Quantitative real time-PCR

Relative quantification of miR-187 in all cell lines and eight sample breast cancer tumours was carried out using a TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA; Assay ID 001193) for miR-187. RNA was extracted from formalin-fixed paraffin-embedded tumours using the RNeasy FFPE kit (Qiagen, Velno, Netherlands) and from cell lines using the MirVana miRNA isolation kit (Ambion, Austin, TX, USA). cDNA was synthesised using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and real-time PCR was carried out on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All techniques were carried out as per manufacturer's instructions. Relative expression of miRNA was determined using QBase software (24) by the $2^{-\Delta\Delta Ct}$ method. Expression of miR-187 was normalised to an endogenous reference RNA (RNU6B, Assay ID 001093; Applied Biosystems, Foster City, CA, USA).

In vitro functional analysis

In vitro functional analysis was carried out on MCF7 and SKBR3 cells ectopically expressing miR-187 to model different aspects of tumour progression. All *in vitro* assays were carried out in triplicate and repeated on three independent occasions. Figures shown are representative examples.

MTT proliferation assay

Cellular proliferation was measured over 6 days by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colourimetric assay. Cells were seeded in quadruplicate in four 96 well plates at a density of 2×10^3 per well. On Days 0, 2, 4 and 6, one plate was removed from the incubator and 50 µl 5 mg/ml MTT solution (Sigma-Aldrich, St Louis, MO, USA) added to each well, to a final concentration of 1.25 mg/ml. Following incubation for 3 hours, the media/MTT solution was aspirated and formazan crystals dissolved in 200 µl DMSO (Sigma-Aldrich, St Louis, MO, USA). Absorbance was measured at 540 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) and cell growth over the 6 day period graphed.

MTT drug response assay

Cell death in response to drug treatment was also measured by MTT assay as detailed above. In brief, cells were seeded in a 96 well plate at a density of 2 X 10^3 cells per well. On Day 0, media was aspirated and replaced with media containing the desired concentration of drug (fulvestrant, Sigma-Aldrich, St Louis, MO, USA; or 4-hydroxytamoxifen, Merck, Whitehouse Station, NJ, USA) or vehicle (ethanol). Cells were treated for 8 days, with the drug-containing media refreshed on Day 4. On Day 8, cell viability was assessed and represented as a percentage of the vehicle control.

Scratch wound migration assay

Horizontal lines were drawn across all wells of a 24 well plate and cells were plated at a density of 1.2 X 10^5 (MCF7) or 1 X 10^5 (SKBR3) cells per well. At Time 0, a scratch was made perpendicular to this line using a P200 micropipette tip. Cells were then washed with phosphate-buffered saline (PBS) and media replaced with serum-free media. Photomicrographs of scratched areas were taken at T0, 24, 48, 56 and 72 for MCF7 cells and T0 and T48 for SKBR3 cells and were analysed using TScratch (25).

Soft agar colony formation assay

Agarose (1%) and phenol red-free DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) or DMEM (Sigma-Aldrich, St Louis, MO, USA) containing 2X additives [20% FBS (Sigma-Aldrich, St Louis, MO, USA), 2% penicillin/streptomycin (50 units/ml; Gibco, Invitrogen, Carlsbad, CA, USA), 2% L-glutamate (2 mM; Gibco, Invitrogen, Carlsbad, CA, USA)] were equilibrated to 40^{o}C in a waterbath. Equal volumes were mixed together and 2 ml transferred to each well of a 6 well plate. Agarose (0.7%) and phenol red-free DMEM (MCF7 cells) or DMEM (SKBR3 cells) containing 2X additives (as above) were then equilibrated to 40^{o}C in a waterbath. Cells were trypsinised, counted and 8 X10^4 cells resuspended in 4 ml media with 2X additives. The same volume of 0.7% agarose was then added to the cell suspension and mixed. This mixture was then plated into three separate wells at a volume of 2 ml per well. Once the agarose had set, 2 ml media containing 1X additives was added to each well.

Plates were incubated at 37^{o}C for 14 days and colonies counted (per field of view) in triplicate for each well. A colony was classed as a group of cells ≥ 50 μm in diameter.

Spheroid invasion assay

Formation of three dimensional spheroids and implantation into collagen was carried out as previously described (26). Briefly, agarose-coated plates were prepared by dispensing 50 μl 1.5% sterile agarose into each well of a 96-well plate. Cells were trypsinised, counted, and 4 X 10^3 cells were plated in each well in 150 μl media. Spheroids were then allowed to form over the next 4-6 days. Subsequently, a 12-well plate was prepared with 1 ml 1.5% agarose/well. To prepare the collagen, 4.36 μl 1 M sodium hydroxide, 25 μl 10X MEM (Gibco, Invitrogen, Carlsbad, CA, USA), 2.5 μl L-glutamine solution (2 mM; Gibco, Invitrogen, Carlsbad, CA, USA), 2.5 μl penicillin/streptomycin solution (50 units/ml; Gibco, Invitrogen, Carlsbad, CA, USA), 35.63 μl PBS, 5 μl FBS (Sigma-Aldrich, St Louis, MO, USA) and 175 μl collagen (5 mg/ml; Invitrogen, Carlsbad, CA, USA) were combined on ice to give 250 μl collagen/cell line. Spheroids were harvested and resuspended in the prepared collagen which was then dispensed slowly into a well already coated in 1.5% agarose (1 well/cell line). The collagen was allowed to set for 30 minutes and media containing 2% FBS added to the well. Invasion was then monitored over a period of 14 days and quantified using ImageJ (27).

Western blotting

Cells were harvested into radioimmunoprecipitation assay (RIPA) buffer for protein extraction. Following rotation for 20 minutes at 4 ^{o}C, samples were centrifuged at 20,817 x g for 20 minutes to remove cellular debris. Protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay (Pierce, Illinois, USA). 20 μg protein samples were separated by SDS-PAGE on 12% gels under reducing conditions and then transferred to nitrocellulose membrane (Whatman, GE Healthcare Life Sciences, Wauwatosa, WI, USA). Blots were blocked in tris-buffered saline (TBS) containing 0.1% Tween and 5% w/v bovine serum albumin (BSA) before

incubating overnight with primary antibodies at 4^{o}C (1:500 MMP3, 1:500 MMP11, 1:1000 MMP13, 1:10,000 MMP21, EpiSelect MMP sampler kit, #5006-1, Epitomics, now Abcam, Cambridge, UK; 1:2000 β-actin, Sc-81178, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5000 β-catenin, Ab32572, and 1:8000 E-cadherin, Ab15148, Abcam, Cambridge, UK; 1:500 N-cadherin, #4061, Cell Signaling Technogy, Danvers, MA, USA; 1:5000 Vimentin 1:5000, #550513, BD Biosciences, Franklin Lakes, NJ, USA). Following overnight incubation, blots were washed with (TBS) containing 0.1% Tween and incubated in secondary antibody solution for one hour at room temperature (1:5000 anti-mouse or anti-rabbit-HRP; Dako, Glostrup, Denmark, or 1:2000 anti-rabbit-HRP; Cell Signaling Technology, Danvers, MA, USA). Blots were then washed again and developed using the the ECL Western Blotting Substrate kit (Pierce, now part of Thermo Fisher Scientific, Waltham, MA, USA).