



# **Downregulation of the MicroRNA-200 Family Induces Epithelial to Mesenchymal Transition**

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To my mother, Chris, who has given me all  
the love, support, encouragement, and  
opportunity to achieve my goals

And to William and Mignon  
for their guidance



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## Abstract

Epithelial to mesenchymal transition (EMT) is a reversible developmental morphogenesis associated with cancer invasion and metastasis. Essential for the transition is the downregulation of E-cadherin, an adherens junction protein whose transcription is repressed by E-box binding proteins, such as Snail, Slug, Twist, ZEB1, and ZEB2. MicroRNA microarrays showed the miR-200 family and miR-205 are dramatically downregulated during TGF- $\beta$ - and Pez-induced EMT (Gregory *et al.* 2008). A search for possible targets of these miRNAs using TargetScan (Grimson *et al.* 2007) revealed multiple putative miR-200 and miR-205 binding sites in the 3' untranslated regions (UTRs) of *ZEB1* and *ZEB2*. We hypothesised that *ZEB1* and *ZEB2* are negatively regulated by miR-200 and miR-205 at the post-transcriptional level. To test this, the *ZEB1* and *ZEB2* 3'UTRs were amplified from human genomic DNA, cloned downstream of a renilla luciferase (RL) reporter then transfected into the Pez-induced model of EMT in Madin-Darby canine kidney (MDCK) cells (Wyatt *et al.* 2007). Expression of RL-ZEB1/2 was over five-fold lower in epithelial MDCK cells that had high endogenous miR-200 and miR-205 levels compared to mesenchymal MDCK-Pez cells, which were devoid of the miRNAs. Co-transfection of the reporters with synthetic miRNAs (Pre-miR-200a, -200b, -205) into MDCK-Pez cells inhibited RL-ZEB1/2. Conversely, co-transfection of the reporters with antisense inhibitors to the miRNAs (Anti-miR-200a, -200b, -205) into MDCK cells augmented expression of RL-ZEB1/2. Mutagenesis of all miR-200b sites in the *ZEB1* and *ZEB2* 3'UTRs effectively relieved inhibition by endogenous and ectopic miR-200b. These results established miR-200 and miR-205 as negative regulators of *ZEB1* and *ZEB2*.

## Abstract

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Next, we sought to determine whether miR-200 participates in cancer invasion *in vivo*. Previous studies reported EMT-like changes, including upregulation of ZEB1 and ZEB2 and loss of E-cadherin, at the invasive front of carcinomas (Aigner *et al.* 2007a; Brabletz *et al.* 2001; Hlubek *et al.* 2007a; Spaderna *et al.* 2006). Since loss of the miR-200 family is an essential step in the progression of EMT, we predicted these miRNAs would be downregulated at the invasive front of carcinomas where EMT takes place. A method for *in situ* hybridisation of miRNAs in formalin-fixed paraffin-embedded tissues was developed, and along with immunofluorescence and immunohistochemistry, was used to assess expression of miR-200 and EMT markers in human colorectal adenocarcinomas. Laser capture microdissection and qPCR were used to quantify and compare levels of miR-200 in normal colon epithelium, the tumour core, and the invasive front. Expression of miR-200 was significantly reduced at the invasive front of a subset of colorectal adenocarcinomas displaying degraded basement membranes and markers of EMT in leading edge malignant cells. In contrast, miR-200 expression was maintained across normal epithelium and in carcinomas with intact basement membranes, where no signs of EMT were evident. In regional lymph node and vascular metastases, miR-200 expression was strong, suggesting miR-200 is required to recapitulate the primary tumour phenotype at the metastatic site. These data implicate the miR-200 family in the progression of invasion and metastasis, and add further support to the roles of EMT and MET in the metastasis cascade.

## Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Emily L. Paterson, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Emily Paterson

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## Abbreviations

$\alpha\beta6$	alpha-5 and beta-6 integrins
$\alpha$ SMA	alpha smooth muscle actin
$\delta$ EF1	see ZEB1
4T07	Mouse mammary gland carcinoma cell line
4T1	Mouse mammary gland carcinoma cell line
5-FU	5-Fluorouracil
67NR	Mouse mammary gland tumour cell line
168FARN	Mouse mammary gland tumour cell line
A549	Human lung carcinoma cell line
ABC	Avidin-biotin complex
ACPS	Australian Clinicopathology Staging
AE1/AE3	Pan-cytokeratin antibody
Ago	Argonaute
AJCC	American Joint Committee on Cancer
Akt	v-akt murine thymoma viral oncogene homologue
APC	Adenomatosis polyposis coli
Aspc-1	Human pancreatic adenocarcinoma cell line
BAX	Pro-apoptotic Bcl2-associated X protein
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BCL2	B-cell lymphoma 2
<i>BIRC5</i>	Survivin gene
Bmi1	Polycomb ring finger protein
BMP	Bone morphogenic protein
bp	Base pair
BRAF	v-raf murine sarcoma viral oncogene homologue B1
BSA	Bovine serum albumin
BT-549	Invasive ductal carcinoma cell line
CaCo-2	Human colorectal adenocarcinoma cell line
CAF	Carcinoma-associated fibroblast
<i>CCND1</i>	Gene encoding Cyclin D1
CD	Cluster of differentiation glycoprotein

## Abbreviations

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<i>CDH1</i>	Gene encoding E-cadherin (cadherin Type 1)
Cdk	Cyclin-dependent kinase
Cdx2	Caudal-type homeobox 2
CEA	Carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK1	Casein kinase 1
CK-20	Cytokeratin-20
CMV	Cytomegalovirus
<i>COL8A2</i>	Collagen alpha-2 (VIII) chain
COX-2	Cyclooxygenase-2
CpG	Cytosine-phosphate-Guanine
CSC	Cancer stem cell
CT	Computed tomography
CtBP	C-terminal binding protein
<i>CTNNB1</i>	Gene encoding $\beta$ -catenin
DAB	3,3' Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCC	Deleted in colon cancer
DDR2	Discoidin domain receptor tyrosine kinase 2
DGCR8	DiGeorge syndrome region gene 8
DLD-1	Human colon adenocarcinoma cell line
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DSH	Dishevelled
dsRBP	Double-stranded RNA binding protein
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGF-CFC	Epidermal growth factor - Cripto, Frl-1, and Cryptic
EGFR	Epidermal growth factor receptor
EMT	Epithelial to Mesenchymal Transition

EndMT	Endothelial to mesenchymal transition
endo-shRNA	Endogenous short hairpin RNA
EPHB3	Ephrin type-B receptor 3
EPLIN	Epithelial protein lost in neoplasm
ERK/MAPK	Extracellular signal-regulated kinase/mitogen-activated protein kinase
FAP	Familial adenomatous polyposis
FAP-1	Fas-associated phosphatase-1
FCS	Foetal calf serum
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FGFBP	Fibroblast growth factor binding protein
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FN1	Fibronectin
FOBT	Faecal occult blood test
FOG2	Zinc finger protein ZFPM2
FSP1	Fibroblast specific protein 1
GDF	Growth and differentiation factor
GPR43R	G-protein coupled receptor 43
GRB2	Growth factor receptor-bound protein 2
GSK3 $\beta$	Glycogen synthase kinase-3 $\beta$
GTPase	Guanosine triphosphatase
H&E	Haematoxylin and eosin
<i>hBUB1</i>	Budding uninhibited by benzimidazoles 1 homologue
<i>hBUB1<math>\beta</math></i>	Budding uninhibited by benzimidazoles 1 homologue beta
HCT 116	Human colon carcinoma cell line
HEK 293	Human embryonic kidney epithelial cell line
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HMGA2	High mobility group protein 2
HMLE	Human mammary epithelial cells
HNPCC	Hereditary non-polyposis colorectal cancer
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1

## Abbreviations

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HPAF-II	Human pancreatic adenocarcinoma cell line
HRP	Horseradish peroxidase
HT-29	Human colon adenocarcinoma cell line
<i>HUGL2</i>	Human giant larvae homologue 2
IBC	Inflammatory breast cancer
IBD	Inflammatory bowel disease
Id2	Inhibitor of DNA binding 2
IGF-IIR	Insulin-like growth factor II receptor
IgG	Immunoglobulin G
IgH	Immunoglobulin H
IL-6	Interleukin-6
ILK	Integrin-linked kinase
IUCC	International Union for Cancer Control
ISH	<i>In situ</i> hybridisation
JNK	c-Jun N-terminal kinase
KHSRP	KH-type splicing regulatory protein
Ki67	Antigen identified by monoclonal antibody Ki-67
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue
L1-CAM	L1 cell adhesion molecule
LAMC2	Laminin subunit $\gamma$ 2
LEF	Lymphoid enhancer-binding factor 1
Lgl2/LLGL2	Lethal giant larvae homologue 2
LNA	Locked nucleic acid
LOH	Loss of heterozygosity
LRP	Low-density lipoprotein receptor-related proteins
<i>LS</i>	<i>Lynch syndrome</i>
MAPK	Mitogen-activated protein kinase
MCF7	Human mammary gland adenocarcinoma cell line
MCSC	Mesenchymal cancer stem cell
MDA-MB-231	Human mammary gland adenocarcinoma cell line
MDA-MB-361	Human mammary gland adenocarcinoma cell line derived from brain metastasis
MDCK	Madin-Darby canine kidney cell line

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MEF-2	Mouse embryonic fibroblast cell line
MEK	Mitogen-activated protein kinase kinase
MET	Mesenchymal to epithelial transition
MiaPaCa-2	Human pancreatic carcinoma cell line
miR	MicroRNA
miRNA	MicroRNA
MLH1	mutL homologue 1
MLH3	mutL homologue 3
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MMR	Mismatch repair
MQ-H <sub>2</sub> O	Milli-Q water
MRF	Microglial response factor
MRI	Magnetic resonance imaging
MSH2	mutS homologue 2
MSH6	mutS homologue 6
MSI	Microsatellite instability
MSI-H	Microsatellite instability-high
MSI-L	Microsatellite instability-low
MSS	Microsatellite stable
MT1-MMP	Membrane-type MMP (also know as MMP-14)
MUTYH	mutY Homologue ( <i>E. coli</i> )
MWS	Mowat-Wilson Syndrome
NBT	Nitro blue tetrazolium chloride
NCCN	National Comprehensive Cancer Network
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NMuMG	Normal murine mammary gland epithelial cells
NRE-A	Negative regulatory element A
NSAID	Non-steroidal anti-inflammatory drug
NSCC	Non-stem cancer cell
NSCLC	Non-small cell lung cancer
nt	Nucleotide
NZF/CZF	N/C-terminal zinc finger cluster

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## Abbreviations

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Oct4	POU domain, class 5, transcription factor 1
p53	Tumour protein 53
Panc-1	Ductal pancreatic epithelioid carcinoma cell line
PATJ	Pals-1 associated tight junction protein
P-body	Processing body
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PET	Positron emission tomography
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PKP3	Plakophilin 3
PMS1	Postmeiotic segregation increased 1
PMS2	Postmeiotic segregation increased 2
PPCD	Posterior polymorphous corneal dystrophy
PRC1/2	Polycomb repressor complex 1/2
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
P-S	Penicillin-Streptomycin
PTEN	Phosphatase and tensin homologue
qPCR	Quantitative real-time polymerase chain reaction
RAF	Murine sarcoma viral oncogene homologue
RAS	Rat sarcoma viral oncogene homologue
REST	RE-1 silencing transcription factor
RhoA	Ras homologue gene family, member A
RISC	RNA-induced silencing complex
RL	Renilla luciferase
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
SHC1	Src homology 2 domain containing transforming protein 1
Shh	Sonic hedgehog
SIP1	Smad interacting protein 1

siRNA	Small interfering RNA
SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1
SK-OV-3	Human ovarian adenocarcinoma cell line
SMAD	Mothers against decapentaplegic homologue
SMURF1	Smad ubiquitylation regulatory factor 1
<i>SNAIL</i>	Gene encoding Snail
<i>SNAIL2</i>	Gene encoding Slug
snoRNA	small nucleolar RNA
SOS	Son of Sevenless
Sox2	SRY (sex determining region Y)-box 2
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue
ssDNA	Single-stranded deoxyribonucleic acid
Suz12	Suppressor of zeste 12 homologue
SW480	Human colon adenocarcinoma cell line
SW620	Human colon adenocarcinoma lymph node metastasis cell line
T47D	Human mammary gland ductal adenocarcinoma
TAK1	TGF- $\beta$ -activated kinase 1
TCF	T cell factor
TCF/LEF	T cell factor/lymphoid enhancer-binding factor
TEMTIA	The EMT International Society
TFIIIA	Transcription factor IIIA
TGF- $\beta$	Transforming growth factor $\beta$
TGF- $\beta$ -RI	TGF- $\beta$ receptor type I
TGF- $\beta$ -RII	TGF- $\beta$ receptor type II
TGF- $\beta$ -RI-KI	TGF- $\beta$ type I receptor kinase inhibitor
THBS1	Thrombospondin 1
TIP	Tubular invasion pole
T <sub>m</sub>	Melting temperature
TNF- $\alpha$	Tumour necrosis factor $\alpha$
TNM	Tumour-node-metastasis
TP53	Tumour protein 53 (p53)
TRAF6	Tumour necrosis factor $\alpha$ receptor-associated factor 6
<i>TUBB3</i>	Class III $\beta$ -tubulin

## Abbreviations

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uPA	Urokinase plasminogen activator
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WAVE3	Wiskott-Aldrich syndrome protein family member 3
Wnt	Wingless-type MMTV integration site family
WTX	Wilms tumour suppressor protein
ZEB1/2	Zinc finger E-box binding homeodomain protein 1/2
Zfhx1b	Zinc finger E-box-binding homeobox 2 (ZEB2)
ZO-1	Zona occludens 1