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# TMPRSS4 regulates levels of integrin $\alpha 5$ in NSCLC through miR-205 activity to promote metastasis

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**Background:** TMPRSS4 is a membrane-anchored protease involved in cell migration and invasion in different cancer types including lung cancer. TMPRSS4 expression is increased in NSCLC and its inhibition through shRNA reduces lung metastasis. However, molecular mechanisms leading to the protumorigenic regulation of TMPRSS4 in lung cancer are unknown.

**Methods:** miR-205 was identified as an overexpressed gene upon TMPRSS4 downregulation through microarray analysis. Cell migration and invasion assays and *in vivo* lung primary tumour and metastasis models were used for functional analysis of miR-205 overexpression in H2170 and H441 cell lines. Luciferase assays were used to identify a new miR-205 direct target in NSCLC.

**Results:** miR-205 overexpression promoted an epithelial phenotype with increased E-cadherin and reduced fibronectin. Furthermore, miR-205 expression caused a G<sub>0</sub>/G<sub>1</sub> cell cycle arrest and inhibition of cell growth, migration, attachment to fibronectin, primary tumour growth and metastasis formation *in vivo*. Integrin  $\alpha 5$  (a proinvasive protein) was identified as a new miR-205 direct target in NSCLC. Integrin  $\alpha 5$  downregulation in lung cancer cells resulted in complete abrogation of cell migration, a decreased capacity to adhere to fibronectin and reduced *in vivo* tumour growth, compared with control cells. TMPRSS4 silencing resulted in a concomitant reduction of integrin  $\alpha 5$  levels.

**Conclusion:** We have demonstrated for the first time a new molecular pathway that connects TMPRSS4 and integrin  $\alpha 5$  through miR-205 to regulate cancer cell invasion and metastasis. Our results will help designing new therapeutic strategies to inhibit this novel pathway in NSCLC.

Lung cancer is a critical problem in public health. It represents the most frequent tumour type in men and the second in women, and the 5-year survival rate remains inferior to 20% (Jemal *et al*, 2010). More than 60% of NSCLC patients present locally advanced, unresectable or metastatic (stage III/IV) tumours at the time of diagnosis, which fatally concludes in death within few months after diagnosis. In spite of the advent of targeted therapies, which has been a breakthrough for NSCLC management, only a small percentage of patients will benefit from them. Therefore, there is a need for identifying new potential therapeutic targets against which more effective treatments may be developed.

Altered expression of proteases is currently considered as a hallmark of cancer, as malignant cells need proteolytic activities to promote growth, motility and invasion (Roy *et al*, 2009). Type II transmembrane serine proteases (TTSPs) are characterised by the presence of an N-terminal transmembrane domain that anchors these proteases to the plasma membrane. TTSPs include 18 members that can be divided into four groups. Examples of TTSPs related to cancer are hepsin, matriptase, HAT/DESC and TMPRSS4 (Szabo and Bugge, 2008).

TMPRSS4 comprises a short N-terminal cytoplasmic domain, a transmembrane domain and a large extracellular domain that

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contains the catalytic activity. TMPRSS4 is synthesised as an inactive zymogen that needs to be activated (Netzel-Arnett *et al*, 2003). However, both the substrates and activators of this serine protease are largely unknown. TMPRSS4 participates in the regulation of cellular signalling events at the plasma membrane and in the extracellular matrix (Hooper *et al*, 2001). TMPRSS4 is upregulated in pancreatic, colon, lung, ovary and thyroid cancers, where it has been suggested as a diagnostic marker (Kebebew *et al*, 2005). In a previous work, we demonstrated that TMPRSS4 is highly expressed in lung tumours compared with normal lung, particularly in squamous cell carcinomas (SCC) as compared with adenocarcinomas (AC). We also demonstrated that high TMPRSS4 mRNA levels in SCC are associated with poor prognosis (Larzabal *et al*, 2011). Moreover, a knockdown strategy to reduce TMPRSS4 levels in lung tumour cells resulted in a significant impairment of metastasis in animal models (Larzabal *et al*, 2011).

Studies of biological activity in colon cancer have reported that elevated TMPRSS4 expression induces epithelial-to-mesenchymal transition (EMT) of cancer cells and promotes metastasis (Jung *et al*, 2008; Kim *et al*, 2010). Further analysis of TMPRSS4-mediated signalling in colon cancer cells suggested that multiple downstream signalling pathways are activated. These included Src, ERK1/2, AKT, FAK and Rac1, resulting in E-cadherin down-regulation and induced expression of integrin  $\alpha 5\beta 1$ , a critical adhesion molecule involved in the acquisition of an EMT phenotype and cell motility (Maschler *et al*, 2005). Inhibition of PI3K or Src with specific compounds decreases cell invasiveness and actin rearrangement mediated by TMPRSS4. Moreover, functional blockade of integrin  $\alpha 5\beta 1$  demonstrated that this integrin has an important role in TMPRSS4-mediated effects (Kim *et al*, 2010). In spite of these data, the molecular regulation of TMPRSS4 in cancer cells is poorly understood.

The aim of this study was to identify molecular mechanisms involved in the protumorigenic regulation of TMPRSS4 in NSCLC. Through a microarray analysis, we discovered that the *miR-205* gene (MIR205HG) was consistently overexpressed upon TMPRSS4 downregulation. We demonstrate here that overexpression of miR-205 promotes an epithelial phenotype and inhibits tumour cell migration and metastasis formation in lung cancer models. Moreover, we have identified integrin  $\alpha 5\beta 1$  (a proinvasive protein) as a new miR-205 direct target in NSCLC; we also show a novel molecular mechanism that connects TMPRSS4 with integrin  $\alpha 5\beta 1$  through miR-205.

## MATERIALS AND METHODS

**Cell culture.** All human lung cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in RPMI (Sigma, Madrid, Spain) with 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Lonza, Basel, Switzerland), at 37 °C and 5% CO<sub>2</sub> atmosphere.

H358 and H441 cell clones with a reduction of TMPRSS4 (shTMP4) and their corresponding controls (carrying the empty vector; shCtrl) were previously described by our group (Larzabal *et al*, 2011).

Immortalised normal human bronchial epithelial cells (HBECs) have been previously characterised (Ramirez *et al*, 2004). These cell lines were kindly provided by Dr J.D. Minna (University of Texas Southwestern Medical Center, Dallas, TX, USA). Cells were maintained in keratinocyte serum-free medium supplemented with human recombinant epidermal growth factor (EGF) and bovine pituitary extract (Life Technologies, Carlsbad, CA, USA).

**miRNA transfection.** H2170, H441 and A549 cell lines were transfected with miRNASelect pEP-miR Null control vector or

miRNASelect pEP-hsa-miR-205 vector (Cell Biolabs, San Diego, CA, USA) using GeneJuice reagent (Merck Millipore, Darmstadt, Germany). Transfected cell clones were selected with puromycin (2  $\mu\text{g ml}^{-1}$ ).

**Integrin  $\alpha 5$  inhibition.** We used integrin  $\alpha 5$  shRNA lentiviral particles (Santa Cruz Biotechnology, Dallas, TX, USA) to inhibit integrin  $\alpha 5$  expression in the H2170 cell line. Infection with shRNA was performed with polybrene according to the manufacturer's protocol. Stable clones were selected with 1  $\mu\text{g ml}^{-1}$  puromycin. The integrin  $\alpha 5$  expression was analysed by western blot and qPCR.

**RNA extraction and quantitative real-time PCR.** RNA isolation and qPCR was carried out as previously described (Larzabal *et al*, 2011). Assays were performed to quantify mRNA levels of human E-cadherin, fibronectin, ZEB1/ZEB2 and integrin  $\alpha 5$ . The primer sequences are shown in Supplementary Table 1.

To detect mature miR-205 expression, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA. For the quantification of this miRNA, stem-loop RT of 20 ng total RNA was run using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with specific primers for miR-205 and RNU48 as endogenous control (Applied Biosystems). Subsequently, the qPCR amplifications were performed with TaqMan 2  $\times$  Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and specific primers for miR-205 and RNU48 (Applied Biosystems). Relative miR-205 levels were normalised to RNU48 expression. Data are given as  $2^{-\Delta\Delta\text{Ct}}$  or  $2^{-\Delta\text{Ct}}$ .

**Proliferation and clonogenic assays.** To determine proliferation of H2170 and H441 cells and their miR-205 expressing clones, 1200 cells per well were seeded in 96-well plates. Ninety-six hours after plating, MTT assays were performed following the manufacturer's protocol (Sigma).

To evaluate the clonogenic potential of miR-205 expressing clones, 500 cells per well were plated into six-well plates in adherent conditions. After 10 days in culture, colonies were fixed with 4% buffered formalin (Panreac, New Jersey, NY, USA) and stained with 2% crystal violet. The number of colonies per well was determined.

**Anchorage-independent growth assay.** Soft-agar assay was used to determine the ability of miR-205-expressing clones to grow under anchorage-independent conditions. Each well of a six-well plate was coated with culture medium containing 0.6% agarose. Upon solidification, a layer of 0.3% agar in culture medium carrying 2000 cells per well was placed on top of the agarose. Colonies were stained with MTT and counted after 2 weeks of incubation.

**Cell cycle analysis.** For cell cycle distribution analysis, adherent and floating cells were fixed in 70% ethanol for 1 h (or until analysis) at 4 °C. Cells were then rehydrated in PBS, treated with RNase A (0.2 mg ml<sup>-1</sup>, Sigma) for 1 h at 37 °C and stained with 7AAD (0.02 mg ml<sup>-1</sup>, Sigma). Cells were analysed in a flow cytometer (FACSCalibur, BD). Percentages of cells in the Sub-G<sub>0</sub>, G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases were determined.

**Migration assays.** Migration assays were conducted in a Boyden chamber for the H2170 and A549 cell lines. A total of 15 000 cells per well in serum-free medium were seeded in the upper transwell of 24-well plates (Costar, Tewksbury, MA, USA). Medium with 10% of serum was used as a chemoattractant and was placed in the lower compartment. After 48 h, cells in the top chamber were wiped with a cotton swab, and cells in the lower compartment were fixed with 4% formalin and stained with crystal violet. The number of migratory cells was evaluated with a Leica DMIL LED microscope using the LAS EZ software (Leica Microsystems, Wetzlar, Germany).

H441 cells did not migrate through the transwell in Boyden chambers (unpublished observations). For this reason, we examined migration of this cell line with an *in vitro* model of wound healing. Cells were grown until confluence, and a 20- $\mu$ l micropipette tip was used to create a linear scratch in the monolayer. Pictures of the wounds were taken right after the scratching and 24 h later with a Nikon Eclipse photomicroscope (Nikon, Kingston, UK) using the ACT-2U1.6 software (Nikon). The empty surface between the wound edges was measured with the TScratch analysis software (Zurich, Switzerland).

**Adhesion assay.** Single-cell suspensions were rinsed with serum-free medium supplemented with 0.5% BSA, and 30 000 cells per well were seeded in 96-well plates precoated with 3% BSA (control), fibronectin (20  $\mu$ g ml<sup>-1</sup> per well) or collagen type I (50  $\mu$ g ml<sup>-1</sup> per well). After 30 min incubation to let cells adhere to the substrates at 37 °C, wells were washed with PBS to remove the floating cells. Attached cells were fixed in 4% paraformaldehyde and stained with 0.5% toluidine blue. To obtain the adherent cell number, stained cells were treated with 1% SDS, and the absorbance at 595 nm was measured using a Sunrise spectrophotometer (Tecan, San Jose, CA, USA).

**E-cadherin and fibronectin immunofluorescence.** Control cells and miR-205-expressing cell clones were grown in slide chambers until confluence. Cells were fixed by immersion in acetone:methanol (1:1) solution for 10 min at -20 °C. Then, non-specific binding sites were blocked with 2% BSA, and slides were incubated with primary anti-E-cadherin (BD Bioscience, San Jose, CA, USA) or anti-fibronectin (Abcam, Cambridge, UK) antibodies at 1:100 dilution for 2 h at room temperature. Slides were then incubated in darkness with the secondary antibody (488 Alexa Fluor dye, Invitrogen) for 1 h at room temperature. For nuclei staining, samples were incubated with 0.1 mg ml<sup>-1</sup> DAPI. A hydrophilic mounting medium (SlowFade antifade) was used, and coverslips were sealed with nail polish. For visualisation and acquisition of images, an Axio Imager Z1 (ZEISS) epifluorescence microscope equipped with a CoolSnap CCD camera was used.

**Microarray hybridisation and bioinformatic analysis.** Transcriptional profiling was done with the Affymetrix Human Gene 1.0 ST microarray according to the manufacturer's protocol. Both background correction and normalisation were done with RMA (Robust Multichip Average) algorithm (Irizarry *et al*, 2003). Then, a filtering process was performed to eliminate low expression probe set intensities for each probe, recorded in CEL files and data were analysed. The analysis of significant gene expression changes in shTMP4 samples compared with expression in control cells was performed by *Z*-score transformation of fold-change distributions (Cheadle *et al*, 2003). A threshold of  $|z| > 2.3$  ( $P$ -value < 0.01) was set to select genes for further analysis. The selection of genes differentially expressed in miR-205-overexpressing cells with respect to control cells was performed using the criteria based on the fold-change value. Probe sets were selected as significant using a cutoff value of 2. The set of differentially expressed genes was compared with the miRNA-predicted targets stored in public databases (Betel *et al*, 2008; Griffiths-Jones *et al*, 2008; Friedman *et al*, 2009). As a result, putative microRNA target genes with altered expression were identified.

**Western blotting.** Protein extraction and western blot were carried out as previously described (Ponz-Sarvisé *et al*, 2011). The primary antibodies (diluted at 1:1000) were as follows: anti-human ITG $\alpha 5$  (Sigma), anti phospho-Src (Cell Signaling, Danvers, MA, USA) and anti-Src (Cell Signaling). An anti-human  $\beta$ -actin antibody (Sigma) at 1:10 000 dilution was used as a loading control. Horseradish peroxidase-labelled secondary antibodies (GE Healthcare, Waukesha, WI, USA) against the corresponding primary antibodies were used. Immunoreactive bands were

visualised by a chemiluminescent method using the Lumi-lightPLUS kit (Roche, Palo Alto, CA, USA).

**Generation of H2170 TGL-expressing cells for *in vivo* imaging.** Amphopack-293 packaging cells (Clontech, Madison, WI, USA) cultured with DMEM and 10% fetal bovine serum were transfected with the pSFG<sub>NES</sub>TGL purified plasmid as previously described (Larzabal *et al*, 2011). After 48 h in culture, virus containing supernatant was added to parental H2170 cells and H2170-miR-205-expressing cell clones. To increase the efficiency of infection, 7.5  $\mu$ g ml<sup>-1</sup> polybrene (Sigma) was added to the virus containing supernatants. Vector containing GFP + cells were sorted by FACS (FACSaria Ilu). More than 90% of the cells were confirmed to express the reporter construct (Supplementary Figure 1A).

***In vivo* experiments.** Animal studies were carried out according to the ethical guidelines established by our Institution (University of Navarra), under an approved animal protocol (069/11). For the lung metastasis model,  $1.5 \times 10^6$  H2170 cells containing the control vector (miR-Scr) or the miR-205 overexpressing clones (miR-205#2 or miR-205#3) infected with the TGL reporter vector were injected in the tail vein in NOD SCID ILR $\beta 2$  null (NSG) mice. Four mice per group were used. Lung tumour development was monitored by detection of luciferase photon emission, using a Xenogen (Xenogen Inc., Alameda, CA, USA) (IVIS 100 series) living image system. Anaesthetised mice were injected intraperitoneally with 3 mg of D-Luciferin (Promega, Fitchburg, WI, USA) diluted in PBS, 5 min before light capture. The image acquisition time was in the range of 5 min. Data analyses were performed with the Living Image Igor Pro (Xenogen) software coupled to the IVIS system. Photon counts were calculated for each mouse using a circular region of interest.

To study the effect of miR-205 on primary tumour growth,  $10 \times 10^6$  H2170 cells and their corresponding miR-205-overexpressing clones (miR-205#2 or miR-205#3) were injected subcutaneously into the flanks of the mice in a 1:1 PBS/Matrigel solution (BD). A similar experiment was conducted to assess whether knockdown of ITG $\alpha 5$  (shITG $\alpha 5$  H2170 cells) would decrease primary tumour growth with respect to controls. Tumour volumes were calculated with the formula:  $V = \text{length} \times (\text{width})^2/2$ .

**Immunohistochemistry.** Tumours were excised, fixed, paraffin-embedded and cut in 5  $\mu$ m thick sections, which were placed on slides. Slides were deparaffinised, rehydrated and treated for 12 min with 3% H<sub>2</sub>O<sub>2</sub> (Panreac) in water to quench the endogenous peroxidase activity. For caspase-3, antigen retrieval was performed by heating the sections in a microwave oven in 1 mM EDTA (pH 8). Tissues were then blocked with normal goat serum in TBS (30 min, RT). Incubation with the following primary antibodies was done overnight at 4 °C: anti-cleaved caspase-3 Asp175 (Cell Signaling) at 1:200 dilution, anti-PCNA (Clone PC10; Dako, Glostrup, Denmark) at 1:400 dilution and anti-integrin  $\alpha 5$  (Sigma) at 1:50 dilution. After incubation with Envision anti-rabbit or anti-mouse system (Dako), peroxidase activity was developed with DAB (3,3'-diaminobenzidine; Dako). Finally, slides were counterstained with haematoxylin, dehydrated and mounted with DPX (VWR).

**Renilla luciferase assay.** The renilla luciferase constructs with the 3'-untranslated region (UTR) of ITG $\alpha 5$  were made with the following oligonucleotides:

ITG $\alpha 5$  3'UTR-F: 5'-CTAGAGAGGAGCTGGGGATCCCTCCG  
CCCTGTGAAGGAACCATGCACTGTGAAGGACCCTTGTGC-3'

ITG $\alpha 5$  3'UTR-R: 5'-GGCCGCACAAGGGTCTTTCACAGTGC  
ATGGGGCGGAGGGATCCCCAGCTCCTCT-3'

ITG $\alpha 5$ -3'UTR-Mut-F: 5'-CTAGAGAGGAGCTGGGGATCCCT  
CAACCCCCAGACACTTGAGGTAGCCCTTGTGC-3'

ITG $\alpha 5$ -3'UTR-Mut-R: 5'-GGCCGCACAAGGGCTACCTCAA  
GTGTCTGGGGTTGAGGATCCCCAGCTCCTCT-3'

These oligonucleotides contain the target site sequence or the mutated version (indicated in bold letters), which is complementary to the seed sequence of miR-205. Hybridised oligonucleotides were ligated into the *XbaI*–*NotI* site of the renilla reporter vector (pRL-SV40; Promega). GeneJuice reagent was used to co-transfect the renilla-ITG $\alpha 5$ -3'UTR vector or the Renilla-ITG $\alpha 5$ -3'UTR-mut, and a control vector containing firefly luciferase (pGL3-basic; Promega), together with either pre-miR-205 or scramble precursor-miR vectors. Renilla luciferase activity was measured 48 h after transfection with the Dual-Luciferase System (Promega) in a Berthold Luminometer (Lumat LB 9507).

**Statistical analysis.** Statistical differences between groups were examined with the Student's *t*-test or ANOVA for unpaired parametric variables and the Mann–Whitney *U*-test or Kruskal–Wallis for unpaired non-parametric variables. Normality was analysed with the Shapiro–Wilks test. Data were processed with the SPSS statistical software (SPSS-IBM Inc., Chicago, IL, USA) (version 17.0 for Windows SPSS) and GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). *P*-values lower than 0.05 were considered as statistically significant.

**RESULTS**

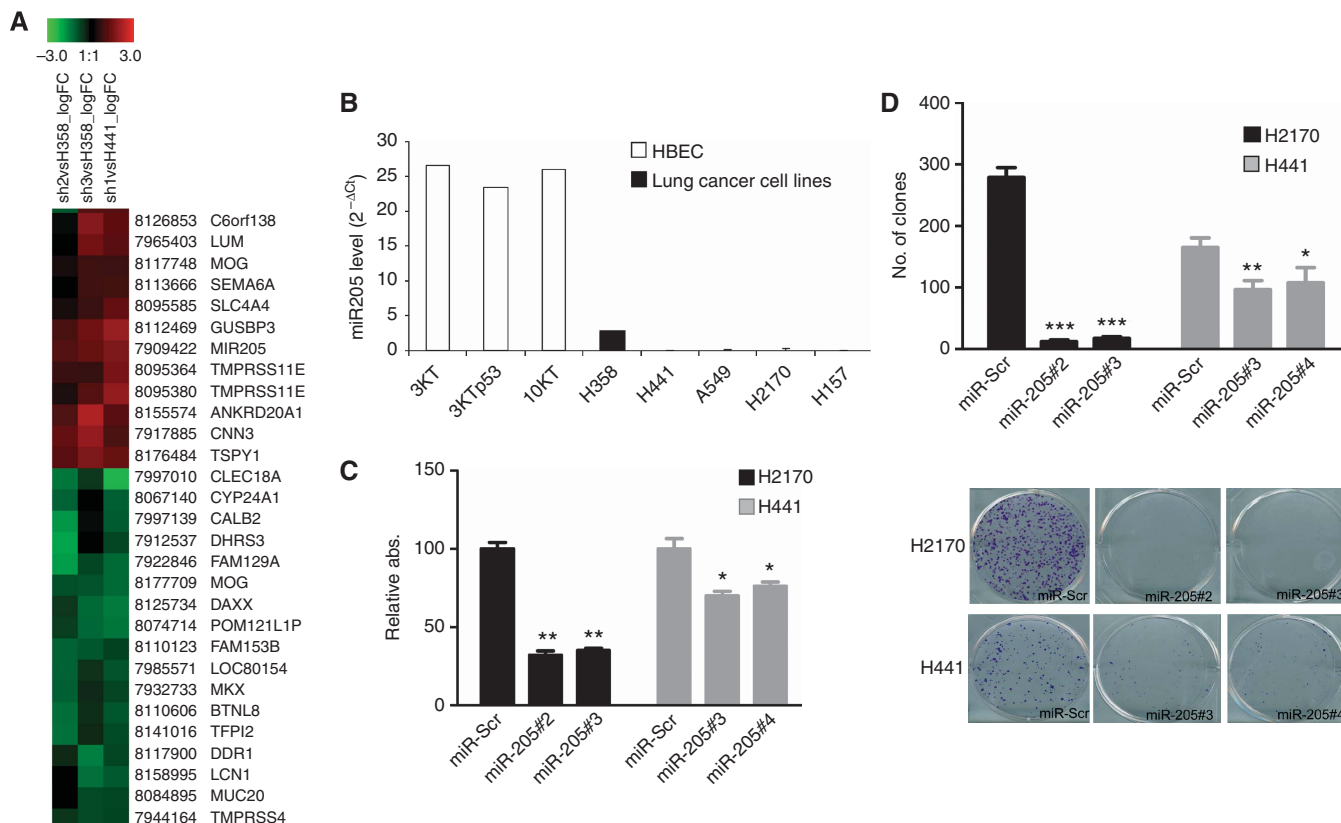
**TMPRSS4 knockdown upregulates miR-205 expression.** Previous studies have shown that TMPRSS4 has a role in cell migration and invasion (Kim *et al*, 2010). We have demonstrated that TMPRSS4 expression is increased in NSCLC and that its inhibition through shRNA reduces lung metastasis (Larzabal *et al*, 2011). Because

molecular mechanisms activated by TMPRSS4 are unknown, we used an shRNA strategy to knockdown this protease in H358 and H441 cells, in search of genes with an altered expression. Clones H358 shTMP4-2 and shTMP4-3 and H441 shTMP4 (with a reduced TMPRSS4 expression) were selected for the microarray analysis. We have previously shown that these clones have reduced proliferative and migratory ability compared with their respective controls (H358 shCtrl and H441 shCtrl) (Larzabal *et al*, 2011).

A list of 287 probes was found to be deregulated by > two-fold in the three cell clones analysed. From those, 176 probes corresponded to non-annotated sequences. We then focused our analysis in those genes whose expression trend was the same (either upregulation or downregulation) in the three clones (Figure 1A). Twenty-nine genes followed this pattern (12 of them overexpressed and 17 of them underexpressed). As expected, TMPRSS4 was one of the downregulated genes of this set. Other downregulated genes that have a role in cancer cell adhesion included DDR1 and CLEC18A. DDR1 has been shown to promote lung cancer metastasis to the bone, and its expression correlates with poor prognosis in NSCLC patients (Valencia *et al*, 2012).

One of the upregulated genes found in the list was MIR205HG. This microRNA has been described as a tumour suppressor gene in different tumour types (Song and Bu, 2009; Wu *et al*, 2009; Majid *et al*, 2011), a reason whereby we decided to further study its role in lung cancer. miR-205 expression in H358 and H441 shCtrl and shTMP4 cell clones was validated by qPCR (Supplementary Figure 1B).

**miR-205 is downregulated in lung cancer cell lines.** We first determined mRNA levels of miR-205 in a panel of immortalised



**Figure 1.** (A) Hierarchical cluster diagram of differentially expressed genes in H358 and H441 shTMP4 cell clones compared with their respective controls (shCtrl). (B) Expression of miR-205 by qPCR in immortalised human lung cells (HBEC) and in lung cancer-derived cell lines. The RNA was isolated when cells reached 70% confluency in all cases. (C) An MTT assay performed 72 h after plating. An inhibition in the proliferation of H2170 and H441 cells transfected with miR-205 was observed. (D) Clonogenic assays showed a lower number of clones in H2170 and H441 cells with high miR-205 expression compared with controls. Representative images are shown. Data and error bars: mean  $\pm$  s.d. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001. All these experiments were repeated at least three independent times.

non-malignant human lung cells and human lung cancer cell lines. Cancer cell lines did not express miR-205 (or had very low expression, as in the case for H358 cells) in comparison with immortalised HBEC cells (Figure 1B and Supplementary Table 2), suggesting a tumour suppressor role for miR-205 in lung cancer.

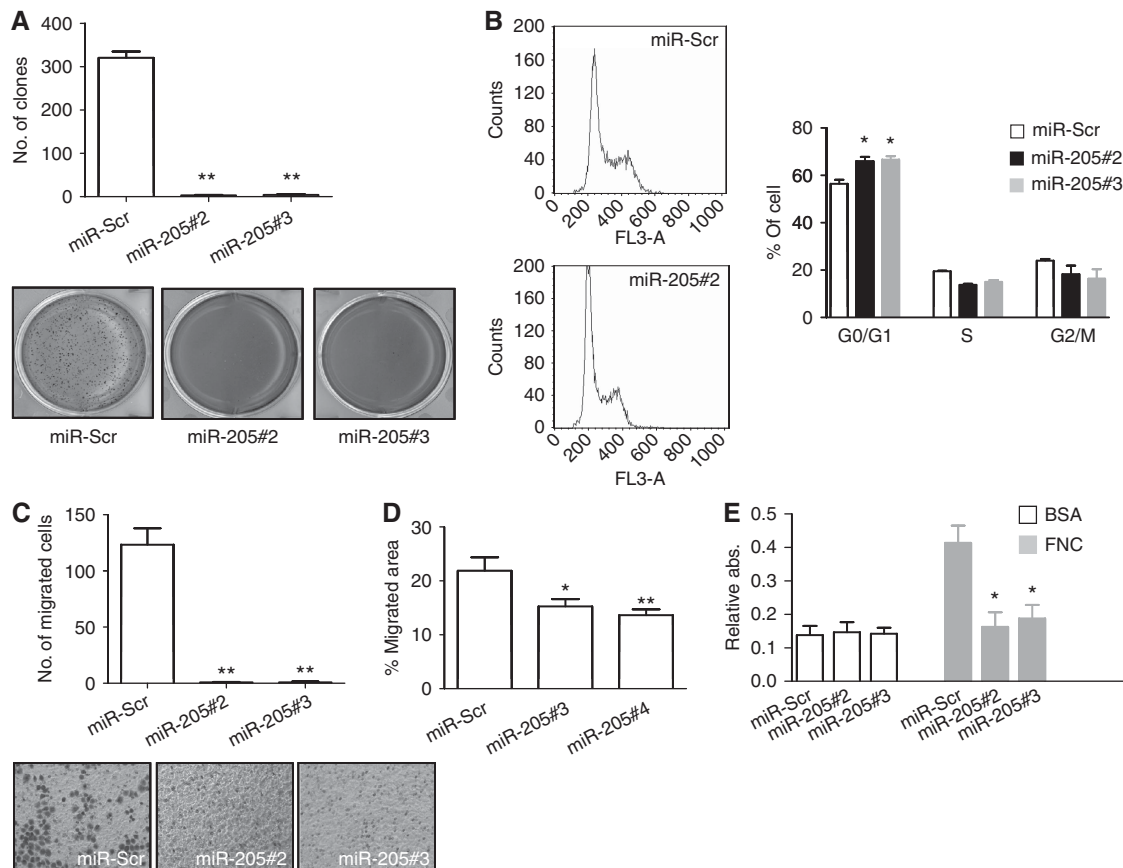
**miR-205 regulates cell proliferation, cell cycle progression and anchorage-independent growth in lung cancer cell lines.** The effects of miR-205 on lung cancer were studied by overexpressing miR-205 precursor molecules (pre-miR-205) in H2170 and H441 cell lines. Overexpression was confirmed by real-time PCR, and two clones for H2170 (miR-205#2 and #3) and for H441 (miR-205#3 and #4) were selected for functional assays (Supplementary Figures 1C and D).

Initially, we assessed whether ectopic expression of miR-205 had a biological effect on proliferation and clonogenicity. MTT proliferation assays indicated that cell clones overexpressing miR-205 had a reduction in cell proliferation rates compared with cells transfected with scramble vector (miR-Scr) in both cell lines tested (Figure 1C). Furthermore, the overexpression of miR-205 decreased clonogenic capacity of H2170 and H441 cell lines (Figure 1D). These data indicate that miR-205 inhibits cell growth in lung cancer cell lines. As anchorage-independent growth is strongly correlated with tumorigenicity, we then determined whether miR-205 would alter cell growth in soft agar. H2170 cell clones were plated in soft agar and incubated for 2 weeks before counting the number of colonies. As shown in Figure 2A, the ability to form

colonies was almost abolished in miR-205-overexpressing clones compared with scramble control. The same results were observed in the H441 cell line (Supplementary Figure 1E).

A cell cycle analysis by flow cytometry was performed to determine whether the decrease in cell proliferation due to miR-205 upregulation was associated with a cycle arrest. Overexpression of miR-205 produced a significant increase in the percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phase (from  $56.44 \pm 1.72\%$  to  $66.03 \pm 1.77\%$ ;  $P < 0.05$ ), along with a decrease in the S phase (Figure 2B), indicating that miR-205 induced a cell cycle arrest in the H2170 cell line. Similar results were observed in the H441 cell line (Supplementary Figure 1F).

**miR-205 inhibits cell migration and adhesion to fibronectin and promotes an epithelial phenotype in lung cancer cells.** We next investigated whether miR-205 would modify cell migration in lung cancer cells. For this purpose, H2170 control cells or clones with elevated miR-205 levels were subjected to a migration assay. As shown in Figure 2C, 48 h after plating, upregulation of miR-205 completely blocked the ability of cells to migrate. Migration assays in Boyden chamber could not be performed with the H441 cell line because, in our hands, these cells did not migrate through the transwells. To further confirm the effect of miR-205 on migration through transwells, A549 cells with miR-205 overexpression or control cells (carrying the empty vector) were also used. As shown in Supplementary Figure 1G, similar results to those found for H2170 cells were obtained. In addition, as an alternative to this assay using H441 cells, an *in vitro* wound healing assay was



**Figure 2.** (A) Soft agar assays demonstrated less capacity of H2170 miR-205-overexpressing clones to grow in non-adherent conditions than control cells. (B) Representative cell cycle distribution profiles of H2170 control cells (miR-Scr) and a miR-205-overexpressing clone (miR-205#2). Cell cycle distribution showed an arrest in the G<sub>0</sub>/G<sub>1</sub> phase after miR-205 overexpression in the H2170 cell line, as revealed by an increase in the proportion of cells in this phase and a decrease in the S phase. (C) Migration of H2170 cells in Boyden chambers was strongly reduced by overexpression of miR-205. (D) Migration of H441 cells in wound healing assays was impaired as well in clones with high miR-205 levels. (E) Adhesion assay of H2170 miR-Scr, miR-205#2 and miR-205#3 clones to fibronectin (FNC). BSA was used as a negative control. Data and error bars show mean  $\pm$  s.d. \* $P < 0.05$ . \*\* $P < 0.01$ . All these assays were repeated at least three independent times.

performed to analyse migration in these cells. Figure 2D and Supplementary Figure 2A show that overexpression of miR-205 caused a reduction in the ability of H441 cells to migrate.

To study the effects of miR-205 on cell–matrix adhesion, we cultured H2170 cells on different substrates. miR-205 overexpression decreased cell adhesion to fibronectin (Figure 2E) but not to collagen type I (Supplementary Figure 2B). A similar tendency was found for H441 cells, although results did not reach statistical significance (data not show).

Different studies have demonstrated that miR-205 regulates EMT by targeting ZEB2 (Gandellini *et al*, 2009; Matsushima *et al*, 2011). As shown in Figure 3A, ZEB1 and ZEB2 mRNA levels were strongly reduced in miR-205-overexpressing cells. The analysis of EMT markers by qPCR and immunofluorescence revealed a marked increase in E-cadherin along with a pronounced decrease in fibronectin in H2170 cells with miR-205 overexpression (Figures 3B–D). The effect of miR-205 on E-cadherin and fibronectin expression was similar (although less remarkable) in H441 cells compared with H2170 cells (Supplementary Figures 2C and D). These results show that miR-205 inhibits EMT and cell motility and promotes an epithelial phenotype.

**miR-205 reduces primary tumour growth and lung metastasis.**

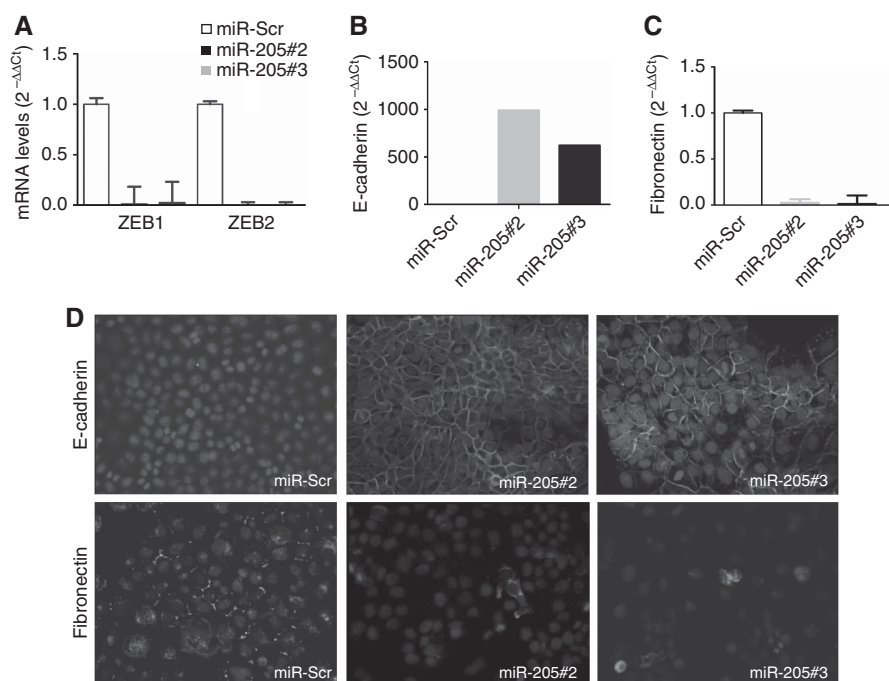
An *in vivo* assay was performed to investigate the role of miR-205 on primary tumour growth. Overexpression of miR-205 resulted in a significant reduction in tumour volumes ( $P < 0.05$ ) in animals xenotransplanted with both miR-205#2 and miR-205#3 H2170 clones, compared with mice injected with the miR-Scr control (Supplementary Figure 2E).

Furthermore, we used a xenograft model to analyse the effect of miR-205 on lung metastasis. In this case, animals were injected with the H2170 clones in the tail vein and monitored by bioluminescence until day 21. At day14th and 21st, a significant increase in light emission in the H2170 miR-Scr group was observed in comparison with the group injected with miR-205-overexpressing cells (Figures 4A and B). At the end of the study

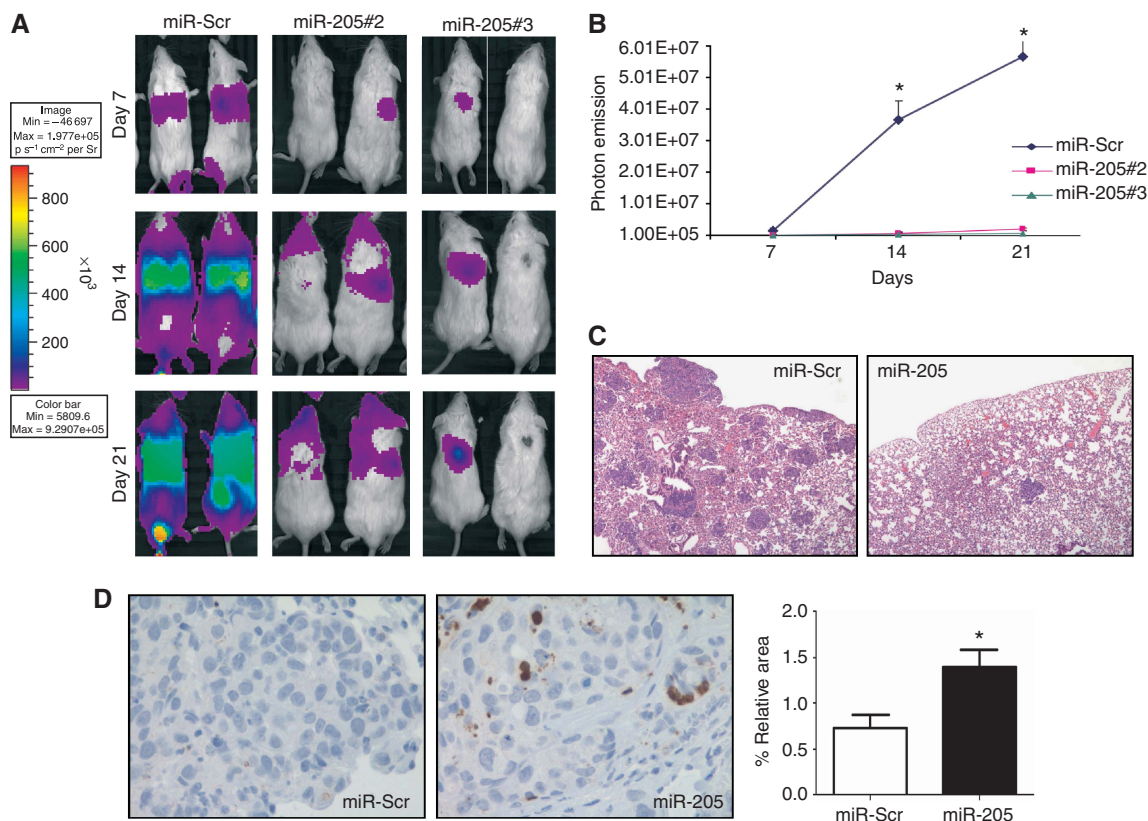
(day 21), animals were killed and lungs were examined for metastatic lesions. Histological analysis confirmed a less tumour burden in mice injected with cells that overexpressed miR-205 (Figure 4C). Immunohistochemical analysis of active caspase-3 revealed a higher proportion of apoptotic cells in tumours with increased levels of miR-205 (Figure 4D).

**Identification of miR-205 targets.** We wanted to address the molecular mechanism that causes miR-205 effects in lung cancer. We hypothesised that miR-205 could reduce the expression of target genes that induce EMT and inhibit cell invasion. To identify miR-205 target genes, we compared mRNA expression profiles in H2170 cells overexpressing miR-205 with those of controls (miR-Scr). By microarray analysis, 796 probes displayed a >two-fold reduction in expression as a result of miR-205 upregulation compared with controls. We searched within our gene list for putative-predicted miR-205 targets using publicly available and commonly used programs, such as TargetScan4, miRBase, PicTar and miRanda. Among the candidates, many were related with cell–cell and cell–matrix adhesion, cell invasion and metastasis (a selected list can be found in Supplementary Table 3). Importantly, one of the genes was integrin  $\alpha 5$  (ITG $\alpha 5$ ), whose 3'-UTR sequence had a perfect complementarity with the seed sequence of miR-205 (Figure 5A).

Because ITG $\alpha 5$  is overexpressed in lung cancer (Dingemans *et al*, 2010), it promotes invasion of cancer cells (Hood and Cheresch, 2002) and its expression has been linked to TMPRSS4 in colon cancer (Kim *et al*, 2010), we focused our attention on this gene. We analysed whether endogenous ITG $\alpha 5$  mRNA and protein levels decreased when miR-205 was overexpressed. We found that miR-205 upregulation in H2170 or H441 lung cancer cells caused a significant reduction in endogenous ITG $\alpha 5$  mRNA levels (Figure 5B for H2170 cells and Supplementary Figure 2F for H441 cells) and protein levels (Figure 5C for H2170 cells and Supplementary Figure 2G for H441 cells). Moreover, Src signalling pathway activated by integrins was downregulated in H2170 cells



**Figure 3.** (A) ZEB1 and ZEB2 mRNA levels were decreased in H2170 cells with an upregulation of miR-205. GAPDH was used as internal control. (B, C) Expression of E-cadherin (B) and fibronectin (C) in H2170 control cell (miR-Scr) and miR-205-overexpressing cell clones measured by qPCR. GAPDH was also used as internal control. (D) Representative immunofluorescence images ( $\times 400$ ) of E-cadherin and fibronectin in H2170 miR-Scr, miR-205#2, and miR-205#3 clones. All the experiments were repeated at least three independent times.



**Figure 4.** (A) Representative luminometric images of mice injected in the tail vein with  $5 \times 10^5$  H2170 control or miR-205-expressing clones. (B) Photon emission quantification revealed a significant reduction in tumour burden in the groups injected with miR-205#2 and miR-205#3 cell clones. (C) Representative histological images of lungs from mice injected with H2170 miR-Scr or miR-205 overexpressing cells ( $\times 40$ ). Numerous tumours are seen in control mice but not in the animals injected with cells that express miR-205. (D) Quantification of active caspase-3 in lung tumours. Measurements are given as relative area occupied by positive signals with respect to reference area. Significant increase in caspase-3 was found in mice injected with miR-205-overexpressing cells. Representative IHC images of caspase-3 immunostaining ( $\times 200$ ) in tumour samples are shown. Data and error bars: mean  $\pm$  s.d. \* $P < 0.05$ .

with an overexpression of miR-205 (Figure 5D). To determine whether ITG $\alpha 5$  was a direct target of miR-205, we constructed renilla-based reporters that contained the wild-type or the mutated miR-205 target sequences of the ITG $\alpha 5$  3'-UTR. Ectopic expression of miR-205 inhibited the expression of the reporter vector containing the wild-type sequence of ITG $\alpha 5$  3'-UTR but not the reporter vector containing the mutation of the seed-miR-205 binding site in the two cell lines tested (Figure 5E for H2170 cells and Supplementary Figure 2H for H441 cells). These data show that ITG $\alpha 5$  is a direct target of miR-205 and that miR-205 can reduce ITG $\alpha 5$  mRNA and protein levels.

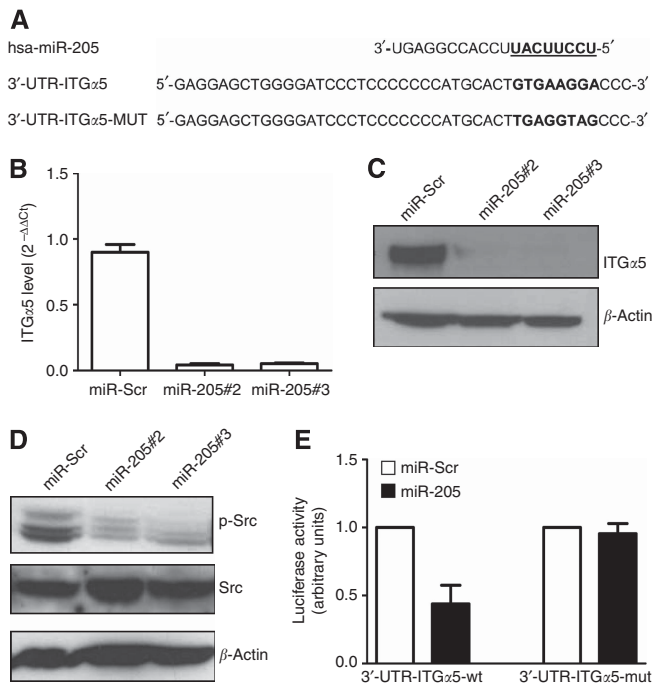
**Knockdown of ITG $\alpha 5$  in lung cancer cells reduces cell migration and cell-matrix adhesion.** To examine whether ITG $\alpha 5$  expression could mediate miR-205 regulation of cell proliferation and migration, shRNAs against ITG $\alpha 5$  were transfected in H2170 cells. ITG $\alpha 5$  shRNA efficiently decreased ITG $\alpha 5$  expression, as detected by western blot analysis (Figure 6A). ITG $\alpha 5$  depletion resulted in a complete abrogation of cell migration (Figure 6B) but no changes in cell proliferation or cell cycle distribution were observed (data not shown).

As integrins are major adhesion molecules involved in cell-ECM interactions, we studied the effects of ITG $\alpha 5$  on cell-matrix adhesion. After culture of these cells on different substrates, we observed that ITG $\alpha 5$  levels modified the adhesiveness to fibronectin (Figure 6C) but not to collagen type I (data not shown). All these results provide evidence that inhibition of ITG $\alpha 5$  by

miR-205 is responsible, at least in part, for the antitumorigenic effect observed in NSCLC cells with miR-205 upregulation.

**Knockdown of ITG $\alpha 5$  inhibits tumour growth *in vivo*.** To further explore whether ITG $\alpha 5$  knockdown would be responsible for the inhibition of tumour growth observed following miR-205 reconstitution, an *in vivo* experiment was performed. As shown in Figure 6D, subcutaneous xenotransplantation of H2170 cells with ITG $\alpha 5$  depletion caused a significant tumour shrinkage ( $P < 0.05$ ) in comparison with control cells.

**Relationship between TMPRSS4 and ITG $\alpha 5$  expression.** Having demonstrated that reduction of TMPRSS4 increases miR-205, which targets ITG $\alpha 5$ , we studied levels of ITG $\alpha 5$  in shTMP4 cell clones. Figure 6E shows that, in agreement with previous results, ITG $\alpha 5$  levels were lower in shTMP4 clones than in controls, in the three cell lines tested. We also wondered whether there would be a correlation between ITG $\alpha 5$  and TMPRSS4 expression in cell lines. qPCR analysis for ITG $\alpha 5$  was conducted in 30 lung cancer cell lines and its expression was compared with that of TMPRSS4 in our previous publication (Larzabal *et al*, 2011). As shown in Figure 6F, 22 out of 30 cell lines expressed ITG $\alpha 5$ . Eleven of the TMPRSS4 (+) cells expressed ITG $\alpha 5$  as well, but some TMPRSS4 (-) cells were positive for ITG $\alpha 5$  (Supplementary Table 4). This shows that ITG $\alpha 5$  may be regulated by other proteins in addition to TMPRSS4. On the basis of our findings, we propose a molecular mechanism through which TMPRSS4, regulating miR-205 expression, can modulate ZEB1, ZEB2 and ITG $\alpha 5$  levels to inhibit EMT and metastasis in lung cancer (Figure 7).



**Figure 5.** (A) Sequence alignment of human miR-205 and the 3'-UTR of ITG $\alpha$ 5. The seed sequence (in bold letters) of miR-205 (top), 3'-UTR of ITG $\alpha$ 5 (middle) and mutant 3'-UTR of ITG $\alpha$ 5 (bottom) to generate the renilla reporter constructs is shown. (B) miR-205 overexpression reduced ITG $\alpha$ 5 mRNA levels in the H2170 cell line. (C) miR-205 overexpression abrogated ITG $\alpha$ 5 protein levels in H2170 cells. Protein expression was normalised to  $\beta$ -actin. (D) Phospho-Src in the H2170 cell clones. (E) Luciferase assay shows that miR-205 inhibited wild type but not mutated ITG $\alpha$ 5-3'-UTR reporter activity. All these experiments were repeated at least three independent times.

**DISCUSSION**

TMPRSS4 has been shown to increase cell motility and invasion, to induce EMT and to promote metastasis in different cancer models, through multiple downstream signalling pathways involving ERK, AKT, Src and Rac1 (Kim *et al*, 2010). TMPRSS4 is upregulated in some cancer types, such as colon, pancreas, thyroid and lung (Wallrapp *et al*, 2000; Kebebew *et al*, 2005; Kim *et al*, 2010; Larzabal *et al*, 2011). Thus, this serine protease has emerged as a promising diagnostic and therapeutic target in cancer. Nonetheless, the molecular regulation of TMPRSS4 leading to these effects is still unknown. In the present study, we have identified a new molecular mechanism whereby TMPRSS4 regulates integrin  $\alpha 5$  levels through the expression of miR-205. Because ITG $\alpha$ 5 is strongly involved in the protumorigenic effects elicited by TMPRSS4, the data reported here about this novel molecular axis will help designing therapeutic strategies to inhibit this pathway. Moreover, in the present study we confirm the tumour suppressor role of miR-205 in lung cancer.

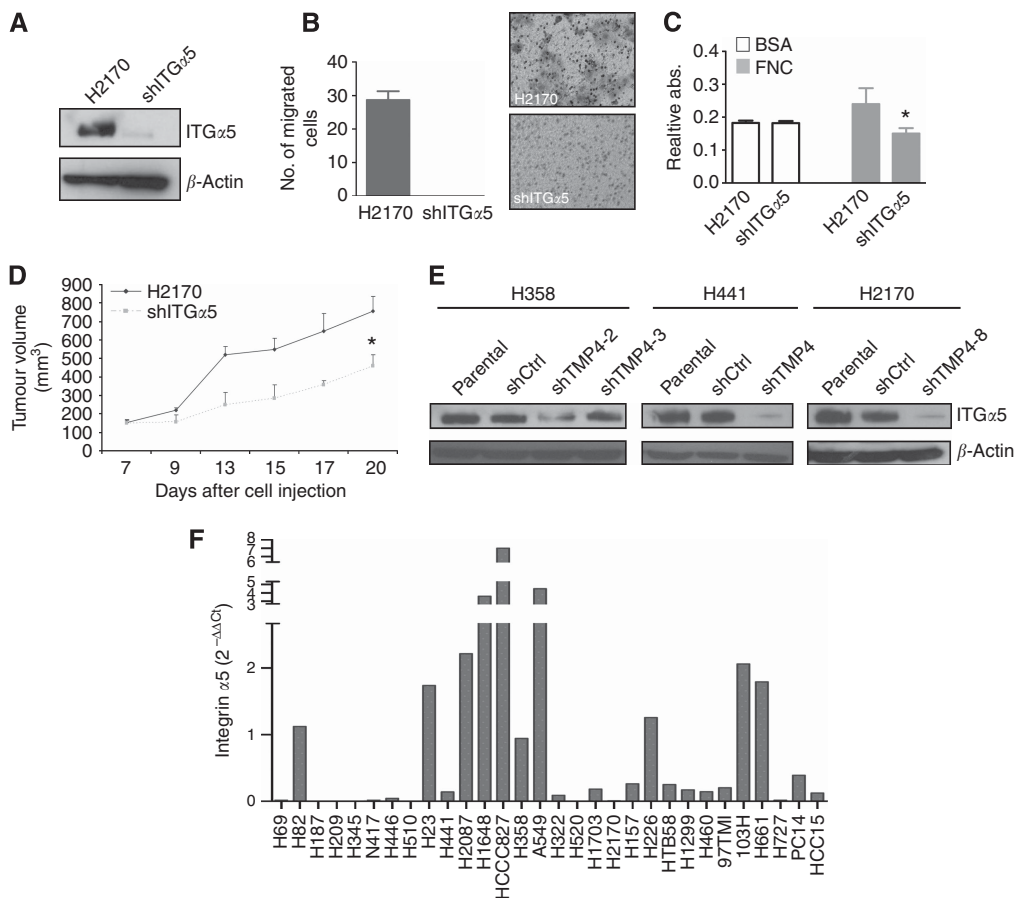
In order to elucidate the mechanism of action elicited by TMPRSS4 during EMT and metastasis formation, we conducted a microarray analysis where we compared gene expression profiles after TMPRSS4 inhibition in H358 and H441 lung cancer cell lines, with those of mock-transfected cells. As expected, the majority of genes with a differential expression were related with cell invasion and motility, in keeping with the functional role described for this serine protease in some tumour types (Jung *et al*, 2008; Kim *et al*, 2010). One of the upregulated genes was miR-205, a microRNA that has been described as a tumour suppressor (Song and Bu, 2009; Wu *et al*, 2009; Majid *et al*, 2011).

MicroRNAs (miRs) are small, non-coding RNAs that negatively regulate gene expression via translational repression or messenger RNA degradation (Bartel, 2004). MicroRNAs are involved in biologic and pathologic processes, including cell differentiation, proliferation and apoptosis (Kloosterman and Plasterk, 2006). Accumulating evidence indicates that deregulation of miRs is associated with the development of human cancers and suggests a causal role of miRs in tumour initiation and progression, as they can function as oncogenes or tumour suppressors (Caldas and Brenton, 2005; Chen, 2005). Recently, studies have shown that miR-205 regulates EMT through the modification of E-cadherin and other adhesion proteins (Bracken *et al*, 2009; Gibbons *et al*, 2009). Expression of the miR-200 family and miR-205 has been shown to be reduced in cells undergoing EMT. These miRs act by directly targeting the 3'-UTR sequence of ZEB1 and SIP1 (Gregory *et al*, 2008; Park *et al*, 2008). miR-205 has also been shown to suppress metastatic spread of human breast cancer xenografts in nude mice (Iorio *et al*, 2009) and to exert a tumour suppressor role by targeting HER3 receptor and VEGF-A in breast cancer (Iorio *et al*, 2009; Wu and Mo, 2009) and Src in renal cancer (Majid *et al*, 2011). In lung cancer, overexpression of miR-205 in cell lines hinders cell migration and invasion (Song and Bu, 2009) and its inhibition results in the acquisition of cancer stem cell and EMT properties, which favors tumour progression (Tellez *et al*, 2011). Consistent with these reports, we demonstrate that miR-205 was markedly downregulated in lung cancer cell lines compared with non-malignant HBEC lung epithelial cells. Its overexpression strongly reduced cell proliferation and clonogenic survival and caused G<sub>0</sub>/G<sub>1</sub> cell cycle arrest. In addition, we show that forced expression of miR-205 in lung cancer cells impaired migratory and invasive capabilities. Furthermore, we demonstrate that miR-205 expression reduced lung metastasis formation and promoted an epithelial phenotype by inducing E-cadherin and decreasing fibronectin levels. However, the role of miR-205 in lung cancer remains controversial, since some studies indicate a high expression of this miRNA in lung cancer compared with normal lung tissue (Yanaihara *et al*, 2006; Markou *et al*, 2008; Lebanony *et al*, 2009). Interestingly, expression of miR-205 has been described as a biomarker to distinguish between AC and SCC (Hamamoto *et al*, 2013; Jiang *et al*, 2013). It is worth noticing though, that exposure of HBEC cells to tobacco carcinogens silences miR-205 expression through epigenetic mechanisms, leading to a dedifferentiation programme (Tellez *et al*, 2011). Moreover, epigenetic silencing of miRNAs with tumour suppressor features, including miR-205, is emerging as a common hallmark of human tumours (Lujambio *et al*, 2008).

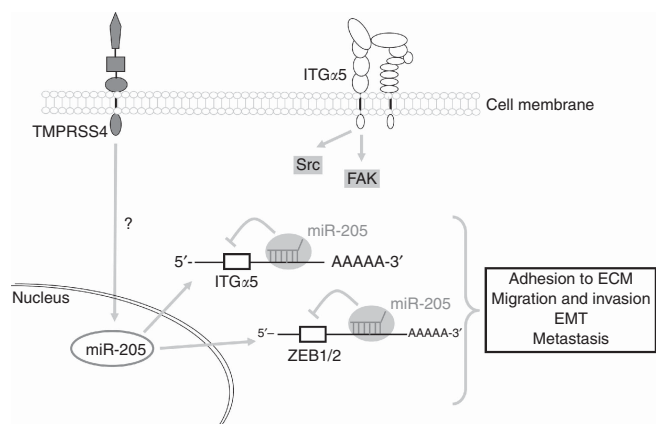
Each miRNA has the potential to target hundreds of genes, which harbour sequences in their 3'-UTRs that are complementary to the seed region of the miRNA (Lim *et al*, 2005). Different targets of miR-205 have been described including, as previously mentioned, ZEB1 and SIP1 (Gregory *et al*, 2008), ERBB3 and VEGF-A (Wu *et al*, 2009), LDL receptor protein 1 (Song and Bu, 2009) and PKC $\epsilon$  (Gandellini *et al*, 2009). In the present study, we have identified and validated for the first time ITG $\alpha$ 5 as a new target of miR-205 in cancer.

Integrins are a family of cell adhesion proteins that activate diverse intracellular signalling molecules and reorganise the actin cytoskeleton to regulate attachment, survival and motility (Giancotti and Ruoslahti, 1999; Hood and Chersesh, 2002). In NSCLC, expression of particular integrins has been shown to predict the clinical course and prognosis of patients (Adachi *et al*, 2000; Gogali *et al*, 2004). ITG $\alpha$ 5 binds to integrin  $\beta 1$  to give rise to the  $\alpha 5\beta 1$  heterodimer. Fibronectin stimulates the proliferation of lung cancer cells through  $\alpha 5\beta 1$  integrin receptor-mediated signalling; thus knockdown of this integrin reduces tumour burden (Roman *et al*, 2010). High expression of ITG $\alpha$ 5 is associated with lower overall survival in patients with early stages of NSCLC





**Figure 6.** (A) Analysis of ITG $\alpha 5$  by western blot in H2170 cells after infection with the shRNA targeting ITG $\alpha 5$ . (B) Migration of H2170 cells was completely blocked after inhibition of ITG $\alpha 5$ . (C) The inhibition of ITG $\alpha 5$  resulted in a decrease in adhesion to fibronectin (FNC). BSA was used as a negative control. (D) Tumour volumes in mice injected with H2170 cells with reduced or wild-type levels of ITG $\alpha 5$ . A delay in tumour growth was found in cells depleted in ITG $\alpha 5$ . (E) Expression of ITG $\alpha 5$  in H358, H441 and H2170 cell lines after transfection with control shRNA or TMPRSS4-specific shRNA. Protein expression was normalised to  $\beta$ -actin. (F) Analysis of ITG $\alpha 5$  expression in a panel of 30 lung cancer cell lines measured by qPCR. Twenty-two cell lines were positive for this gene, 10 of which showed high expression. GAPDH was use as internal control. All the *in vitro* experiments were repeated at least three independent times.



**Figure 7.** Proposed mechanism to show how TMPRSS4 may regulate ITG $\alpha 5$  through miR-205 to exert its biological effects. ECM, extracellular matrix.

adhesion and tumour growth. These data suggest that ITG $\alpha 5$  may constitute a target worth studying in patients with NSCLC.

A close relationship between acquisition of EMT features and ITG $\alpha 5$  expression has been observed. Inhibition of E-cadherin in ovarian cancer cells causes upregulation of ITG $\alpha 5$  (Sawada *et al*, 2008). ZEB2, a transcription factor that represses E-cadherin, upregulates ITG $\alpha 5$  levels through cooperation with Sp1 to induce EMT and invasion in cancer cells (Nam *et al*, 2012). In this line, Kim *et al* (2010) pointed to TMPRSS4 as a new regulator of EMT in colon cancer and suggested that Src, FAK and ERK, which are major downstream effectors of integrins, appeared to be key signalling molecules involved in cell invasion and in the cadherin switch, presumably via regulation of ITG $\alpha 5$  expression. In agreement with these results, we show a downregulation of ITG $\alpha 5$  in H358, H441 and H2170 lung cancer cell lines with an inhibition of TMPRSS4, which confirms the regulation of ITG $\alpha 5$  by TMPRSS4. Therefore, it appears that targeting the novel TMPRSS4/miR-205/ITG $\alpha 5$  axis may be a promising strategy to inhibit EMT and metastasis in NSCLC. In this regard, an antibody targeting integrin  $\alpha 5\beta 1$  (volociximab, PDL Biopharma) has been developed and is being currently tested in phase II clinical trials for solid tumours (particularly for renal carcinoma).

In summary, our results provide evidence for the existence of a new molecular connection between two membrane-anchored proteins (ITG $\alpha 5$  and TMPRSS4) that cooperate to foster tumour growth, metastasis and migration, through miR-205. This new

(Dingemans *et al*, 2010). Moreover, high ITG $\alpha 5$  levels have been associated with poor prognosis in NSCLC patients with negative lymph nodes (Adachi *et al*, 2000). We show here that 22 out of 30 lung cancer cell lines analysed expressed ITG $\alpha 5$ . Furthermore, inhibition of this integrin diminishes cancer cell migration,

intracellular signalling pathway appears to have an important role in the development of lung cancer. TMPRSS4 blockade in tumour cells causes an overexpression of miR-205, resulting in an inhibition of the transcription factors ZEB1 and ZEB2, and ITG $\alpha 5$ , which leads to a loss of EMT features. This, in turn, decreases cell–matrix interaction and cell invasiveness, hindering cell migration and metastasis formation. Biological or pharmacological approaches to block TMPRSS4 and ITG $\alpha 5$  may constitute an interesting novel approach to inhibit lung cancer.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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