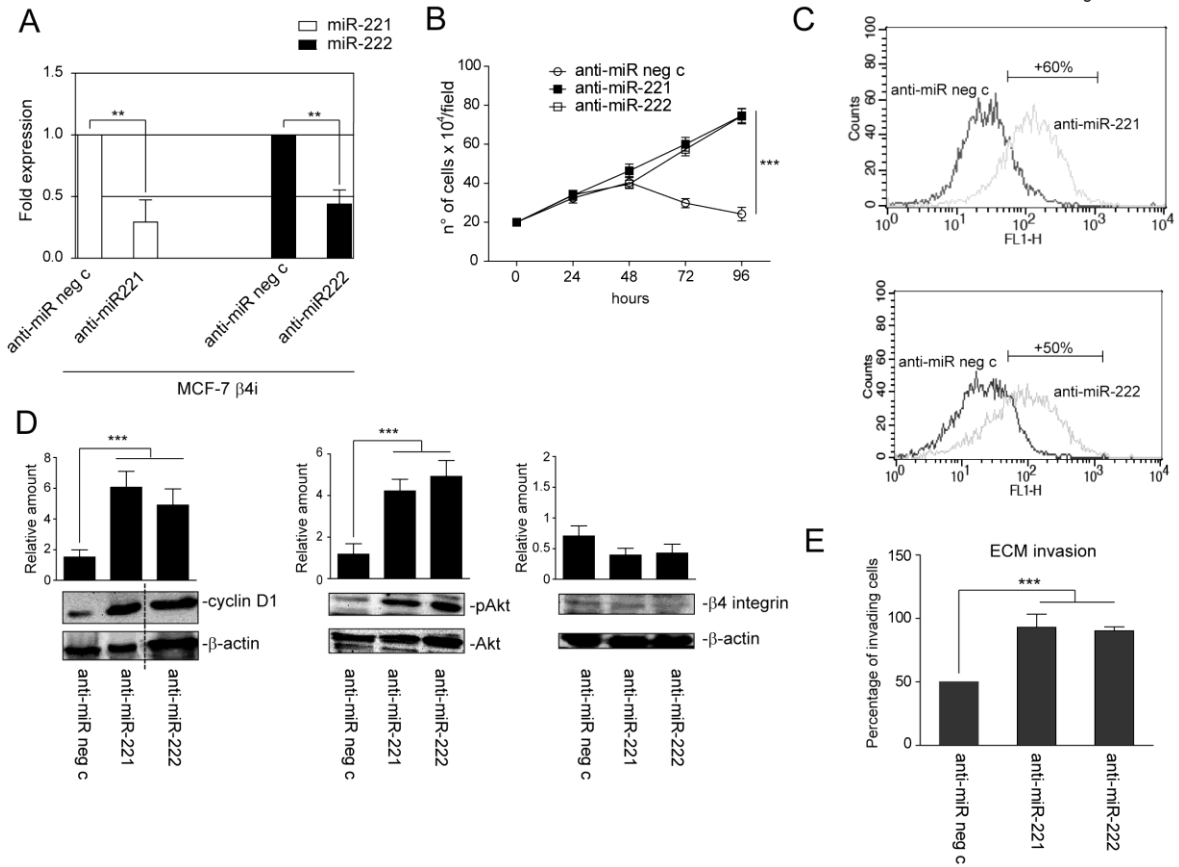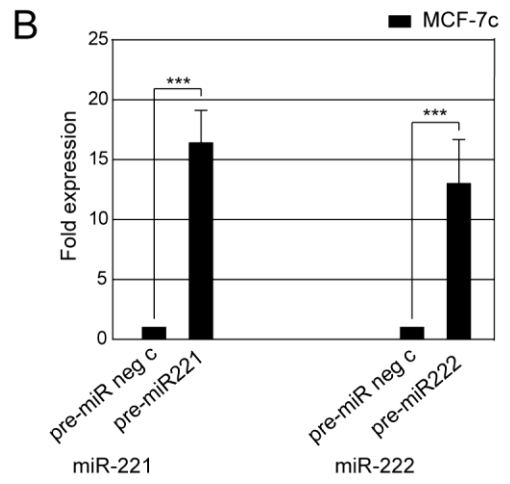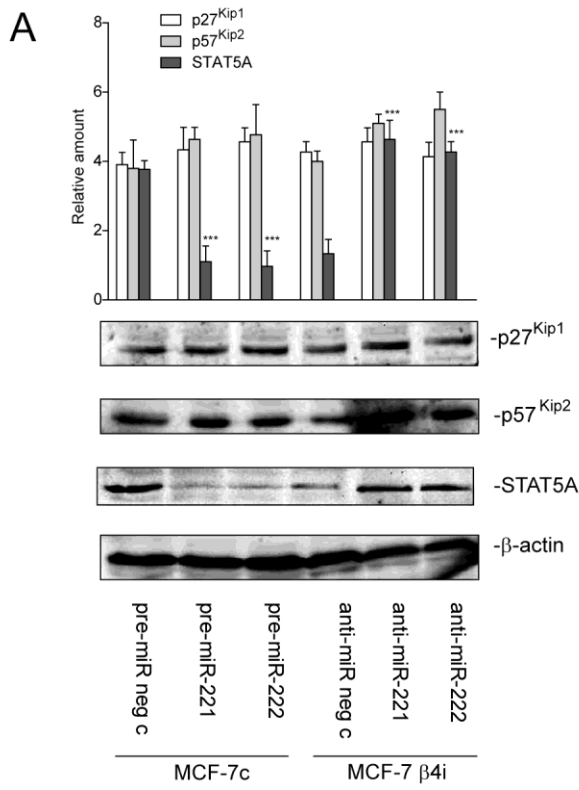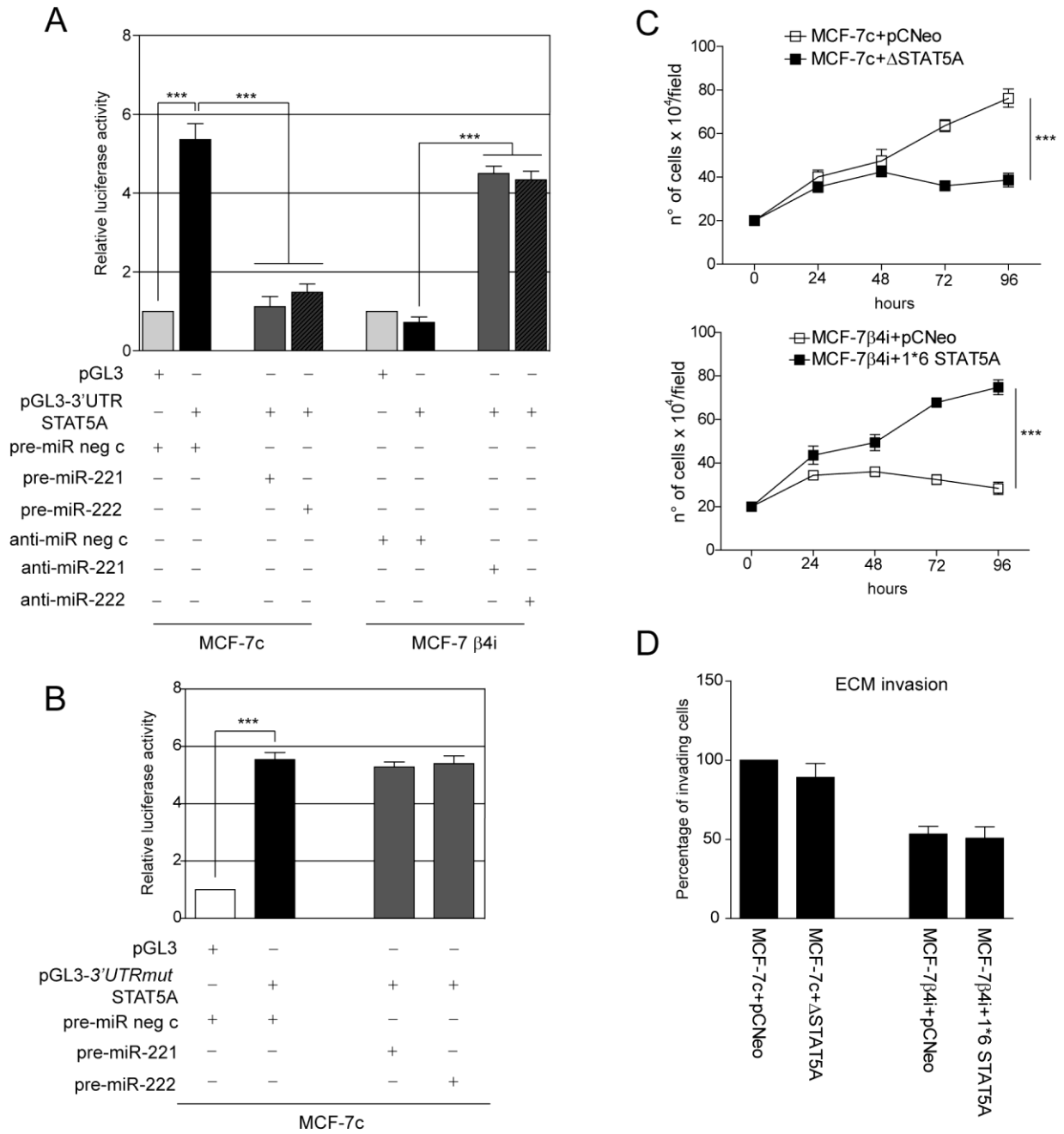
E

G0/G1
S
G2/M

F

pre-miR neg c

pre-miR-221

pre-miR-222

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

A

B

C

D

E

F