MicroRNA-125a Reduces Proliferation and Invasion of Oral Squamous Cell Carcinoma Cells by Targeting Estrogen-related Receptor α

IMPLICATIONS FOR CANCER THERAPEUTICS

Background: ESRRA is frequently up-regulated in several cancers. However, the mechanism underlying its up-regulation remains elusive.

Results: Down-regulation of tumor suppressor miR-125a causes up-regulation of ESRRA in OSCC.

Conclusion: Down-regulation of miR-125a is a novel mechanism for up-regulation of ESRRA in OSCC.

Significance: Targeting miR-125a via a synthetic mimic adds novelty to OSCC therapeutics.

This article has been withdrawn by the authors. In Fig. 3B, the actin immunoblots from patient samples 101 and 128 were duplicated. In Fig. 7B, a portion of the image showing cells co-transfected with pcDNA3.1(+) and pmiR-125a was reused in Fig. 8B showing cells co-transfected with pESRRA-3[prime]UTR-S and pmiR-125a. Additionally, portions of the image of cells co-transfected with pESRRA-3[prime]UTR-M1 and pmiR-125a from Fig. 7B were reused in Fig. 8B as cells co-transfected with pmiR-125a and pESRRA-3[prime]UTR-M or pESRRA-3[prime]UTR-AS. Given these errors, the withdrawing authors strongly believe that the best course of action is to withdraw the article from the journal to maintain the high standard and rigor of scientific literature.

The ESRRA (estrogen-related receptor alpha) gene, located on chromosome 11q13, codes for a 423-amino acid-long protein.

The abbreviations used are: ESRRA, estrogen-related receptor α; OSCC, oral squamous cell carcinoma; miRNA, microRNA; PI, propidium iodide; qRT, quantitative RT; ESRRA, estrogen-related receptor α; TS, target site; AZA, 5-aza-2’-deoxycytidine.
ESRRA has been shown to transcriptionally regulate the expression of several genes, such as WNT11 (wingless-related murine mammary tumor virus integration site 11), CCNE1 (cyclin E1), OPN (osteopontin), and OPG (osteoprotegerin), involved in cell cycle, metastasis, and metabolism. The transcriptional activity of ESRRA is influenced by mitogenic signals controlled by ERBB2 (v-erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2) and EGF receptor (18). A model proposed by Ariazi et al. (18) suggests that the homodimer of ERBB2 signals phosphorylation of MEK/MAPK and PI3K/Akt, which in turn increases the transcriptional activity of ESRRA (18). It has also been shown that mTORC1 (mTOR complex 1) can regulate the activity of ESRRA through ubiquitin-mediated degradation via transcriptional control of the ubiquitin-proteasome pathway (19). Furthermore, constitutive activation of mTORC1 signaling in TSC2 (tuberous sclerosis 2) null mouse embryonic fibroblasts results in an increased level of ESRRA (19).

ESRRA along with its co-activator PGC-1α (peroxisome proliferator-activated receptor γ co-activator 1-α) binds to its own promoter and autoregulates its expression (20). Furthermore, post-translational modifications (e.g., phosphorylation, acetylation, and sumoylation) of ESRRA are known to regulate its activity, such as DNA binding and interaction with co-activators PGC-1α and PGC-1β (11). However, despite its roles in different cellular functions and tumorigenesis, the mechanism underlying its up-regulation in different cancers still remains elusive.

MicroRNAs (miRNAs) are a class of small ~22-nucleotide-long endogenous noncoding RNAs that regulate the expression of genes at the post-transcriptional level by interacting with their 3′UTRs in a sequence-specific manner (21), and in turn regulate a wide range of cellular functions. They are known to regulate the expression level of oncogenes and tumor suppressors. Downregulated miRNAs (e.g., miR-20a, miR-135b, and miR-125a) can act as tumor suppressor genes or genes that control cell differentiation or apoptosis (23), whereas downregulated miRNAs (e.g., let-7) function as tumor suppressor genes or genes that control cell differentiation or apoptosis (24). The only miRNA known to regulate ESRRA is miR-137 (15).

In recent years, miRNAs have been used as therapeutic targets in several cancers (27). For example, MRX34, which is a liposomal formulation of the tumor suppressor miR-34, has entered phase I clinical trials for primary liver cancer patients (28).

Here, we report the identification of a tumor-suppressor microRNA-125a (miR-125a) that targets and regulates the expression of ESRRA in OSCC. Based on our in vitro and in vivo experiments, we suggest that the restoration of miR-125a levels by the use of a synthetic mimic would be a novel therapeutic strategy to treat OSCC and other cancers.

**MATERIALS AND METHODS**

**In Silico Identification of miRNAs Targeting ESRRA**—We used a consensus approach by employing a total of five established miRNA target prediction programs (Table 1) to identify
miRNAs targeting the 3' UTR of ESRRA (GenBank™ accession number NM_004451.3).

Plasmid Constructs—To generate constructs in the pcDNA3-EGFP vector expressing pre-miR sequences of miR-16, miR-107, miR-125a, miR-125b, and miR-137, PCR primers were designed using miRNA-specific DNA sequences retrieved from the UCSC Genome Bioinformatics site. PCR was performed using a standard laboratory procedure and human genomic DNA as a template. Different restriction enzyme sites were incorporated in forward and reverse primers to facilitate directional cloning (supplemental Table S1). To generate constructs with the 3' UTR of ESRRA, the pmiR-REPORT vector (Invitrogen) was used as a template. The site-directed mutagenesis was carried out by miR-Q, a method developed by Sharbati-Tehrani et al. (31) for the expression analysis of miRNAs. 5 S RNA was used as an internal control for quantitative RT-PCR analysis of miR-125a and miR-137 was carried out by miR-Q, a method developed by Sharbati-Tehrani et al. (31) for the expression analysis of miRNAs. 5 S RNA was used as an internal control for quantitative RT-PCR analysis of miR-125a and miR-137 was carried out by miR-Q, a method developed by Sharbati-Tehrani et al. (31) for the expression analysis of miRNAs.

| Serial no. | Pt. no. | Age (year)/sex | Site of the tumor | TNM | Tob usage | ESRRA expression | miR-125a expression |
|------------|---------|----------------|------------------|-----|-----------|------------------|--------------------|
| 1          | 62      | 35/F           | SCC BM           | T3N0M0 | Tob chewing | Up               | Down               |
| 2          | 65      | 55/M           | BM               | T2N1Mx | Tob chewing | No change        | Down               |
| 3          | 67      | 38/F           | BM               | T2N1M0 | Tob chewing | No change        | Down               |
| 4          | 68      | 65/M           | Lower alveolus   | T2N2M0 | Tob chewing | No change        | Down               |
| 5          | 79      | 67/F           | BM               | T3N1Mx | Tob chewing | Up                | Down               |
| 6          | 101     | 48/F           | BM               | T2N1Mx | Tob chewing | Up                | Down               |
| 7          | 109     | 45/M           | SCC BM           | T3N1M0 | Tob chewing | Up                | Down               |
| 8          | 110     | 45/M           | BM               | T2N1M0 | Smoking and alcohol | Up | No change |
| 9          | 128     | 60/F           | SCC BM           | T2N0M0 | Tob chewing | No change        | Down               |
| 10         | 135     | 41/M           | BM               | T2N0M0 | Betel quid chewing | Up | No change |
| 11         | 140     | 70/M           | Lip              | T4N1M0 | Tob chewing | Up                | Down               |
| 12         | 155     | 60/F           | SCC BM           | T3N0Mx | Tob chewing | Up                | Down               |
| 13         | 173     | 56/F           | SCCBM            | T3N0M0 | Tob chewing | Up                | Down               |
| 14         | 174     | 70/F           | SCC BM           | T2N0M0 | Tob chewing | Up                | Down               |
| 15         | 175     | 72/F           | RMT              | T4N1M0 | Tob chewing | Up                | Down               |
| 16         | 179     | 58/F           | Maxilla          | T3N0M0 | Tob chewing | Up                | No change           |
| 17         | 183     | 50/M           | Tongue anterior 2/3 | T1N1M0 | Tob chewing | Up                | No change           |
| 18         | 191     | 45/F           | Lower alveolus   | T2N0M0 | Tob chewing | Up                | Down               |
| 19         | 196     | 67/M           | Tongue           | T4N3M0 | Nil         | Up                | Down               |
| 20         | 226     | 55/F           | BM               | T4N2M0 | Nil         | No change         | Down               |
as a normalizing control to equalize the amount of cDNA generated from each sample. Following equalization of 5 S RNA, the same amounts of cDNA templates were used for RT-PCR to check the expression of miR-125a and miR-137. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining on an UV trans-illuminator (Bangalore Genei, Bangalore, India).

The qRT-PCR analysis was carried out to quantitate the expression of miR-125a, MCPH1, and ESRRA in an ABIprism 7900HT sequence detection system (Applied Biosystems, Foster City, CA), using the Dynamo SYBR Green Mix (Finnzymes, Espoo, Finland). The analysis was performed using the SDS2 software (Applied Biosystems, Foster City, CA). GAPDH or 5 S RNA was used as a normalizing control (23). The fold change was calculated using the following equation: $\Delta Ct_{\text{gene}} = Ct_{\text{gene}} - Ct_{\text{normalizing control}}$, where $Ct$ is the cycle threshold value, and $\Delta Ct$ represents the gene expression normalized to GAPDH or 5 S RNA. The statistical significance of the difference in mRNA expression was assessed by two-tailed unpaired $t$ test using the GraphPad PRISM5 software (GraphPad Software Inc., San Diego). A probability value of $p \leq 0.05$ was considered to be significant. Details of the RT-PCR primers are available on request from the authors.

Western Hybridization—Whole protein lysates, isolated from cells and tissue samples using the CelllyticTM M Cell Lysis Reagent (Sigma), were resolved on SDS-PAGE and then transferred to PVDF nylon membranes (Pall Corp., Port Washington, NY). The membrane was blocked using 5% fat-free milk powder in 1× PBST, and the signal was visualized using 1:750 dilution of an anti-ESRRA antibody (catalog no. sc-32790, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation of the membrane with a horseradish peroxidase-linked secondary antibody (Bangalore Genei, Bangalore, India), the signal was detected using the ImmobilonTM western chemiluminescent HRP substrate (Millipore, Billerica, MA) and x-ray films. The anti-β-actin antibody (catalog no. A5441, Sigma) was used to ensure equal loading of lysates.

Cell Proliferation Assay—The rate of cell proliferation (absorbance at 450 nm) was quantitated using a CHEMICON® BrdU cell proliferation assay kit (Millipore Corp.) as described previously (23). In brief, after 48 h of transfection of cells in 96-well plates with different constructs, miR-125a mimic (200 nM), miR-125a inhibitor (800 nM), or scrambled oligonucleotides (800 nM) (Dharmacon, Carlsbad, CA), cells were incubated with BrdU for 20 h in a 37 °C incubator with 5% CO$_2$, fixed for 30 min at room temperature, and then incubated overnight with a
MicroRNA-125a regulates ESRRA

BrdU detection antibody. Cells were then washed and incubated in the goat anti-mouse IgG, peroxidase-labeled solution. Following washing, cells were incubated with the substrate, and the plate was read at 450 nm using a Bio-Rad™ microplate reader (Bio-Rad). Effective concentrations of miR-125a mimic (200 nM) and miR-125a inhibitor (800 nM) were determined by Western blotting of protein lysates from cells transfected with different dilutions of miR-125a mimic (viz. 50, 100, and 200 nM) and miR-125a inhibitor (viz. 200, 400, and 800 nM).

Cell Cycle Analysis and Detection of Caspase-3 Activation—For cell cycle analysis, cells were stained with propidium iodide (PI) (Sigma) and analyzed by a FACSCalibur™ flow cytometer (BD Biosciences), as described previously (23). The caspase-3 activation was determined using a CaspGLOW™ fluorescein active caspase-3 staining kit (Biovision, Mountain View, CA).

Soft Agar Colony Forming Assay—The soft agar colony forming assay was performed using 2,000 cells for each plate, as described previously (23). Briefly, cells were transiently transfected with different constructs separately, followed by plating colonies were counted in three random microscopic fields under a ×10 objective, and the images were captured using an Olympus CKX41 phase contrast microscope (Olympus Optical Co., Tokyo, Japan).

Cell Invasion Assay—The cell invasion assay was performed using a Biocoat™ Matrigel™ invasion chamber (BD Biosciences). Briefly, 5,000 cells were transiently transfected with different constructs separately, followed by plating them in 35-mm dishes with 0.35% noble agar (Difco, Mumbai, India). At the end of 10 days, colonies were counted in three random microscopic fields under a ×10 objective, and the number of cells that had invaded through the Matrigel matrix. The graph was plotted for the number of cells that had invaded per microscopic field.

In Vivo Assay for Tumor Growth—To see the effect of miR-125a via targeting ESRRA on tumor growth, 2 × 10⁶ SCC084 cells were transfected separately with miR-125a mimic (200 nM), miR-125a inhibitor (800 nM), or scrambled oligonucleotides (800 nM). 24 h after transfection, cells from each transfection were suspended separately in 150 μl of incomplete DMEM and then subcutaneously injected into the right flank of a female BALB/c athymic 6-week-old nude mouse. Four nude mice were used in each transfection experiment. Tumors (xenografts) of measurable size appeared 10 days after injection. Tumor growth was monitored, and its volume was measured using a digital caliper every 3 days until 30 days. Tumor growth was monitored, and its volume was measured using a digital caliper every 3 days until 30 days. Tumor growth was monitored, and its volume was measured using a digital caliper every 3 days until 30 days. Tumor volume (V) was calculated as follows: $V = l \times w^2 \times 0.5$, where $l$ and $w$ represent length and width, respectively. Excised tumors were weighed at the end of 30 days. All nude mice experiments were performed following approval from the institutional animal ethics committee.

RESULTS

Identification of miR-125a as a Regulator of ESRRA—To predict the miRNAs that may target the 3′UTR of ESRRA and reduce its level, we employed five miRNA target prediction programs (viz. DIANA-microTv3.0, Microcosm, microRNA, TargetScan, and PicTar) and identified several miRNAs that had potential target sites in its 3′UTR. For example, miR-137 was predicted to target ESRRA by all five programs (Table 1). However, we decided not to work with this miRNA as it has already been reported to target ESRRA. Next, we wanted to see how many other miRNAs are predicted to target ESRRA by 4/5 programs. The analysis identified miR-16, miR-107, miR-125a (miR-125a-5p), and miR-125b (miR-125b) as potential miRNAs targeting ESRRA (Table 1). To validate this, we cloned these miRNAs in pcDNA3-EGFP and overexpressed in SCC084 cells. The results showed that only miR-125a reduced the level of ESRRA (Fig. 1A). Furthermore, miR-125a down-regulated the level of ESRRA in SCC084 and SCC131 cells in a dose-dependent manner (Fig. 1B). Of note, the level of ESRRA transcript remained unchanged in both SCC084 and SCC131 cells (Fig. 1C), suggesting its translational repression by miR-125a.
As a control, we also transfected miR-137 in SCC084 and SCC131 cells and analyzed its effect on the levels of ESRRA protein and transcript (Fig. 1, A, D, and E). As expected, the levels of ESRRA protein and transcript were reduced in both cell lines (Fig. 1, A, D, and E). Our bioinformatics analysis predicted two putative target sites (TSs) for the miR-125a seed sequence (SD) within the 3′UTR of ESRRA from 312 to 319 nucleotides (TS1) and 536 to 542 nucleotides (TS2). The ClustalW alignment showed that TS1 is highly conserved, whereas TS2 is poorly conserved across different species (Fig. 2 A).

**Confirmation of Target Sites for miR-125a in the 3′UTR of ESRRA**—To confirm whether miR-125a binds directly to the 3′UTR of ESRRA and to underscore the functionality of two predicted TSs, we co-transfected pmiR-125a and pmiR-REPORT-3′UTR-S containing the intact 3′UTR of ESRRA in a sense orientation or pmiR-REPORT-3′UTR-S and the empty vector pcDNA3-EGFP in SCC084 cells and quantitated the luciferase activity. The results showed a significantly reduced luciferase reporter activity in cells co-transfected with pmiR-125a and pmiR-REPORT-3′UTR-S as compared with cells co-transfected with pmiR-REPORT-3′UTR-S and pcDNA3-EGFP (Fig. 2B). Furthermore, when we co-transfected pmiR-125a and pmiR-REPORT-3′UTR-AS containing a full-length 3′UTR of ESRRA in an antisense orientation and thus lacking the miR-125a TSs in cells, there was no significant reduction in the luciferase reporter activity as compared with cells co-transfected with pmiR-REPORT-3′UTR-S and pcDNA3-EGFP (Fig. 2B), confirming that miR-125a binds to the 3′UTR of ESRRA.

To determine whether both or only one of the TSs in the 3′UTR of ESRRA is required for the binding of miR-125a, we mutated each of the two TSs by site-directed mutagenesis. We then co-transfected the pmiR-REPORT-3′UTR-M1F construct containing the mutated TS1 and the wild-type TS2, pmiR-REPORT-3′UTR-M2F construct containing the wild-type TS1 and the mutated TS2 or pmiR-REPORT-3′UTR-MF construct containing both the mutated TSs separately in SCC084 cells with pmiR-125a and quantitated the luciferase activity. The results showed that the luciferase activity was significantly increased in cells co-transfected with single mutants (pmiR-REPORT-3′UTR-M1F or pmiR-REPORT-3′UTR-M2F) and pmiR-125a as compared with cells co-transfected with pmiR-REPORT-3′UTR-S and pmiR-125a. We also observed a significant increase in the luciferase activity in cells co-transfected with pmiR-REPORT-3′UTR-MF and pmiR-125a in comparison with cells co-transfected with either of the two single mutant constructs (pmiR-REPORT-3′UTR-M1F or pmiR-REPORT-3′UTR-M2F) and pmiR-125a as compared with cells co-transfected with either of the two single mutant constructs (pmiR-REPORT-3′UTR-M1F or pmiR-REPORT-3′UTR-M2F) and pmiR-125a (Fig. 2B). These observations clearly indicated that miR-125a regulates ESRRA expression by interacting with both of its TSs in a site-specific manner. As expected, co-transfection of pmiR-REPORT-3′UTR-S and pmiR-137 in SCC084 cells showed a significant down-regulation of luciferase activity in comparison with cells
miR-125a Is Down-regulated in OSCC Samples with Up-regulation of ESRRA—After validating ESRRA as a target of miR-125a by bioinformatics and in vitro assays, we analyzed the levels of miR-125a and ESRRA in a panel of OSCC samples by qRT-PCR and Western blotting, respectively. The results showed down-regulation of miR-125a in 16/20 OSCC samples (viz. patient numbers 183, 65, 67, 101, 128, 62, 68, 79, 109, 155, 173, 179, 140, 175, 196, and 226) as compared with their matched normal oral tissues (Fig. 3A). Furthermore, the level of ESRRA was up-regulated in 15/20 OSCC samples (viz. patient numbers 183, 101, 110, 128, 135, 174, 191, 62, 68, 79, 155, 173, 140, 175, and 196) as compared with normal oral tissues (Fig. 3B). As expected, a majority of 11/15 OSCC samples (viz. patient numbers 183, 101, 110, 128, 62, 68, 79, 155, 173, 140, 175, and 196) with a low expression of miR-125a showed a high level of ESRRA (Fig. 3). However, of 4/15 OSCC samples (viz. patient numbers 110, 135, 174, and 191) showed an up-regulated level of ESRRA; two samples, patient numbers 174 and 191, showed an up-regulation of miR-125a, and two other samples, patient numbers 110 and 135, had no difference in the expression of miR-125a between normal oral tissues and OSCC samples (Fig. 3). Taken together, these results suggest that miR-125a-mediated post-transcriptional targeting of ESRRA is a novel mechanism for its up-regulation in OSCC.

miR-125a Regulates Cell Proliferation and Apoptosis by Targeting ESRRA—As stated above, ESRRA is known to positively regulate cancer cell proliferation. Therefore, we wanted to test whether overexpression or reduced expression of miR-125a in OSCC cells has a functional relevance in cell growth and proliferation as a consequence of reduced or increased levels of ESRRA, respectively. To this end, we transfected both SCC084 and SCC131 cells with synthetic miR-125a mimic and miR-125a inhibitor separately and quantitated the rate of cell proliferation (absorbance at 450 nm) by the BrdU assay. The levels of miR-125a and ESRRA were assessed by qRT-PCR and Western blotting, respectively (Fig. 4A). As expected, cells transfected with miR-125a mimic showed a reduced level of ESRRA as compared with cells transfected with mock (scrambled oligonucleotides), and the cells transfected with miR-125a inhibitor showed an increased level of ESRRA as compared with mock (Fig. 4A). Furthermore, the BrdU assay showed that the ectopic overexpression of miR-125a via an miR-125a mimic significantly reduced the rate of cell proliferation, whereas the miR-125a inhibitor separately and quantitated the rate of cell proliferation (absorbance at 450 nm) by the BrdU assay. The levels of miR-125a and ESRRA were assessed by qRT-PCR and Western blotting, respectively (Fig. 4), and an increase in the expression of miR-125a between normal oral tissues and OSCC samples (Fig. 3). Taken together, these results suggest that miR-125a-mediated post-transcriptional targeting of ESRRA is a novel mechanism for its up-regulation in OSCC.
subjected them to flow cytometry. The results showed an accumulation of the sub-G₁ population (indicative of cell death) in cells transfected with miR-125a mimic as compared with cells transfected with mock (Fig. 4C). To assess the type of cell death, we assessed the rate of apoptosis (% apoptotic cells) in miR-125a mimic and miR-125a inhibitor-transfected SCC084 and SCC131 cells by the caspase-3 activation assay, and the results demonstrated that the cells transfected with miR-125a mimic showed a significantly increased rate of apoptosis, whereas the cells transfected with miR-125a inhibitor showed a significantly decreased rate of apoptosis as compared with mock-transfected cells (Fig. 4D). The above observations suggest that miR-125a negatively regulates the rate of OSCC cell proliferation in both the cell lines by increasing the rate of apoptosis via targeting ESRRA.

Function of ESRRA Is Dependent on the Presence or Absence of Its 3’UTR—To assess the role of ESRRA 3’UTR in the regulation of its expression and hence its function, the different ESRRA constructs without 3’UTR (pESRRA), with the wild-type 3’UTR in a sense orientation (pESRRA-3’UTR-S), with mutated 3’UTR in a sense orientation (pESRRA-3’UTR-M1, pESRRA-3’UTR-M2, and pESRRA-3’UTR-M), with the wild-type 3’UTR in an antisense orientation (pESRRA-3’UTR-AS), or the vector pcDNA3.1(+) were transiently co-transfected separately with miR-125a and other ESRRA constructs in comparison with cells co-transfected with pcDNA3.1(+) vector alone. As expected, the results showed a reduced level of ESRRA in cells co-transfected with pESRRA-3’UTR-S and miR-125a as compared with cells co-transfected with pESRRA and miR-125a (Figs. 5A and 6A). Furthermore, the level of ESRRA was increased in cells co-transfected with pESRRA-3’UTR-AS or any mutant 3’UTR constructs as compared with cells transfected with the pcDNA3.1(+) vector alone (Figs. 5A and 6A).

As expected, the rate of cell proliferation was significantly increased in cells co-transfected with pESRRA and miR-125a as compared with cells transfected with pcDNA3.1(+) (Figs. 5B and 6B). This is because the pESRRA construct lacked the 3’UTR, and therefore the TSs for miR-125a were not available. Furthermore, the rate of cell proliferation was significantly
MicroRNA-125a regulates ESRRA

As mentioned above, ESRRA is known to increase cancer cell invasion. We therefore analyzed the effect of miR-125a-mediated knockdown of ESRRA on anchorage-independent growth and invasion of SCC084 and SCC131 cells. We co-transfected pmir-125a and different ESRRA constructs without 3’UTR (pESRRA), with 3’UTR in a sense orientation (pESRRA-3’UTR-S), with mutated 3’UTR (pESRRA-3’UTR-M1, pESRRA-3’UTR-M2, and pESRRA-3’UTR-M) or pESRRA-3’UTR-AS (Figs. 5B and 6B), confirming that miR-125a regulates cell growth and proliferation in part, by directly targeting the 3’UTR of ESRRA.

The PI staining showed that co-transfection of pESRRA-3’UTR-S and pmir-125a accumulated more cells in sub-G1 than co-transfection of other constructs separately with pmir-125a (Figs. 5C and 6C). The caspase-3 assay showed that the rate of apoptosis was significantly increased in cells co-transfected with pESRRA-3’UTR-S and pmir-125a as compared with cells co-transfected with other constructs separately with pmir-125a (Figs. 5D and 6D). These experiments further reinforced that miR-125a regulates cell growth and proliferation by directly targeting ESRRA, and this function depends on the presence or absence of 3’UTR in ESRRA.

FIGURE 6. miR-125a regulates rates of cell proliferation and apoptosis via targeting the 3’UTR of ESRRA in SCC131 cells. A, Western blot analysis of lysates from cells co-transfected with pmir-125a and different ESRRA constructs. Note the reduced level of ESRRA in cells co-transfected with pmir-125a and pESRRA-3’UTR-S in comparison with its 3’UTR. B, quantitative analysis of PI stained cells co-transfected with pmir-125a and different ESRRA constructs. Note the increase in sub-G1 population in cells co-transfected with pmir-125a and other ESRRA constructs in comparison with cells co-transfected with pmir-125a and pESRRA-3’UTR-S. **, p < 0.01, and ***, p < 0.001. B, quantitative analysis of cell proliferation by the BrdU assay. C, FACS analysis of PI-stained cells co-transfected with pmir-125a and different ESRRA constructs. Note the decrease in sub-G1 population in cells co-transfected with pmir-125a and pESRRA-3’UTR-S in comparison with cells co-transfected with pmir-125a and other ESRRA constructs. D, assessment of the rate of apoptosis by the caspase-3 assay. Note the significantly increased rate of apoptosis in cells co-transfected with pmir-125a and pESRRA-3’UTR-S in comparison with cells co-transfected with pmir-125a and other ESRRA constructs with 3’UTR. **, p < 0.01, and ***, p < 0.001.
Overall, the above observations suggested that ESRRA has a 
definite role in the regulation of cell proliferation, apoptosis, 
anchorage-independent growth, and invasion of OSCC cells, 
and miR-125a is exercising its tumor suppressor role, in part, by 
targeting it.

5-Aza-2’-deoxycytidine Treatment Up-regulates miR-125a—
Methylation of the miR-125a promoter has been shown previ-
ously (32). To understand the mechanism underlying the 
down-regulation of miR-125a, we speculated that the promoter 
of miR-125a is methylated in OSCC cell lines SCC084 and 
SCC131. To test this possibility, we first analyzed the endoge-
 nous levels of miR-125a and ESRRA in SCC084 and SCC131 
cells. The results showed similar levels of miR-125a and ESRRA 
in both the cell lines (Fig. 9A). We then treated SCC084 and 
SCC131 cells with 5-aza-2’-deoxycytidine (AZA), a methyl-
transferase inhibitor, and DMSO (vehicle control). Total RNA 
was isolated from the cells after 3 and 5 days of the treatment, 
and the qRT-PCR analysis was performed to assess the level of 
miR-125a. The results showed a significantly increased level of 
miR-125a in AZA-treated cells as compared with DMSO-
treated cells from both the cell lines (Fig. 9B), suggesting that 
the promoter methylation is one of the mechanisms for down-
regulation of miR-125a in OSCC. Methylation of the MCPH1 
promoter is known in OSCC (33). Therefore, we also assessed 
the level of MCPH1 in AZA-treated cells as a positive control. 
As expected, the level of MCPH1 was significantly increased in 
AZA-treated cells as compared with DMSO-treated cells from 
both the cell lines (Fig. 9C).

Effect of miR-125a-mediated Regulation of ESRRA on Its 
Transcriptional Targets—As mentioned above, ESRRA is a well 
known transcription factor and is known to transcriptionally 
regulate WNT11 and OPN. To see the effect of miR-125a-me-
diated regulation of ESRRA on its transcriptional targets, we 
transfected miR-125a mimic and miR-125a inhibitor in 
SCC084 and SCC131 cells and quantitated the level of WNT11 
and OPN by qRT-PCR (Fig. 10). As expected, the level of 
WNT11 was significantly reduced in miR-125a mimic trans-
ferred cells as compared with mock-transfected cells (Fig. 10A).
Further, the level of WNT11 was significantly increased in miR-125a inhibitor-transfected cells as compared with mock-transfected cells (Fig. 10A). Similarly, the level of OPN was significantly reduced in miR-125a mimic-transfected cells as compared with mock-transfected cells (Fig. 10B). Furthermore, the level of OPN was significantly increased in the miR-125a inhibitor-transfected cells as compared with mock-transfected cells (Fig. 10B).

Restoration of miR-125a Levels Significantly Suppresses Tumor Growth in Vivo—Based on our in vitro studies, we hypothesized that the restoration of miR-125a levels via a synthetic miR-125a mimic and in turn reducing the level of ESRRA in OSCC cells might have an anti-tumor effect in vivo. To address this critical question, we injected equal numbers of SCC084 cells pretransfected with miR-125a mimic, miR-125a inhibitor, or mock separately into the right flanks of nude mice. The mice were observed for xenograft (tumor) growth until 30 days. As expected, nude mouse xenografts with miR-125a mimic had significantly reduced weight and volume, whereas xenografts with miR-125a inhibitor had a significant increase in both weight and volume in comparison with xenografts with mock (Fig. 11, A–C). Furthermore, we also assessed the level of ESRRA in xenografts by Western blotting. As expected, the level of ESRRA was reduced in xenografts with the miR-125a mimic, although its level was increased in xenografts with the miR-125a inhibitor in comparison with xenografts with mock (Fig. 11D), further confirming that miR-125a was responsible for the reduced tumor growth in nude mice via targeting the 3’UTR of ESRRA.

DISCUSSION

Using a combination of in silico, in vitro, and in vivo studies, we have shown for the first time that miR-125a post-transcriptionally regulates the level and function of ESRRA. The only other miRNA known to regulate the level of ESRRA is miR-137 in breast cancer cell lines (15). Our results also show that miR-137 targets ESRRA in OSCC cell lines (Fig. 1). miR-125a, located on chromosome 19q13.3, is frequently down-regulated in several human cancers such as medulloblastoma (34) and ovarian (35), breast (36), lung (37), and stomach (38) cancers. It has been documented that the EGF receptor signaling suppresses the expression of miR-125a, leading to cancer metastasis in ovarian (39) and lung cancers (37). Also, miR-125a has been shown to be the negative regulator of epithelial to mesenchymal transition in ovarian cancer (39). Interestingly, it is also reported to be one of the frequently down-regulated miRNAs in OSCC cell lines as compared with an immortalized human oral keratinocyte cell line (40). Furthermore, it is significantly down-regulated in saliva samples from OSCC patients as compared with normal controls (41).

Promoter methylation has been shown to be the mechanism underlying down-regulation of miR-125a in acute myeloid leukemia (32). Our results of the AZA-treated OSCC cells show that the promoter methylation is one of the mechanisms for down-regulation of miR-125a in OSCC (Fig. 9B). Previous reports suggest that down-regulation of miR-125a correlates with poor prognosis of gastric cancer and hepatocellular carcinoma, indicating its clinical significance in tumor progression.
All these lines of observations strongly suggest that miR-125a functions as a tumor suppressor miRNA in human cancers, including OSCC. Of note, miR-125a is involved in the pathogenesis of breast and gastric cancers via targeting of ERBB2 and ERBB3 (43). It is also known to target the MMP11 and VEGF genes and regulates cell proliferation and metastasis of hepatocellular carcinoma (42). However, no target has been identified for miR-125a in OSCC until now. Ours is the first study to document the involvement and role of miR-125a in the pathogenesis of OSCC. The expression analysis of miR-125a in OSCC samples showed that it is down-regulated in a majority (16/20) of OSCC patient samples as compared with their normal counterparts (Fig. 3A).

Although up-regulation of ESRRA has been shown earlier in breast, colorectal, prostate, and ovarian cancers (4–8), ours is the first study to show its up-regulation in OSCC (Fig. 3B). As expected, we also observed an inverse correlation between the levels of miR-125a and ESRRA in 11/15 OSCC patient samples (Fig. 3), suggesting a key role for miR-125a in tumorigenesis of OSCC via targeting ESRRA. However, we did not observe any correlation in four OSCC samples (viz. patient numbers 110, 135, 174, and 191), which could be either due to tumor heterogeneity or the involvement of an alternative mechanism. Interestingly, miR-125a down-regulated ESRRA at the protein level in SCC084 and SCC131 cells but not at the mRNA level (Fig. 1C). On the contrary, miR-125a has been shown to regulate the ERBB2, ERBB3, and VEGF genes at both the mRNA and protein levels (42, 43). Furthermore, similar to our observation, Parisi et al. (44) and Guo et al. (36) have also shown that miR-125a does not affect the mRNA levels of its target genes DIES1 and HuR, but it affects the mRNA levels of their target genes is complex and may vary with their targets. For example, our results show a transcript and protein level difference in the complex nature of the regulation by miR-125a and its target gene. Moreover, we also observed that the target mRNAs were either degraded or not degraded (22).

We further investigated whether the miR-125a-mediated knockdown of ESRRA is reflected on cell proliferation, apoptosis, anchorage-independent growth, and invasion of OSCC cells transiently transfected with different ESRRA constructs (Figs. 5–8). Ectopic overexpression of miR-125a via a synthetic mimic not only led to the knockdown of ESRRA but also reversed the effect of ESRRA by inhibiting cell proliferation, accelerating apoptosis, and reducing growth of nude mouse xenografts (Figs. 4 and 11). Given the crucial role of ESRRA in the regulation of energy metabolism, it is not surprising that it can accelerate cancer cell proliferation. Until now, many reports have suggested its potential involvement in the regulation of cancer cell proliferation (11–14). Additionally, Wu et al. (45) have demonstrated that an inverse agonist of ESRRA, XCT-790, can also induce cell death in a multidrug resistance cell line HepG2 (human liver hepatocellular carcinoma cell line). Similarly, our results showed that a reduced level of ESRRA via treating the OSCC cells by the miR-125a mimic increased the rate of apoptosis (Fig. 4D).

We further investigated the effect of miR-125a-mediated knockdown on the level of ESRRA on anchorage-independent cell growth, which is one of the hallmarks of malignant transformation of cells. Our results showed that miR-125a regulates the anchorage-independent growth of SCC084 and SCC131 cells largely by targeting ESRRA (Figs. 7A and 8A). This result is in agreement with an earlier report wherein the treatment with XCT790 is shown to impede the colony number in KSR1 (Kinase Suppressor of Ras1) null mouse embryonic fibroblasts derived from 13.5-day-old embryos (46).
We also investigated the effect of a reduced level of ESRRA on cell invasion. The results showed that miR-125a regulates the invasive ability of SCC084 and SCC131 cells largely by targeting ESRRA (Figs. 7B and 8B). This finding is in agreement with an earlier report where ESRRA has been shown to promote cell migration and invasion of breast cancer cells (15). It has been recently shown that RNAi-mediated inhibition of ESRRA results in a significantly reduced tumor burden, ascites formation, and metastatic peritoneal implants in vivo in an orthotopic model of ovarian cancer (47). Furthermore, Lam et al. (47) have shown that targeted inhibition of ESRRA inhibits the expression of SNAI1 (Snail family zinc finger 1) and in turn attenuates epithelial to mesenchymal transition in ovarian cancer cells. The mechanisms underlying these effects can be attributed to the ESRRA-driven regulation of several genes involved in cancer progression and development such as WNT11, CCNE1, OPN, and OPG, which are the crucial genes involved in increasing the proliferative and migratory capacity of cells (17, 48, 49).

Furthermore, OPN is a well known gene involved in the regulation of proliferation, migration, and invasion of OSCC cells (49). Our results showed that miR-125a-mediated targeting of ESRRA indeed regulates the levels of WNT11 and OPN (Fig. 10), which in turn regulate OSCC cell proliferation and invasion. Collectively, our findings highlight a key role that miR-125a plays in OSCC tumorigenesis by targeting ESRRA. However, it is well known that a particular miRNA may target several genes, and similarly a given gene may be targeted by several miRNAs (36). Therefore, miR-125a is exerting its tumor suppressor function, in part, by targeting ESRRA.

Studies on miRNAs showing loss of function (e.g. let-7 and miR-34) in a broad range of tumors suggest that they play a crucial role in tumor suppression (28). Therefore, a replacement therapy of these miRNAs would provide a new therapeutic strategy. The most accepted way to re-express an miRNA is via introducing a synthetic miRNA mimic, which can mimic the function of miRNA and is highly stable due to modifications.

**FIGURE 10.** Effect of miR-125a-mediated down-regulation of ESRRA on its transcriptional targets. A, qRT-PCR analysis to assess the level of WNT11 transcript in cells transfected with mock, miR-125a mimic, and miR-125a inhibitors. B, qRT-PCR analysis to assess the level of OPN transcript in cells transfected with mock, miR-125a mimic, and miR-125a inhibitor. *, p < 0.05, and **, p < 0.01.

**FIGURE 11.** Restoration of miR-125a level suppresses tumorigenicity in vivo. A, effect of miR-125a mimic and miR-125a inhibitor on SCC084 cell-derived xenografts in nude mice. Top panel, photographs of nude mice showing tumor growth on day 30 after injection. Bottom panel, excised xenografts on day 30. B, effect of miR-125a mimic and miR-125a inhibitor on weight of xenografts on day 30. C, effect of miR-125a mimic and miR-125a inhibitor on volume of xenografts during a time course of 30 days. D, Western blot analysis to assess the level of ESRRA in xenografts on day 30. N1, N2, M1, M2, I1, and I2 are different nude mice. *, p < 0.05; **, p < 0.01, and ns, nonsignificant.
(viz. 2’-O-methoxyethyl and 2’-fluoro) in it. Furthermore, the mimic-based replacement therapy is unlikely to show nonspecific off-target effects as mimics are designed to behave similarly to their natural counterparts (50). Being accessible from outside, miRNA mimics could be easily and directly injected at the OSCC tumor sites. Thus, our preclinical study in nude mice could provide a platform for designing a therapeutic strategy for the treatment of OSCC via the use of an miR-125a mimic.

In summary, the results of this study show for the first time that ESRR

ACKNOWLEDGMENTS—We thank the patients for their participation in this study. We also thank Dr. M. I. Rather for critically reading the manuscript and suggestions. We are grateful to Prof. Eric R. Fearon and four anonymous reviewers for their suggestions to improve the manuscript.

REFERENCES

1. Tremblay, A. M., and Giguère, V. (2007) The NR3B subgroup: an overview. Nucl. Recept. Signal. 5, e009–e009

2. Giguère, V., Yang, N., Segui, P., and Evans, R. M. (1988) Identification of a new class of steroid hormone receptors. Nature 331, 91–94

3. Sladek, R., Bader, J. A., and Giguère, V. (1997) The orphan estrogen-related receptor α is a transcriptional repressor of the medium-chain acyl coenzyme A dehydrogenase gene.

4. Ariazi, E. A., Clark, G. M., and Mertz, J. E. (2007) Estrogen-related receptor α and estrogen-related response elements: Potentially favorable biomarkers, respectively. J. Natl. Cancer Inst. 99, 6510–6518

5. Cavallini, A., Notarnicola, M., Visconti, A., Minervini, F., Gualberto, A., and Gualberto, G. (2003) In vivo and in vitro expression of estrogen-related receptor α and expression in normal and malignant breast tissue. J. Clin. Endocrinol. Metab. 88, 4124–4130

6. Cheung, C. P., Yu, S., Wong, K. B., Chan, L. W., Lai, F. M., Wang, X., Suetsugi, M., Chen, S., and Chan, F. K. (2003) Expression and functional study of estrogen receptor-related receptors in human prostatic cells and tissues. J. Clin. Endocrinol. Metab. 90, 1830–1844

7. Sun, P., Sehouli, J., Denkert, C., Mustea, A., Kösngen, D., Koch, I., Wei, L., and Lichtenegger, W. (2005) Expression of estrogen receptor-related receptors, a subfamily of orphan nuclear receptors, as new tumor biomarkers in ovarian cancer cells. J. Mol. Med. 83, 457–467

8. Bernatchez, G., Giroux, V., Lassalle, T., Carpentier, A. C., Rivard, N., and Giguère, V. (2004) The NR2B subgroup: an overview. J. Biol. Chem. 279, 27–36

9. Suzuki, T., Miki, Y., Moriya, T., Shimada, N., Ishida, T., Hirakawa, H., Inoue K., Ouchi, Y., Kitamura, T., Muramatsu, M., and Inoue, S. (2007) Inverse agonist XCT-790 arrests A549 lung cancer cell population growth by inducing mitochondrial reactive oxygen species production. Cell Prolif. 43, 103–113

10. Wang, J., Wang, Y., and Wong, C. (2010) Oestrogen-related receptor α inverse agonist XCT-790 arrests A549 lung cancer cell population growth by inducing mitochondrial reactive oxygen species production. Cell Prolif. 43, 103–113

11. Mori, T., Sawada, M., Kuroboshi, H., Tatsumi, H., Katsuyama, M., Iwasaki, K., and Kitawaki, J. (2011) Estrogen-related receptor α expression and function are associated with vascular endothelial growth factor in human cervical cancer. Int. J. Gynecol. Cancer 21, 609–615

12. Zhao, Y., Li, Y., Lou, G., Zhao, L., Xu, Z., Zhang, Y., and He, F. (2012) miR-137 targets estrogen-related receptor α and impairs the proliferative and migratory capacity of breast cancer cells. PLoS One 7, e39102

13. Debois, G., Chahrou, G., Perry, M. C., Sylvain-Drolet, G., Muller, W. I., and Giguère, V. (2010) Transcriptional control of the ERBB2 ampiclon by ERRe and PGC-1α promotes mammary gland tumorigenesis. Cancer Res. 70, 10277–10287

14. Debois, G., and Giguère, V. (2013) Oestrogen-related receptors in breast cancer: control of cellular metabolism and beyond. Nat. Rev. Cancer 13, 27–36

15. Ariazi, E. A., Kraus, R. J., Farrell, M. L., Jordan, V. C., and Mertz, J. E. (2007) Estrogen-related receptor α transcriptional activities are regulated in part via the ErbB/HER2 signaling pathway. Mol. Cancer Res. 5, 71–85

16. Chaverey, C., Giroux, S., Rousseau, F., and Giguère, V. (2003) Estrogen-related receptor α (ERRA) promoter directs ERα expression. Mol. Cancer 2, 279, 18504–18510

17. Pathare, S. M., Gerstung, M., Beerenwinkel, N., Schäffer, A. A., Kannan, S., Einspanier, R. (2008) miR-Q: a novel quantitative RT-PCR approach for microRNA quantification. BMC Mol. Biol. 9, 10

18. Ariazi, E. A., Clark, G. M., and Mertz, J. E. (2007) Estrogen-related receptor α and estrogen-related response elements: Potentially favorable biomarkers, respectively. J. Natl. Cancer Inst. 99, 6510–6518

19. Sun, P., Sehouli, J., Denkert, C., Mustea, A., Köngsen, D., Koch, I., Wei, L., and Lichtenegger, W. (2005) Expression of estrogen receptor-related receptors, a subfamily of orphan nuclear receptors, as new tumor biomarkers in ovarian cancer cells. J. Mol. Med. 83, 457–467

20. Bernatchez, G., Giroux, V., Lassalle, T., Carpentier, A. C., Rivard, N., and Carrier, J. C. (2013) The ERRα metabolic nuclear receptor controls growth of colon cancer cells. Carcinogenesis 34, 2253–2261

21. Suzuki, T., Miki, Y., Moriya, T., Shimada, N., Ishida, T., Hirakawa, H., Ohuchi, N., and Sasano, H. (2004) Estrogen-related receptor α in human breast carcinoma as a potent prognostic factor. Cancer Res. 64, 4670–4676

22. Fujimura, T., Takahashi, S., Urano, T., Kumagai, J., Ogushi, T., Horie-Inoue K., Ouchi, Y., Kitamura, T., Muramatsu, M., and Inoue, S. (2007) Increased expression of estrogen-related receptor α (ERRα) is a negative prognostic predictor in human prostate cancer. Int. J. Cancer 120, 2325–2330

23. Bianco, S., Lanvin, O., Tribollet, V., Macari, C., North, S., and Vanacker, J. M. (2009) Modulating estrogen receptor-related receptor-α activity inhibits cell proliferation. J. Biol. Chem. 284, 23286–23292

24. Gandhadi, M. K., Frazier, C. R., Hartenstein, J. S., Cloix, J. F., Bernier, M., and Wainer, I. W. (2010) Identification and characterization of estrogen receptor-related receptor α and γ in human glioma and astrocytoma cells. Mol. Cell. Endocrinol. 315, 314–318

25. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. (1999) Cancer statistics, 1999. CA Cancer J. Clin. 49, 8–31

26. Pathare, S. M., Gerstung, M., Beerenwinkel, N., Schäffer, A. A., Kannan, S., Pai, P., Pathak, K. A., Borges, A. M., and Mahimkar, M. B. (2011) Clinico-pathological and prognostic implications of genetic alterations in oral cancer. Oncol. Rep. 26, 1003–1010

27. Ariazi, E. A., Clark, G. M., and Mertz, J. E. (2007) Estrogen-related receptor α and estrogen-related response elements: Potentially favorable biomarkers, respectively. J. Natl. Cancer Inst. 99, 6510–6518

28. Bouché, A. (2013) First microRNA mimic enters clinic. Nat. Biotechnol. 31, 577–577

29. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 3 Vol. 2, pp. 13.19–13.25, Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY
MicroRNA-125a regulates ESRRA

33. Venkatesh, T., Nagashri, M. N., Swamy, S. S., Mohiyuddin, S. M., Gopinath, K. S., and Kumar, A. (2013) Primary microcephaly gene MCPH1 shows signatures of tumor suppressors and is regulated by miR-27a in oral squamous cell carcinoma. *PloS One* 8, e54643

34. Ferretti, E., De Smaele, E., Po, A., Di Marcotullio, L., Tosi, E., Espinola, M. S., Di Rocco, C., Riccardi, R., Giangaspero, F., Farcomeni, A., Nofroni, I., Laneve, P., Gioia, U., Caffarelli, E., Bozzoni, I., Srepani, I., and Gulino, A. (2009) MicroRNA profiling in human medulloblastoma. *Int. J. Cancer* 124, 568–577

35. Nam, E. J., Yoon, H., Kim, S. W., Kim, Y. T., Kim, J. H., Kim, J. W., and Kim, S. (2008) MicroRNA expression profiles in serous ovarian carcinoma. *Clin. Cancer Res.* 14, 2690–2695

36. Guo, X., Wu, Y., and Hartley, R. S. (2009) MicroRNA-125a represses cell growth by targeting HuR in breast cancer. *RNA Biol.* 6, 575–583

37. Wang, G., Mao, W., Zheng, S., and Ye, J. (2009) Epidermal growth factor receptor-regulated miR-125a-5p-a metastatic inhibitor of lung cancer. *FEBS J.* 276, 5571–5578

38. Nishida, N., Mimori, K., Fabbi, M., Yokobori, T., Sudo, T., Tanaka, F., Shibata, K., Ishii, H., Doki, Y., and Mori, M. (2011) MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab. *Clin. Cancer Res.* 17, 2725–2733

39. Cowden Dahl, K. D., Dahl, R., Kruijshak, J. N., and Hudson, L. G. (2009) The epidermal growth factor receptor responsive miR-125a represses mesenchymal morphology in ovarian cancer cells. *Neoplasia* 11, 1208–1215

40. Kozaki, K., Imoto, I., Mogi, S., Omura, K., and Inazawa, J. (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. *Cancer Res.* 68, 2094–2105

41. Park, N. J., Zhou, H., Elashoff, D., Henson, B. S., Kastratovic, D., mayor, E., and Wong, D. T. (2009) Salivary microRNA: diagnostic characterization, and clinical utility for oral cancer detection. *PLoS One* 7, e40169

42. Bi, Q., Tang, S., Xia, L., Du, R., Fan, R., Gao, L., Jin, J., Liang, S., Chen, Z., and Xu, G. (2012) Ectopic expression of miR-125a inhibits the proliferation and metastasis of hepatocellular carcinoma by targeting MMP11 and VEGF. *PloS One* 7, e40169

43. Scott, G. K., Goga, A., Bhaumik, D., Berger, C. E., Sullivan, C. S., and Benz, C. C. (2007) Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro- RNA miR-125a or miR-125b. *J. Biol. Chem.* 282, 1479–1486

44. Parisi, S., Battista, M., Musto, A., Navarra, A., Tarantino, C., and Russo, T. (2012) A regulatory loop involving Dies1 and miR-125a controls BMP4 signaling in mouse embryonic stem cells. *FASEB J.* 26, 3957–3968

45. Yu, F., Wang, J., Wang, Y., Kwok, T. T., Kong, S. K., and Wong, C. (2009) Estrogen-related receptor α (ERRα) inverse agonist XCT-790 induces cell death in chemotherapy-resistant cancer cells. *Chem. Biol. Interact.* 181, 236–242

46. Fisher, K. W., Das, B., Kortum, R. L., Chaika, O. V., and Lewis, R. E. (2011) Kinase suppressor of ras 1 (KSR1) regulates PGC1α and estrogen-related receptor α to promote oncogenic ras-dependent anchorage-independent growth. *Mol. Cell. Biol.* 31, 2453–2461

47. Lam, S. S., Mak, A. S., Yam, J. W., Cheung, A. N., Ngan, H. Y., and Wong, A. S. (2014) Targeting estrogen-related receptor α inhibits epithelial to mesenchymal transition and self-renewal properties of ovarian cancer cells. *Mol. Ther.* 22, 2725–2733

48. Ouko, L., Ziegler, T. R., Gu, L. H., Eisenberg, L. M., and Yang, V. W. (2004) Wnt11 signaling promotes proliferation, transformation, and migration of IEC6 intestinal epithelial cells. *J. Biol. Chem.* 279, 26707–26715

49. Meng, X. K., Su, X. L., Hou, J. B., Zhang, N., Li, T., Li, Y., Xie, X., Shi, L. (2014) Osteopontin mediates the tumorigenicity of colon cancer cells. *Cancer Res.* 74, 118

50. Bader, A. G., Brown, D., and Winkler, M. (2010) The promise of microRNA replacement therapy. *Cancer Res.* 70, 7027–7030