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Regulation of miR-200 family microRNAs and ZEB transcription factors in ovarian cancer: evidence supporting a mesothelial-to-epithelial transition

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Introduction

MicroRNAs are small (~22 nt) RNAs that influence gene expression networks by repressing target messenger RNAs (mRNAs) via specific base-pairing interactions in 3' untranslated regions (3'UTRs) (1,2). Knockout or knockdown experiments have shown that in many cases they act as switches in cell differentiation by repressing key genes in organismal development (3). Aberrant miRNA expression has been observed in a wide range of human cancers, and in several cases miRNAs have been shown to have oncogenic or tumor suppressor functions (4).

Aspects of the molecular program that causes epithelial-to-mesenchymal transition (EMT) and its reversion, mesenchymal-to-epithelial transition (MET) are prominent mechanisms in carcinoma progression. In many cancer types, the EMT and MET programs contribute to the dissemination of malignant cells by recapitulating the normal EMT and MET processes that are crucial during early embryonic development (5). In canine kidney cells and in human cancers from patients and cell lines, including in pancreatic, colorectal and breast cancer cells (6-11), the miR-200 sequence group of miRNAs has been found to be involved in regulating EMT. This group comprises hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-141 and hsa-miR-429 which are generated from two transcripts (one derived from chromosome 1 that generates miR-200a/miR-200b/miR-429 and another from chromosome 12 that generates

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miR-141/miR-200c). The members of this group are highly related in sequence (Table 1), especially in the nucleotide 2-8 seed region that determines target specification, indicating that they likely target a similar complement of messenger RNAs. For convenience, we will refer to this sequence group as the miR-200 family, a nomenclature we distinguish from the phylogenetic classification of miRNA families presented in the miRBase database (12).

Among the targets of the miR-200 family are the Zn-finger transcriptional repressors ZEB1 and ZEB2 (also known as AREB6, TCF8, ZFHX1A, δEF1; and SIP1, ZFHX1B, SMADIP1 and KIAA0569, respectively). These transcription factors promote EMT at least in part by repressing the expression of E-cadherin and Lgl-2, which are critical adhesion molecules of epithelial cells (7,9,13-15). In cancer progression, the loss of E-cadherin expression is thought to lead to increased mesenchymal characteristics, migratory behavior and metastasis (5).

Epithelial ovarian cancer is the 5th most fatal cancer for women (16) and the vast majority of ovarian cancers are believed to originate from the ovarian surface, inclusion cysts in the ovarian parenchyma, or from the nearby distal fallopian tube epithelium (17,18). In this study, we focus on ovarian carcinogenesis occurring in cells of the ovarian surface. Recent global miRNA profiling studies have shown differential expression of certain miRNAs in epithelial ovarian cancer cells when compared to normal controls. The miR-200 family miRNAs are among the most differentially regulated miRNAs in some but not all studies (19-22). The profiling studies of Iorio et al. (21) and Nam et al. (19), which used whole ovary as the normal comparison tissue, found several miR-200 family members to be overexpressed in ovarian cancer. The choice of normal control comparison is potentially problematic in those studies because the contribution of cells from the ovarian surface constitutes <1% of the cellular content of the whole ovary, which consists primarily of mesenchymal cells in the ovarian stroma. Dahiya et al. on the other hand did not find miR-200 family miRNAs to be differentially expressed in ovarian cancer (and in fact found them to be underexpressed in ovarian cancer cell lines) using the human ovarian surface epithelial line HOSE-B line as a normal control comparison (20). However, the HOSE-B has been immortalized by the viral oncoproteins E6 and E7 which complicates interpretation. Yang et al., using a combination of different immortalized HOSE samples as well as normal ovary tissue as a comparison, reported moderate overexpression of miR-200a in ovarian cancer (22). Furthermore, a recent study by Zhang et al. using immortalized ovarian surface epithelial cells as normal comparison samples did not report miR-200 as a differentially expressed miRNA (23).

Given the potentially important role of miR-200 family miRNAs in regulating cancer progression and the unresolved results on miR-200 family expression in ovarian carcinogenesis, we investigated expression of this miRNA family and two of its prominent targets, the ZEB1 and ZEB2 transcription factors, focusing on the implications for carcinogenesis in ovarian surface cells. Cells on the ovarian surface are of mesothelial origin, a cell type which co-expresses mesenchymal and epithelial markers (24). In order to determine physiologically relevant expression patterns of miR-200 family miRNAs we studied non-immortalized, early-passage primary cell cultures derived from human ovarian surface “epithelium” (HOSE) as the normal controls and a large panel of 70 ovarian cancer tissues and 15 ovarian cancer cell lines. In order to understand the mechanism of regulation of the miR-200 family and ZEB transcription factors, we investigated miR-200 and ZEB1/2 feedback regulation by mutational analysis of the ZEB2 3’UTR and the miR-200a/141 promoter. Our results, presented below, support a model involving mesothelial-to-epithelial transition that is regulated by a miR-200/ZEB double-negative feedback loop.

Materials and Methods

A detailed description of materials and methods is provided in Supplementary Material. All clinical samples in this study were collected under IRB-approved protocols. Primary human ovarian surface epithelial (HOSE) cells were obtained from normal ovaries of women using a modification of the technique described previously (25) and snap-frozen ovarian epithelial tumor specimens were obtained from the Pacific Ovarian Cancer Research Consortium (POCRC) repository; a subset of the clear cell samples were obtained from the Cedars Sinai Medical Center. RNA was extracted using the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX) and miRNA analysis was carried out using TaqMan miRNA assays (Applied Biosystems, Inc.), using the endogenous control RNU24 for normalization. mRNA expression levels were measured using the TaqMan Gene Expression Assay kit (Applied Biosystems) for ZEB1, ZEB2 and normalized to GUSB. Luciferase reporter assays were carried out in ES-2 cells seeded in 96-well plates. All plasmids were co-transfected with pRL-TK (Promega) for normalization; firefly and renilla luciferase activity was assayed 24 hours following transfection. Knockdown of ZEB1 and ZEB2 was carried out using siGENOME SMART pool Human TCF8 or ZFHX1B (Thermo Fisher Scientific) against ZEB1 (TCF8) or ZEB2 (ZFX1B). siGENOME Control Non-Targeting siRNA #3 (siNT) (Thermo Fisher Scientific) was used as a negative control.

Results

Expression of miR-200 family members in normal ovarian surface epithelial cells and epithelial ovarian cancer

Global miRNA expression profiles of ovarian cancers and ovarian cancer cell lines have shown miR-200 family miRNAs to be strongly overexpressed relative to normal comparisons in some studies (19,21,22) but underexpressed in another (20). We used Taqman qRT-PCR to compare expression of the five miR-200 family members, miR-200a, mirR-200b, miR-200c, miR-141 and miR-429, in the normal and malignant states. As a representation of the normal state, we used early passage cultures of non-immortalized primary HOSE cells isolated from three different individuals. To assess miR-200 family miRNA expression in the malignant state, we analyzed stage III/IV malignant ovarian primary tumors from 70 patients encompassing the three major histological subtypes (56 serous specimens, nine endometrioid specimens and five clear cell specimens) as well as 15 ovarian cancer cell lines (Table S1). The ovarian cancer tissue samples were comprised of at least 70% malignant epithelial cells.

We found expression of the five miR-200 family members to be substantially higher in all three primary tumor types compared to normal HOSE samples (Fig. 1A and Fig. S1). The ovarian cancers showed a wide range in expression of miR-200 family members, which may reflect a diversity of differentiation stages or other physiological states. When ovarian cancer cell lines were compared to HOSE samples, many of the cancer cell lines demonstrated substantially higher expression than HOSE samples (Fig. S2).

ZEB1 and ZEB2 are predicted targets of the miR-200 family miRNAs and are underexpressed in ovarian cancer

To gain insight into genes regulated by miR-200 family miRNAs, we searched for predicted messenger RNA targets using TargetScan, which is a program that predicts miRNA targets based on matches to the conserved seed region of miRNAs (26). The miR-200 family can be divided into two groups based upon seed sequence, which is the 2-8 nucleotide sequence at the 5 end of an miRNA that mediates mRNA targeting. The two highly related seed region groups, which are called miR-141/200a and miR-200b/c/429, differ only at the fourth nucleotide of the seed region (Table 1). At least 602 targets were predicted for the miR-200b/c/429 group and 429 predicted targets were found for the miR-141/200a group. The highest confidence sites,

based on a total score that incorporates not only conservation of the binding sequence and total conserved sites, but factors like local context, are listed in Tables S2 and S3. For both groups, the predicted target with the highest total context score is the zinc finger E-box binding homeobox 2, also known as ZEB2 (GeneID: 9839), SIP1, ZFHX1B, SMADIP1 and KIAA0569. The transcription factor ZEB1, a homolog of ZEB2 (GeneID: 6935), also known as AREB6, TCF8, ZFHX1A and δEF1, is also predicted as a target by TargetScan for the miR-200b/c/429 group. The ZEB1 3'UTR also contains predicted seed region matches for the miR-141/200c seed region group when a longer version of the UTR than the one used by TargetScan is considered (8,11).

We used Taqman qRT-PCR assays to examine the expression of ZEB1 and ZEB2 across our panel of normal and malignant specimens. The primary ovarian HOSE cultures showed high expression levels of ZEB1/2 whereas in the ovarian cancer tissue and ovarian cancer cell line samples, ZEB1/2 expression was substantially diminished (Fig. 1B). Furthermore, a strong inverse relationship between miR-200 family and ZEB1/2 expression was present in all sample types studied (Fig. 2 and Fig. S3). This inverse relationship is consistent with data reported from analysis of cell lines from human and canine kidney, and human lung and breast cancer (8,9,14), and a panel of 60 National Cancer Institute human cancer cell lines (11). However, unlike the expression pattern seen in non-cancer-derived derived cell lines from canine kidney and murine mammary epithelium, which have high miR-200 expression and low ZEB1/2 levels (8,10), the normal HOSE samples have very low or undetectable miR-200 family expression (Fig. 1A and Fig. S1) and high ZEB1/2 expression (Fig. 1B).

Evidence that the miR-200 family targets ZEB1 and ZEB2 in ovarian cancer

Because of the inverse relationship observed between miR-200 family and ZEB1/2 expression and previous studies showing miR-200 family miRNA regulation of ZEB1/2 in other cell types (7-11), we hypothesized that the miR-200 family directly represses ZEB family expression in ovarian cells. As described earlier, both ZEB1 and ZEB2 have multiple predicted binding sites for miR-200 family miRNAs. For example, in the 1,434 bp ZEB2 3'UTR TargetScan identifies five highly conserved and one poorly conserved binding site for miR-200b/c/429, and two highly conserved and one poorly conserved binding site for miR-141/200a (Tables S2 and S3).

To evaluate the activity of the ZEB2 3'UTR miR-200 family binding sites in ovarian cancer cells, we employed a luciferase-ZEB2 3'UTR reporter construct described previously (14) and also generated a version with mutations in all of the predicted highly conserved miR-200 family binding sites. Both wild-type and mutant luciferase-3'UTR reporter constructs were transfected into two ovarian cancer cell lines, 2008 and ES-2, followed by measurement of luciferase activity. The choice of these cell lines for analysis is based on the fact that they show highly divergent miR-200 family and ZEB1/2 expression as well as divergent epithelial vs. fibroblastic morphology (Fig. 3A). The 2008 cells have an epithelial-like morphology, high expression of miR-200 and low or undetectable ZEB1/2 expression (Fig. 3A). ES-2 cells have a more spindle-shaped, mesenchymal-like morphology, low or undetectable miR-200 levels and high ZEB1/2 expression (Fig. 3A). In both cell lines, introduction of a luciferase expression vector with no ZEB2 3'UTR (i.e., pGL3-Control) gave high luciferase expression, as expected (Fig. 3B). When the ZEB2 3'UTR constructs were transfected into the 2008 cells that have high endogenous miR-200 expression, luciferase expression was suppressed, whereas when the mutated ZEB2 3'UTR was transfected repression was relieved (Fig. 3B). No difference was seen between the wild-type and 3'UTR mutant when the plasmids were introduced into ES-2 cells that do not express miR-200 family members, consistent with the hypothesis that the ZEB2 3'UTR is specifically targeted by the miR-200 miRNA family (Fig. 3B).

To confirm that microRNAs of the miR-200 family could repress ZEB2 through the predicted binding sites in its 3'UTR in ovarian cancer cells, the wild-type and mutant ZEB2 3'UTR

luciferase reporters were co-transfected with increasing amounts of the miR-200a and miR-200b expression plasmids. The ES-2 line was used for this experiment as it has undetectable or low levels of endogenous miR-200 family miRNAs. miR-200a and miR-200b were chosen because they represent the two miR-200 seed region sub-families. We observed a dose-dependent reduction in luciferase expression with co-transfection of the miR-200 plasmids with the wild-type luciferase-ZEB2 3'UTR construct, up to a decrease of 56% when five times the molar quantity of miR-200a was co-transfected with the luciferase reporter (Fig. 3C). The same result was seen with the miR-200b expression vector, with a maximum decrease in luciferase activity of 50% (Fig. 3C). Transfection of miR-200a or miR-200b constructs had no effect on luciferase activity when co-transfected with either the plasmid containing mutated miR-200 family binding sites or with the control plasmid lacking the ZEB2 3'UTR (Fig. 3C). Taken together, the results presented above are consistent with ZEB2 3'UTR regulation by miR-200 family miRNAs in ovarian cancer cells.

Evidence that ZEB2 represses miR-200 expression in a double-negative feedback loop

After finding evidence for repression of ZEB2 in ovarian cells by miR-200 family miRNAs, we sought insight into how miR-200 family members are regulated at the transcriptional level. As noted earlier, tumor samples with lower levels of miR-200 tend to have higher expression of ZEB1 and ZEB2. ZEB transcription factors usually act as transcriptional repressors. ZEB1 has been shown to repress the miR-200c/141 promoter in colorectal, breast and pancreatic cells and miR-200b/200a/429 promoter in breast cancer cell line and canine kidney cells (6,7). We hypothesized that ZEB family members might repress miR-200 expression in ovarian cancer cells as well.

According to the Transfac 7.0 database (27), the consensus ZEB1/ZEB2 binding site is N N Y N Y A C C T G W V T (AREB6_01 matrix) or W N W C A C C T G W N N (AREB6_02 matrix). Both ZEB1 and ZEB2 are known to bind to a consensus sequence containing (C/T) ACCTG sites (28). To provide definite reference points for potential ZEB1/2 binding sites, likely transcriptional start sites (TSS) were identified. For the miR-200c/141 promoter, the TSS was found using Acembly (AceView) annotation (29). Since EST data were lacking for the miR-200a/b/429 promoter, we used 500 bp upstream from the start of the most 5'-miRNA gene as the TSS (Table S4). We then looked for transcription factor binding sites in a region 8,000 bp upstream, and 500 bp downstream of the TSS of the miR-200c/141 and 200a/b/429 genes, using TFBS track from UCSC (30). This database contains the location and Transfac Matrix Database score of transcription factor binding sites that are conserved in human, mouse and rat. One conserved ZEB1/2 binding site was found upstream of the first bp of the miR-200a/b/429 gene cluster on chromosome 1, and three conserved binding sites were found upstream of the miR-200c/141 cluster on chromosome 12 (Table S5). Since the miR-200a/b/429 site was far from the TSS, and the miR-200c/141 cluster contained several potential sites near the TSS, we focused on the miR-200c/141 promoter for further analyses.

A fragment of the genomic region 922 bp upstream of the miR-200c/141 TSS containing all three conserved ZEB1/2 binding sites was cloned upstream of the luciferase gene in the pGL3-Enhancer vector (Fig. 4A). When transfected into ES-2 cells, which have high endogenous levels of ZEB1 and ZEB2, the empty control vector showed extremely low luciferase activity whereas addition of the 922-bp upstream region with its putative regulatory sequences produced a 17-fold increase in luciferase activity, establishing that this region acts as a functional promoter (Fig. 4B). Next, we tested the importance of the three conserved ZEB1/2 binding sites by generating various plasmids containing single, double or triple mutations (Fig. 4A). Each single mutation increased luciferase gene expression (Fig. 4B). Mutation of the putative binding site farthest upstream from the TSS, called 1mut (Fig. 4A) caused a 1.7-fold increase in luciferase gene expression, while mutations in the sites closest to the TSS, called

2mut and 3mut, caused 1.3-fold and 1.5-fold increases, respectively, in luciferase activity (Fig. 4B). A double mutant of the 1st and 2nd binding sites showed a 3.2-fold increase over the wild-type, which was almost double the increase of any of the single mutations (Fig. 4B). Interestingly, a double mutant of the 2nd and 3rd sites showed the same 1.5-fold increase as either single mutation, and a triple mutant of all three sites showed the same increase as the double mutant of the 1st and 2nd binding sites, suggesting that the 3rd binding site could be redundant to the 1st and 2nd sites (Fig. 4B). In agreement with our luciferase assay results in ovarian cells, the 3rd ZEB1/2 binding site, called 'Z-box 2' in Burk *et al*. (7) shows less activity than other sites in the colorectal and breast cancer cells. It is also notable that the site farthest upstream (the "1st site") that we studied is one that was previously untested (E-box 1 in Burk *et al*. (7); site 1 in Fig. 4A) but that we found to have a significant regulatory role.

To differentiate whether ZEB1, ZEB2 or both act on the miR-200c/141 promoter, we co-transfected ES-2 cells with the wild-type promoter luciferase plasmid and either a control, non-targeting siRNA (siNT), or siRNA against ZEB1 or ZEB2. We observed a two-fold increase of expression from the miR-200c/141 promoter with siZEB2, suggesting that ZEB2 was acting as a repressor on this promoter (Fig. 4C) whereas siZEB1 transfection did not lead to significantly increased luciferase reporter activity, indicating that ZEB2 rather than ZEB1 is the functional miR-200c/141 transcriptional suppressor in the ES-2 line. The efficacy of both ZEB1- and ZEB2-targeting siRNAs was confirmed by qRT-PCR measurement of ZEB1 and ZEB2 mRNA in transfected samples (Fig. 4C). Interestingly, ZEB1 has been shown to repress miR-200c/141 expression in colorectal (SW480 and HCT116) cell lines and in breast cancer MDA-MB231 cells lines and to repress miR200b/200a/429 in breast cancer MDA-MB231 and canine kidney MDCK (6,7) cells.

Discussion

miR-200 family expression in ovarian cancer

Recent global miRNA analyses of ovarian tissues and cells have yielded conflicting results about the relative levels of miR-200 family members in non-malignant vs. malignant states (19-23). A likely cause of the discrepancies is the varying types of "normal" sample comparisons used. Prior studies have generally used either whole ovary (of which the ovarian surface epithelium comprises <1% of the cells) or human ovarian surface epithelial cell lines immortalized using oncogenes. In our study, we used primary cultures of human cells from the surface of overtly normal ovaries (conventionally referred to as human ovarian surface epithelium (HOSE)) to obtain a more appropriate normal sample for comparison with ovarian cancers as it has been hypothesized that a significant proportion of ovarian cancers arise from ovarian surface epithelial cells. We find that primary cultures of normal HOSE express all five miR-200 family members at relatively low levels and that in comparison, miR-200 family expression rises dramatically in the vast majority of ovarian cancer samples studied. Although our use of primary HOSE cell cultures still carries the caveat that miRNA expression changes may occur upon *in vitro* culturing, our normal HOSE samples come the closest to date to approximating the physiologic non-malignant counterpart cells of epithelial ovarian cancer that develops at the ovarian surface. While laser captured cells from the ovarian surface may provide an even more relevant sample for comparison, at this point the technology to accurately measure miRNAs in such samples requires further development and testing for reliability.

Our data support a model of epithelial ovarian cancer development that includes a mesothelial-to-epithelial transition (Meso-E-T) in ovarian surface cells

The surface of the ovary is continuous with the mesothelial lining of the peritoneal cavity. Therefore, normal cells on the surface of the ovary are of mesothelial origin. Accordingly, cells on the ovarian surface display both epithelial and mesenchymal phenotypes, expressing

epithelial markers such as keratin and mucin in conjunction with mesenchymal proteins like vimentin and N-cadherin (instead of E-cadherin) and tenuous basement membrane attachment (17). Thus, if ovarian carcinomas were to arise from HOSE, HOSE would have to undergo a transition from mesothelial to epithelial cells (17,31-33), which we refer to here as Meso-E-T. The presence of Meso-E-T on the ovarian surface and in inclusion cysts has been suggested based on measurements of the epithelial cell markers, E-cadherin and glycoprotein CA-125. Our data of reduced ZEB1 and ZEB2 and increased miR-200 in ovarian cancers compared to HOSE are in support of a Meso-E-T (Fig. 5). High levels of miR-200 have been associated with E-cadherin-expressing epithelial cells (11) and this is consistent with the increase in E-cadherin known to occur with ovarian carcinogenesis (17). We propose that miR-200 family members help maintain E-cadherin expression in ovarian cancer by repressing ZEB1 and ZEB2, which are known repressors of E-cadherin transcription (10,13,34-36).

Once ovarian cancers have formed, they are believed to follow a similar course of progression as other epithelial cancers; that is, ovarian cancer cells can undergo EMT with diminished E-cadherin protein levels observed in cells in the ascites, as well as increased ZEB2 levels (37-39) (Fig. 5). However unlike most other carcinomas, such as breast, colon cancer and squamous cancers, the loss of E-cadherin in advanced ovarian cancer is caused by proteolytic cleavage from the surface (40), and not through transcriptional silencing. It is not yet established what causes increased expression of ZEB2 in ascites cells. We hypothesize that increased ZEB2 expression in ovarian cancer cells in ascites occurs as a result of a decrease in miR-200 family miRNA expression during ovarian cancer progression (Fig. 5); future studies will be needed to test this hypothesis.

Comparing our results to those of Dahiya *et al*. (20) who used E6/E7 immortalized HOSE cells as the normal control, we find over-expression of miR-200 family miRNAs in ovarian cancer, whereas they did not report differential expression for miR-200 family miRNAs between immortalized HOSE and ovarian cancer. An attractive interpretation of the differing results is that whereas primary normal HOSE express low levels of the miR-200 family miRNAs, transformation by E6 and E7 viral oncoproteins induces miR-200 family expression; if this is true, induction of miR-200 family miRNAs could represent an important step in ovarian carcinogenesis. Future work will be needed to address this hypothesis.

A double negative feedback loop operative in ovarian cancer cells – a robust mechanism to stabilize the epithelial phenotype in ovarian cancer cells

We observed a strong inverse, mutually exclusive relationship between expression of miR-200 family members and ZEB1/ZEB2 expression in our study; in fact not a single one of the 74 samples studied here showed high expression of *both* the miR-200 family and ZEB1/2. Our studies of the regulation of the ZEB2 3'UTR and the miR-200c/141 promoter in ovarian cancer cells established a mechanism for reciprocal regulation between miR-200 family miRNAs and ZEB transcription factors that involves a double-negative feedback loop. This is similar to that described in colorectal and breast cancer cell lines, where Burk *et al*. (2008) (7) found that ZEB1 represses the miR-200c/141 promoter, although the contribution of ZEB2 in those cells is unknown. ZEB1 and ZEB2 are redundant for E-cadherin regulation (8), but have slightly different roles when studied in the context of *Xenopus* embryogenesis (41). Further studies will show if there are tissue-specific differences in the roles of ZEB1 and ZEB2 in miR-200c/141 repression.

The double-negative feedback loop is a design principle that is particularly suited to generate powerful on-off, bistable switches (42) or circuits (43). We propose that the feedback loop described here provides the basis for a regulated switch between mesothelial and epithelial states (44) during ovarian carcinogenesis. Any double-negative feedback regulatory system raises intriguing questions about which part of the system is triggered first to begin neoplastic

progression. Further studies will resolve this "chicken-and-egg" dilemma about what triggers the bistable switch and if the initial targets are miR-200 or ZEB1/2. Although several triggers for EMT are fairly well-established (45), the signals that initiate Meso-E-T require further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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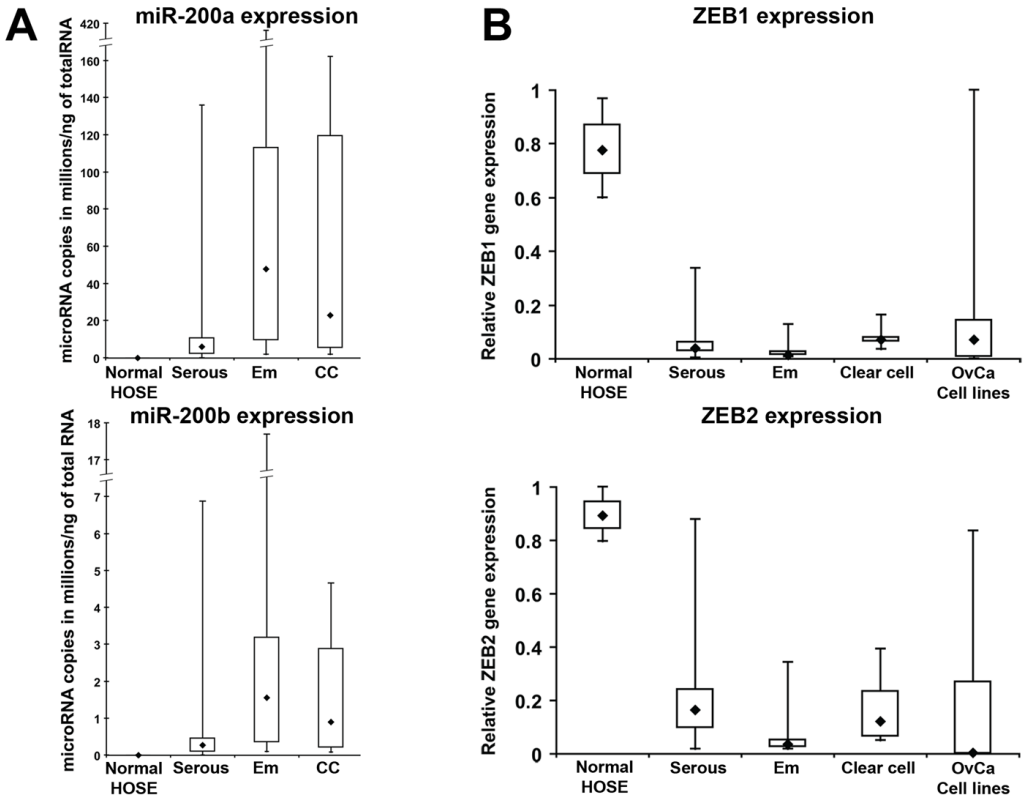

Figure 1. Expression of miR-200 family members and ZEB1/2

(A) Real time qRT-PCR was performed across 70 primary tumor samples, three primary HOSE cell cultures and 15 different ovarian cancer cell lines for miR-200a, miR-200b, miR-200c, miR-141 and miR-429. Representative graphs for miR-200a and miR-200b are in the left column, additional graphs are in Figure S1. miR-200 family expression in HOSE and ovarian cancer cell lines are represented in log scale graphs in Figure S2. (B) The same samples were assayed for ZEB1 and ZEB2 expression (right column). Data are presented as fold change relative to the highest expression among the four HOSE samples.

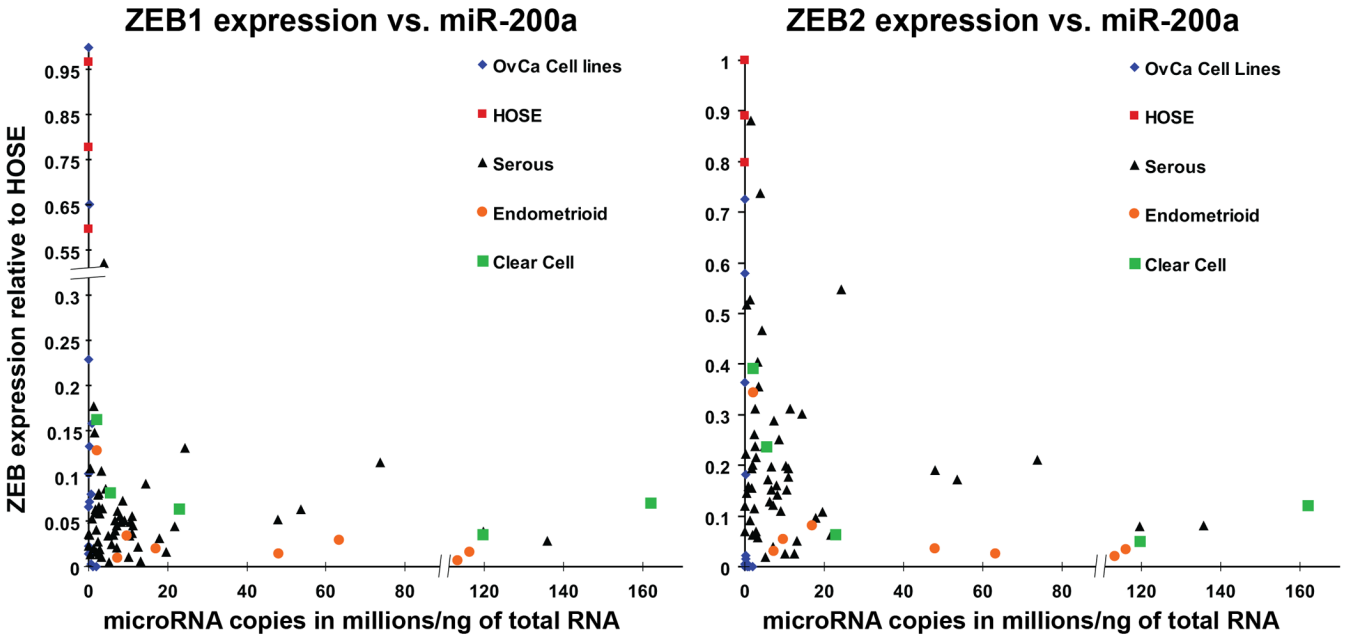

Figure 2.

Expression of transcription factor ZEB1 vs. copy number of miR-200a (left) and miR-200b (right) for each of the primary HOSE, primary ovarian tumor samples and ovarian cell lines (OvCa). Representative graphs are shown here; additional graphs for other miR-200 family members and ZEB2 are in Figure S3.

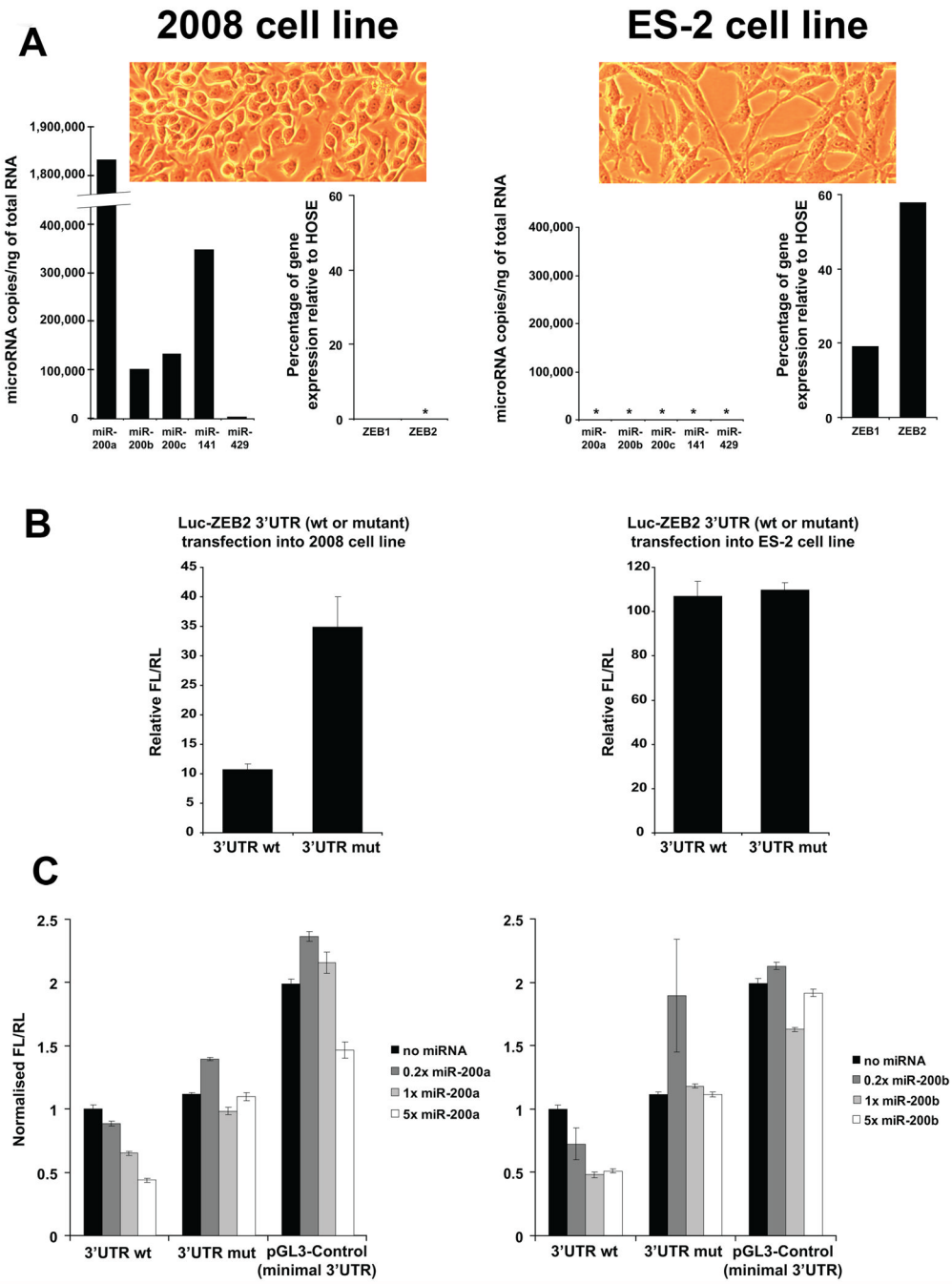

Figure 3. Regulation of ZEB2 by miR-200 family members

(A) Phase-contrast micrographs of 2008 and ES-2 cells, showing epithelial and mesenchymal morphologies, respectively. 2008 cells express high levels of miR-200 (left), whereas ES-2 cells do not express miR-200 (right). The asterisks denote cases in which expression values were below the limit of accurate linear quantitation. (B) 2008 (left) or ES-2 (right) cells were either mock transfected, or transfected with luciferase gene constructs containing either no ZEB2 3' UTR (Control), wild-type ZEB2 3'UTR (wt) or ZEB2 3' UTR with mutations at all conserved, predicted miR-200 binding sites (mut). Results from luciferase assays performed 24h after transfection are shown. (C) ES-2 cells were co-transfected with one of the three luciferase gene constructs described in (A) plus different quantities of pSM30-miR-200a or

pSM30-miR-200b. 1/5x, 1x and 5x indicate the molar quantity of the pSM30-miR expression plasmid relative to the luciferase gene plasmid. Results show the mean and standard error of experiments carried out in triplicate. Firefly luciferase (FL) activity normalized to Renilla luciferase (RL) activity is shown on the y-axis.

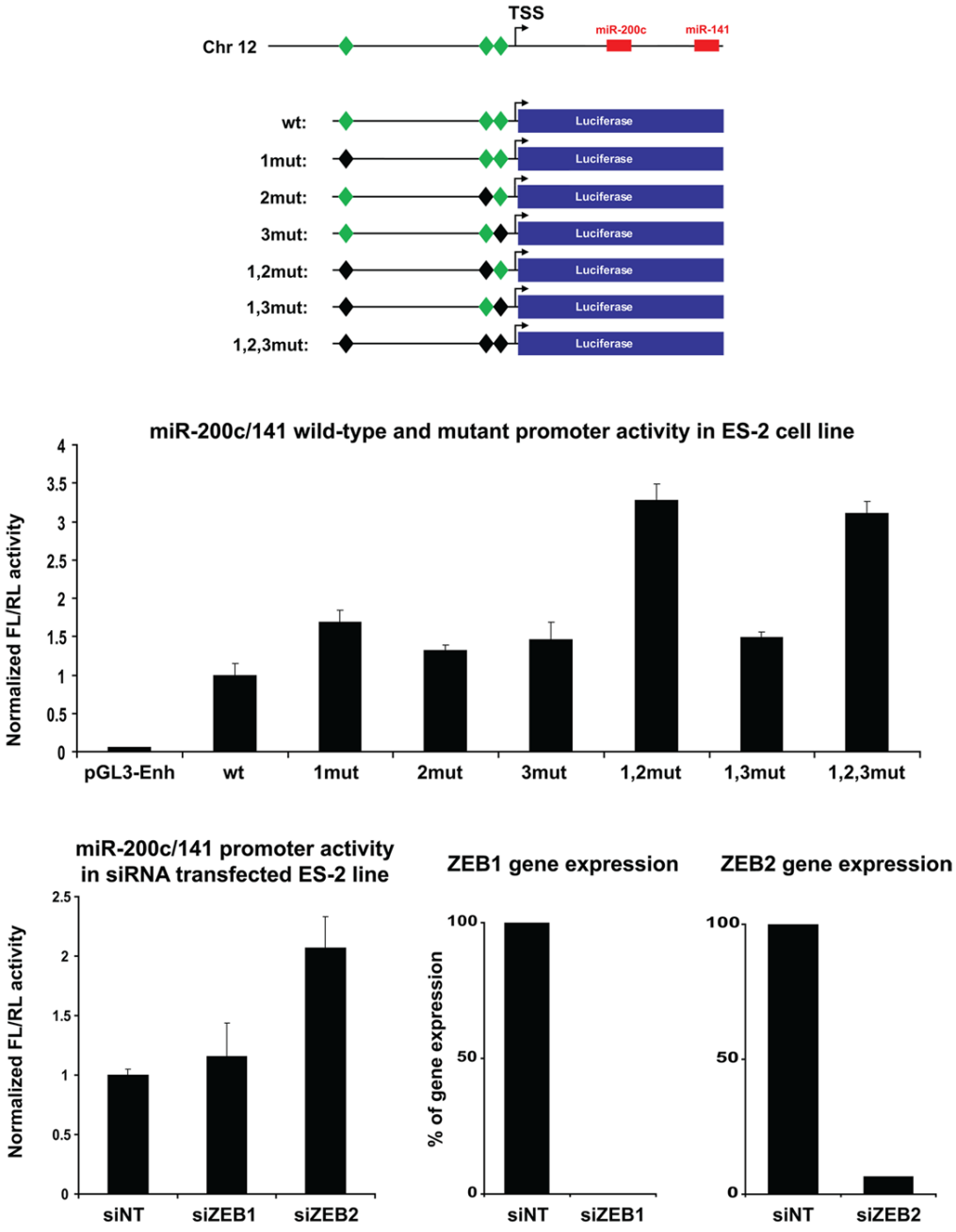

Figure 4. ZEB1, ZEB2 transcription factors regulate miR-200c/141 cluster expression in ovarian cancer

(A) A fragment containing 922-bp of the miR-200c/141 promoter was cloned upstream of the luciferase gene of the pGL3-Enhancer vector. Single or multiple mutations were made in the conserved ZEB1/2 binding sites. Wild-type binding sites are shown in green, and mutated in black. (B) ES-2 cells with high levels of ZEB1 and ZEB2 were transfected with the pGL-3 Control (minimal 3'UTR), the wild-type or mutated miR-200c/141 promoter-luciferase constructs. Experiments were carried out in triplicate, and shown as the means and standard errors of Firefly luciferase activity normalized to Renilla luciferase activity. The experiment was repeated four times and the same expression patterns were seen. (C) Luciferase gene expression in ES-2 cells co-transfected with the wild-type miR-200c/141 promoter-luciferase

construct and control, non-targeting siRNA (siNT) or siRNA against ZEB1 or ZEB2. (D) Real time qRT-PCR for ZEB1 and ZEB2 expression in samples transfected with control siRNA, or siZEB1 and siZEB2.

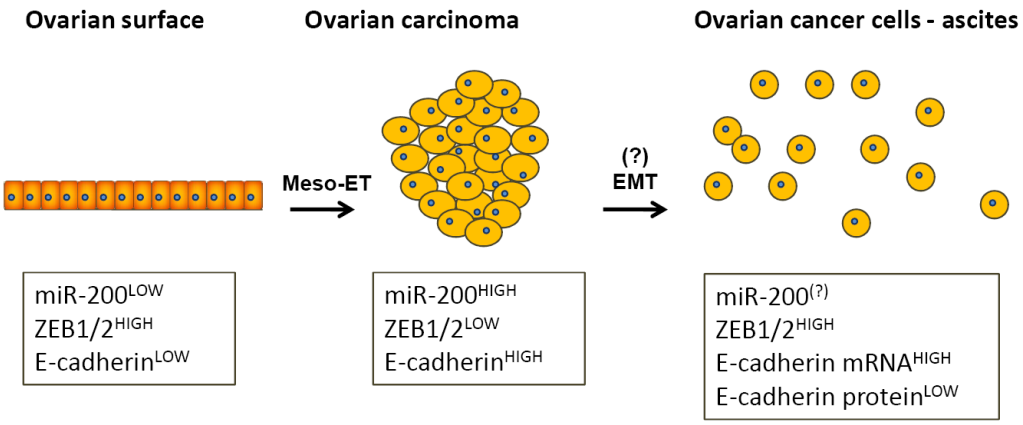

Figure 5. A model of epithelial ovarian carcinogenesis that includes mesothelial-to-epithelial transition

Our model supports the idea that ovarian surface mesothelial cells initially undergo a Mesothelial to Epithelial Transition (Meso-ET), acquiring characteristics of epithelial cells such as high miR-200, low ZEB1/2, and high E-cadherin levels (11,31,32). Subsequently during tumor progression, Epithelial to Mesenchymal Transition (EMT) may occur as has been described in other epithelial cancers (46,47), based in part on a decrease in E-cadherin protein levels mediated by proteolytic cleavage (40). In further support of EMT at later stages of ovarian cancer progression is expression of ZEB2 in cells isolated from effusions (37-39); the expression of the miR-200 family miRNAs in ascites cells has not yet been examined.

Table 1
Sequence alignment of miR-200 family microRNAs

The seven-nucleotide seed regions are underlined; nucleotides that differ across the set of five microRNA family members are distinguished in bold. The family is sub-grouped on the basis of identical seed region.

hsa-miR-200a	UAA**C**ACUG**U**CUGGUAA**C**GAUG**U**
hsa-miR-141	UAA**C**ACUG**U**CUGGUAA**A**GAUG**G**
hsa-miR-200b	UAA**U**ACUG**C**C**U**GGUAA**UGAU**G**A**
hsa-miR-200c	UAA**U**ACUG**C**C**G**GGUAA**UGAU**G**GA**
hsa-miR-429	UAA**U**ACUG**U**C**U**GGUAA**AACC**G**U**