Specificity Protein 1 (Sp1) Maintains Basal Epithelial Expression of the miR-200 Family

IMPLICATIONS FOR EPITHELIAL-MESENCHYMAL TRANSITION

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Background: Epithelial-mesenchymal transition (EMT) is a key process in embryonic development and cancer metastasis. The miR-200 family plays a critical role in enforcing the epithelial state with their expression lost in cells undergoing EMT. EMT can be mediated by activation of the ZEB1 and ZEB2 (ZEB) transcription factors, which repress miR-200 expression via a self-reinforcing double negative feedback loop to promote the mesenchymal state. However, it remains unclear what factors drive reinforcement of this loop that provides to some extent a gene logic circuit that is capable of switching between either of two stable but interconvertible states.

Results: Sp1 activates miR-200 transcription in epithelial cells and prevents EMT.

Conclusion: miR-200 family members require Sp1 to drive basal expression and maintain an epithelial state.

Significance: Defining the mechanisms controlling the epithelial state has implications for understanding early differentiation and for designing interventions to prevent cancer metastasis.

Epithelial-mesenchymal transition (EMT) is required for the specification of tissues during embryonic development and is recapitulated during the metastatic progression of tumors. The miR-200 family plays a critical role in enforcing the epithelial state with their expression lost in cells undergoing EMT. EMT can be mediated by activation of the ZEB1 and ZEB2 (ZEB) transcription factors, which repress miR-200 expression via a self-reinforcing double negative feedback loop to promote the mesenchymal state. However, it remains unclear what factors drive and maintain epithelial-specific expression of miR-200 in the absence of EMT-inducing factors. Here, we show that the transcription factor Specificity Protein 1 (Sp1) binds to the miR-200b–200a–429 proximal promoter and activates miR-200 expression in epithelial cells. In mesenchymal cells, Sp1 expression is maintained, but its ability to activate the miR-200 promoter is perturbed by ZEB-mediated repression. Reduction of Sp1 expression caused changes in EMT-associated markers in epithelial cells. Furthermore, we observed co-expression of Sp1 and miR-200 during mouse embryonic development wherein miR-200 expression was only lost in regions with high ZEB expression. Together, these findings indicate that miR-200 family members require Sp1 to drive basal expression and to maintain an epithelial state.

Epithelial cells represent a mature stage of cellular differentiation; in the adult, the vast majority of epithelial cells remain epithelial until they die. Yet epithelial cells can retain the option of a change in differentiation state, which can be activated in the adult during wound healing or during the malignant progression of epithelial cell-derived cancers (carcinomas) (1). This change of differentiation state, known as epithelial-mesenchymal transition (EMT), has an important role during embryological development, where it enables cell migrations and tissue remodeling, and establishes various cellular lineages in the developing tissues and organs (2). The EMT process is reversible both in embryos and in cancer, and cells cultured in vitro can be switched between epithelial and mesenchymal states by manipulations of EMT-regulating microRNAs and/or transcription factors (3). Reversible EMT-like changes also occur during ES cell differentiation (4) and iPS cell establishment (5–7), and may also be important in allowing the establishment of cancer metastases (8). Thus the molecular controls that determine whether cells reside in the epithelial or mesenchymal state are of great interest.

Several transcription factors are able to prompt epithelial cells to undergo EMT, including ZEB1, ZEB2, Snail, Slug, and Twist (9–14). Interestingly, most of these factors are repressors and are reported to bind the same DNA motif, CACCTG, sometimes referred to as the E-box or Z-box (although the term E-box is also used to refer to the less specific motif CANNTG, which also binds a variety of other factors) (15). Key genes affected by the EMT-promoting factors include E-cadherin (9–11, 16, 17) and the two loci encoding the miR-200 family members (18–21).

The members of the miR-200 family of microRNAs are expressed in epithelial cells and prevent EMT by targeting ZEB1 and ZEB2 (18–21). ZEB1 and ZEB2 reciprocally repress the miR-200 genes (21, 22), creating a double negative feedback loop that provides to some extent a gene logic circuit that is capable of switching between either of two stable but inter-

The abbreviations used are: EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition; ES, embryonic stem; iPS, induced pluripotent stem; ZEB, zinc finger E-box-binding protein; Sp1, specificity protein 1; TSS, transcription start site.

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This article contains supplemental Tables S1–S3.

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changeable states (epithelial and mesenchymal) (3, 23). While it is clear that factors such as ZEB1 can repress miR-200 gene expression, allowing the mesenchymal gene expression program to be established, we were interested to know what drives miR-200 expression in epithelial cells. Because miR-200 expression is highly specific to epithelial cells, it is possible that one or more epithelial-specific activators may be important in driving this expression. However, our investigations reported here indicate that expression of the miR-200b~200a~429 gene is driven primarily by a ubiquitous activating transcription factor, Sp1, that is expressed in nearly all cells and tissues (24). This finding is consistent with the proposition that the epithelial state is a default state (25, 26), while the mesenchymal state requires active intervention by inducible regulators. Such a situation has ramifications for understanding the mechanisms controlling early differentiation and for designing interventions to prevent cancer metastasis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Generation of Stable Cell Lines**—All cell lines were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The MDCK-Pez and MDCK-vector stable cell lines were generated as previously described (27).

**Extraction of RNA and PCR Analysis**—Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer’s instructions. Messenger RNA and microRNA analysis was carried out as previously described with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 snRNA levels, respectively (18). The primers used for mRNA qPCR are listed in supplemental Table S1.

**Generation of miR-200b~200a~429 Promoter Constructs**—The miR-200b~200a~429~321/+/+ 19 promoter construct was generated as previously described (22). Mutations to the AP-2 and Sp1 binding sites were made in this construct using the QuikChange multi-site-directed mutagenesis kit (Stratagene). A −399/+31 E-cadherin promoter fragment (28) was PCR amplified from MCF-7 genomic DNA and cloned into the Nhel (pcDNA3.1, Sp1, or AP-2) mega) and in some cases 100 ng of expression vectors AQuikChange multi-site-directed mutagenesis kit (Stratagene).

**Transfection and Reporter Assays**—For reporter assays, 6 × 10⁴ cells were plated in 24-well plates and co-transfected with 200 ng of miR-200b~200a~429 promoter firefly luciferase reporter plasmids, 100 ng of pRL-TK Renilla plasmid (Promega) and in some cases 100 ng of expression vectors (pcDNA3.1, Sp1, or AP-2α) using Lipofectamine 2000 (Invitrogen). After 48 h of incubation, cells were lysed in Passive Lysis Buffer (Promega), and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) using the GloMax Multi Detection System Luminometer (Promega). All reporter assays are shown as relative luciferase activities (averaged ratios of firefly luciferase/Renilla ± S.E.) and combined from at least three separate experiments. The pcDNA3.1-Sp1 expression vector was kindly supplied by Maciej Pietrzak of the Polish Academy of Sciences. The pCMX-AP-2α was donated by Nicholas Saunders of Diamantina Institute, University of Queensland, Australia. For siRNA experiments, cells were transfected in suspension with 20 nM control, Sp1 (Stealth siRNAs, Invitrogen), ZEB1, and ZEB2 (22) siRNAs using Lipofectamine RNAiMAX (Invitrogen). Cells were harvested 72 h later for downstream processing or retransfected when prolonged knockdown of these factors was required.

**Chromatin Immunoprecipitation**—ChIP assays were conducted with 1 × 10⁶ MCF-7 cells per reaction cross-linked with 1% formaldehyde as previously described (29). Cells were lysed for 10 min, diluted, and sonicated on ice water (30 s on and off intervals for 18 min) on the Diagenode Bioruptor sonicator. Immunoprecipitations were performed overnight with 1 µg of Sp1 (Santa Cruz sc-H225) or ZEB1 (Santa Cruz E20X), and captured for 2 h with protein A and protein G beads (Invitrogen, Dynal magnetic beads). Bead and immune complexes were washed of nonspecific binding three times with RIPA buffer and then twice in TE buffer before elution for 2 h at 68 °C with vortexing (Eppendorf Thermomixer). Supernatants were collected, and DNA collected using phenol-chloroform extraction. Real-time PCR of miR-200b~200a~429 and E-cadherin promoter regions were performed using primers listed in supplemental Table S3.

**Immunofluorescent Staining for E-cadherin and Sp1**—MDCK and MCF10A cells were transfected with siRNA as described above, plated onto fibronectin-coated chamber slides (BD Biosciences), and stained for E-cadherin and Sp1 as previously described (18). Nuclei were observed by co-staining with 4′,6-diamidino-2-phenylindole (DAPI). Cells were visualized on an Olympus IX81 microscope, and pictures were taken using a Hamamatsu Orca camera. Images were analyzed with Xcellence software (Olympus).

**In Situ hybridization**—E13.5 embryos were fixed in 4% formaldehyde for 24 h and then were embedded in paraffin. Sagittal sections (4 µm) were placed on polyline glass slides for analysis. For in situ hybridization, sections were de-waxed by washing in xylene for 15 min, rehydrated through graded series of ethanol solution for 5 min, each and rinsed in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) twice for 5 min. Tissues were permeabilized by treatment with proteinase K at 6.7 µg/ml in buffer (50 mM Tris, pH 7.5, 5 mM EDTA) for 5 min. The reaction was stopped by incubating sections in 4% formaldehyde for 15 min and rinsed in PBS 3× for 5 min. Sections were pre-washed in 1-methylimidazole buffer and fixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as described previously (30). Sections were acetylated to reduce background staining by incubating for 10 min in 0.25% acetic anhydride in 0.1% triethanolamine and rinsed 3× for 5 min in PBS. Sections were air-dried after dehydration by washing in increasing ethanol gradient for 5 min each. Probes (Exiqon, double-DIG-labeled LNA probes for miR-200b, scrambled control, and snRNA-U6 at 2 µl/slide) were denatured at 90 °C before dilution in 23 µl of hybridization buffer (Roche) and applied to the slide. Sections were covered with glass coverslips, sealed with rubber cement, and incubated overnight at 53 °C to allow hybridization of probe to internal microRNA. Coverslips were removed, and slides were washed in 5× saline-sodium citrate buffer (SSC, pH 7.0) for 5 min, twice 5 min in 1× SSC, and twice 5 min in 0.2× SSC all at 53 °C. Sections were rinsed in PBS, washed with levamisole buffer (0.24 mg/ml levamisole, 0.1 M Tris, pH 7.5, 0.15 M NaCl) and blocked (1/5th fetal calf serum,
1/5th 10% Roche blocking powder in maleate buffer, 3/5th maleate buffer) for 1 h at room temperature. Alkaline phosphatase-conjugated anti-DIG was applied to sections at 1:1000 dilution for 4 h at room temperature. Sections were then rinsed in PBST and color developed in buffer (0.1M Tris, pH 9.5, 0.1M NaCl, 0.05M, MgCl2, 0.24 mg/ml levanosiole, 1% Tween-20, 20 μl NBT/BCIP (Roche)) until complete. Reactions were stopped by washing in TE pH 8.0 and sections dried, dipped in xylene, and mounted in xylene-based mounting medium. Images were captured on the Hamamatsu Nanozoomer.

**Immunohistochemistry**—Embryos were fixed, and sections cut as described above. Sections were de-waxed by washing in xylene 3× for 5 min and rehydrated by washing in 100% ethanol twice for 10 min, 95% ethanol twice for 10 min, MQ-H2O twice for 5 min, and PBS for 5 min. For antigen unmasking, slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min and cooled for 20 min. Once cooled, sections were washed 3× in water for 5 min, bleached in 1% hydrogen peroxide for 10 min, and rinsed 3× in water for 5 min. Sections were blocked in 10% horse serum at room temperature before antibodies were applied (1:400 ZEB1 (31), Sp1 1:200 (sc-H225X; Santa Cruz)). Slides were rinsed in PBS before secondary antibody was applied (1:500 biotin α-rabbit). The ABC and DAB kits (Vector Laboratories) were used to visualize ZEB1 and Sp1 expres-
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**RESULTS**

**Sp1, but Not AP-2α, Activates the miR-200b−200a~429 Proximal Promoter**—The expression of the miR-200 family is epithelial specific with the miR-200b−200a~429 cluster regulated by a promoter region located ~4 kb upstream of the sequence encoding miR-200b (22). Using reporter gene assays, we have previously identified a −321/+19 bp segment of the miR-200b promoter (hereafter called miR-200b promoter) that is sufficient to drive strong epithelial-specific expression (Fig. 1, A and C and Ref. 22). This specificity of expression appears to be largely governed by the presence of repressive binding sites for the ZEB transcription factors, which when mutated allow expression of the miR-200b promoter in mesenchymal cells to near epithelial levels (Fig. 1C and Ref. 22). These data suggest that the miR-200b promoter may be positively regulated by factors present in both epithelial and mesenchymal cells, but whose activity is perturbed by ZEB binding in mesenchymal cells.

To identify factors likely to be involved in transcriptional activation of the miR-200b promoter, we searched for potential transcription factor binding sites using the TRANSFAC database (32). This analysis indicated the presence of several Activator Protein 2 alpha (AP-2α) and Specificity Protein 1 (Sp1) binding sites within the proximal promoter (Fig. 1A). Examination of Sp1 and AP-2α levels in a panel of breast cancer cell lines and in an MDCK EMT model system (27), showed they were expressed at broadly similar levels in epithelial and mesenchymal cells, in contrast to the reciprocal cell type-specific expression of the miR-200b primary transcript and ZEB1 in these cell types (Fig. 1B).

We initially selected AP-2α as a candidate factor for further investigation, as AP-2α has been reported in several contexts to activate expression of another epithelial-specific gene, E-cadherin (33–36). To determine whether AP-2α could activate the miR-200b promoter, we co-transfected the miR-200b promoter luciferase reporter along with an AP-2α expression vector into either epithelial canine MDCK cells or its mesenchymal derivative, MDCK-Pez cells (described in Ref. 27). As shown in Fig. 2A, although ectopic expression of AP-2α activated the E-cadherin promoter, it was unable to activate the miR-200b promoter in either cell type. To rule out the possibility that AP-2α activity may already be maximal in these cells and not further enhanced by exogenous AP-2α, we mutated each of the three predicted AP-2α binding sites in the miR-200b promoter. Mutation of these sites had no effect on promoter activity indicating that AP-2α was not responsible for activity of the miR-200b gene (Fig. 2A).

In contrast to AP-2α, ectopic expression of Sp1 significantly increased miR-200b promoter and E-cadherin activity in epithelial and mesenchymal cells (Fig. 2B). To verify this was a direct effect on the miR-200b promoter we mutated the predicted Sp1 binding sites and examined the effect on activity of the promoter in epithelial MDCK and MCF-7 cells. Mutation of each site reduced promoter activity (Fig. 3A), while mutation

**FIGURE 2. Sp1, but not AP-2α, activates the miR-200 promoter.** Wild type and AP-2 site mutant −321/+19 miR-200b promoter or 399/+31 E-cadherin promoter reporters were co-transfected with control or AP-2α expression vectors (A) or Sp1 expression vector (B) into epithelial MDCK and mesenchymal MDCK-Pez cell lines. Data are expressed as relative luciferase activity ± S.E. from three independent experiments relative to the wild type −321/+19 miR-200b reporter. Statistical significance (**, p < 0.01; ***, p < 0.001) was determined relative to indicated controls transfection using unpaired two-tailed Student’s t test.
Sp1 Specifically binds to this region (Fig. 4). Binding was observed coincident with the location of the miR-200b promoter, we performed ChIP-PCR to demonstrate that Sp1 is able to drive expression of the miR-200b promoter in mesenchymal cells (Fig. 3). These results suggest that binding of Sp1 with the miR-200b promoter in mesenchymal cells may be prevented by ZEB binding to the nearby E-box sites. To examine this, we performed ChIP-PCR for ZEB over the same regions and found that ZEB specifically interacted within the −321/+19 miR-200b proximal promoter in MDA-MB-231 cells, but not in MCF-7 cells (Fig. 4A). Together, these data suggest that the binding of ZEB1 to the miR-200b promoter prevents binding of Sp1. Interestingly, this pattern of Sp1 and ZEB1 binding was also observed at the E-cadherin proximal promoter (Fig. 4B), suggesting an interplay between these factors in controlling epithelial gene expression may be more widespread.

Reduction in Sp1 Levels Decreases miR-200 Levels and Causes Changes in EMT Marker Expression—Having shown that Sp1 binds to the miR-200b promoter, we next wanted to confirm whether endogenous Sp1 activates miR-200b promoter activity and expression in epithelial cells. To do this, we first co-transfected an Sp1 siRNA along with the wild type and Sp1 mutant miR-200b promoter reporter constructs into MCF-7 cells. Knockdown of Sp1 significantly reduced promoter activity of the wild type but not the Sp1 mutant construct (Fig. 5A), indicating Sp1 may be required for the basal expression of the miR-200b gene. As miR-200 is a critical enforcer of the epithelial phenotype, we reasoned that loss of Sp1 may trigger a
reduction in miR-200 levels and induction of EMT. To test this hypothesis, we utilized two epithelial cell lines (MCF10A and MDCK), which are sensitive to undergoing EMT. Knockdown of Sp1 in either cell line by 40% resulted in a reduction of miR-200b expression and up-regulation of the mesenchymal markers ZEB1 and/or ZEB2 (Fig. 5, B and C). Although we did not observe a change in E-cadherin mRNA in the total population of cells, we found by immunofluorescence microscopy that cells with reduced Sp1 levels also exhibited loss or internalization of junctional E-cadherin (Fig. 5D). These data indicate that reduction in Sp1 levels leads to down-regulation of miR-200 and changes in EMT marker expression.

Sp1 Activates miR-200b Expression in the Absence of ZEB Binding in Mesenchymal Cells—The above reporter experiments suggest Sp1 can activate the miR-200b promoter in mesenchymal cells when ZEB binding is abrogated by ZEB site mutation. To confirm that endogenous Sp1 can drive expression of miR-200b in mesenchymal cells, we knocked down Sp1 concurrently with ZEB and examined their effect on miR-200 expression. Treatment of either MDA-MB-231 cells or TGF-β-induced MDCK cells (MDCK-TGF) (3) with ZEB siRNAs caused a significant increase in miR-200b levels as expected (Fig. 6, A and B). This de-repression and induction of miR-200b was markedly reduced after co-transfection with an Sp1 siRNA. These data indicate that Sp1 is a basal activator of miR-200 expression in both epithelial and mesenchymal cells, but whose activity is perturbed by the presence of the ZEB transcriptional repressors in mesenchymal cells.

miR-200b and Sp1 Are Co-expressed and Inversely Correlated with ZEB1 during Mouse Embryonic Development—miR-200 and its homolog, miR-8, have been shown to facilitate many developmental processes including eye formation and adipogenesis (37), body size in Drosophila (38), olfactory biogenesis in rats and zebrafish (39), osmoregulation in zebrafish (40), and eye and cranial cartilage development in Xenopus (41). To determine the relationship between miR-200, Sp1, and Zeb1 during mouse embryonic development we surveyed their expression in various embryonic tissues at E13.5 using sagittal formalin-fixed paraffin embedded (FFPE) sections by in situ hybridization for miR-200b and immunohistochemistry for Sp1 and Zeb1. As expected from reports indicating epithelial-specific expression of miR-200, miR-200b was expressed in epithelial tissues of the stomach, duodenum, kidney, olfactory epithelium, cochlea, lungs, and skin (Fig. 7). In these organs, we...
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A

MCF-7

Relative Luciferase activity

pGL3-basic

miR-200b (-321/+19) wildtype

miR-200b (-321/+19) Sp1 mutant

Control siRNA

Sp1 siRNA

B

MCF10A

E-cadherin

ZEB2

miR-200b

Relative RNA

Sp1

control

siRNA

control

Sp1

control

Sp1

control

Sp1

C

MDCK

E-cadherin

ZEB1

ZEB2

Pri-miR-200b-a-429

miR-200b

Relative RNA

Sp1

control

Sp1

control

Sp1

control

Sp1

D

DAPI

E-cadherin

Sp1

Merge

MDCK

Negative siRNA

Sp1 siRNA

MDCK

Negative siRNA

Sp1 siRNA

MCF10A

Negative siRNA

Sp1 siRNA
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DISCUSSION

The ability of cells to transition between epithelial and mesenchymal states underlies developmental processes, pluripotency, and tumor progression and is tightly regulated by numerous signaling pathways and downstream effectors. In recent years, the miR-200 family have been proven to be powerful enforcers of the epithelial state. While various signaling pathways and mesenchymal transcription factors are known to contribute to reversal of EMT (or MET). By analysis of the epithelial-specific miR-200b−200a~429 promoter (22), we identified Sp1 as an essential activator of miR-200 expression. We find Sp1 induces miR-200 expression through multiple sites while reduction of its expression can result in a reduction in epithelial and gain in mesenchymal properties.

These findings present intriguing implications for our understanding of how epithelial cell plasticity is controlled. It is well known that autocrine signaling pathways (such as TGF-β and PDGF) are able to drive EMT and act to maintain cells in a mesenchymal state (42–44). Mechanistically, one pathway that contributes to this process is the TGF-β/ZEBOmiR-200 signaling network, whereby TGF-β stimulates ZEB production to effect miR-200 loss (3, 21, 23). Upon sustained TGF-β exposure, a self-reinforcing tripartite loop can be initiated between these factors, which enhance autocrine TGF-β signaling and maintains cells within a mesenchymal state (3). Significant per-
Sp1 Regulates Epithelial Expression of miR-200

miR-200b Sp1 Zeb1

brain

cochlea

duodenum

kidney

lung

olfactory epithelium

skin

stomach

FIGURE 7. MiR-200b and Sp1 are co-expressed and inversely correlated with Zeb1 during mouse embryonic development. Paraffin sections of E13.5 mouse embryos were assayed for miR-200 expression by in situ hybridization using LNA probes, or Sp1 or Zeb1 expression using immunohistochemistry. Scale bars indicate 100 μm. Black arrows indicate positive staining, and red arrows indicate negative staining regions.

turbations of any one of these factors can cause a break in “mesenchymal” signaling with cells reverting to an epithelial phenotype (3). These previous studies suggested that perhaps cells normally reside in an “epithelial default” state (25, 26), which is stable but able to be altered by factors that initiate mesenchymal signaling. However, the mechanistic evidence for these observations has not been defined. Our finding here that the basal transcription factor, Sp1, is a primary driver of miR-200 expression fits well with the “epithelial default” model of epithelial plasticity. We show that Sp1 maintains basal miR-200 expression in both epithelial and mesenchymal cells, but in mesenchymal cells this capacity is likely overruled by competition with transcriptional repressors such as ZEB. In addition to miR-200, Sp1 has previously been shown to be an important transcriptional activator of E-cadherin (45), with re-expression of Sp1 increasing E-cadherin levels and suppressing metastasis in a lung adenocarcinoma model (46). Thus, Sp1 may promote an epithelial state by activating the expression of several key epithelial enforcing genes.

Sp1 has also been shown in some cases to enhance the expression of mesenchymal factors. For example, Sp1 can support TGF-β-induced EMT through activation of Snail expression (47), and can also acts as a cofactor with the TGF-β effector Smad complexes (48). Sp1 can also contribute to the up-regulation of MMP9 and Id-1 genes in mesenchymal cells (49, 50). A recent analysis of global cis regulatory elements (CREs) during EMT found Sp1 sites are among a select group of CREs frequently observed in genes that are differentially regulated during EMT (51). These findings reflect a complex role for Sp1 in EMT and suggest that its function may be context dependent and regulated by its interaction with other cofactors.

Sp1 is widely expressed in almost every cell type in the developing mouse; however, its expression level varies considerably across tissues (52). Mice lacking Sp1 expression die at day 11 of gestation through cell autonomous defects indicating its essential role in early development (53). We investigated miR-200 expression in relation to Sp1 and Zeb1 expression is several tissues from E13.5 mouse embryos and found Sp1 and miR-200 expression were coincident in all tissues tested, whereas miR-200 was lost in Zeb1-expressing cells. The inverse correlation of miR-200 and Zeb1 are consistent with the observations made in olfactory (39), palate (54), and inner ear (55) tissues as well as at the invasive front of colorectal cancer (56). Thus, we propose that Sp1 maintains basal expression of miR-200 in most cell types, and the absence of miR-200 is primarily triggered by the expression of ZEB and/or other repressing factors.

In addition to Sp1, the miR-200 family have been reported to be positively regulated by the p53/p63/p73 family (57–59), p53 family members have been shown to bind to regions outside of the minimal epithelial miR-200b−200a−429 promoter and contribute to its activation (58, 59). Similarly, the miR-200c−141 promoter is also activated by p53, with p53 loss causing reduction of miR-200c expression and EMT (57). We have found that Sp1 can also activate the miR-200c−141 promoter; however this effect may be indirect as binding of endogenous Sp1 has not been detected in its promoter region. Interestingly, in breast cancer, p53 reduction or mutation can augment loss of miR-200 levels, but penetrance is often incomplete and is dependent on activation of other signaling pathways (such as HGF signaling via MET) for a full EMT (60, 61). This would be in keeping with the notion that activation of mesenchymal signaling pathways are needed to sustain repression of miR-200. Sp1 and p53 have also been reported to interact (62, 63) and may cooperatively enforce miR-200 expression in epithelial cells. Sp1 also plays roles in the maintenance of methylation-free CpG islands (53, 64) and may prevent DNA hyper-methylation of the miR-200 promoters, which contributes to their repression during EMT (3, 65, 66). Furthermore, the expression of the miR-200b−200a−429 transcript can also be stimulated through an alternative downstream promoter and an upstream enhancer.

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element (67, 68), which may cooperatively bind Sp1 to enhance the activity of the epithelial-specific miR-200b promoter. Very recently, an Sp1-related family member KLF5 has also been shown to activate miR-200 expression and maintain epithelial characteristics of keratinocyte and breast cell lines (69). Together, these studies suggest that the Sp1/KLF family play important roles in enforcing miR-200 expression and the epithelial phenotype. Furthermore, these data lend support to the hypothesis that the epithelial phenotype is maintained as the default cellular state, and that generation of a mesenchymal state requires active intervention by inducible regulators. These findings have implications for our understanding of developmental and pathological scenarios that involve cell plasticity.

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REFERENCES

1. Brabletz, T. (2012) To differentiate or not—routes towards metastasis. Nat. Rev. Cancer 12, 425–436
2. Thiery, J. P., Acloque, H., Huang, R. Y., and Nieto, M. A. (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139, 871–890
3. Gregory, P. A., Bracken, C. P., Smith, E., Bert, A. G., Wright, J. A., Roslan, S., Morris, M., Wyatt, L., Farshid, G., Lim, Y. Y., Lindeman, G. J., Thompson, M. F., Drew, P. A., Khew-Goodall, Y., and Goodall, G. J. (2011) An autocrine TGFβ-ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition. Mol. Biol. Cell 22, 1686–1698
4. Eastham, A. M., Spencer, H., Soncin, F., Ritson, S., Merry, C. L., Stern, P. L., Barrio, M. G., Portillo, F., and Nieto, M. A. (2000) The transcription factor ZEB1-SIP1 and the microRNA-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and SIP1. J. Biol. Chem. 275, 2256–2266
5. Li, R., Ni, S., Zhou, T., Qi, J., Wang, L., He, S., Liu, J., Li, F., Chen, D., Yon, Q., Qin, D., Pan, G., Chen, J., Pei, D., and Zheng, H. (2013) Sequential introduction of reprogramming factors reveals a time-sensitive requirement for individual factors and a sequential EMT-MET mechanism for optimal reprogramming. Nat. Cell Biol. 15, 829–838
6. Tsai, I. H., Donaher, J. L., Murphy, D. A., Chau, S., and Yang, J. (2012) Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 22, 725–736
7. Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huybrechts, D., and van Roy, F. (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol. Cell 7, 1267–1278
8. Battle, E., Sancho, E., Francí, C., Domínguez, D., Monfar, M., Baulida, J., and García De Herreros, A. (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat. Cell Biol. 2, 84–89
9. Cano, A., Pérez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, F., and Nieto, M. A. (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. 2, 76–83
10. Savagner, P., Yamada, K. M., and Thiery, J. P. (1997) The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J. Cell Biol. 137, 1403–1419
11. Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R. A. (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117, 927–939
12. Eger, A., Aigner, K., Sonderegger, S., Dampier, B., Oehler, S., Schreiber, M., Berx, G., Cano, A., Beug, H., and Foisner, R. (2005) DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene 24, 2375–2385
13. Peinado, H., Olmeda, D., and Cano, A. (2007) Snail and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7, 415–428
14. Grotekeca, M. L., and Frisch, S. M. (2000) Evidence for a function of ChBp in epithelial gene regulation and anoikis. Oncogene 19, 3823–3828
15. Hajra, K. M., Chen, D. Y., and Fearon, E. R. (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res. 62, 1613–1618
16. Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsukin, A., Farshid, G., Vadas, M. A., Khew-Goodall, Y., and Goodall, G. J. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. 10, 593–601
17. Park, S. M., Gaur, A. B., Lengyel, E., and Peter, M. E. (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
18. Korpal, M., Lee, E. S., Hu, G., and Kang, Y. (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J. Biol. Chem. 283, 14910–14914
19. Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., and Brabletz, T. (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 9, 582–589
20. Bracken, C. P., Gregory, P. A., Kolesnikoff, N., Bert, A. G., Wang, J., Shann, M. F., and Goodall, G. J. (2008) A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res. 68, 7846–7854
21. Gregory, P. A., Bracken, C. P., Bert, A. G., and Goodall, G. J. (2008) MicroRNAs as regulators of epithelial-mesenchymal transition. Cell Cycle 7, 3112–3118
22. Wierstra, I. (2008) Sp1: emerging roles—beyond constitutive activation of TATA-less housekeeping genes. Biochem. Biophys. Res. Commun. 372, 1–13
23. Frisch, S. M. (1997) The epithelial cell default-phenotype hypothesis and its implications for cancer. Bioessays 19, 705–709
24. Polyak, K., and Weinberg, R. A. (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat. Rev. Cancer 9, 265–273
25. Wyatt, L., Wadham, C., Crocker, L. A., Lardelli, M., and Khew-Goodall, Y. (2007) The protein tyrosine phosphatase Pez regulates TGFβ signaling and organ development. J. Cell Biol. 178, 1223–1235
26. Bussemakers, M. J., Girolli, L. A., van Bokhoven, A., and Schalken, J. A. (1994) Transcriptional regulation of the human E-cadherin gene in human prostate cancer cell lines: characterization of the human E-cadherin gene promoter. Biochem. Biophys. Res. Commun. 203, 1284–1290
27. Lim, Y. Y., Wright, J. A., Attema, J. L., Gregory, P. A., Bert, A. G., Smith, E., Thomas, D., Lopez, A. F., Drew, P. A., Khew-Goodall, Y., and Goodall, G. J. (2013) Epigenetic modulation of the miR-200 family is associated with transition to a breast cancer stem-cell-like state. J. Cell Sci. 126, 2256–2266
28. Pena, J. T., Sohn-Lee, C., Rouhanifard, S. H., Ludwig, J., Hafner, M., Michailov, A., Lim, C., Holoch, D., Berninger, P., Zavolan, M., and Tuschl, T. (2009) miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. Nat. Methods 6, 139–141
29. Costantino, M. E., Stoeckert, R. P., Smith, G. E., and Darling, D. S. (2002) Cell-specific phosphorylation of Zfhx transcription factor. Biochem. Biophys. Res. Commun. 296, 368–373
30. Matys, V., Kel-Margoulis, O. V., Frice, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., Voss, N.,
Sp1 Regulates Epithelial Expression of miR-200

Stegmaier, P., Lewicki-Potapov, B., Saxel, H., Kel, A. E., and Wingender, E. (2006) TRANSFAC and its module TRANScopel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res. 34, D108–D110

33. Batsché, E., Muchardt, C., Behrens, J., Hurst, H. C., and Crémiès, C. (1998) RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. Mol. Cell. Biol. 18, 3647–3658

34. West-Mays, J. A., Sivak, J. M., Papagiotas, S. S., Kim, J., Nottoli, T., Williams, T., and Fini, M. E. (2003) Positive influence of AP-2a transcription factor on cadherin gene expression and differentiation of the ocular surface. Differentiation 71, 206–216

35. Sumigama, S., Ito, T., Kajiyama, H., Shibata, K., Tamakoshi, K., Kikkawa, F., Williams, T., Tainsky, M. A., Nomura, S., and Mizutani, S. (2004) Suppression of invasion and peritoneal carcinomatosis of ovarian cancer cells by overexpression of AP-2a. Oncogene 23, 5496–5504

36. Schwartz, B., Melnikova, O. V., Tellez, C., Mourad-Zeidan, A., Blehm, K., Zhao, Y. J., McCarty, M., Adam, L., and Bar-Eli, M. (2007) Loss of AP-2a results in deregulation of E-cadherin and MMP-9 and an increase in tumorigenics of colon cancer cells in vivo. Oncogene 26, 4049–4058

37. Kennell, J. A., Gerin, I., MacDougall, O. A., and Cadigan, K. M. (2008) The microRNA miR-8 is a conserved negative regulator of Wnt signaling. Proc. Natl. Acad. Sci. U.S.A. 105, 15417–15422

38. Knouf, E. C., Garg, K., Arroyo, J. D., Correa, Y., Sarkar, D., Parkin, R. K., Wurz, K., B’Orian, K. C., Godwin, A. K., Urban, N. D., Ruzzo, W. L., Gentleman, R., Drescher, C. W., Swisher, E. M., and Tewari, M. (2012) An integrative genomic approach identifies p73 and p63 as activators of miR-200 microRNA family transcription. Nucleic Acids Res. 40, 499–510

39. Herschkowitz, J. I., Zhao, W., Zhang, M., Usary, J., Murrow, G., Edwards, D., Knezevic, J., Greene, S. B., Darr, D., Troester, M. A., Hilsenbeck, S. G., Medina, D., Perou, C. M., and Rosen, J. M. (2012) Comparative oncomicrosequencing identifies breast tumors enriched in functional tumor-initiating cells. Proc. Natl. Acad. Sci. U.S.A. 109, 2778–2783

40. Lagger, G., Dötzthöfer, A., Scheuttenbruger, B., Haideweg, E., Simbeck, E., Tischler, J., Chiocca, S., Suske, G., Rotheneder, H., Wintersberger, E., and Seiser, C. (2003) The tumor suppressor p53 and histone deacetylase 1 are antagonistic regulators of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 gene. Mol. Cell. Biol. 23, 2669–2679

41. Valero, E., Jordà, M., Vinyals, A., Marazuela, A., Cubillo, E., Olmeda, D., and Hung, J. S. (2012) miR-200b regulates cell migration via Zeb1 family during mouse palate development. Histochem. Cell Biol. 137, 459–470

42. Attema, J. L., Bert, A. G., Lim, Y. Y., Kolesnikoff, N., Lawrence, D. M., Pillman, K. A., Smith, E., Drew, P. A., Kessl, D., and Shannon, F. and...
Goodall, G. J. (2013) Identification of an Enhancer That Increases miR-200b—200a—429 Gene Expression in Breast Cancer Cells. PloS ONE 8, e75517

68. Wee, E. J., Peters, K., Nair, S. S., Hulf, T., Stein, S., Wagner, S., Bailey, P., Lee, S. Y., Qu, W. J., Brewster, B., French, J. D., Dobrovic, A., Francis, G. D., Clark, S. J., and Brown, M. A. (2012) Mapping the regulatory sequences controlling 93 breast cancer-associated miRNA genes leads to the identification of two functional promoters of the Hsa-mir-200b cluster, methylation of which is associated with metastasis or hormone receptor status in advanced breast cancer. Oncogene 31, 4182–4195

69. Zhang, B., Zhang, Z., Xia, S., Xing, C., Ci, X., Li, X., Zhao, R., Tian, S., Ma, G., Zhu, Z., Fu, L., and Dong, J. T. (2013) KLF5 activates microRNA 200 transcription to maintain epithelial characteristics and prevent induced epithelial-mesenchymal transition in epithelial cells. Mol. Cell Biol. 33, 4919–4935

70. Wiklund, E. D., Bramsen, J. B., Hulf, T., Dyrskjøt, L., Ramanathan, R., Hansen, T. B., Villadsen, S. B., Gao, S., Ostenfeld, M. S., Borre, M., Peter, M. E., Ørntoft, T. F., Kjems, J., and Clark, S. J. (2011) Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. Int. J. Cancer 128, 1327–1334

Sp1 Regulates Epithelial Expression of miR-200