Mutant p53 (p53-R248Q) functions as an oncogene in promoting endometrial cancer by up-regulating REGγ

Huihui Wang a,1, Wei Bao b,1, Feizhou Jiang a, Qi Che a, Zheng Chen a, Fangyuan Wang a, Huan Tong a, Chenyun Dai c, Xiaoying He b, Yun Liao d, Binya Liu d, Jing Sun d, Xiaoping Wan d,*

a Department of Obstetrics and Gynecology, Shanghai First People's Hospital affiliated with Shanghai Jiao Tong University, Shanghai, China
b Department of Obstetrics and Gynecology, International Peace Maternity and Child Health Hospital affiliated with Shanghai Jiao Tong University, Shanghai, China
c Center Laboratory of Shanghai First People's Hospital affiliated with Shanghai Jiao Tong University, Shanghai, China
d Department of Obstetrics and Gynecology, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, China

ARTICLE INFO

Article history:
Received 24 November 2014
Received in revised form 11 February 2015
Accepted 12 February 2015

Keywords:
EC
Mutant p53
REGγ
Proliferation
Invasion
EMT

ABSTRACT

P53 mutation plays a pivotal role in tumorigenesis of endometrial cancer (EC), here we report that the gain-of-function mutant p53-R248Q targets the proteasome activator REGγ to promote EC progression. Increased p53 expression significantly correlated with high pathological grade and lymph node metastasis in EC specimens. Manipulation of p53-R248Q in EC cells caused coincident changes in REGγ expression, and chromatin immunoprecipitation coupled with PCR further indicated that p53-R248Q bound to the REGγ gene promoter at a p53 responsive element. Silencing of REGγ in EC cells attenuated the cell proliferation, migration and invasion abilities, whereas overexpression of p53-R248Q rescued these activities. Overexpression of REGγ also induced an epithelial–mesenchymal transition phenotype. Moreover, a mouse xenograft tumor model showed that REGγ promoted tumor growth, further demonstrating a p53-R248Q–REGγ oncogenic pathway. Finally, examination of EC and normal endometrium specimens confirmed the oncogenic role of REGγ, in that REGγ was more highly overexpressed in p53-positive specimens than in p53-negative specimens. Our data suggest that REGγ is a promising therapeutic target for EC with the p53-R248Q mutation.

© 2015 Elsevier Ireland Ltd. All rights reserved.

Introduction

Endometrial cancer (EC) is one of the most common gynecological malignancies, ranking 4th among expected new cancer cases in women in the United States in 2015, with 10,170 deaths estimated [1]. The risk of metastasis, recurrence and resistance to chemotherapy is increasing in a comparatively small proportion of EC patients [2] although the majority of cases are curable [3]. This subset of patients fail to respond to the conventional cytotoxic and radiation therapies [4]. Therefore, it is crucial to elucidate the molecular pathways driving this disease.

The tumor suppressor gene TP53, which encodes p53, is mutated in more than half of human tumors and affects tumor development, progression and response to therapy [5–7]. P53 mutation occurs in approximately 20%–50% in endometrioid adenocarcinomas and up to 90% in papillary serous and clear cell carcinomas [8–13], but it is still unclear how mutant p53 contributes to this disease. Although there has been no published data of p53-R248Q mutation frequency in EC, the overall frequency obtained from a p53 database of 25,902 different human cancers is 3.5%, second only to R175H (4.6%) among all hot spot’ mutations [14], and this gain-of-function (GOF) mutant appears to accelerate onset of multiple tumor types [15] and shows a significant association with shorter patient survival [16]. Our study focuses on the endometrioid subtype of EC which constitutes about 90% of this disease [17] and we try to reveal whether p53-R248Q exerts a certain influence over it.

Cancer cells are able to tolerate cellular stresses like oxygen stress, DNA damage and aneuploidy through activation of the proteasome pathways [18–20]. REGγ (also called PA28γ, PSME3 or Ki antigen) is a subunit of the 11S activator, which binds and activates the 20S proteasome to degrade specific proteins [21,22], such as cell-cyclce inhibitors and SRC-3, in a ubiquitin and ATP-independent manner [23–25]. REGγ is reported to be overexpressed in thyroid [26], colorectal [27], breast [28] and laryngeal cancers [29], and is predicted to be associated with multiple oncogenic pathways [30]. The fact that proteasome inhibitors induce apoptosis of EC cells [31] suggests that REGγ-associated proteasome activity plays a role in EC.
A p53 binding site exists within the REGγ promoter and REGγ transcription can be inhibited by wild-type p53 binding and activated by mutant p53 binding [32], thus prompting us to investigate whether the p53 GOF mutant operates through modulation of REGγ in EC.

Here we report that REGγ promotes oncogenic properties of EC cells, and this function is promoted by mutant p53-R248Q.

Materials and methods

Tissue collection

EC and normal endometrium tissues were obtained from patients who underwent surgery at the Shanghai Jiao Tong University affiliated International Peace Maternity and Child Health Hospital (IPMCH, Shanghai, China) from 2011 to 2013. The tissue samples were embedded in paraffin for sectioning and histological evaluation. The stages and histological grades of the tumors were determined according to the criteria of the 2009 International Federation of Gynecology and Obstetrics (FIGO) surgical staging system [33]. None of the patients underwent neoadjuvant or endocrine therapy before surgery. The research project was approved by the Human Investigation Ethical Committee of IPMCH.

Cell culture

The human EC lines HEC-1B, Ishikawa, AN3CA and SPEC-2 were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (HyClone, Waltham, MA) with 10% (v/v) fetal bovine serum (FBS) (Gibco, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO2.

Transfection with small interfering RNA (siRNA) and plasmids

The siRNA targeting REGγ (siREGγ) and p53 (sip53) and the negative control (siNC) were purchased from Genepharma (Shanghai, China). The REGγ overexpression plasmid pCMV/EGFP/Neo-REGγ (exREGγ) and the empty plasmid (exNC) were purchased from Genechem (Shanghai, China). The REGγ overexpression plasmid pCMV/EGFP/Neo-R248Q (exR248Q) was purchased from Biscien (Guangzhou, China). The cells were transfected with the siRNA or plasmid or co-transfected in OptiMEM (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (11668-019; Invitrogen) as described by the manufacturer. The sequences of the RNA oligonucleotides are provided in Supplementary Table S1.

Transfection with lentivirus

Lentivirus carrying small hairpin RNA (shRNA) targeting REGγ (shREGγ; Genepharma) or p53 (shp53; Genepharma) was transfected into cells at 70% confluency in 12-well culture plates at a multiplicity of infection of 5 with polybrene (5 μg/ml; Genepharma). The medium was replaced with fresh medium after 24 h of transfection and cell were incubated for another 72 h before analysis of mRNA or protein expression. The sequences of the shRNAs are provided in Supplementary Table S1.

Quantitative reverse transcription PCR (qRT-PCR), western blot and immunohistochemistry (IHC)

Total RNA extraction, qRT-PCR, western blot and IHC were performed as described previously [34]. The primers used for qRT-PCR are listed in Supplementary Table S2. Antibodies used in western blot are: REGγ (700180; 1:1000; Invitrogen), p53 (75523; 1:1000; Cell Signaling Technology, Danvers, MA), E-cadherin (3879; 1:1000; Cell Signaling Technology) or Vimentin (3195; 1:100; Cell Signaling Technology) in the HEC-1B cell line. A 392bp p53-binding site was identified –2 kb upstream of the REGγ transcription start site previously [32] and by qRT-PCR in this study. Enrichment was calculated using the comparative Ct method. IgG was used as negative control. The primers are listed in Supplementary Table S2. The amplification products were detected by 2% agarose gel electrophoresis with ethidium bromide staining.

Colony formation assay

Cells were seeded into six-well plates (200 cells/well) and the plates were incubated at 37 °C with 5% CO2 for two weeks to form colonies. The cell colonies were fixed with 4% methanol and stained with 5% crystal violet.

Cell invasion assay

Cells were seeded into Matrigel coated transwell chambers (BD Biosciences, Shanghai, China; 2 × 104 cells/chamber) in 200 μl serum-free DMEM/F12 (HyClone). A volume of 600 μl DMEM/10% FBS was added into the lower chamber. After 48 h, cells attached to the underside of the chamber membrane were fixed with 4% methanol and stained with 5% crystal violet.

Wound-healing assay

Wound-healing assay was performed as described previously [35]. The migration index was defined as the distance traveled by the cell monolayer relative to the gap made by the pipette tip at 0 h.

Chromatin immunoprecipitation (ChIP)–PCR and agarose gel electrophoresis

ChIP assays were performed as previously described [36] using anti-p53 (2527; 1:50; Cell Signaling Technology) in the HEC-1B cell line. A 392 bp p53-binding site was identified –2 kb upstream of the REGγ transcription start site previously [32] and by qRT-PCR in this study. Enrichment was calculated using the comparative Ct method. IgG was used as negative control. The primers are listed in Supplementary Table S2. The amplification products were detected by using a confocal microscope (Leica TCS SP8).

Cell immunofluorescence

Cells were seeded into 35 mm confocal dishes and cultured in DMEM/10% FBS overnight. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with goat serum before incubation with antibodies against E-cadherin (3195; 1:1000; Cell Signaling Technology) or Vimentin (5741; 1:1000; Cell Signaling Technology) at 4 °C overnight. The cells were then incubated with tetra-ethyl rhodamine isothiocyanate-conjugated secondary antibody (114768; 1:200; Jackson ImmunoResearch, West Grove, PA) and counterstained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Beyotime) before being analyzed using a confocal microscope (Leica TCS SP8).

Peritoneal disseminated tumor growth assay and xenograft tumor-formation assay

Female athymic nude mice 5–6 weeks of age were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Science. For peritoneal disseminated tumor growth assay, mice were randomly divided into two groups (five in each group). HEC-1B cells transfected with shNC or shREGγ were injected into peritoneally into each group at 2 × 106 cells per mouse. After five weeks of injection, the mice were euthanized and laparotomy was performed to detect any disseminated tumors that ≥1 mm in diameter. The volume of dissemination was measured by the sum of all disseminated tumors. For xenograft tumor-formation assay, mice were randomly divided into three groups (five in each group). HEC-1B cells transfected with shNC, shREGγ or shp53 were injected subcutaneously into each group at 1 × 107 cells per mouse. Tumor size was measured every week for six weeks. The tumor volume above was calculated as follows: tumor volume (mm3) = (longest diameter) × (shortest diameter)2 × 0.5. At endpoint the tumors were removed, fixed with formalin and sectioned to perform Hematoxylin and eosin (H&E) and IHC staining. These experiments were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Department of Laboratory Animal Science at Shanghai Jiao Tong University School of Medicine.

Hoechst 33342 staining for cultured cells and tissue specimens

Cultured cells were fixed with 4% paraformaldehyde and tissue sections (4 μm thick) of the paraffin-embedded specimens were deparaffinized and dehydrated before they were stained with 10 μg/ml Hoechst 33342 (Beyotime) for 15 min at room temperature in the dark, and were observed and imaged by a fluorescence microscope (Leica DMI 3000B).

Statistical analysis

Data were analyzed by the Statistical Package for Social Sciences software, version 17.0 (Chicago, IL) using the unpaired Student’s t-test for comparison between two groups and the 2 × 2 tables to compare the categorical data. P ≤ 0.05 was considered statistically significant. Each experiment was performed at least three times independently.
Results

Mutant p53 expression in EC tissues

EC tumors harboring p53 mutations tend to show strong p53 immunoreactivity, whereas wild-type p53 tumors show no or only weak staining [8]. We carried out p53 immunohistochemistry in 81 endometrioid adenocarcinoma specimens and analyzed correlations with clinicopathological parameters. We found 35% (28/81) of the specimens showed positive nuclear p53 staining, and its expression positively correlated with tumor grade and lymph node metastasis (P < 0.001; Supplementary Table S3). Moreover, we noted three distinct staining patterns in the specimens, with positive staining throughout or within a portion of all endometrial glands or only in a subset of endometrial glands (Fig. 1).

Mutant p53-R248Q functions as an upstream signal of REGγ and directly binds to the REGγ promoter

Because the p53 GOF mutant is known to promote cancer progression including enhancing transcription of REGγ in breast and colon cancers [32], we examined p53 and REGγ expression in four EC cell lines by western blot (Fig. 2A). HEC-1B cells harbor the R248Q mutation [36] and showed relatively strong staining. REGγ levels varied across the four cell lines and the highest level was also detected in HEC-1B. As the R248Q mutation correlates with malignancy [15,16,37–39], we speculated that the high levels of the two proteins in HEC-1B indicated that p53-R248Q enhances REGγ functions in EC.

To elucidate whether p53-R248Q influences REGγ expression, we examined REGγ mRNA and protein after manipulating p53-R248Q levels by p53-R248Q siRNA knockdown in HEC-1B cells and p53-R248Q overexpression from a plasmid in Ishikawa cells [32]. Consistent with the findings above, the invasion ability of HEC-1B and Ishikawa cells was significantly reduced when REGγ was knocked down or recovered in the exR248Q co-transfected cells (Fig. 3D). However, the expression of REGγ upon siREGγ and siREGγ+exR248Q transfection appears not significantly different (Fig. 3E), indicating overexpression of p53-R248Q in REGγ-depleted cells may have rescued cell proliferation, migration and invasion by targeting other pathways than REGγ.

Depletion of REGγ in HEC-1B and Ishikawa cells reduced cell proliferation rate. However, the proliferation rate recovered when cells were transfected simultaneously with exR248Q (Fig. 3A). To determine whether the declined cell proliferation upon siREGγ treatment is due to increased apoptosis, we performed Hoechst 33342 staining. At 48 h after transfection, the apoptotic cells showing condensed or fragmented nuclei in the siREGγ group exceeded those in the siNC group (Supplementary Fig. S1). In a colony formation assay, colonies of siREGγ cells were significantly fewer than those of siNC cells, and overexpression of R248Q rescued the number of colonies (Fig. 3B). A wound-healing assay of HEC-1B cells showed the weakened migration ability of siREGγ cells, which was regained when cells were co-transfected with exR248Q (Fig. 3C). Consistent with the findings above, the invasion ability of HEC-1B and Ishikawa cells was significantly reduced when REGγ was knocked down and recovered in the exR248Q co-transfected cells (Fig. 3D). However, the expression of REGγ upon siREGγ and siREGγ+exR248Q transfection appears not significantly different (Fig. 3E), indicating overexpression of p53-R248Q in REGγ-depleted cells may have rescued cell proliferation, migration and invasion by targeting other pathways than REGγ.

Furthermore, we also tested whether REGγ overexpression could drive enhanced malignance. The Ishikawa cells showing lower REGγ levels than HEC-1B (Fig. 2A) were transfected with exREGγ and the up-regulation of REGγ was verified by western blot (Supplementary Fig. S2A). Cell proliferation and invasion abilities were significantly promoted by REGγ overexpression as shown in Supplementary Fig. S2B–D, further confirming the oncogenic function of REGγ in EC cells.

Because p53-R248Q is known to induce EMT in EC cells [40], we next examined whether this induction is another downstream process of REGγ. Several EMT-characteristic proteins were...
Fig. 2. Mutant p53-R248Q transactivates REGγ. (A) P53 and REGγ levels in four EC cell lines examined by western blot. GAPDH was included as an internal control. (B) qRT-PCR and (C) western blots of p53 and REGγ level in sip53-transfected p53-R248Q-carrying HEC-1B cells. Values in (B) are the mean ± SD from at least three independent experiments. GAPDH in (C) was included as an internal control. (D) qRT-PCR and (E) western blots of p53 and REGγ level in p53-R248Q-overexpressing Ishikawa cells. Values in (D) are the mean ± SD from at least three independent experiments. GAPDH in (E) was included as an internal control. (F) Schematic representation of the p53 responsive element in the REGγ promoter and the three pairs of primers used for ChIP–PCR. TSS: transcription start sites. (G) Immunoprecipitated DNA fragments of HEC-1B cells were examined by qRT-PCR with the primer pairs shown in (F). IgG was used as the negative control. (H) Agarose gel electrophoresis of the amplicons in ChIP–PCR. (I) Western blots of p53-R248Q and REGγ in siREGγ-transfected HEC-1B cells and (J) R248Q-overexpressing Ishikawa cells. GAPDH was included as an internal control. ***P ≤ 0.001.
Fig. 3. REGγ promotes proliferation, migration and invasion of EC cells. (A) Assessment of cell proliferation in HEC-1B and Ishikawa cells transfected with siNC, siREGγ or siREGγ + exR248Q. (B) Left: Colony-formation assay of HEC-1B and Ishikawa cells transfected with siNC, siREGγ or siREGγ + exR248Q. Right: Graphical representation of the mean (±SD) number of colonies in three independent experiments. (C) Left: Wound-healing assay of HEC-1B cells transfected with siNC, siREGγ or siREGγ + exR248Q. Right: Graphical representation of the migration index. (D) Left: Cell invasion assay of HEC-1B and Ishikawa cells transfected with siNC, siREGγ or siREGγ + exR248Q. Right: Graphical representation of the mean (±SD) number of invasive cells in three independent experiments (HPF: high-power field). (E) Western blots of REGγ level in siNC, siREGγ and siREGγ + exR248Q transfected HEC-1B and Ishikawa cells. GAPDH was included as an internal control. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ns P > 0.05.
analyzed by western blot in HEC-1B cells (Fig. 4A). The mesenchymal markers Vimentin, Snail1 and N-cadherin decreased, whereas the epithelial marker E-cadherin increased in REGγ-depleted cells, and co-transfection with exR248Q reversed these trends. We further confirmed these results by E-cadherin and Vimentin immunofluorescence (Fig. 4B and Supplementary Fig. S3). To test whether REGγ potentiates EC metastasis in vivo, we performed peritoneal dissemination assay. HEC-1B cells stably transfected with shREGγ or shNC were injected peritoneally into nude mice. Pathological anatomy analysis of the disseminated tumors was carried out at five weeks after injection, which revealed a significant abrogation of tumor growth in the shREGγ group compared with the shNC group (Fig. 4C and D). REGγ expression was verified to be depleted in shREGγ disseminated tumors, relatively weaker staining of Vimentin and stronger staining of E-cadherin were detected in shREGγ tumors than shNC controls (Fig. 4E), demonstrating the metastasis-facilitating function of REGγ in vivo.

The R248Q–REGγ pathway in a xenograft model

To further evaluate the oncogenic role of REGγ in EC and to verify REGγ is regulated by p53-R248Q, we performed a xenograft tumor growth assay in nude mice subcutaneously injected with shRNA transfected HEC-1B cells. After six weeks, the average size of tumors in the shREGγ and shp53-R248Q groups was significantly smaller than that in the shNC group (Fig. 5A–C). To determine the effect of REGγ on proliferation and apoptosis, we performed IHC of Ki67 and PCNA and Hoechst 33342 staining in the tumor tissues. Substantially weaker Ki67 and PCNA staining was detected in shREGγ and shp53-R248Q tumor tissues than in the shNC controls (Fig. 5D and E), the positive-staining cells were quantified and indicated a significant suppression of proliferation upon REGγ and p53-R248Q depletion (Supplementary Fig. S4A and B). Also, significantly increased apoptotic cells were detected in shREGγ and shp53-R248Q xenografts (Fig. 5D and E and Supplementary Fig. S4C and D). Furthermore, REGγ immunoreactivity was greatly reduced in shp53 tumor tissues compared to the shNC tumor tissues (Fig. 5E), confirming that the p53-R248Q mutant regulates REGγ expression in vivo.

REGγ expression in EC tissues and its correlation with mutant p53 expression

We examined REGγ expression by IHC in 81 endometrioid adenocarcinoma samples and 46 normal endometrial tissues. REGγ was predominantly localized to the nucleus of endometrial epithelial cells. Weak or no staining was detected in normal endometrium, whereas moderate to strong staining was seen in EC tissues (Fig. 6A). In other words, the immunoreactivity scores of EC tissues were significantly higher than those of normal endometrium (Fig. 6B). Correlation analysis of REGγ levels with clinicopathological parameters of these specimens showed that significantly higher REGγ expression was detected in cases with lymph node metastasis (P = 0.003) and high pathological grade (P = 0.011) (Supplementary Table S4).

We then classified these specimens into p53-positive or -negative and REGγ-positive or -negative staining groups. Among the 28 p53-positive specimens, 26 (93%) were REGγ-positive and 2 (7%) were REGγ-negative, whereas among the 35 p53-negative specimens, 33 (94%) were REGγ-positive and 2 (6%) were REGγ-negative. A typical example of the positive correlation between p53 and REGγ expression is shown in Fig. 6C. A significantly higher proportion of p53-positive specimens were REGγ-positive compared with the p53-negative group (P = 0.008; Fig. 6D).

Discussion

Recent studies of mutant p53 have mainly focused on mutation hot spots at residues 175, 248, 249, 273 and 282 [14]. Mutations at these residues can interfere with the tumor suppression function of p53 through either dominant-negative or GOF effects, both of which are related to poor cancer prognosis [41,42]. P53 GOF mutations have not yet been extensively studied in EC, but the R175H, R248Q and R273H mutants have been examined. The R175H mutation increases the invasiveness of EC cells by activating the EGFR/PI3K/Akt pathway [43], and the R175H, R248Q and R273H mutations induce EMT through modulation of the miR-130b–ZEB1 axis [40].

In this study, we found that the p53-R248Q bound to the promoter and up-regulated transcription of REGγ, which was shown to have oncogenic functions in EC (Fig. 7). It is speculated that p53-R248Q may control transcription of REGγ by modulating other transcription factors, which will need more work to do before the exact mechanism is elaborated, and our work mainly focused on how the p53-R248Q–REGγ pathway functions in EC progression. We found deletion of REGγ in HEC-1B and Ishikawa cells reduced cell proliferation, migration and invasion, and transfection of cells with p53-R248Q partly restored these malignancy-related properties (Fig. 3A–D). The R248Q mutation also appears to promote EMT (Fig. 4A and B), somewhat consistent with previous findings [40]. Moreover, our western blot showed that the REGγ protein level in REGγ-depleted cells could not be rescued despite p53-R248Q overexpression (Fig. 3E). Since p53-R248Q has been proved to be a GOF mutant, we speculate these results indicate the overexpressed p53-R248Q may rescue malignance of the REGγ-depleted cells through other unknown pathways. It is reported that p53-R248Q can activate the miR-130b–ZEB1 axis [40], we cannot exclude this pathway in our experiments and we will verify it in future studies.

Our work is the first report of a p53 GOF mutant operating via the proteasome pathway in EC. The proteasome pathway is essential for basal protein turnover and degradation of misfolded proteins in normal cells, but in cancer cells, which frequently produce an excessive amount of non-functional proteins, protein turnover is up-regulated to maintain the malignant phenotype [44]. Therefore, targeting excessive proteasome activity is a strategy of some cancer treatments. For example, bortezomib is an FDA-approved proteasome inhibitor that has demonstrated broad anti-tumor activities in many malignancies, including inducing cell apoptosis in EC [45,46].

It was reported REGγ undergoes intranuclear redistribution at mitosis [47] and post-translational modification may involve its subcellular localization [48]. REGγ was found to be predominantly localized to the nucleus of EC specimens (Fig. 6A), consistent with published literatures about other cancers [26–29], but the underlying mechanism of its nuclear-localization remains largely unknown. REGγ was highly expressed in human EC tissues and positively correlated with pathological grade and lymph node metastasis (Supplementary Table S3). This is the first study to address the role of REGγ in EC, suggesting that the REGγ–20S proteasome pathway may play an oncogenic role by contributing to the degradation of proteins that normally prevent malignant transformation of endometrial epithelium, one of which may be wild-type p53 [49]. The finding that REGγ did not promote degradation of the mutant p53-R248Q, possibly due to a more stable conformation resulting from the point mutation, supports a hypothesis in which stabilized p53-R248Q up-regulates REGγ, which may in turn degrade wild-type p53 in EC, but further studies are necessary to verify this.

Proteasome inhibition has been shown to contribute to E-cadherin stabilization and Snail1 suppression [50,51], indicating that excessive proteasome activity may lead to aberrant degradation of
Fig. 4. REGγ induces EMT of EC cells. (A) Levels of EMT marker proteins in HEC-1B cells transfected with siNC, siREGγ or siREGγ + exR248Q analyzed by western blot. (B) Immunofluorescence of E-cadherin and Vimentin in HEC-1B cells transfected with siNC, siREGγ or siREGγ + exR248Q. (C) Laparotomy of nude mice to explore disseminated tumors of >1 mm in diameter. Arrows and the circled area show solitary tumors and tumor cluster separately. (D) Volume of disseminated tumors in shNC and shREGγ group. *P ≤ 0.05.

epithelial proteins and up-regulation of mesenchymal proteins through as yet unknown pathways. This is consistent with our findings with the proteasome activator REGγ in EC, but the molecular mechanisms by which REGγ induces EMT are still unknown.

On principle, the p53 antibody is unable to identify mutant p53 from wild-type one since it is not designed to be mutation site-specific. However, mutant p53 is reported to own a much longer half-life and to be more stable compared with wild-type one, the

Fig. 5. Tumorigenicity in a nude mouse xenograft model. (A) Tumors of mice injected with shNC-, shREGγ- and shp53-R248Q-transfected HEC-1B cells removed six weeks after injection. (B) Weights of shNC, shREGγ and shp53-R248Q tumors at endpoint. Values are the mean ± SD of five tumors in each group. (C) Volumes of each tumor measured each week for six weeks. Values are the mean ± SD of five tumors in each group. (D) H&E and immunohistochemical staining of REGγ, Ki67 and PCNA and Hoechst 33342 staining in the shNC and shREGγ xenograft tumors. (E) H&E and immunohistochemical staining of p53, REGγ, Ki67 and PCNA and Hoechst 33342 staining in the shNC and shp53-R248Q xenograft tumors.

wild-type p53 is vulnerable to degradation which makes it usually undetectable by antibodies [52–55], thus making it feasible to probe mutant p53 using the p53 antibody with only slight or even no influence of wild-type one. We detected positive p53 staining in 35% of our endometrioid adenocarcinoma specimens, primarily in higher-grade (G2–G3) tumors and those associated with lymph node metastasis (Supplementary Table S4), which agrees with previous reports [8–10]. Although p53 mutation frequency is higher in non-endometrioid EC, our data indicate that it could also be a GOF oncogene for endometrioid EC; however larger numbers of patients and long-term follow-up of appropriate clinical indices will help to confirm these correlations. Moreover, we found that significantly more of the p53-positive specimens were positive for REGγ than the p53-negative specimens (Fig. 6D), but as the p53 antibody was not R248Q-specific, it is possible that other p53 mutations are involved in the modulation of REGγ. It should be noted that there are still 66% p53-negative EC specimens showing positive REGγ staining (Fig. 6D). In fact, REGγ is reported to be regulated by other signals than mutant p53, the most studied is miR-7 [56–58], a tumor suppressor in many cancers. Therefore we suppose REGγ could be up-regulated by some undetermined molecules in these p53-negative specimens and cells. Besides, the sample size of our p53-positive EC (n = 28) didn’t equal that of p53-negative ones (n = 53), so these results need to be reconfirmed with a better-matched and larger sample set.

In conclusion, our experiments reveal that p53 correlates with malignance of EC and that the p53-R248Q mutant appears to target REGγ to promote EC progression. This represents a novel GOF mechanism for a p53 mutant in EC. We propose that targeting the REGγ proteasome pathway may be helpful in the treatment of ECs carrying the p53-R248Q mutation.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (grant numbers 81272885, 81172476, 81472427, 81402134 and 81001154).

Conflict of interest

None.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.02.028.
Fig. 7. Schematic diagram of the p53-R248Q–REGγ pathway in EC. P53-R248Q binds to the promoter of REGγ to enhance formation of the REGγ–20S proteasome by up-regulating transcription of REGγ in EC cells, inducing more degradation of tumor-suppression proteins, which accelerates cell proliferation, facilitates migration and invasion, induces EMT and suppresses cell apoptosis.

References

[24] X. Li, L. Amazit, H. Ohsaki, K. Yanoh, T. Kawanishi, Expression of proteasome activator REGgamma in human laryngeal carcinoma and its role in regulating transcription of REGγ in EC cells, inducing more degradation of tumor-suppression proteins, which accelerates cell proliferation, facilitates migration and invasion, induces EMT and suppresses cell apoptosis.


