Abstract. In most types of human cancer, inactivation of pRb/E2F complexes occurs, and released E2Fs initiate transcription of genes required for cell cycle progression. Evidence reveals that phosphorylated pRb deregulates E2F-1, and the levels of E2F-1 expression can accurately predict prognosis of oral squamous cell carcinoma (OSCC). Paradoxically, numerous reports indicate that E2F-1 is also capable of inducing apoptosis under certain cellular circumstances. In the present study, lentivirus-mediated shRNA was used to downregulate endogenous E2F-1 expression in order to study the function of E2F-1 in the pRb/E2F-1 pathway in the OSCC cell line Tca8113, and to investigate the alteration of Tca8113 cells in proliferation and apoptosis. The data from real-time quantitative RT-PCR and Western blot analysis showed that E2F-1-shRNA led to the inhibition of endogenous E2F-1 mRNA and protein expression, and E2F-1 may be associated with proliferation and apoptosis pathways. Growth kinetics data showed that Tca8113-E2F-1-shRNA cells presented more active proliferation properties than Tca8113-NC cells, and flow cytometry data demonstrated that the percentages of cells in the G1 phase, G2 phase and undergoing apoptosis differed between groups. In conclusion, silencing of E2F-1 inhibits proliferation and induces apoptosis. E2F-1 may also be involved in multi-level regulation networks; therefore, its role in OSCC requires further clarification.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the oral cavity, comprising more than 80% of cases of oral cancer (1). Although diagnostic and surgical techniques for treatment of OSCC have advanced in recent years, post-surgery survival rates have not improved. The overall 5-year survival rate of patients with OSCC has not significantly increased, and overall and disease-free survival rates remain at approximately 50% (2).

One characteristic of all cancers is the ability to proliferate beyond the normal limits and constraints. To do this, the cell must acquire new properties, such as being able to deregulate cell proliferation and suppress cell death (3,4). It is possible that multi-level regulation networks lead to the new properties of the mutated cells in their cell cycle. A nuclear protein called E2F-1 plays a key role in cell cycle control in most human cancers (4).

E2F-1 belongs to the E2F family that includes 8 genes (E2F-1 to E2F-8) to encode 9 distinct proteins in mammalian cells. E2F-1 is a cellular component that is required for the early region transforming protein (E1A) of adenovirus to mediate transcriptional activation of the viral E2 promoter (5). Several other E2F family members have now been isolated, either through homology to E2F-1 or through binding to Rb-related proteins. In G0 and early G1 phases, the vast majority of E2F proteins are in complexes with Rb and related proteins (6), that inhibit the transcriptional activation capacity of E2F factors and, in certain cases, convert E2F factors to repressors of transcription. pRB can be phosphorylated and dissociates from E2F by upstream regulators in this pathway, such as p16 silencing, amplification of cyclin D1 or cdk4 (7), then activated E2Fs result in activation of E2F-responsive genes (e.g. those involved in DNA synthesis, cell cycle control, pocket protein expression, etc.)

E2F-1 regulates cell cycle progression through checkpoints in the cell cycle and plays a dual role in cancer development with the capacity to act both as a tumor suppressor and as an oncogene (8). As an oncogene, E2F-1 induces many genes, such as Cdc2, Cdc25, cyclin E, which are regulated in a cell-specific manner and have E2F-binding sites as their promoters to express directly in quiescent cells to enter into DNA synthesis (9-11). As a suppressor gene, E2F1 also has the capacity to induce apoptosis, partly through induction of and cooperation with the p53-dependent pathway or p53-independent pathway (12). Overexpression of E2F-1 is detected in head and neck cell carcinoma and is associated with increased disease-free and overall survival (13).

Overexpression of E2F-1 is associated with increased disease-free survival in OSCC (14), however, the pathways involving E2F-1 are both multiple and interactive, and although numerous recent advances have elucidated these processes, many questions remain. Our previous research demonstrated

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a significant impact of E2F-1 overexpression on cell cycle progression and proliferation in an in vitro cell model of OSCC (15). The aim of the present study was to clarify the effects of E2F-1 knockdown on biological characteristics of OSCC in vitro. In particular, we were interested in E2F-1 as a target for the development of therapeutic approaches in the treatment of this disease.

Materials and methods

Cell culture. Tca8113 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Gaithersburg, MD, USA) with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) (1% Pen/Strep). The cells were cultured in an incubator at 5% CO₂ and at 37˚C, with the replacement of medium every 3 days.

Constructs and production of lentivirus. The lentiviral vectors expressing short hairpin RNA (shRNA) were successfully constructed, as previously described. The effectively selected E2F-1 shRNA and negative control oligonucleotide (NC) sequences were designed according to the Sigma-Aldrich website (http://www.sigmaaldrich.com/sigma-aldrich/areas-of-interest/life-science/functional-genomics-and-rna/shrna/productoverview.html) as follows: E2F-1-shRNA, sense: 5'-CAGGATGGATATGAGATGGGACTCGAGTCATCTCATATATCCATCCTGAGATTG-3'; Negative control shRNA, sense: 5'-CCTAAGGTGTAAGTCGCCCTCCTCGGACGAGCGACACTTAACCTTAGG-3'. All of the above sequences were designed according to the Sigma-Aldrich website (http://www.sigmaaldrich.com/sigma-aldrich/areas-of-interest/life-science/functional-genomics-and-rna/shrna/productoverview.html) as follows: E2F-1-shRNA, sense: 5'-CAGGATGGATATGAGATGGGACTCGAGTCATCTCATATATCCATCCTGAGATTG-3'; Negative control shRNA, sense: 5'-CCTAAGGTGTAAGTCGCCCTCCTCGGACGAGCGACACTTAACCTTAGG-3'.

Infection of lentivirus. OSCC cells (Tca8113) were transduced with the above two types of lentivirus particles. The transduced Tca8113 cells were then selected by puromycin (2 µg/ml), and puromycin-resistant colonies were then picked, expanded and analyzed separately. Tca8113 cells transduced with lentivirus-mediated shRNA targeting E2F-1 were named Tca8113-E2F-1-shRNA cells. Tca8113 cells transduced with lentivirus-mediated shRNA (NC) were named Tca8113-NC cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of E2F-1 mRNA expression. The cells (stably transduced) were harvested after selection by puromycin. Total RNA was extracted from cells using the RNeasy kit (Qiagen Inc., Valencia, CA, USA). The reverse transcription reaction was performed using high-capacity cDNA synthesis kit (Applied Takara Bio Inc., Madison, WI, USA). The products of PCR were checked by agarose gel electrophoresis and the abundance of each mRNA was determined and normalized to that of β-actin mRNA.

Real-time quantitative polymerase chain reaction (real-time PCR) analysis. Real-time PCR was performed using SsoFast™ EvaGreen® Supermix with ROX kit (Bio-Rad, Hercules, CA, USA) and the ABI 7300 real-time PCR system. Primers used in this experiment were as follows: E2 promoter-binding factor-1 (E2F-1), 5'-ACCCTGCAGAGCAGATGTT-3' (forward) and 5'-TTTGCTCTAAGGGAGATGGA-3' (reverse); renin-angiotensin system protein (pRb), 5'-TTCTCACGAAACCTGGCAGAATTG-3' (forward) and 5'-CAGTTGTCACCAAAAGTCTGAG-3' (reverse); cyclin E, 5'-TTTGCAGGATCCAGATAGAAG-3' (forward) and 5'-CACAGACGGCATTTATGCCTTCAAG-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TTCTAGAGACCCAGCTG-3' (forward) and 5'-TGTAACCAGGTGTCGATA-3' (reverse). Real-time PCR reaction conditions were: 95˚C for 30 sec; followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Values (mean ± SD) were determined from 4 independent experiments.

Western blot analysis. Tca8113-E2F-1-shRNA cells and Tca8113-NC cells were collected after a 7-day culture, washed twice with cold phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer (Beyotime, Institute of Biotechnology, Haimen, Jiangsu, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell debris was eliminated by centrifugation at 12,000 rpm for 10 min. Protein concentrations were determined using the Bio-Rad protein assay kit (Pierce, Rockford, IL, USA). Proteins (50 µg per lane) were loaded on a 12% SDS-polyacrylamide gel for electrophoresis, and then transferred onto PVDF membranes (Millipore Co., Bedford, MA, USA) at 300 mA for 1 h in a blotting apparatus (Bio-Rad). Membranes were blocked at room temperature for 2 h with blocking solution (5% w/v skim milk, 0.01 mol/l PBS, 0.1% Tween-20). The membranes were incubated overnight at 4˚C with primary polyclonal antibodies against E2F-1 (1:1500; Abcam, Cambridge, MA, USA), cyclin E (1:500; Bioworld, Atlanta, GA, USA), p14 (1:500; Bioworld), p53 (1:500; Bioworld) and monoclonal antibody against β-actin (1:500; Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China). Subsequently, the membranes were rinsed with PBST (0.1% Tween-20 in 0.01 mol/l PBS), incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at 1:5,000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for an additional 1 h, visualized by SuperSignal® West Pico Chemiluminescent Substrate (Thermo, Rockford, IL, USA) and exposed to Kodak X-ray films. The relative intensity of the bands was digitalized and evaluated using Image-Pro Plus 5.0 (Media Cybernetics, Bethesda, MD, USA). β-actin served as the internal control in these experiments.

Cell proliferation and survival assay. MTT assay was performed to determine the viability and proliferation of the Tca8113 cells. The Tca8113-E2F-1-shRNA and Tca8113-NC cells were seeded into 96-well plates at 2x10³ cells/ml and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) (1% Pen/Strep). The cell groups were cultured for
1, 2, 3, 4, 5, 6 and 7 days. Following each culture time, 20 µl MTT (5 mg/ml) was added to each well, and the cells were incubated at 37˚C for an additional 4 h. The reaction was then terminated by lysing with 150 µl DMSO for 5 min. Optical densities were determined on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

**Cell cycle analysis by flow cytometry.** For cell cycle analysis, Tca8113 cells (1x10^6) were washed twice with ice-cold PBS, treated with trypsin, and then fixed in 70% cold ethanol at 4˚C for 30 min. The cell pellet was incubated in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature for 30 min, and then analyzed by flow cytometry using a FACscan (Becton-Dickinson, Mountain View, CA, USA). The data were analyzed with the MultiCycle for Windows (Phoenix Flow Systems, San Diego, CA, USA).

**Apoptosis assay by flow cytometry.** Apoptotic cells were determined using the Annexin V-FITC Apoptosis Detection kit (Shenzhen Jingmei Biotech Co., Ltd., Shenzhen, Guangdong, China) and an EPICS XL-MCL flow cytometer (Becton-Dickinson, Mountain View, CA, USA). The data were analyzed with the MultiCycle for Windows (Phoenix Flow Systems, San Diego, CA, USA).

**Statistical analysis.** Data are expressed as the mean ± standard error of the mean (SEM) analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The Student’s t-test was used to measure statistical significance between the two treatment groups. Multiple comparisons were performed with one-way analysis of variance (ANOVA). Data were considered significant at P<0.05.

**Results**

**Inhibitory effect of the expression of E2F-1 mRNA and protein.** The sequences of E2F-1-shRNA were transduced to Tca8113 cells by lentiviral vector leading to notable inhibition of E2F-1 mRNA and protein expression. The results of agarose gel electrophoresis, qRT-PCR and Western blotting showed that E2F-1 mRNA and protein in Tca8113-E2F-1-shRNA cells were downregulated significantly, compared with the Tca8113-NC cells (P<0.05; Fig. 1).

**Cell growth and proliferation in transfected Tca8113 cells.** As shown in Fig. 2, following an initial lag phase, cells began to grow exponentially at day 3 in the Tca8113-NC group and at day 4 in the Tca8113-E2F-1-shRNA group. In the logarithmic phase, population doubling time of Tca8113-NC cells (33.4 h) was shorter than that of Tca8113-E2F-1-shRNA cells (49.7 h), indicating a quick growth rate of Tca8113-NC cells. The proliferation potential of Tca8113-E2F-1-shRNA and Tca8113-NC cells was further assessed by growth kinetics in vitro. A representative histogram of flow cytometry revealed that the distribution of Tca8113-E2F-1-shRNA cells in the G1 and G2 phases was different from that of the Tca8113-NC cells, but the number of cells in the S phase showed no differences between the two groups (Fig. 2). The percentage of Tca8113-E2F-1-shRNA cells in G1 phase (G1, 36.60±4.98%) was markedly lower than that in
the Tca8113-NC cells (G1, 58.53±0.57%, P<0.05), whereas the percentage of Tca8113-E2F-1-shRNA cells in the G2 phase (G2, 31.54±4.88%) was notably higher than that in Tca8113-NC cells (G2, 8.92±1.23%, P<0.05), demonstrating downregulation of the proliferation capacity of Tca8113-E2F-1-shRNA cells.

**Apoptosis in transfected Tca8113 cells.** In addition to the function of E2F-1 to induce proliferation, E2F-1 also leads to apoptosis through multiple pathways. To study the alteration of apoptosis in Tca8113 cells after downregulation of E2F-1, cells were stained with Annexin V-FITC and PI, and then subsequently analyzed by flow cytometry. The cells undergoing early apoptosis are shown in the lower right quadrant (Annexin V+/PI−), and the cell undergoing late apoptosis or necrosis are shown in the upper right quadrant (Annexin V+/PI+). As shown in Fig. 3, the percentage of Tca8113-E2F-1-shRNA cells with Annexin V+/PI− staining was significantly higher than that of the Tca8113-NC cells (P<0.05). This finding indicates that inhibition of E2F-1 was able to induce apoptosis in Tca8113 cells.

**Alteration of the regulation of cyclin E, p14 and p53 in Tca8113 cells upon downregulation of E2F-1.** Real-time PCR findings were consistent with the results of Western blot analysis and revealed that the gene expression of cyclin E, p14 and p53 was significantly downregulated in Tca8113-E2F-1-shRNA cells (Fig. 4A, P<0.05), while Rb showed no significant differences. By contrast, Tca8113-NC cells, and the protein expression of cyclin E, p14 and p53 were decreased in the Tca8113-E2F-1-shRNA group (Fig. 4B and C, P<0.05), accompanied by no differences of pRb between Tca8113-E2F-1-shRNA and Tca8113-NC cells.

**Discussion**

Numerous findings suggest that E2F-1 has a tissue-specific effect that enables it to induce proliferation as an oncogene, while also stimulating apoptosis as a tumor-suppressor gene in different cancers and cell lines. Enforced expression of E2F-1 in vitro has been shown to cause not only cell cycle progression but also apoptosis in a number of cell types (16,17). In this study, downregulation of E2F-1 in Tca8113 cells by a transfected lentivirus led to significant inhibition of cell growth and
caused cell cycle arrest in the G2/M phase. Moreover, we also detected a higher level of apoptosis than in control Tca8113 cells. To examine the role of E2F-1 in pathways controlling cell proliferation and apoptosis, we investigated its negative regulator (pRb) and target genes for proliferation (cyclin E) and apoptosis (p14ARF, p53). Notably, both mRNA and protein expression levels of cyclin E, p14ARF and p53 were found to be significantly decreased, while the expression of pRb revealed no marked alteration with the knockdown of expression of E2F-1 in Tca8113-E2F-1-shRNA cells. This study showed that E2F-1 not only regulates downstream target gene cyclin E but also induces the p14/MDM2/p53-dependent pathway; E2F-1 stimulates cell growth in OSCC cell line Tca8113.

The cell cycle is controlled by numerous mechanisms, including the regulation of cyclin-dependent kinases (CDK) by cyclins, CDK inhibitors and phosphorylation events (18). Mechanistically, it is known that E2F-1 activates a variety of genes required for S phase entry, DNA replication and synthesis (19,20). Consistent with this, overexpression of E2F can also bypass G1 phase arrest imposed by various stimuli. Transcriptional activity of E2F-1 has been found to be activated in cooperation with deregulation of the pRb/E2F-1 complex in almost all human cancers, due to the mutation or deletion of pRb, or upstream regulators in this pathway, such as p16 silencing, amplification of cyclin D1 or cdk4 (21).

When quiescent cells are stimulated by growth factors or mitogenic signals, the D-type cyclins are the first to be activated. D-type cyclins associate with CDK4 or CDK6 and function at the early G1 phase. CDK4 associates with the D-type cyclins to mediate the phosphorylation of the retinoblastoma (Rb) family protein (22). This event inactivates the ability of the pRb/E2F complex to release E2Fs and then initiates transcription of genes required for S phase entry. Many of the upregulated genes encode proteins involved in DNA replication and cell-cycle progression, such as DNA polymerases, minichromosome maintenance complex components (MCMs), cdc6 and cyclin E (23). In this study, E2F-1 downregulation via RNAi did not change the status of pRb in the Tca8113-E2F-1-shRNA cells, thus the inactivated pRb/E2F-1 complex may have little effect on the progression of the cell cycle. Cell cycle analysis demonstrated that the ratio of Tca8113-E2F-1-shRNA cells at the G1 phase was lower than that in the Tca8113-NC cells, while the proportion of cells in the S phase showed no significant differences. It may be concluded that knockdown of E2F-1 downregulates cyclin E to alter the activity of the cyclin E-CDK2 complex but not the state of S phase in Tca8113-E2F-1-shRNA cells. In the E2F family, E2F1, E2F2 and E2F3a, which interact only with pRB, constitute a subfamily and are often referred to as the ‘activator E2Fs’, due to the fact that they appear to function mainly in activating gene expression. In line with the regulation of many proliferation-related genes by E2F, overexpression of E2F1, E2F2 or E2F3a induces quiescent immortalized cells to enter the S-phase (24). Different activator E2Fs appear to have partially overlapping, but distinct, roles in the regulation of cell proliferation. Both E2F1 and E2F3 are required for cell-cycle entry, but only E2F3 is required for continued cell proliferation (25). Combined loss of E2F1, E2F2 and E2F3 completely abolishes the ability of cells to progress through the cell cycle and proliferate (26). Therefore, activator E2Fs are associated with roles in G1-S transition and only downregulation of E2F-1 may have some effect on the alteration of S phase.

Components of the checkpoint mechanism include sensors, mediators, transducers and effectors, which act together in
different phases of the cell cycle (27). The first checkpoint, found at the G1/S transition, is compromised in many malignant cells, due to mutations in various tumor-suppressor genes, including retinoblastoma protein (Rb) and p53. Cells deficient in the G1 checkpoint are dependent on the S and G2 checkpoints for DNA repair. p53-dependent arrest of cells in the G1 phase of the cell cycle is an important component of the cellular response to stress. Evidence also suggests that p53 controls entry into mitosis when cells enter G2 with damaged DNA or when they are arrested in the S phase, due to depletion of the substrates required for DNA synthesis. Part of the mechanism by which p53 blocks cells at the G2 checkpoint involves inhibition of Cdc2, the cyclin-dependent kinase required to enter mitosis (28). In the present study, accompanied by E2F-1 knockdown, expression of p53 decreased in Tca8113-E2F-1-shRNA cells, the cell growth was reduced significantly and the cell cycle was arrested in G2/M. While it is possible that p53 also contributed to the initial arrest, it is clear that additional pathways can cause arrest when p53 is missing.

Indeed, mechanisms of G2 arrest are controlled on multiple levels and are related to a variety of effector molecules. Checkpoint kinase-1 and checkpoint kinase-2 are the checkpoint transducer kinases that function downstream in the DNA-damage checkpoint signalling pathway. Chk2, expressed throughout the cell cycle, is activated in the presence of DNA damage (29). By contrast, Chk1, preferentially expressed during S and G2, has constitutive activity that is amplified in the presence of DNA damage (30). Chk1 and Chk2 can be phosphorylated by ATR and ATM respectively, and significant crosstalk exists between the ATM/Chk2 and ATR/Chk1 pathways (31). In addition, another transducer kinase called MAPKAP kinase-2 (MK2) is directly involved in phosphorylating effectors CDC25B and C, and in maintaining G1, S, and G2 checkpoints (32) and its response is essential for the survival of p53-deficient cells (33).

Apart from its ability to induce proliferation, E2F-1 was also found to be capable of inducing apoptosis, as was shown by determining that both p14ARF and p53 were downregulated upon E2F-1 mRNA interference. Several studies have identified p14ARF as a key molecule that links Rb/E2F1 and the p14ARF-MDM-p53 apoptosis pathway and this has stimulated research involving the process of E2F-1-regulated apoptosis (34,35). However, analysis of the percentage of apoptotic cells by flow cytometry showed that the percentage of Tca8113-E2F-1-shRNA cells undergoing apoptosis was markedly higher than that in Tca8113-NC cells. It follows that the apoptosis of Tca8113-E2F-1-shRNA cells involves other mechanisms. We believe that other apoptotic mechanisms exist in Tca8113-E2F-1-shRNA cells that exhibit lower expression of E2F-1.

E2F-1-mediated cell death has been studied in depth, however, an apoptotic role for E2F-1 in cancer has yet to be fully defined and loss of E2F-1, for example, in Myc-mediated lymphomagenesis, does not necessarily lead to apoptosis resistance (36). In this situation, the inactivation of E2F-1 leads instead to the inhibition of the enhanced cell cycle progression caused by c-Myc, indicating that targeting of E2F-1 itself may prove beneficial (4). The pathways involving E2F-1 and apoptosis are multiple and interactive. Downregulation of E2F-1 may affect the interaction among molecules in the network of the cell cycle and may indirectly induce apoptosis by other mechanisms.

In conclusion, it is evident from this study that E2F-1 may be involved in pathways of proliferation and apoptosis; however, downregulation of E2F-1 can reduce proliferation of Tca8113 cells more significantly in vitro. These findings suggest that the role of E2F-1 in OSCC requires further clarification and the multiple mechanisms involved in gene therapies targeting E2F-1 still need to be fully elucidated.

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