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A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells

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The embryonic programme ‘epithelial–mesenchymal transition’ (EMT) is thought to promote malignant tumour progression. The transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1) is a crucial inducer of EMT in various human tumours, and was recently shown to promote invasion and metastasis of tumour cells. Here, we report that ZEB1 directly suppresses transcription of microRNA-200 family members miR-141 and miR-200c, which strongly activate epithelial differentiation in pancreatic, colorectal and breast cancer cells. Notably, the EMT activators transforming growth factor β 2 and ZEB1 are the predominant targets downregulated by these microRNAs. These results indicate that ZEB1 triggers a microRNA-mediated feedforward loop that stabilizes EMT and promotes invasion of cancer cells. Alternatively, depending on the environmental trigger, this loop might switch and induce epithelial differentiation, and thus explain the strong intratumourous heterogeneity observed in many human cancers.

Keywords: EMT; feedback loop; invasion; microRNA; ZEB1

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INTRODUCTION

Increasing evidence indicates that aberrant activation of the embryonic programme ‘epithelial–mesenchymal transition’ (EMT) promotes tumour cell invasion and metastasis (Bex *et al*, 2007).

EMT allows detachment of cells from each other and increases cell mobility, both of which are necessary for tumour cell dissemination. Metastases often recapitulate the differentiated phenotype of the primary tumour; therefore, EMT seems to be transiently activated by the inductive tumour environment at the invasive tumour edge, but is reversed in growing metastases (Brabletz *et al*, 2001, 2005). Activators of EMT, such as transforming growth factor (TGF) β , tumour necrosis factor α (TNF α) and hepatocyte growth factor, are produced by infiltrating cells or the tumour cells themselves, and trigger expression of EMT-inducing transcriptional repressors (Thiery & Sleeman, 2006). These include members of the Snail family, the basic helix–loop–helix family, Goosecoid and members of the ZFH family (zinc-finger E-box binding homeobox (ZEB)1 and ZEB2; Barrallo-Gimeno & Nieto, 2005; Hugo *et al*, 2007; Peinado *et al*, 2007). Recently, we described that ZEB1 is a crucial EMT activator in human colorectal and breast cancer, and suppresses expression of basement membrane components (Spaderna *et al*, 2006) and cell polarity factors (Aigner *et al*, 2007; Spaderna *et al*, 2008). Expression of ZEB1 promotes metastasis of tumour cells in a mouse xenograft model, indicating a role of ZEB1 in invasion and metastasis of human tumours (Spaderna *et al*, 2008).

MicroRNAs (miRNAs) are small non-coding RNAs that can silence their cognate target genes by specifically binding and cleaving messenger RNAs or inhibiting their translation (Bartel, 2004). miRNAs regulate diverse cellular processes and some miRNAs have been shown to function as either tumour suppressors or oncogenes (Esquela-Kerscher & Slack, 2006). Recent important examples are the oncogenic miR-10b, which promotes metastasis (Ma *et al*, 2007), and miR-335/miR-126, which suppress metastasis in breast cancer (Tavazoie *et al*, 2008).

Owing to these important regulatory functions of miRNAs, it is of prime interest to know how their expression is regulated by upstream factors. Here, we address this point and focus on

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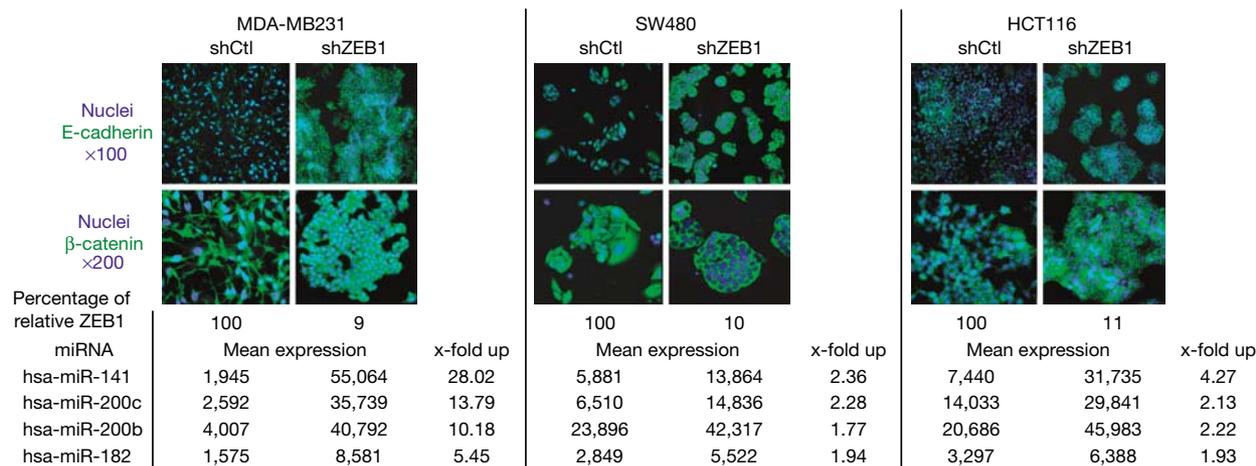


Fig 1 | Knockdown of ZEB1 enhances expression of microRNAs. Growth patterns and staining for E-cadherin and β -catenin of characteristic short hairpin control (shCtl) and shZEB1 knockdown clones of breast (MDA-MB231) and colorectal (SW480 and HCT116) cancer cell lines used for the microRNA (miRNA) expression array screen. The mean relative ZEB1 expression levels of each two clones are indicated. The mean miRNA expression values (two hybridizations of each two independent sh control or shZEB1 clones per cell line) are shown. Knockdown of ZEB1 resulted in upregulation of the indicated miRNAs in all three cell lines. ZEB1, zinc-finger E-box binding homeobox 1.

the activation and stabilization of EMT in cancer cells. We investigated whether aberrant expression of the crucial EMT activator ZEB1 and the control of potential EMT-regulatory miRNAs are linked and can synergize to promote malignant tumour progression.

RESULTS

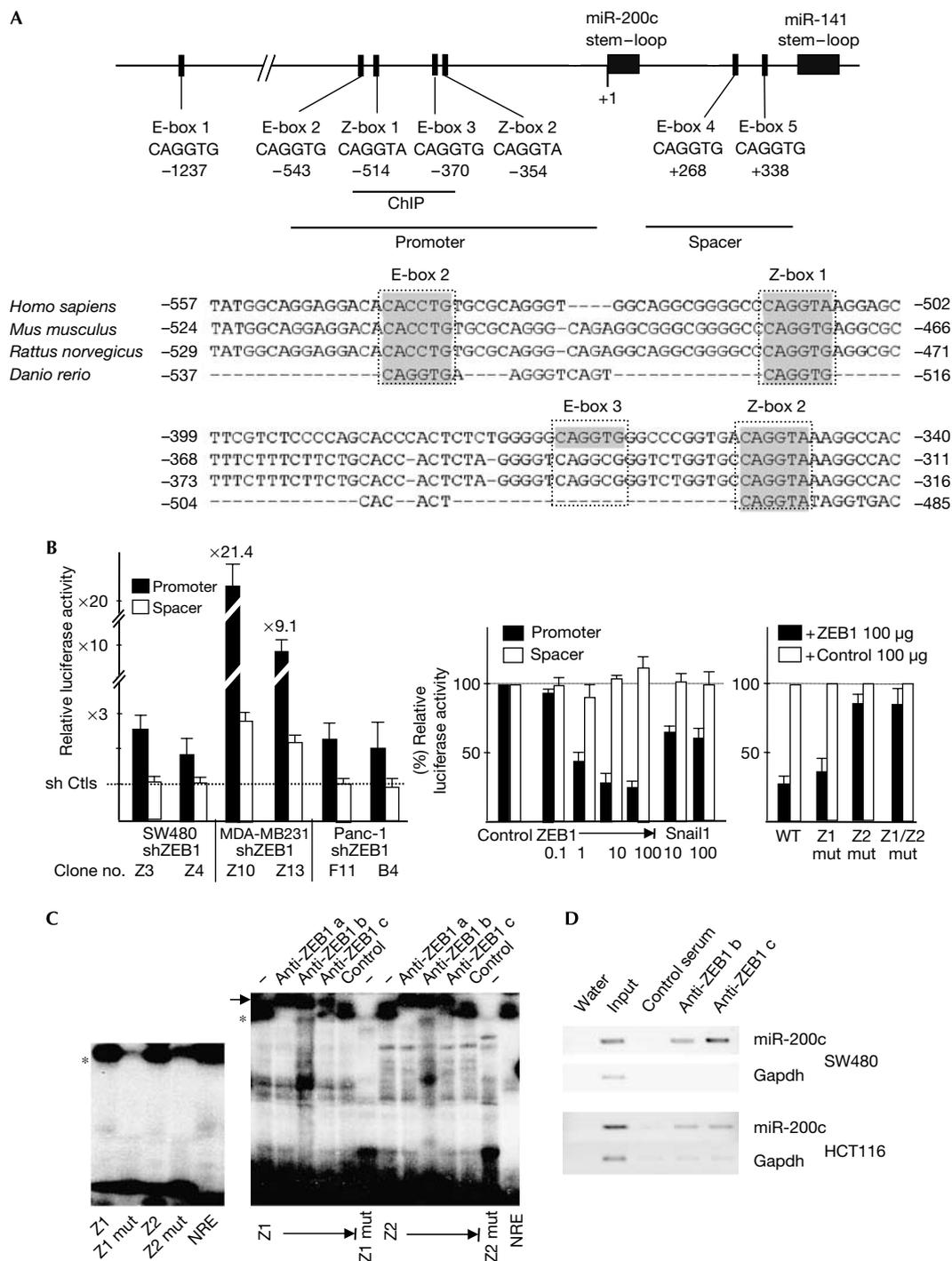
ZEB1 directly suppresses transcription of miRNAs

TGF β and TNF α have been shown to induce an EMT in differentiated colorectal cancer cells (Bates *et al*, 2005). We observed activation of EMT in differentiated pancreatic (HPAF2), colorectal (DLD1) and breast cancer (MCF7) cell lines by TGF β /TNF α . Thus, ZEB1 was a crucial intracellular transmitter of this EMT, as its expression was upregulated by both cytokines and its knockdown partly prevented EMT (supplementary Fig 1A,B online). Direct epithelial target genes suppressed by ZEB1, such as E-cadherin, cell polarity factors and basement membrane components, have been described previously (Grootclaes & Frisch, 2000; Aigner *et al*, 2007; Spaderna *et al*, 2008). Here, we investigated whether ZEB1 also affects expression of miRNAs and if detected miRNAs themselves are candidate regulators of EMT.

A miRNA expression microarray screen was used to analyse SW480 and HCT116 colorectal and MDA-MB231 breast cancer cell clones with stable short hairpin RNA-mediated knockdown of ZEB1 (shZEB clones) in comparison with control knockdown (shCtl) clones. We have shown previously that stable knockdown of ZEB1 in these clones led to a reversal of the fibroblastoid phenotype towards an epithelial differentiation in the sense of a mesenchymal–epithelial transition (Spaderna *et al*, 2008). Out of 743 human, rat and mouse miRNAs included on the microarray screen, we detected average upregulation (>1.75-fold) after ZEB1 knockdown of 79 miRNAs (10.59%) in at least one cell line, 17 miRNAs (2.27%) in two cell lines and only 4 miRNAs (0.53%) in all three cell lines (Fig 1; supplementary Table 1 online). The strongest effect was observed for the undifferentiated breast cancer

cell line MDA-MB231. Notably, three of the four miRNAs upregulated in all cell lines (miR-141, miR-200b and miR-200c) belong to the highly conserved miRNA-200 family, which was recently linked to the induction of epithelial differentiation (Hurteau *et al*, 2007; Gregory *et al*, 2008; Park *et al*, 2008). Further analyses focused on miR-141 and miR-200c, which showed strongest upregulation after knockdown of ZEB1 in all three cancer cell lines.

The array data were validated by using real-time PCR for miR-141 and miR-200c, which showed a strong increase after knockdown of ZEB1 in undifferentiated pancreatic, colorectal and breast cancer cell lines (supplementary Fig 1C online). Next, we investigated whether ZEB1 directly suppresses transcription of the *miR-141* and *miR-200c* miRNA genes. Both miRNAs map closely on human chromosome 12p13.31 and the stem–loop sequences are separated by only a 338-base-pair spacer sequence (Fig 2A). This spacer and the putative promoter 600bp upstream from the hsa-miR-200c stem–loop contain six putative binding sequences for ZEB1, two of which were restricted to ZEB factors (Z-box 1 and 2, CAGGTA). The remaining four were perfect E-boxes (E-box 1–4, CAGGTG), which, in addition to ZEB1, represent putative binding sites for other EMT activators, such as Snail factors. Note that the overall miRNA gene structure and the two Z-boxes, as well as E-box 2, are highly conserved in evolution from zebrafish to human (Fig 2A). All the conserved ZEB1-binding sequences are located within the putative promoter, whereas the two E-boxes in the spacer sequence are not conserved. After cloning of the putative promoter (–683 to –67 relative to first nucleotide of the miR-200c stem–loop) and the spacer (+66 to +338) into a luciferase reporter vector, ZEB1-dependent activity was assessed. shRNA-mediated knockdown of ZEB1 in all undifferentiated colorectal, breast and pancreatic cancer cell lines tested resulted in an enhanced promoter activity when compared with shCtl cell clones (Fig 2B, left). On the contrary, the promoter activity was suppressed by ZEB1 overexpression in a dose-dependent manner.



Similar effects, although to a lesser extent, were also observed after Snail1 overexpression, which can bind to only two of the four potential ZEB1-binding sites within the putative promoter (Fig 2B, middle). The expression level of ZEB1 had no significant effect on the transcriptional activity of the spacer sequence. Mutation of the two highly conserved Z-boxes showed that Z-box 2 confers the strongest repressive function by ZEB1 (Fig 2B, right) and also made

the promoter activity insensitive to stable knockdown of ZEB1 (supplementary Fig 1D online). A direct binding of ZEB1 to the two conserved ZEB1 sites (Z-boxes) was shown by electromobility shift assay by using recombinant DNA-binding domain of ZEB1 and nuclear extracts from SW480 colorectal cancer cells (Fig 2C). By applying chromatin immunoprecipitation (ChIP) with chromatin from SW480 or HCT116 colorectal cancer cells, we could

Fig 2 | ZEB1 directly suppresses transcription of miR-141 and miR-200c. (A) Schematic of the genomic organization of hsa-miR-141 and hsa-miR-200c on chromosome 12p13.31, and alignment of the highly conserved Z- and E-boxes. The putative ZEB1 binding sites, the cloned promoter and spacer sequence, as well as the region amplified for ChIP, are indicated. All numbers are relative to the start of the hsa-miR-200c stem-loop sequence. (B) Left panel: stable ZEB1 knockdown in the indicated undifferentiated colorectal (SW480), breast (MDA-MB231) and pancreatic (Panc-1) cell clones enhances the activity of the putative promoter, but does not or only weakly enhances the activity of the spacer sequence. The mean values of the corresponding short hairpin control (shCtl) clones were set to 1. Middle panel: overexpression of ZEB1 and to a lesser extent that of Snail in HCT116 cells repress the activity of the promoter but not the spacer sequence in a dose-dependant manner. The absolute transcriptional activity of the promoter was 55-fold higher than the activity of the spacer sequence. Right panel: mutations (mut) of the Z-boxes render the promoter less sensitive to suppression by ZEB1. (C) Electromobility shift assay using recombinant DNA-binding domain of ZEB1 (left panel) or nuclear extracts from SW480 colorectal cancer cells (right panel) and the indicated labelled probes. Mutation of the two conserved Z-boxes (Z1, Z2) strongly reduced the specific binding complex (asterisk). Three different antisera against ZEB1, but not control serum, supershifted (arrow) a specific complex (asterisk), which is missing in the mutated probes, indicating that it contains ZEB1. The known ZEB1-binding site of the interleukin-2 promoter (NRE) was used as a positive control. (D) ChIP with two different ZEB1 antisera shows *in vivo* binding of ZEB1 to the putative promoter in the indicated colorectal cancer cell lines. ChIP, chromatin immunoprecipitation; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type; ZEB1, zinc-finger E-box binding homeobox 1.

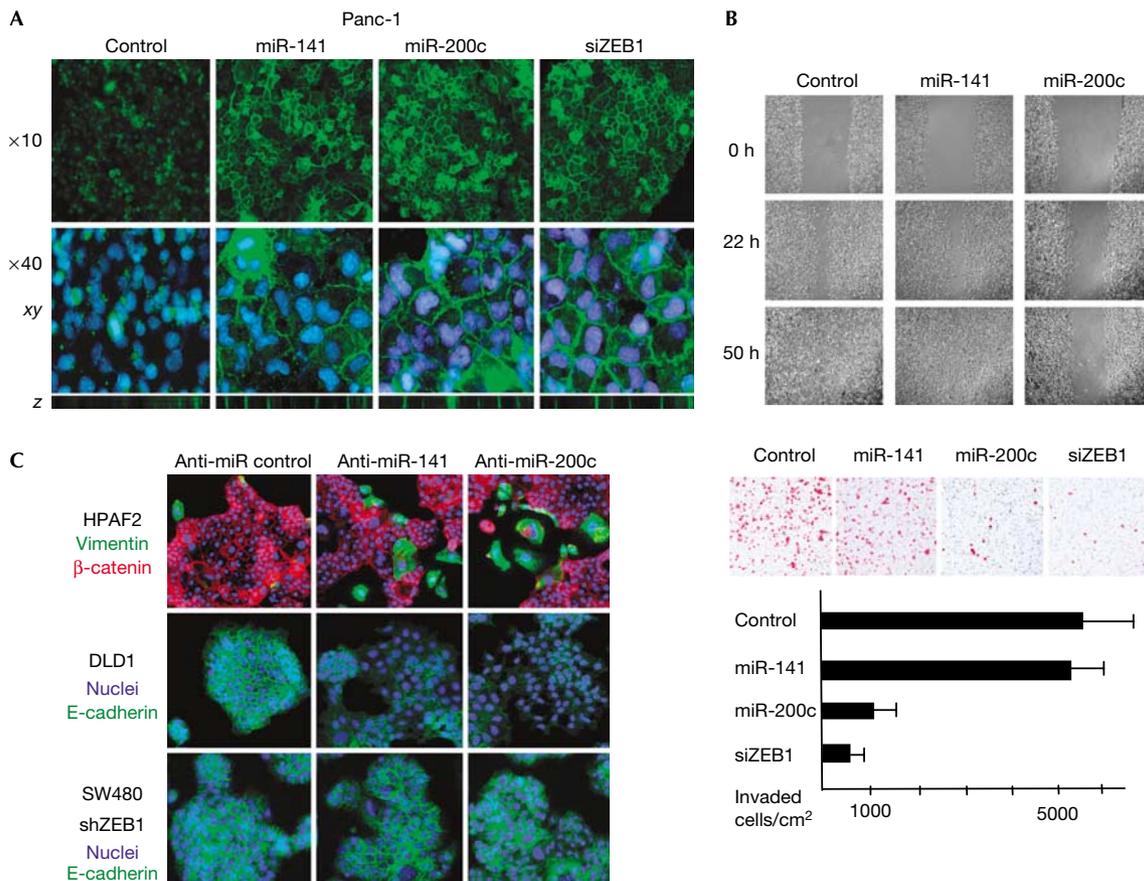


Fig 3 | miR-141 and miR-200c induce mesenchymal-epithelial transition. (A) Overexpression of both microRNAs (miRNAs) in undifferentiated cancer cell lines induces a mesenchymal-epithelial transition, indicated by upregulation of E-cadherin (green), partial membranous translocation of E-cadherin and restoration of basal-apical polarity (see z-mode). The effect was stronger for miR-200c and comparable with the effect of a ZEB1 knockdown. (B) Upper panel: miR-200c suppressed migration of MDA-MB231 cells. Lower panels: picture and quantification of a Matrigel invasion assay. Transfection with miR-200c strongly suppressed invasion of MDA-MB231 into Matrigel, comparable with short interfering (si) RNA-mediated knockdown of ZEB1. (C) Differentiated cancer cell lines transfected with miRNA inhibitors against miR-141 and miR-200c show increased cell scattering, upregulation of vimentin (HPAF2) or reduced expression of E-cadherin (DLD1). By contrast, the epithelial phenotype induced by stable knockdown of ZEB1 (SW480 shZEB1) cannot be reversed by miRNA inhibitors. sh, short hairpin; ZEB1, zinc-finger E-box binding homeobox 1.

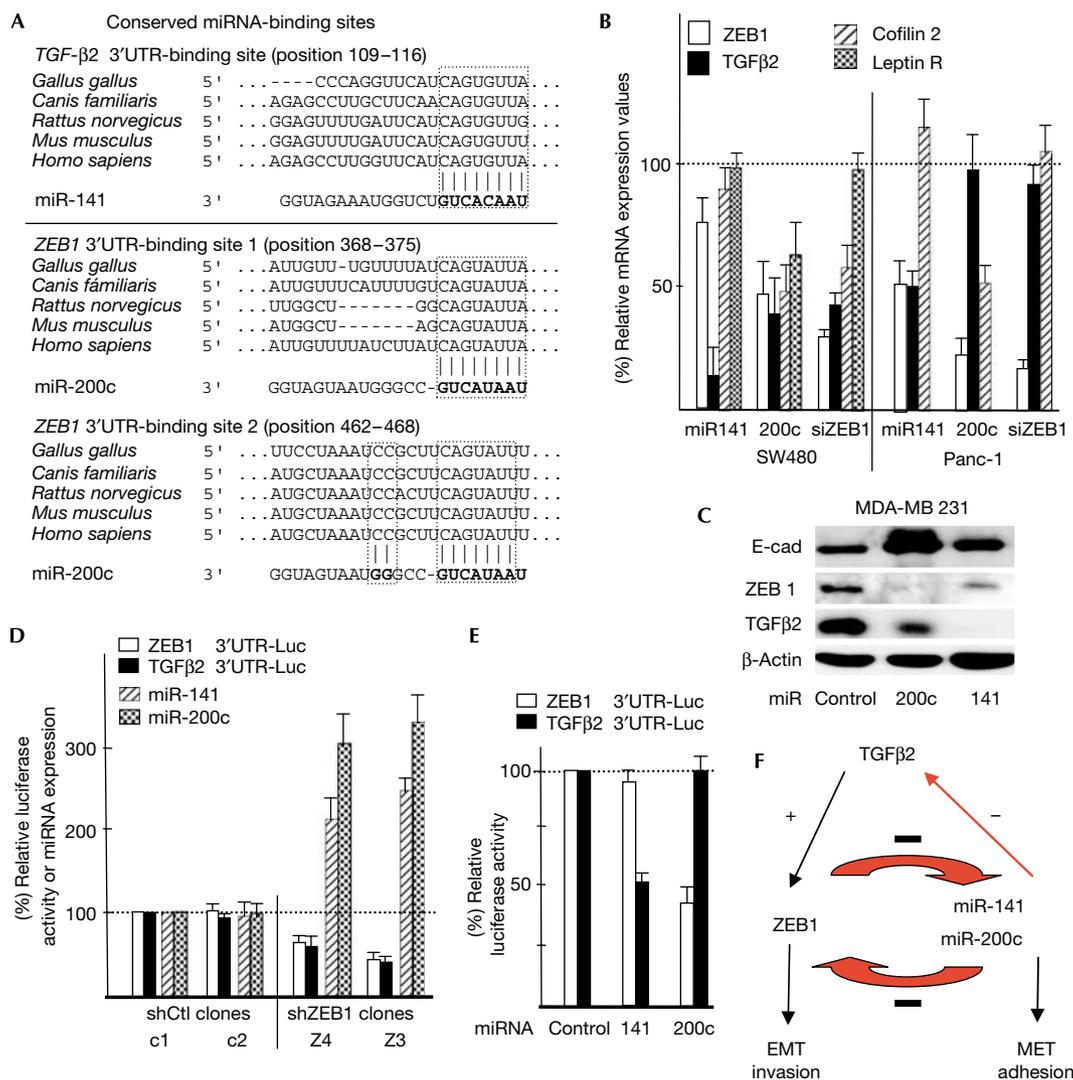


Fig 4 | miR-141 and miR-200c reduce expression of factors promoting epithelial–mesenchymal transition and invasion. (A) Predicted duplex formation of TGFβ2 3'UTR with the miR-141 stem–loop and of two ZEB1 3'UTR regions with the miR-200c stem–loop. The putative recognition sites are highly conserved among various species. (B) Real-time reverse transcription–PCR of various putative target factors after transfection of the indicated miRNAs or short interfering (si) RNA in undifferentiated cancer cells. Note strongest reduction of TGFβ2 expression after miR-141 transfection and that of ZEB1 after miR-200c transfection. (C) Confirmation of the results on protein levels by immunoblots. (D) Luciferase reporter assay showing reduced activity of 3'UTR-luciferase reporter constructs in the indicated SW480 short hairpin ZEB1 (shZEB1) clones with increased levels of miR-141 and miR-200c compared with shCtl clones. (E) Overexpression of miR-141 and miR-200c in SW480 cells leads to decreased activity of TGFβ2 3'UTR-luciferase and ZEB1 3'UTR-luciferase constructs, respectively. (F) Model of a feedforward loop linking ZEB1 and counteracting miRNAs, in particular miR-200c. As ZEB1 and both miRNAs repress each other, they are linked in a feedforward loop. Depending on the initial signal, this loop could stabilize either mesenchymal or epithelial differentiation. In tumours, environmental TGFβ could trigger upregulation of ZEB1, promoting a self-enhancing loop in tumour cells, finally resulting in EMT and invasion. Once the initial signal breaks down, the loop could induce a reversal of the epithelial phenotype. This might explain the strong phenotypic heterogeneity often seen within individual tumours and metastases. EMT, epithelial–mesenchymal transition; MET, mesenchymal–epithelial transition; miRNA, microRNA; TGF, transforming growth factor; UTR, untranslated region; ZEB1, zinc-finger E-box binding homeobox 1.

show that endogenous ZEB1 binds to the native promoter region (Fig 2D). These data indicate that the transcriptional repressor ZEB1 can directly suppress expression of both miR-141 and miR-200c by binding to their putative common promoter.

miRNAs targeted by ZEB1 are inhibitors of EMT

Next, we investigated whether the two miRNAs suppressed by ZEB1 represent direct EMT regulators. We showed that miR-200c and miR-141 are strong inducers of an epithelial phenotype,

which was also recently reported by other groups during the course of our work (Hurteau *et al*, 2007; Gregory *et al*, 2008; Park *et al*, 2008). Transient overexpression of both miR-141 and miR-200c resulted in the induction of epithelial differentiation of undifferentiated cancer cells (Fig 3A; supplementary Fig 2A,B online). In particular, the overall effect of miR-200c was comparable with the strong effect of ZEB1 knockdown. Notably, miR-200c, and to a lesser extent miR-141, also led to increased E-cadherin expression and cell–cell adhesion, as well as reduced spreading of normal human fibroblasts (supplementary Fig 2C online). Overexpression of miR-200c strongly reduced cancer cell migration and invasion into Matrigel, indicating a potential inhibitory role in malignant tumour progression (Fig 3B; supplementary Fig 2D online). On the contrary, treatment of differentiated cancer cell lines HPAF2 and DLD1 with inhibitors of miR-200c, and to a lesser extent of miR-141, resulted in a mesenchymal transition as indicated by cell scattering, upregulation of vimentin and reduced expression of E-cadherin (Fig 3C). Knowing that the breast cancer cell line MDA-MB231, which almost completely lost both miRNAs, is derived from a basal type of breast cancer, we analysed various types of human breast cancer. As predicted from using cancer cell lines, we confirmed that basal types of breast cancers, characterized by an undifferentiated phenotype and poor clinical prognosis, show reduced expression of both miRNAs compared with the common ductal invasive type of breast cancer (supplementary Fig 2E online).

To explain further the mechanisms by which both miRNAs induce epithelial differentiation, we searched for putative target genes on the basis of the predicted mRNA recognition sequence of the conserved stem–loop sequences by using the TargetScan search programme (Lewis *et al*, 2003). Notably, among the highest scored target genes for miR-200c were *ZEB1* and *ZEB2*, which confirms recent findings published during the course of our work (Hurteau *et al*, 2007; Gregory *et al*, 2008; Park *et al*, 2008). In addition, a putative target of miR-141 is *TGFβ2*, indicating that both miRNAs are functionally linked by affecting different members of the same EMT-inducing pathway. We further selected the putative target factors leptin receptor and cofilin 2 because they are known promoters of malignant tumour progression (Attoub *et al*, 2000; Wang *et al*, 2007). The predicted miRNA binding sites in the 3′ untranslated region (UTR) were highly conserved during evolution (Fig 4A; supplementary Fig 3A online). We could show that overexpression of miRNAs led to reduced expression of *ZEB1*, *TGFβ2* and the other candidate genes in undifferentiated cancer cells (Fig 4B,C; supplementary Fig 3B online). As predicted, miR-141 had the strongest inhibitory effect on *TGFβ2* and miR-200c on *ZEB1* expression, as shown by RNA and protein levels. On the contrary, treatment of differentiated cancer cell lines HPAF2 and MCF7 with inhibitors of miR-200c or miR-141 resulted in a change in the expression of characteristic genes, including an increase in *ZEB1* and *TGFβ2* expression (supplementary Fig 3C online). A negative regulation of *ZEB1* and *TGFβ2* expression by miR-200c and miR-141 was indicated after cloning of their highly conserved, putative mRNA 3′UTR target sequences in a luciferase reporter vector. After transfection into SW480 shZEB1 clones with reduced *ZEB1* and enhanced expression of both miRNAs, both reporters showed reduced luciferase activity in two independent shZEB1 clones compared with shCtl clones (Fig 4D). Overexpression of miR-141 and

miR-200c in SW480 cells led to a reduced activity of *TGFβ2*-3′UTR and *ZEB1*-3′UTR constructs, respectively (Fig 4E). On the contrary, selective inhibition of miR-141 and miR-200c in SW480 shZEB1 cells by specific anti-miRNAs resulted in a selective increase in the activity of *TGFβ2*-3′UTR and *ZEB1*-3′UTR constructs, respectively (supplementary Fig 3D online).

DISCUSSION

By applying a miRNA expression array screen for various human cancer cells, we detected several miRNAs suppressed by the EMT inducer *ZEB1*. The most prominent effect was on members of the miR-200 family. *ZEB1* directly suppressed transcription of two members closely linked on human chromosome 12, miR-141 and 200c, by binding to at least two highly conserved sites in their putative promoter. In confirmation with the data published during the course of our work (Hurteau *et al*, 2007; Gregory *et al*, 2008; Park *et al*, 2008), the detected miRNAs induced a mesenchymal to epithelial transition (MET) and inhibited EMT, migration and invasion of undifferentiated cancer cells. We further identified putative target genes, which are known promoters of EMT and malignant tumour progression. One target of miR-200c is *ZEB1* itself, indicating an EMT-enhancing feedforward loop in invading cancer cells. This regulatory loop might be stabilized further by downregulation of miR141, as one of its putative targets is *TGFβ2*. There is increasing evidence that *ZEB1* has crucial effects on various processes of malignant tumour progression (Peinado *et al*, 2007) and promotes metastasis (Spaderna *et al*, 2008). In the light of the important role of *ZEB1* and other EMT inducers, such as Snail (Olmeda *et al*, 2007), Twist (Yang *et al*, 2004) and of EMT as a whole in tumour progression, our data, indicating that *ZEB1* promotes an EMT-stabilizing feedforward loop by suppressing specific miRNAs, add functional evidence for the molecular mechanisms underlying these processes.

Our work addressed the clinically relevant question of how putative tumour-suppressive miRNAs can be inactivated in cancer progression. The fact that both the *ZEB1* binding sites and the overall structure of the *miR-200c* and *miR-141* genes are highly conserved in vertebrates from zebrafish to human suggests that the tumour cells use a long-established regulatory mechanism of miRNA expression. Moreover, the second miRNA cluster of the miR-200 family on human chromosome 1p36 also contains highly conserved putative *ZEB1* binding sites in the upstream sequence, indicating that the whole miR-200 family can be suppressed by *ZEB1* (supplementary Fig 3E online). In addition, both the strong transcriptional inhibition of the two miRNAs by *ZEB1* and their putative tumour-suppressive effect were detected in tumour cell lines of various important cancer entities, namely pancreatic, colorectal and breast cancer. A clinical relevance is indicated by the fact that both miRNAs are lost in the highly aggressive basal type of breast cancer, which, in contrast to the luminal and ductal invasive type, is poorly differentiated, shows no expression of oestrogen and progesterone receptors, and has a worse clinical prognosis (Sempere *et al*, 2007).

Both miRNAs affect the expression of different molecules, which all work in the same proinvasive manner, as is known for *TGFβ2*, *ZEB1*, cofilin and leptin receptor. The differential function of the two miRNAs can synergize, as they are coexpressed, possibly through coactivation by a common promoter. The intriguing fact is that both miRNAs inhibit members of their own

repressing pathway: miR-200c targets ZEB1 and miR-141 targets TGF β 2. Thus, ZEB1 becomes a crucial regulator, as its aberrant expression in cancer might start a self-enhancing feedforward loop by downregulating its own inhibitors miR-141 and miR-200c (Fig 4F). Moreover, if the initial signal breaks down (for example, tumour environmental TGF β), such a loop might as well re-enforce expression of the miRNAs, thereby re-inducing an epithelial phenotype. This might explain the strong phenotypic heterogeneity often seen within individual tumours and metastases. Recently, Liu *et al* (2008) showed that ZEB1 is crucial for TGF β -mediated EMT in various steps of organ development. This important role of ZEB1 points out that the predicted regulatory loop might also have a physiological role in separating mesenchymal from epithelial tissue in development and organogenesis.

In conclusion, we suggest that ZEB1 is a crucial promoter of tumour progression by reducing transcription of both mRNAs and miRNAs. Thus, ZEB1 is a central molecular regulator of a miRNA-mediated feedforward loop, which can re-enforce EMT.

METHODS

miRNA expression microarray screen. A 50 μ g portion of total RNA including small RNAs isolated from 4×10^7 cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) was shipped to Capital Bio (Beijing, China). An expression screening was carried out using CapitalBio Mammalian miRNA Array V2.0 containing 743 human, rat and mouse non-redundant miRNA probes. The microarray data have been deposited on ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) with the accession code E-TABM-461.

DNA constructs. For the hsa-miR-200c promoter reporter plasmid nucleotides -683 to -67, and for the spacer reporter nucleotides +66 to +403 (relative to first nucleotide of miR-200c stem-loop) were cloned into pGL3basic (Promega, Mannheim, Germany). For the 3'UTR reporter plasmids, nucleotides +3,399 to +3,953 of human ZEB1 complementary DNA and nucleotides +1,427 to +1,695 of human TGF β 2 cDNA were amplified and cloned downstream of the luciferase gene in the pMIR-REPORT vector (Ambion, Austin, TX, USA).

Cell culture and standard assays. All cell lines were purchased from ATCC (Manassas, VA, USA). Standard cell culture, transient transfections, reporter assays, electromobility shift assays, immunoblots, transient short interfering RNA (siRNA)-mediated knockdown and quantitative real-time reverse transcription-PCR were carried out as described previously (Brabletz *et al*, 1999, 2004; Hlubek *et al*, 2001). For TGF β /TNF α stimulation 3×10^4 cells per well were seeded in a 12-well plate, transfected at day 1 as indicated, and stimulated with 2 ng/ml TGF β and 10 ng/ml TNF α for 5 days.

Specific assays. *miRNA modulation:* A total of 5×10^4 cells per well were seeded in a 12-well plate. After 24 h, cells were transfected with 30 pmol oligonucleotides for miR-141, miR-200c or control miRNA-16 (Ambion, Austin, TX, USA) using OligofectamineTM Reagent (Invitrogen, Carlsbad, CA, USA) for overexpression, or with 420 nM of specific anti-miRs (Ambion) for inhibition. Cells were cultivated for days before further use. Cell invasion was evaluated using the Chemicon Cell Invasion Assay Kit as described previously (Chemicon International, Millipore, Schwalbach, Germany), using 20,000 transiently (miRNA or siRNA) transfected cells.

Cell migration assay: Cells were transfected with miRNAs and controls as described (Spaderna *et al*, 2006). After reaching confluence, cells were scratched with a pipette tip and the migration potential was observed for up to 50 h.

Quantitative real-time PCR for miRNAs: RNA from cultured cells was extracted using the mirVanaTM PARISTM Kit (Ambion, Austin, TX, USA). Total RNA of formalin-fixed, paraffin-embedded samples of breast carcinomas retrieved from the archives of the Department of Pathology, University of Erlangen was extracted after microdissection using the Total Nucleic Acid Isolation Kit for FFPE (Ambion, Austin, TX, USA). Specific quantitative real-time PCR experiments were carried out using TaqMan[®] MicroRNA Assays for miR-141, miR-200c and control miRNA-16 (Applied Biosystems, Foster City, CA, USA) on a Roche LightCycler 480.

ChIP analysis: The ChIP IT kit (Active Motif, Carlsbad, CA, USA) was applied according to the manufacturer's instructions. A 3 μ g portion of control rabbit antiserum or antisera against ZEB1 was used for immunoprecipitation.

For oligonucleotide sequences, plasmids and antibodies used in this study, see the supplementary information online data.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

- Aigner K *et al* (2007) The transcription factor ZEB1 (Δ EF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. *Oncogene* **26**: 6979–6988
- Attoub S, Noe V, Pirola L, Bruyneel E, Chastre E, Mareel M, Wymann MP, Gespach C (2000) Leptin promotes invasiveness of kidney and colonic epithelial cells via phosphoinositide 3-kinase-, rho-, and rac-dependent signaling pathways. *FASEB J* **14**: 2329–2338
- Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* **132**: 3151–3161
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**: 281–297
- Bates RC, Bellovin DI, Brown C, Maynard E, Wu B, Kawakatsu H, Sheppard D, Oettingen P, Mercurio AM (2005) Transcriptional activation of integrin β 6 during the epithelial-mesenchymal transition defines a novel prognostic indicator of aggressive colon carcinoma. *J Clin Invest* **115**: 339–347
- Berx G, Raspe E, Christofori G, Thiery JP, Sleeman JP (2007) Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer. *Clin Exp Metastasis* **24**: 587–597
- Brabletz T, Jung A, Hlubek F, Lohberg C, Meiler J, Suchy U, Kirchner T (1999) Negative regulation of CD4 expression in T cells by the transcriptional repressor ZEB. *Int Immunol* **11**: 1701–1708
- Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart L, Knuechel R, Kirchner T (2001) Variable β -catenin expression in colorectal cancer indicates a tumor progression driven by the tumor environment. *Proc Natl Acad Sci USA* **98**: 10356–10361
- Brabletz T *et al* (2004) Down-regulation of the homeodomain factor Cdx2 in colorectal cancer by collagen type I: an active role for the tumor environment in malignant tumor progression. *Cancer Res* **64**: 6973–6977

- Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T (2005) Opinion: migrating cancer stem cells—an integrated concept of malignant tumour progression. *Nat Rev Cancer* **5**: 744–749
- Esquela-Kerscher A, Slack FJ (2006) Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer* **6**: 259–269
- Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10**: 593–601
- Grootclaes ML, Frisch SM (2000) Evidence for a function of CtBP in epithelial gene regulation and anoikis. *Oncogene* **19**: 3823–3828
- Hlubek F, Jung A, Kotzor N, Kirchner T, Brabletz T (2001) Expression of the invasion factor laminin γ 2 in colorectal carcinomas is regulated by β -catenin. *Cancer Res* **61**: 8089–8093
- Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW (2007) Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. *J Cell Physiol* **213**: 374–383
- Hurteau GJ, Carlson JA, Spivack SD, Brock GJ (2007) Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. *Cancer Res* **67**: 7972–7976
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell* **115**: 787–798
- Liu Y, El-Naggar S, Darling DS, Higashi Y, Dean DC (2008) Zeb1 links epithelial–mesenchymal transition and cellular senescence. *Development* **135**: 579–588
- Ma L, Teruya-Feldstein J, Weinberg RA (2007) Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* **449**: 682–688
- Olmeda D, Moreno-Bueno G, Flores JM, Fabra A, Portillo F, Cano A (2007) SNAI1 is required for tumor growth and lymph node metastasis of human breast carcinoma MDA-MB-231 cells. *Cancer Res* **67**: 11721–11731
- Park SM, Gaur AB, Lengyel E, Peter ME (2008) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* **22**: 894–907
- Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* **7**: 415–428
- Sempere LF, Christensen M, Silahtaroglu A, Bak M, Heath CV, Schwartz G, Wells W, Kauppinen S, Cole CN (2007) Altered microRNA expression confined to specific epithelial cell subpopulations in breast cancer. *Cancer Res* **67**: 11612–11620
- Spaderna S, Schmalhofer O, Hlubek F, Bex G, Eger A, Merkel S, Jung A, Kirchner T, Brabletz T (2006) A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. *Gastroenterology* **131**: 830–840
- Spaderna S et al (2008) The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res* **68**: 537–544
- Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massague J (2008) Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451**: 147–152
- Thiery JP, Sleeman JP (2006) Complex networks orchestrate epithelial–mesenchymal transitions. *Nat Rev Mol Cell Biol* **7**: 131–142
- Wang W, Eddy R, Condeelis J (2007) The cofilin pathway in breast cancer invasion and metastasis. *Nat Rev Cancer* **7**: 429–440
- Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**: 927–939



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