

## Frequent Downregulation of miR-34 Family in Human Ovarian Cancers

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### Abstract

**Purpose:** The miR-34 family is directly transactivated by tumor suppressor p53, which is frequently mutated in human epithelial ovarian cancer (EOC). We hypothesized that miR-34 expression would be decreased in EOC and that reconstituted miR-34 expression might reduce cell proliferation and invasion of EOC cells.

**Experimental Designs:** miR-34 expression was determined by quantitative reverse transcription-PCR and *in situ* hybridization in a panel of 83 human EOC samples. Functional characterization of miR-34 was accomplished by reconstitution of miR-34 expression in EOC cells with synthetic pre-miR molecules followed by determining changes in proliferation, apoptosis, and invasion.

**Results:** miR-34a expression is decreased in 100%, and miR-34b\*/c in 72%, of EOC with p53 mutation, whereas miR-34a is also downregulated in 93% of tumors with wild-type p53. Furthermore, expression of miR-34b\*/c is significantly reduced in stage IV tumors compared with stage III ( $P = 0.0171$  and  $P = 0.0029$ , respectively). Additionally, we observed promoter methylation and copy number variations at *mir-34*. *In situ* hybridization showed that miR-34a expression is inversely correlated with MET immunohistochemical staining, consistent with translational inhibition by miR-34a. Finally, miR-34 reconstitution experiments in p53 mutant EOC cells resulted in reduced proliferation, motility, and invasion, the latter of which was dependent on MET expression.

**Conclusions:** Our work suggests that miR-34 family plays an important role in EOC pathogenesis and reduced expression of miR-34b\*/c may be particularly important for progression to the most advanced stages. Part of miR-34 effects on motility and invasion may be explained by regulation of MET, which is frequently overexpressed in EOC. *Clin Cancer Res*; 16(4); 1119–28. ©2010 AACR.

Ovarian cancer is the most deadly malignancy and will lead to ~15,000 deaths in the United States in 2009 (1). Although survival has increased slightly over the past 25 years, 5-year survival remains below 50%. A major factor for low survival is our poor understanding of the initiating events that lead to ovarian cancer and how the dis-

ease progresses. Due to asymptomatic development and few screening options, ~70% of women present at late stages of carcinogenesis. At an advanced stage, treatment options are severely limited, with palliative treatment most often administered in the form of debulking surgery and paclitaxel- and platinum-based therapeutics. However, work over the past decade using human cancer samples and mouse models have revealed new insights into the molecular basis of ovarian cancer, particularly its most common form epithelial ovarian cancer (EOC). For example, it is well established that >50% of high-grade serous-type EOCs contain p53 mutations and alterations in the RB pathway (reviewed in refs. 2, 3). Consistently, conditional inactivation of p53 and Rb in the mouse ovarian surface epithelium (OSE) leads to development of poorly differentiated serous ovarian adenocarcinomas (4), whereas K-Ras, Pten, and Wnt/β-catenin are implicated in carcinogenesis of the endometrioid EOC subtype (5, 6).

In recent years, the involvement of small noncoding RNAs, called microRNAs (miRNA), in cancers of many types has become unambiguous, including ovarian cancer (7, 8). Although the precise roles they play during carcinogenesis are still being dissected, it is clear that miRNAs can act as tumor suppressors and oncogenes by regulating

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### Translational Relevance

Diagnosis and treatment of epithelial ovarian cancer (EOC) is particularly difficult due to poor understanding of disease pathogenesis. We show that miR-34 expression is frequently decreased in EOC due to several mechanisms but mainly due to *p53* mutation. Importantly, reconstitution of miR-34 in human ovarian cancer cells results in decreased proliferation and invasion, at least partially by inhibition of the MET oncogene. Taken together, these data suggest that miR-34 family is important for EOC development and may be an attractive candidate for development of novel therapeutic approaches.

processes such as proliferation and the cell cycle, apoptosis, invasion, and metastasis (reviewed in ref. 9). miRNAs have been found to be dysregulated in cancer by DNA copy number changes and epigenetic alterations, altered processing by the miRNA biogenesis machinery, and through altered transcriptional activation. In particular, the transcription factor and tumor suppressor *p53* has been independently shown by several laboratories to directly transactivate genes of the miR-34 family, which is composed of three members (10–15). The gene encoding miR-34a is located on human chromosome 1p36, whereas miR-34b and miR-34c are cotranscribed from one transcription unit on chromosome 11q23. The miR-34 family downregulates numerous important regulatory proteins and thereby presumably mediates tumor suppression (reviewed in ref. 16).

Previously, we have shown that conditional inactivation of *p53* results in miR-34 downregulation in mouse OSE (10). To evaluate the potential roles of miR-34 family in human EOC, we have determined their expression level in a panel of 83 cancer tissues and found that miR-34 expression is frequently decreased in EOC and is associated with metastatic clinical stage and increased expression of receptor protein tyrosine kinase MET. Furthermore, reconstitution of miR-34 expression in EOC cells leads to reduced proliferation and invasion as well as decreased MET levels.

### Materials and Methods

**Clinical samples.** Informed consent was obtained from patients undergoing surgery for ovarian cancer at Fox Chase Cancer Center (Philadelphia, PA) and M.D. Anderson Cancer Center (Houston, TX). Sample collection was done after approval by an Institutional Review Board, and a portion of tumor tissue not required for diagnostic purposes was snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Surgical evaluation was used to determine clinical stage and presence of metastases, whereas histopathologic analysis by gynecologic pathologists was done to assess cancer type and subtype. Only tumors found to contain

over 70% tumor cells were used in the study, and tissue sample and clinical data were available for 83 patients (Table 1). Additional formalin-fixed, paraffin-embedded specimens were obtained from New York-Presbyterian Hospital/Weill Cornell Medical Center (New York, NY).

**miR-34 nomenclature.** miRNA nomenclature has recently been revised such that the miR-34b sequence has been renamed miR-34b\* (i.e., the passenger strand; ref. 17). Nevertheless, “star” or passenger strands of the miRNA duplex have previously been shown to be biologically important (e.g., miR-199a\* and miR-10\*; refs. 18, 19). Although both miR-34b strands are likely to be functional, the miR-34b strand is not predicted to bind the 3'-untranslated region of MET and our studies have therefore focused on the miR-34b\* strand. It is worth noting that our quantitative reverse transcription-PCR (qRT-PCR) data show that both strands are present at equal quantities and are highly correlated (Supplementary Fig. S1); they therefore might be renamed miR-34b-3p and miR-34b-5p, respectively, consistent with mouse miR-34b nomenclature.

**p53 mutation screening.** The IARC protocol<sup>7</sup> was followed for *p53* mutation screening. DNA was isolated by DNeasy Mini kit (Qiagen), and exons 4 to 11, including splice junctions, were amplified by PCR and sequenced with both forward and reverse primers (Supplementary Table S1). In the case sequencing data were unclear, T-vector cloning was done, and three clones were sequenced by T7 and SP6 primers.

**Quantitative reverse transcription-PCR.** Total RNA was isolated using mirVana miRNA Isolation kit (Ambion) according to the manufacturer's protocol, and RNA concentration and purity were determined by NanoDrop analysis. Stem-loop qRT-PCR for mature miR-34 and miR-199a\* miRNAs was done as previously described (20). For MET qRT-PCR, cDNA was prepared from 100 ng total RNA using SuperScript III (Invitrogen) and amplified with Taqman primer/probes. All PCRs were done in triplicate on an ABI 7500 Real-Time PCR System (Applied Biosystems, Inc.), and miRNA and mRNA expression was normalized to *RNU6B* and *GAPDH*, respectively, using the  $2^{-\Delta\Delta C_t}$  method (21).

**MET immunohistochemistry.** Paraffin sections of formalin-fixed tissue were stained according to modified avidin-biotin-peroxidase technique (22). The antibody used for detection of MET was CVD13 from Zymed Laboratories (dilution, 1:200).

**In situ hybridization.** Detection of miR-34 in a panel of serous adenocarcinomas was done by the protocol adapted from Nelson et al. (23). To prevent the loss of miRNAs, we additionally applied 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) fixation as described by Pena et al. (24). In brief, 4- $\mu\text{m}$ -thick sections of formalin-fixed, paraffin-embedded material were deparaffinized, rehydrated, and fixed with EDC. After 1-h prehybridization, a digoxigenin (DIG)-labeled locked nucleic acid (LNA)

<sup>7</sup> <http://www-p53.iarc.fr>

**Table 1. Characteristics of 83 patients with EOC**

Characteristic	Value
Age (y)	
Mean	60.8
Range	28-87
Race (%)	
White	64 (77.1)
African American	5 (6)
Other non-white	6 (7.2)
Unknown	2 (2.4)
Tumor stage (%)	
I	2 (2.4)
II	4 (4.8)
III	46 (55.4)
IV	18 (21.7)
TX	13 (15.7)
Histology (%)	
Serous	62 (74.7)
Mucinous	2 (2.4)
Endometrioid	3 (3.6)
Clear cell	1 (1.2)
Adenocarcinoma, NOS/undifferentiated	4 (4.8)
Mixed	11 (13.3)

Abbreviation: NOS, not otherwise specified.

probe (Exiqon) was hybridized to proteinase K-treated sections at 56 °C for 16 h. Slides were then incubated with anti-DIG-AP antibody (Roche), and miRNA expression was detected by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate method. Methyl green was used for nucleic counterstaining.

**Statistical analysis.** Statistical tests used were two-sided Student's *t* tests, with Welch's correction for unequal variance, where appropriate, using InStat 3.05 and Prism 4.03 software (GraphPad, Inc.).

Other methods, including quantitative PCR, methylation-specific PCR analysis, cell culture experiments, and Western blotting, are described in Supplementary Data.

## Results

**miR-34 expression is reduced in EOC and is correlated with metastatic stage.** To determine miR-34 family expression in EOC, we isolated total RNA from 83 EOC samples and compared expression levels to that in six wild-type OSE primary cell samples (Table 1). We observed significantly reduced expression for all three family members in EOC compared with wild-type (Fig. 1A), with miR-34a most significantly reduced by 21.2-fold ( $P < 0.0001$ ), whereas miR-34b\* and miR-34c were reduced by 2.3-fold ( $P = 0.0172$ ) and 3.4-fold ( $P = 0.0002$ ), respectively. It has been recently reported that Droscha and Dicer expression is

deregulated in EOC (8, 25). To test whether alteration in miR-34 expression can be explained by Droscha/Dicer-mediated global changes in miRNA processing, we determined miR-199a\* expression, which is deregulated in EOC (7, 26). miR-199a\* expression seems to be elevated, although this is not statistically significant (Supplementary Fig. S2). No significant differences in expression of miR-34 family members were detected among different histologic types of EOC, with an exception of significantly reduced miR-34a expression in endometrioid type as compared with serous adenocarcinoma (Supplementary Fig. S3).

Tumor staging is linked to survival, with stage III tumors having tumor cell dissemination in the peritoneum, whereas stage IV tumors have distant metastasis, commonly to liver, and is indicative of poor prognosis. We compared gene expression in stage III and IV tumors and observed significantly reduced miR-34b\* and miR-34c expression in stage IV tumors ( $P = 0.0171$  and  $P = 0.0033$ , respectively; Fig. 1B), suggesting that miR-34b\* and miR-34c may be involved in metastatic progression. Interestingly, however, change in miR-34a expression was not statistically significant ( $P = 0.2574$ ).

**Decreased miR-34 expression is associated with p53 mutation.** Mutation of *p53* is a common event in many human cancers but is particularly common in high-grade serous EOC (3). We therefore took a subset of our serous EOC samples ( $n = 26$ ) and sequenced *p53* exons 4 to 11, where >99% of *p53* mutations are located (Supplementary Table S2). We have found that although miR-34a expression is reduced in both samples with wild-type or mutant *p53*, patients with mutant *p53* show significantly lower expression of all miR-34 family members than patients with wild-type *p53* ( $P = 0.0012$ , 0.0285, and 0.0216 for miR-34a, miR-34b\*, and miR-34c, respectively; Fig. 1C).

**Regulation of miR-34 by promoter methylation and copy number alterations.** Promoters of both *mir-34a* and *mir-34b\*/c* are located in CpG islands, and methylation has been reported to regulate miR-34a expression in several cancer cell lines and primary prostate tumors and melanomas, whereas miR-34b\*/c expression in colorectal cancer is also epigenetically regulated (27, 28). Such methylation has not been reported in ovarian cancer, so we set out to determine the frequency of such methylation by methylation-specific PCR analysis. Methylation at the *mir-34a* and *mir-34b\*/c* loci was observed in 27% (8 of 30) and 47% (14 of 30) of EOC samples, respectively (Fig. 2A). All samples (8 of 8) with *mir-34a* methylation show reduced miR-34a expression, whereas 57% (8 of 14) of samples with methylation at *mir-34b\*/c* show reduced miR-34b\*/c expression. Classification of samples based on *p53* mutation status revealed that 21% (3 of 14) and 50% (7 of 14) of mutant *p53* samples show promoter methylation at *mir-34a* and *mir-34b\*/c*, respectively, whereas for samples with wild-type *p53*, 38% (5 of 13) and 46% (6 of 13) show methylation, respectively.

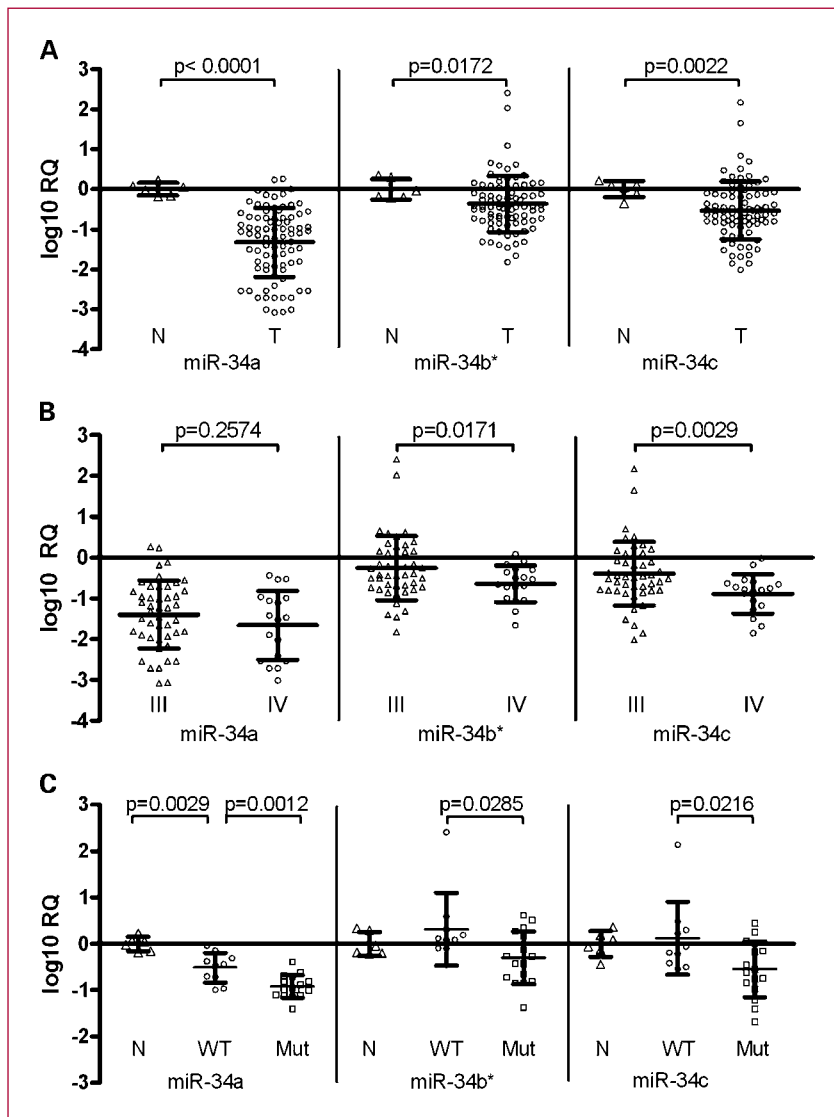
We next raised the question of whether loss of heterozygosity or copy number alterations could be responsible

for reduced miR-34a expression and designed custom Taqman primer and probes to amplify the *mir-34a* locus in a qPCR assay. Reduced copy number at the *mir-34a* locus was observed in 39% (13 of 33) of EOC samples (Fig. 2C), of which 92% (12 of 13) had reduced miR-34a expression. Three of these 12 samples (25%) with reduced *mir-34a* copy number and expression showed no *p53* mutation or promoter methylation. Taken together, reduced miR-34a expression is associated with *p53* mutation, *mir-34a* promoter methylation, and/or copy number variation in 82% (27 of 33) of EOC samples.

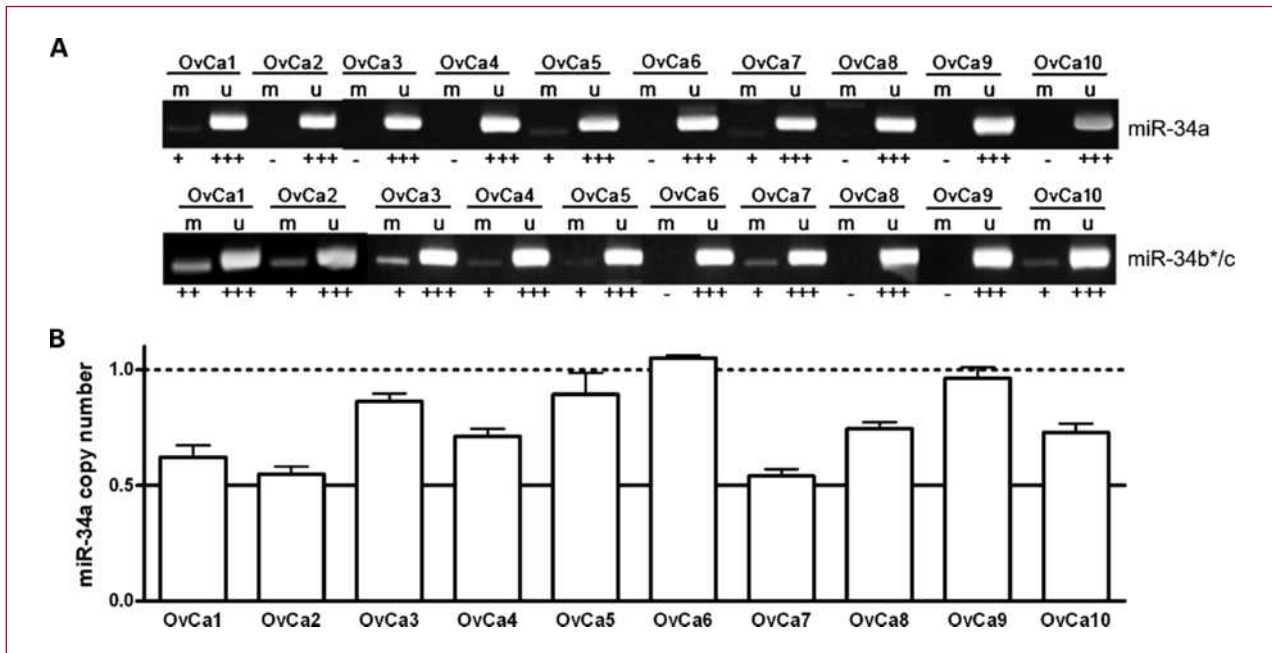
**miR-34 and MET expression in EOC paraffin sections.** To explore miR-34 expression in EOC tissue, we did miRNA *in situ* hybridization with paraffin-embedded tissue sections of human ovarian cancer. In the case of miR-34a probe, we observed positive signal in cytoplasm compared with control probe, whereas U6 small nuclear RNA was exclusively expressed in the nucleus as expected

(Fig. 3A-C). We tested a total of 21 cases of serous EOC with LNA miR-34a probe. Consistent with qRT-PCR data, 85.7% (18 of 21) of cases have weak or undetectable miR-34a expression.

One of the shared targets of miR-34 family is the receptor tyrosine kinase MET according to bioinformatic assessment and luciferase assays (15, 18). Furthermore, the majority of EOCs express elevated levels of MET (29). Thus, we decided to compare MET expression level in parallel sections of 17 cases with semiquantitative immunohistochemical analysis. EOC cases expressing moderate to strong miR-34a (Fig. 3C) had a relatively weak expression of MET (Fig. 3D). On the contrary, low miR-34a-expressing EOC (Fig. 3E) had strong expression of MET (Fig. 3F). Based on semiquantitative analysis, expression of miR-34a and MET had statistically significant inverse correlation ( $r = -0.5898$ ;  $P = 0.0162$ ), confirming that miR-34a might play a role in regulating MET expression in EOC.



**Fig. 1.** miR-34 is downregulated in EOC and associated with metastatic clinical stage. A, qRT-PCR analysis reveals significantly reduced miR-34a, miR-34b\*, and miR-34c expression in a panel of EOC samples ( $n = 83$ ; ○) relative to normal OSE samples ( $n = 6$ ;  $P < 0.0001$ ,  $P = 0.0172$ , and  $P = 0.0022$ , respectively; Δ). B, ovarian cancer stage was determined by surgical evaluation during cryoreduction/debulking surgery. miR-34a, miR-34b\*, and miR-34c expression is shown for stage III ( $n = 46$ ; Δ) and stage IV ( $n = 18$ ; ○) tumors. miR-34b\* and miR-34c expression is significantly decreased in stage IV (distant metastasis) compared with stage III (localized to peritoneum;  $P = 0.0171$  and  $0.0029$ , respectively). C, adenocarcinomas (serous type,  $n = 26$ ) and wild-type OSE samples ( $n = 6$ ) were analyzed for *p53* mutational status by direct sequencing and correlated with miR-34 expression. Whereas both *p53* wild-type (WT; ○) and *p53* mutant (Mut; □) EOC samples show significantly reduced miR-34a expression compared with wild-type OSE (N; Δ), EOC samples with mutant *p53* show most downregulated expression for miR-34a, miR-34b\*, and miR-34c ( $P = 0.0012$ ,  $0.0285$ , and  $0.0216$ , respectively). Bars, SD.



**Fig. 2.** *mir-34* promoter methylation and copy number variations are common in EOC. A and B, representative examples of *mir-34a* and *mir-34b\*/c* promoter methylation determined by methylation-specific PCR with primers specific for methylated (m) and unmethylated (u) DNA (A) and copy number changes at the *mir-34a* locus determined by qPCR (B). Bars, SD.

**miR-34 reduces migration, invasion, and proliferation in EOC cells.** To determine the role of miR-34 in human ovarian cancer, we transfected synthetic miR-34 molecules either separately or in combination into SKOV-3, p53-null human ovarian adenocarcinoma cells (Fig. 4; Supplementary Fig. S4). SKOV-3 cells express low endogenous levels of all three miR-34 family members (8, 10) and are therefore well suited to test functions of miR-34. We transfected SKOV-3 cells with 15 nmol/L miR-34 individually or 5 nmol/L combined and observed reduced amounts of MET protein and mRNA (Fig. 4A and B). Even more significant reduction of MET levels was observed after transfection with 30 nmol/L miR-34 (Fig. 4C).

The miR-34 family has been shown to reduce cell invasion in gastric and hepatocellular carcinoma cells, at least partially through downregulation of MET (18, 30). To examine the role of miR-34 family in invasion and motility in ovarian cancer, we did Transwell motility and Matrigel invasion assays with miR-34 family and/or MET small interfering RNA (siRNA)-transfected SKOV-3 cells. Notably, whereas MET knockdown was observed after MET siRNA treatment, cyclin-dependent kinase 4 (CDK4), which is another target of miR-34 family, was not affected by MET siRNA but only after miR-34 family transfection (Fig. 4C). As expected, individual miR-34 reconstitution by transfection caused significant reduction in motility and invasion in the presence of the MET ligand, hepatocyte growth factor (Fig. 4D and E; Supplementary Fig. S4). However, when

miR-34 and MET siRNA were transfected together, no further reduction was observed, showing that MET downregulation by miR-34 is largely responsible for the reduced invasion.

Next, we asked whether miR-34 family reconstitution reduces cell proliferation because miR-34 family also can target cell cycle-related genes such as *CDK4* (Fig. 4C). Transfection of SKOV-3 cells with either 15 nmol/L miR-34a, miR-34b\*, or miR-34c reduced proliferation by ~30% compared with control-transfected cells ( $P = 0.0044$ ,  $0.0042$ , and  $0.0106$ , respectively; Fig. 4F). We next treated cells with a combination of 5 nmol/L of each miR-34 family member to determine whether additional suppression could be achieved due to sequence, and presumably target, differences of miR-34 family members. Although the percentage of proliferative cells was reduced to 20.7% ( $P = 0.0021$ , compared with control), the difference in reduction compared with miR-34c transfection individually was not statistically significant ( $P = 0.0876$ ). Additionally, we assessed the amount of apoptosis in miR-34-transfected cells by determination of cleaved caspase-3 staining (Supplementary Fig. S5). No significant change in number of apoptotic cells was observed in p53 mutant SKOV-3, consistent with miR-34-induced apoptosis being p53 dependent (31).

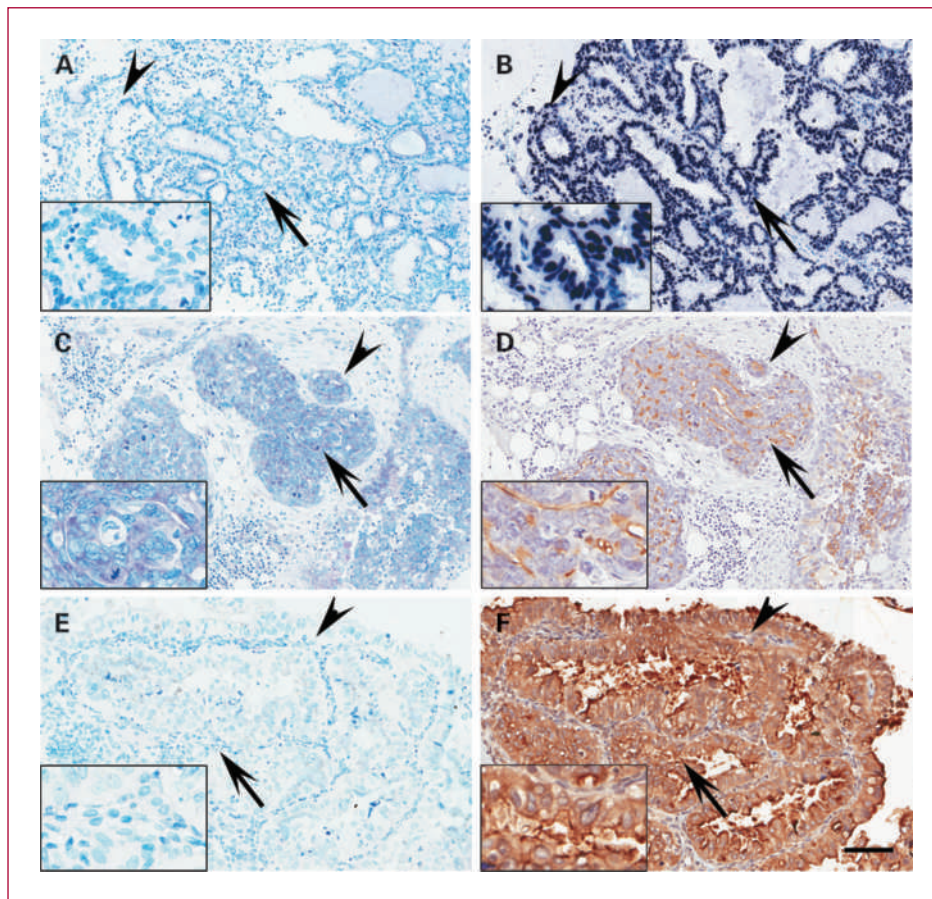
## Discussion

Previously, we showed that miR-34b-5p and miR-34c expression is reduced in a p53-dependent manner in a

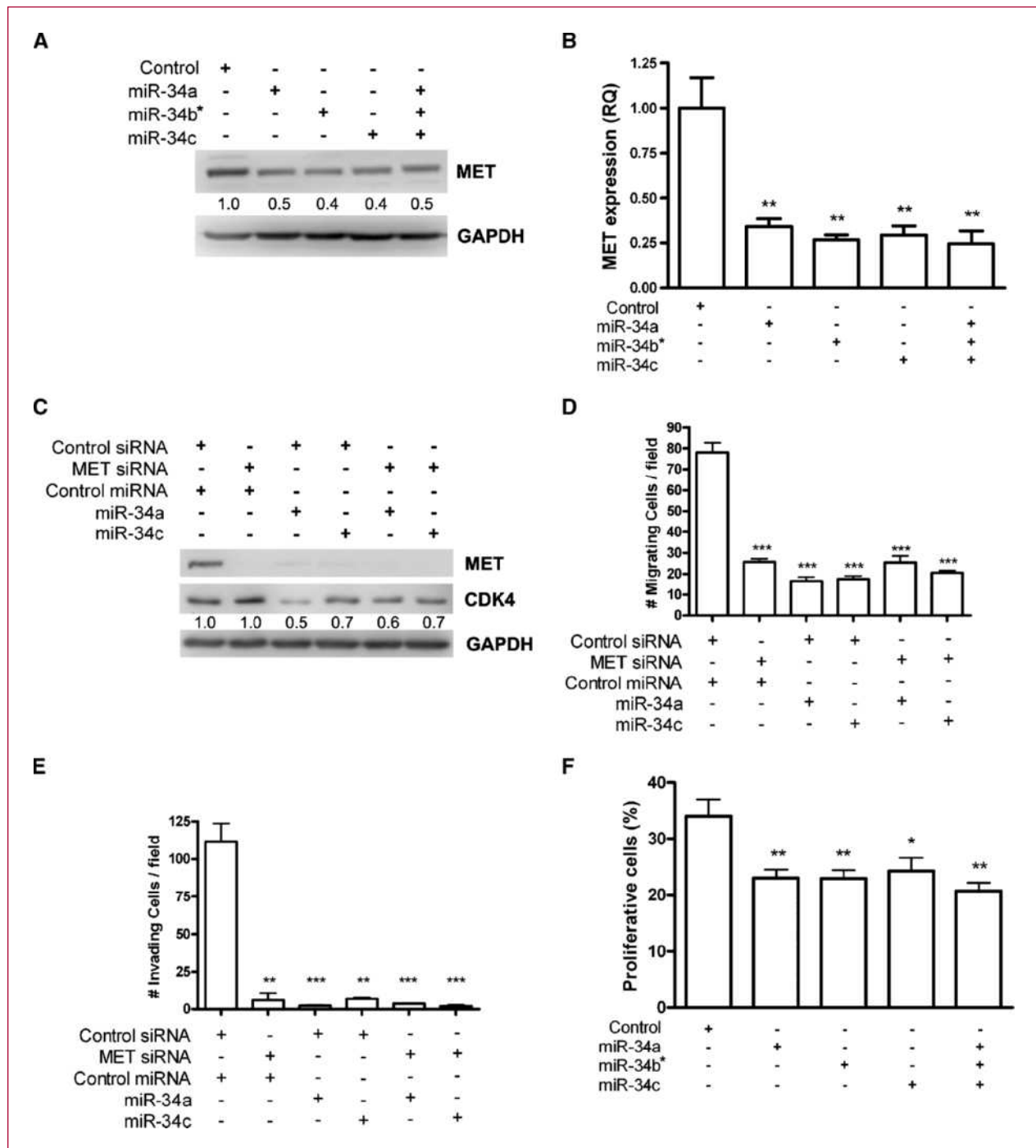
mouse model of EOC, whereas others also reported reduced miR-34 expression in a variety of cell lines and mouse models (10–15). These results led us to question the involvement of miR-34 in human EOC, and we show here that miR-34 family expression is also significantly reduced in human EOC, particularly in patients with *p53* mutations.

Recently, the involvement of Drosha and Dicer in EOC has been reported, linking reduced expression of these proteins to poor outcome (25). Yet, although reduced Drosha/Dicer processing might be expected to lead to globally decreased miRNA expression, this does not seem to account for all miRNA expression defects, given that miR-199a\* expression is modestly increased (Supplementary Fig. S2). Moreover, unlike miR-34a, miR-34b\*/c expression is not reduced in tumors with wild-type *p53*. This discrepancy between miR-34a and miR-34b\*/c expression suggests that, in addition to shared *p53*-dependent transactivation, control mechanisms unique to miR-34a are altered in tumors with wild-type *p53*. Underlining differences between the two *mir-34* loci is our observation that expression of miR-34b\*/c, but not expression of miR-34a, is significantly associated with stage IV distant metastatic disease (Fig. 1B).

To understand the cause of these differences, we investigated the role of promoter methylation and copy number variations of *mir-34*. *mir-34* promoter methylation has been reported in several tumor types (27, 28), whereas megabase pair deletions at chromosome 1p36 containing the *mir-34a* locus have been identified in seven low-grade serous carcinomas (32) and in neuroblastoma (33). Indeed, promoter methylation was observed at *mir-34a* and *mir-34b\*/c* in 27% and 47% of EOC samples, respectively. Furthermore, reduced *mir-34a* copy number was observed in 39% of samples. However, there was no direct correlation between methylation or copy number and miR-34 expression levels. There are several possible explanations that may account for these data. Firstly, although our *p53* sequencing data identified *p53* mutations with high confidence, mutation in genes that regulate *p53* may be involved, such as MDM2, which posttranslationally silences *p53* through ubiquitinylation (34, 35), whereas *p53* may itself be epigenetically silenced (36). Secondly, many miRNAs, including miR-34a, have been shown by Chang et al. (37) to be suppressed by c-Myc, an oncogene frequently overexpressed in multiple tumor types, including EOC. Additionally, it is likely that additional transcription factors regulate miR-34 expression. Very recently,



**Fig. 3.** miR-34a expression is inversely associated with MET expression in EOC. Sections of formalin-fixed, paraffin-embedded human EOC specimens were hybridized with DIG-labeled LNA control probe (A), U6 small nuclear RNA probe (B), and miR-34a probe (C and E) or immunostained with MET antibody (D and F). C and D, EOC with strong miR-34a expression shows relatively low level of MET expression. E and F, on the contrary, EOC with weak miR-34a expression has strong staining for MET. Insets show high magnification of areas indicated with arrows. Similar structures of parallel sections (A and B, C and D, and E and F) are indicated with arrowheads. Methyl green and hematoxylin were used for counterstaining of *in situ* hybridization and immunostaining, respectively. Scale bar, 100  $\mu$ m.



**Fig. 4.** miR-34 reconstitution decreases migration, invasion, and proliferation in EOC cells. A and B, individual miR-34 family member (15 nmol/L) or entire miR-34 family (5 nmol/L each) was transfected, and statistically significant reduction in mRNA was observed for each treatment (miR-34a,  $P = 0.028$ ; miR-34b\*,  $P = 0.0017$ ; miR-34c,  $P = 0.0022$ ; entire miR-34 family,  $P = 0.0020$ ), although reconstitution of entire miR-34 family does not further downregulate MET expression. C, MET siRNA and/or indicated miR-34 precursor molecule (30 nmol/L) was transfected in SKOV-3 cell line. Transfected cell lysates were probed against MET, CDK4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in Western blot analysis. MET siRNA induced strong knockdown of MET protein with no effect on CDK4, whereas miR-34 downregulated CDK4 as well as MET. D and E, MET siRNA and/or miR-34 transfection induced significant reduction of cell migration (MET siRNA,  $P = 0.0005$ ; miR-34a,  $P = 0.0003$ ; miR-34c,  $P = 0.0003$ ; MET siRNA and miR-34a,  $P = 0.0008$ ; MET siRNA and miR-34c,  $P = 0.0003$ ) and invasion (MET siRNA,  $P = 0.0013$ ; miR-34a,  $P = 0.0009$ ; miR-34c,  $P = 0.0011$ ; MET siRNA and miR-34a,  $P = 0.0009$ ; MET siRNA and miR-34c,  $P = 0.0009$ ). F, quantitative assessment of proliferation by bromodeoxyuridine incorporation 48 h after transfection of SKOV-3 cells with either 15 nmol/L synthetic miR-34a, miR-34b\*, or miR-34c pre-miR individually or 5 nmol/L of each pre-miR concurrently (miR-34a,  $P = 0.0044$ ; miR-34b\*,  $P = 0.0042$ ; miR-34c,  $P = 0.0106$ ; miR-34 in combination,  $P = 0.0021$ ). Bars, SD.

Christoffersen et al. (38) studied human primary hTERT-immortalized TIG3 fibroblasts and observed p53-independent transcription of miR-34a. Oncogene-induced senescence mediated through B-RAF activation induces miR-34a expression in cells treated with a p53 siRNA or a p53 dominant-negative variant. Through chromatin immunoprecipitation experiments, the authors show that the ETS oncogene family member and transcription factor ELK1 binds a conserved region in the *mir-34a* promoter.

Clearly, future studies are required to obtain a more complete understanding of the regulation of miR-34 expression in both normal development and disease. However, the observation of B-RAF-induced miR-34a expression through oncogene-induced senescence raises an important question about the etiology of EOC. It has been hypothesized that low- and high-grade serous tumors have distinct precursor lesions (3, 39), with p53 mutations rarely found in low-grade tumors but common in high-grade tumors. Interestingly, and further emphasizing the differing molecular defects between the two tumor types, activating B-RAF mutations are found exclusively in low-grade serous EOC (40, 41). Together, this suggests that activation of miR-34a transcription by B-RAF/ELK1 and p53 in low-grade serous EOC induces senescence and prevents progression to high-grade disease. In contrast, a lack of B-RAF mutations combined with frequent p53 mutation in high-grade serous EOC would seem to largely eliminate miR-34 expression, and subsequently, tumor suppression capability is lost. Additionally, whereas p53 mutation has no effect on *miR-34b\*/c* methylation, *miR-34a* methylation is more common in samples with wild-type p53, consistent with a requirement for diminished p53-miR-34 activity to progress to carcinoma. Together, these observations suggest that high-grade tumors arise from a population of cells with mutated p53 but wild-type B-RAF.

Our data showing correlation of miR-34b\*/c expression with metastatic disease suggest that these two miRNAs will be useful as a prognostic marker. This observation is in a good agreement with a recent report that low miR-34a levels are correlated with increased probability of relapse in non-small cell lung carcinoma (42) and reinforces the importance of decreased miR-34 expression. Future studies based on complete follow-up data will determine if miR-34 expression correlates with survival of EOC patients.

Our functional studies of miR-34 reconstitution suggest therapeutic applications too. miRNAs represent attractive candidates for gene therapy approaches for several reasons. Computationally, individual miRNAs have been predicted to target tens or hundreds of mRNAs for translational repression. Indeed, one miRNA may regulate many targets. For example, in the case of miR-223 in neutrophils, hundreds of proteins are directly repressed, which has a significant effect on phenotype, despite protein repression being relatively modest (43, 44). It is noteworthy that microarray experiments done after miR-34 reconstitution in cancer cell lines revealed highly significant altera-

tions that are clustered to important biological pathways (e.g., cell cycle pathway genes; refs. 12, 15). These data suggest that reconstituted expression of a downregulated miR-34 might reset or otherwise induce normal function of these gene networks.

Due to its large number of transcriptional targets, p53 has also been an attractive candidate for gene therapy. It was therefore disappointing that p53 gene therapy in EOC failed in phase II/III clinical trials (45). Yet, whereas p53 therapy may have failed due to p53 degradation through MDM2 and dominant-negative tetramer formation, gene therapy with a p53-independent miR-34 transgene under control of a strong promoter would not be expected to face either of these problems. Furthermore, miR-34 may be more attractive than p53 due to its small size, making it more amenable to packaging in viral and nonviral technologies. No less of importance, several EOCs do not harbor p53 mutations but express low levels of miR-34, which could be corrected. Thus, it would be interesting to study the consequence of miR-34 delivery to EOC cells with wild-type p53. However, currently characterized EOC cell lines carrying wild-type p53, such as the serous adenocarcinoma cell line A2780 and clear cell carcinoma cell line TOV-21G (15, 18), show high levels of expression of endogenous miR-34.

MET is one of few common targets for all three miR-34 family members. Taking into account that the majority of EOCs express elevated levels of MET (29), we have tested the role of all miR-34 family members in repression of MET in EOC cells by using Western blotting, invasion, and motility assays, finding that all three members inhibit MET. Small-molecule MET inhibitors are now under clinical trials for several cancers (46), and Sawada et al. (47) showed that MET siRNA could reduce adhesion, invasion, metastasis, and tumor burden in intraperitoneal ovarian cancer xenograft model but did not affect proliferation. On the contrary, miR-34 therapeutics may have advantages compared with siRNA approach because miR-34 is capable of regulating cell proliferation as well as invasion through targeting several target genes in addition to MET, such as MYC and E2F3. This is underscored by our experiment showing reduced CDK4 protein after miR-34 treatment, but not MET siRNA.

Taken together, our data show the frequent reduction of miR-34 family expression in EOC and its functional properties as an inhibitor of proliferation and invasion. This miRNA family is therefore an attractive candidate gene for further studies aimed at better understanding of disease pathogenesis and development of novel therapeutic approaches.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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