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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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ABSTRACT

Objective. Our objective was to characterize the expression and function of the miR-200 family of microRNAs (miRNA) in ovarian carcinogenesis.

Methods. We used qRT-PCR to examine expression of the miR-200 miRNA family and its predicted targets, the ZEB1 and ZEB2 transcriptional repressors, in primary cultures of normal cells from the surface of the ovary and in a panel of 70 ovarian cancer tissues and 15 ovarian cancer cell lines. We studied the mechanisms of regulation of miR-200 miRNAs and ZEB transcription factors in ovarian cells using 3′ UTR luciferase reporters, promoter luciferase reporters and siRNAs.

Results. miR-200 family members are expressed at low or negligible levels in normal ovarian surface cells and substantially increase in expression in ovarian cancer, whereas expression of ZEB1 and ZEB2 shows the opposite pattern. There is reciprocal repression between miR-200 family members and ZEB transcription factors, creating a double negative regulatory feedback loop resembling that reported in other cancer cell types. In contrast to epithelial cells from other sites, expression levels of miR-200 miRNAs and ZEB1/2 in cells from the ovarian surface are more consistent with a mesenchymal cell phenotype, potentially reflecting the mesothelial origin of the ovarian surface.

Conclusion. Analysis of ovarian cancer tissues suggests that ovarian surface cells acquire a more epithelial miR-200-ZEB1/2 phenotype as they undergo transformation, switching from a miR-200 familyLOW and ZEB1/2HIGH state to a miR-200 familyHIGH and ZEB1/2LOW phenotype. Collectively, our data support the mesothelial-to-epithelial (Meso-E-T) model for development of ovarian cancers that arise from ovarian surface cells, as has been proposed previously on the basis of studies of protein markers.

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 miR-141/miR-200c). The members of this group are highly related in sequence (Table 1), especially in the nucleotide 2–8 seed

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Table 1
Sequence alignment of miR-200 family microRNAs.

hsa-miR-200a	UAACACUGUCUGGUAACGAUGU
hsa-miR-141	UAACACUGUCUGGUAAAGAUGG
hsa-miR-200b	UAAUACUGCCUGGUAAUGAUGA
hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA
hsa-miR-429	UAAUACUGUCUGGUAAAACCGU

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 subgrouped on the basis of identical seed region.

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 fifth 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 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 that coexpresses 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 cotransfected with pRL-TK (Promega) for normalization; firefly and renilla luciferase activity was assayed 24 h 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, miR-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, 9 endometrioid specimens, and 5 clear cell specimens) as well as 15 ovarian cancer cell lines (Table S1). The ovarian cancer tissue samples were composed 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