Epidermal Growth Factor Receptor and Mutant p53 Expand an Esophageal Cellular Subpopulation Capable of Epithelial-to-Mesenchymal Transition through ZEB Transcription Factors

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Abstract

Transforming growth factor-β (TGF-β) is a potent inducer of epithelial to mesenchymal transition (EMT). However, it remains elusive about which molecular mechanisms determine the cellular capacity to undergo EMT in response to TGF-β. We have found that both epidermal growth factor receptor (EGFR) overexpression and mutant p53 tumor suppressor genes contribute to the enrichment of an EMT-competent cellular subpopulation among telomerase-immortalized human esophageal epithelial cells during malignant transformation. EGFR overexpression triggers oncogene-induced senescence, accompanied by the induction of cyclin-dependent kinase inhibitors p15INK4B, p16INK4A, and p21. Interestingly, a subpopulation of cells emerges by negating senescence without loss of EGFR overexpression. Such cell populations express increased levels of zinc finger E-box binding (ZEB) transcription factors ZEB1 and ZEB2, and undergo EMT on TGF-β stimulation. Enrichment of EMT-competent cells was more evident in the presence of p53 mutation, which diminished EGFR-induced senescence. RNA interference directed against ZEB resulted in the induction of p15INK4B and p16INK4A, reactivating the EGFR-dependent senescence program. Importantly, TGF-β-mediated EMT did not take place when cellular senescence programs were activated by either ZEB knockdown or the activation of wild-type p53 function. Thus, senescence checkpoint functions activated by EGFR and p53 may be evaded through the induction of ZEB, thereby allowing the expansion of an EMT-competent unique cellular subpopulation, providing novel mechanistic insights into the role of ZEB in esophageal carcinogenesis.

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Introduction

Esophageal squamous cell carcinoma (ESCC) is among the deadliest cancers known (1) and is a paradigm for the investigation for all types of squamous cell cancers. Its high mortality rate is attributed to diagnosis at an advanced stage characterized by invasion and metastases to local lymph nodes and remote organs, as well as lack of curative therapy. Genetic lesions associated frequently with ESCC include inactivation of tumor suppressors p53 and p16INK4A and overexpression of cyclin D1 and epidermal growth factor receptor (EGFR; ref. 2) in addition to telomerase activation (3). EGFR overexpression and p53 mutations are particularly common in premalignant lesions (4–6). The presence of p53 mutations is positively correlated with EGFR overexpression (7).

Epithelial to mesenchymal transition (EMT) occurs during fundamental biological and disease processes including development and cancer (8). EMT in cancer leads to the loss of cell-cell adhesion and cell polarity as well as altered cell–extracellular matrix interactions, resulting in invasion and metastasis (8, 9). EMT is also associated with resistance to anticancer agents such as EGFR inhibitors (10). Although transforming growth factor (TGF)-β is one of the most potent EMT inducers present in the tumor microenvironment (11), EMT is not the sole consequence of TGF-β-mediated stimulation. It remains unknown about what determines the cellular capacity to undergo EMT in response to TGF-β (12). Among the transcription factors essential in EMT are zinc finger E-box binding proteins ZEB1 (a.k.a. δEF1) and ZEB2 (a.k.a. SIP1; ref. 13). ZEB1 and ZEB2 (ZEB) are critical regulators of TGF-β-mediated signaling through physical interaction with the SMAD proteins to recruit coactivators and corepressors (14). ZEB are implicated in EMT in several tumor types (9). Zeb1-deficient mouse embryonic fibroblasts undergo
premature replicative senescence and ectopic E-cadherin expression (15). However, the precise roles of ZEB in EMT remain to be elucidated.

Cellular senescence is induced by eroded telomeres, oncogene-induced DNA damage and epigenetic derepression of the INK4A/ARF (16, 17). Senescent cells exhibit flat and enlarged cell morphology as well as proliferative arrest accompanied by increased senescence-associated β-galactosidase (SABG) activity and upregulation of cell cycle inhibitors such as p15INK4B, p16INK4A, and p21 (CDKN1A). In primary human esophageal epithelial cells, telomerase (hTERT) activation overcomes replicative senescence, establishing a nontransformed immortalized diploid cell line EPC2-hTERT, which maintains functionally intact p53 and p16INK4A (18). Ectopically expressed p16INK4A alone induces senescence, whereas activation of oncogenes such as Ha-RasG12V and AKT also induce senescence in EPC2-hTERT cells (18–20), indicating senescence as a critical barrier function against oncogene-induced malignant transformation in human esophageal cells. Recently, EMT has been implicated in the early stages of carcinogenesis to bypass oncogene-induced senescence (21). However, it remains unclear how cellular senescence functions may be inactivated during EMT associated with malignant transformation.

We have shown recently that EGFR overexpression and p53 mutations (p53R175H and p53V143A) are necessary and sufficient to transform EPC2-hTERT cells, leading to increased cell motility, anchorage-independent growth, and tumor formation in nude mice (22). Herein, we have investigated how cell motility, anchorage-independent growth, and tumor formation are induced by EMT in EPC2-hTERT cells, leading to increased senescence-associated β-galactosidase (SABG) activity. Transient transfection was carried out using the FuGENE 6 transfection reagent (Roche Applied Science) to produce replication-incompetent viruses. Cells were infected as in retrovirus-mediated gene transfer and flow sorted for the green fluorescent protein–brightest cells (top 20%).

**Materials and Methods**

**Cell lines and monolayer culture.** EPC1-hTERT and EPC2-hTERT, established from independent primary cultures of normal human esophageal epithelial cells, and their derivatives were grown in Keratinocyte serum–free medium (Invitrogen) at 37°C in a 5% CO2 atmosphere as previously described (18, 20, 22, 23). HCE7, an ESCC cell line, was grown as previously described (24). Countess Automated Cell Counter (Invitrogen) was used to count cells with 0.2% trypsin blue dye to exclude dead cells. Cells were treated with 5 ng/mL of recombinant human TGF-β (R&D Systems) reconstituted in 4 mmol/L HCl containing 0.1% bovine serum albumin (BSA). AG 1478 (Calbiochem) was reconstituted in 0.1% dimethyl sulfoxide (DMSO) and used at 100 nmol/L. Phase-contrast images were acquired using a Nikon Eclipse TS100 microscope. Spindle-shaped cells were scored by counting at least 100 cells per high-power field (n = 6) under light microscopy.

**Retrovirus- and Lentivirus-mediated gene transfer.** Retroviral vectors expressing EGFR in pFB-Neo and/or either p53R175H or p53V143A in pBABE-puro were stably transduced into EPC1-hTERT and EPC2-hTERT cells as previously described (20, 22, 23). Stable cell lines were established by drug selection for 7 days with 300 μg/mL of G418 (Invitrogen) for pFB-Neo and 1 μg/mL of Puromycin (Invitrogen) and pBABE-puro.

The lentiviral pGIPZ vectors expressing short hairpin RNA (shRNA) directed against human ZEB1 designated ZEB1-A and ZEB1-B (clone ID # V2LHS_116663 and V2LHS_116659), ZEB2 designated ZEB2-A and ZEB2-B (V2LHS_234331 and V2LHS_268826), or a nonsilencing scramble sequence (Open Biosystems) were transfected into HEK-293T cells with Trans-It-Transfection Reagent (Open Biosystems) to produce replication-incompetent viruses. Cells were infected as in retrovirus-mediated gene transfer and flow sorted for the green fluorescent protein–brightest cells (top 20%).

**Transient transfection and dual-luciferase assays.** Transient transfection was carried out using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Briefly, 1 × 105 cells were seeded per well in 24-well plates 24 hours before transfection. Four hundred nanograms of the luciferase-reporter constructs p15PG1S1-lac (25) containing the p15INK4B promoter (0.75 kb; gift of Dr. Dr. Xiao-Fan Wang, Duke University, Durham, NC) or PGL3-p16 (26) containing the p16INK4A promoter (2.3 kb; gift of Dr. James W. Rocco, Massachusetts General Hospital, Boston, MA) were transfected along with 5 ng of phRL-SV40-Renilla luciferase vector (Promega) to calibrate the variation of transfection efficiencies among wells. Cells were incubated for 48 hours before cell lysis. Luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega) and the ORION Microplate Luminometer (Berthold Detection Systems USA). The mean of firefly luciferase activity was normalized with the cotransfected Renilla luciferase activity. Transfection was carried out at least thrice and variation between experiments was not >15%.

**5-Bromo-2′-Deoxyuridine incorporation assays.** Cell proliferation was assessed using the Cell Proliferation ELISA kit (Roche) with bromodeoxyuridine (BrdU) labeling for 2 hours before fixation. All experiments were performed in triplicate.

**SABG assays.** The Senescence β-Galactosidase Staining kit (Cell Signaling) was used to stain senescent cells, which were scored by counting at least 100 cells high-power field (n = 6) under light microscopy.

**RNA isolation, cDNA synthesis, and real-time reverse transcription-PCR.** RNA extraction and cDNA synthesis were performed as previously described (27). Real-time reverse transcription-PCR was done with Taqman Gene Expression Assays (Applied Biosystems) for CDH1 (Hs00170423_m1), CDH2 (Hs00983062_m1), ZEB1 (Hs00232783_m1), ZEB2 (Hs00207691_m1), SNAI1 (Hs00195591_m1), SNAI2 (Hs00161804_m1), TWIST1 (Hs00361186_m1), and CDKN1A (Hs00355782_m1) using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). SYBR green reagent (Applied Biosystems) was used to quantitate mRNA for β-actin as described (27). The relative level of each mRNA was normalized to β-actin as an internal control.

**Immunofluorescence.** Cells grown in chamber slides (Nalge Nunc) precoated with BD Matrigel Matrix (BD Biosciences) were fixed in 1:1 methanol/acetone for 10 minutes at −20°C.
and blocked with 1% BSA for 30 minutes. Slides were incubated with mouse anti-E-cadherin (1: 200; BD Biosciences) or mouse anti-vimentin (1:10,000; Novus Biologicals) overnight at 4°C and then with appropriate Cy2- or Cy3-conjugated secondary antibody (1: 400; Jackson ImmunoResearch) for 1 hour at room temperature. Nuclei were counterstained by 4′,6-diamidino-2-phenylindole (1:10,000; Invitrogen). Stained objects were examined with a Nikon Microphot microscope and imaged with a digital camera.

**Western blot analysis.** Whole-cell lysates were prepared as described (20, 22). Nuclear extracts were purified as previously described (28). Briefly, cells were washed twice with PBS, resuspended in buffer A [10 mmol/L HEPES-KOH (pH 7.8), 10 mmol/L KCl, 0.1 mmol/L EDTA (pH 8.0), 0.1% NP40, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL peptatin] and vortexed vigorously. Following centrifugation at 5,000 rpm for 1 minute, the nuclear pellets were resuspended in buffer C [50 mmol/L HEPES-KOH (pH 7.8), 0.4 mol/L KCl, 0.1 mmol/L EDTA (pH 8.0), 5 mmol/L MgCl2, 20% Glycerol, 1 mmol/L DTT, 0.5 mmol/L PMSF, and 2 μg/mL peptatin] and mixed gently at 4°C for 30 minutes. Following centrifugation at 15,000 rpm for 15 minutes, the supernatant was recovered as nuclear extracts.

Twenty micrograms of denatured protein were fractionated on a NuPAGE Bis-Tris 4% to 12% gel (Invitrogen). Following electrophoresis, Immobilon-P membranes (Millipore) were incubated with primary antibodies listed in Supplementary Table S1 and then with the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare). Table S1 and then with the appropriate horseradish peroxidase conjugated secondary antibody (GE Healthcare).

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**Statistical analysis.** Data from triplicate and hexaduplicate experiments are presented as mean ± SEM and were analyzed by two-tailed Student's t test. P < 0.05 was considered significant.

**Results**

**EGFR overexpression and p53 mutation promote the enrichment of an EMT-competent subpopulation of cells.**

EGFR overexpression and concurrent expression of mutant p53 transform EPC2-hTERT cells, conferring invasive characteristics as previously described (22). EMT was suggested by gene expression profiling of EPC2-hTERT-EGFR-p53R175H cells grown in organotypic three-dimensional culture, a form of human tissue engineering.7 When cells were treated with TGF-β in monolayer culture, >90% of EPC2-hTERT-EGFR-p53R175H cells exhibited spindle-shaped cell morphology within 3 weeks (Fig. 1A and B). This was accompanied by the loss of E-cadherin as well as the induction of mesenchymal markers such as N-cadherin and vimentin (Fig. 1C and D), indicating EMT. EMT was also induced in EPC2-hTERT-EGFR-puro cells, yet to a limited extent (30–40%; Fig. 1A–D). In fact, the frequency of EMT reached plateau despite

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Figure 1. EGFR and mutant p53 enrich the EMT-competent subpopulation of cells. EPC2-hTERT cell derivatives carrying the indicated genotypes were stimulated with or without TGF-β1. A, phase-contrast images were taken 14 d after TGF-β treatment. Arrows, spindle-shaped cells suggesting EMT. Scale bar, 100 μm. B, spindle-shaped cells were scored at the indicated time points after TGF-β stimulation. **, P < 0.001 versus neo-puro; *, P < 0.05 versus neo-puro; ns, not significant versus neo-puro; †, P < 0.001 versus EGFR-puro (n = 6). C, cells were double stained for E-cadherin (green) and vimentin (red). Note the presence of E-cadherin–negative and vimentin-positive spindle-shaped cells (arrow), suggesting spontaneous EMT (top). Scale bar, 100 μm. D, E-cadherin (CDH1) and N-cadherin (CDH2) mRNA levels in A. **, P < 0.001 versus neo-puro with TGF-β (+); #, P < 0.001 versus indicated genotype with TGF-β (−) (n = 3).
ZEB and the microRNA (miR)-205 and miR-200 family negatively regulate each other (29–32). In fact, these miR species were sharply suppressed on TGF-β1-induced EMT and that miR-200b, miR-141, and miR-205 were downregulated significantly in EPC2-hTERT-EGFR-p53R175H cells before TGF-β1 treatment (Supplementary Fig. S3). Thus, these miRs likely have a role in ZEB expression in EGFR-overexpressing cells. However, we cannot conclude whether suppression of these miRs led to the induction of ZEB or vice versa.

**ZEB1 and ZEB2 are expressed in the cells negating EGFR-induced senescence.** We next aimed at delineating how EGFR overexpression may lead to the enrichment of the cells expressing ZEB1 and ZEB2. We have noticed that a small subset of EPC2-hTERT-EGFR-puro cells exhibit proliferative arrest and morphology compatible with senescence corroborated by the SABG activity without TGF-β stimulation (Supplementary Fig. S4A). In addition, Western blotting detected the upregulation of cyclin-dependent kinase inhibitors (CDKI) p15INK4B, p16INK4A, and p21 in EPC2-hTERT-EGFR-puro cells (Supplementary Fig. S4B). We suspected that EGFR overexpression may trigger senescence. In fact, senescence was observed in 30% to 40% of EPC2-hTERT cells shortly after drug selection on retrovirus-mediated transduction of EGFR, but not a control empty vector (Fig. 3A and B). ZEB1 and ZEB2 were found to be upregulated as p15INK4B and p16INK4A were downregulated reciprocally in such a cell population (Fig. 3C and D). Moreover, induction of ZEB1 and ZEB2 was accelerated when EGFR was transduced in EPC2-hTERT cells along with p53R175H, alleviating EGFR-mediated senescence and CDKI upregulation (data not shown; Supplementary Fig. S4A and B). ZEB was also induced following EGFR transduction in EPC1-hTERT, an independently established immortalized human esophageal cell line (Supplementary Fig. S4C). These observations suggested that EGFR overexpression may allow expansion of a subset of cells negating senescence and expressing ZEB1 and ZEB2, which may have a role in facilitating EMT.

**ZEB1 and ZEB2 promote TGF-β-mediated EMT by suppressing senescence.** To address the role of ZEB1 and ZEB2 in TGF-β-mediated EMT, we targeted ZEB in EPC2-hTERT-EGFR-p53R175H cells by RNA interference. Stable knockdown of either ZEB1 or ZEB2 resulted in the upregulation of p15INK4B and p16INK4A, and p21 in EPC2-hTERT-EGFR-puro cells (Supplementary Fig. S4B). We suspected that EGFR overexpression may trigger senescence. In fact, senescence was observed in 30% to 40% of EPC2-hTERT cells shortly after drug selection on retrovirus-mediated transduction of EGFR, but not a control empty vector (Fig. 3A and B). However, actively proliferative cells emerged without losing EGFR and predominated over the senescent cells eventually (Fig. 3A–C). Interestingly, ZEB1 and ZEB2 were found to be upregulated as p15INK4B and p16INK4A were downregulated reciprocally in such a cell population (Fig. 3C and D). Moreover, induction of ZEB1 and ZEB2 was accelerated when EGFR was transduced in EPC2-hTERT cells along with p53R175H, alleviating EGFR-mediated senescence and CDKI upregulation (data not shown; Supplementary Fig. S4A and B). ZEB was also induced following EGFR transduction in EPC1-hTERT, an independently established immortalized human esophageal cell line (Supplementary Fig. S4C). These observations suggested that EGFR overexpression may allow expansion of a subset of cells negating senescence and expressing ZEB1 and ZEB2, which may have a role in facilitating EMT.

**Figure 2.** ZEB1 and ZEB2 expression is associated with EGFR overexpression. EPC2-hTERT cell derivatives carrying the indicated genotypes were stimulated with or without TGF-β1 for 14 d. A, relative mRNA levels of ZEB1, ZEB2, and SNAI1 were determined. **, *P < 0.001 versus neo-puro; *, P < 0.05 versus neo-puro (n = 3). B and C, ZEB1 and ZEB2 proteins were determined by Western blotting using nuclear extracts in B and whole-cell lysates in C. Arrowheads, specific bands for ZEB1 and ZEB2. Arrows, nonspecific bands.
By contrast, TGF-β induced EMT in scrambled shRNA-transduced control cells (Fig. 4C and D). Interestingly, ZEB1 knockdown resulted in partial inhibition of ZEB2, despite the lack of homology between the ZEB1 shRNAs and ZEB2 mRNA (Fig. 4A). Such an effect has been observed by others (29, 33) and may be accounted for in part by the derepression of the miR-200 family (32). Thus, our data indicate the possibility that ZEB1 may influence the ZEB2 expression level. Therefore, ZEB1 and/or ZEB2 is/are required for EPC2-hTERT-EGFR-p53R175H cells to undergo EMT in response to TGF-β and that ZEB may prevent EGFR from activating cellular senescence checkpoint functions through the suppression of p15INK4B and p16INK4A.

**Senescence prevents TGF-β from inducing ZEB in the EMT-competent cells.** Cellular senescence on ZEB knockdown was associated with reactivation of CDKI (Fig. 4B and C). Impaired EMT in such ZEB knockdown cells (Fig. 4C and D) thus can be attributed to suppressed ZEB-dependent transcriptional regulation of EMT markers such as E-cadherin.
N-cadherin, and vimentin. However, it remains unclear whether senescence per se affects the EMT processes including the TGF-β–stimulated ZEB augmentation observed in EPC2-hTERT-EGFR-p53<sup>R175H</sup> cells (Fig. 2A and C).

To determine whether senescence can block EMT, we established EPC2-hTERT-EGFR-p53<sup>V143A</sup> cells, in which temperature-sensitive mutant p53<sup>V143A</sup> gains a tertiary conformation similar to wild-type p53 and DNA binding as...
well as transcriptional activities at 32.5°C (34). When EPC2-hTERT-EGFR-p53V143A cells were exposed to 32°C, massive senescence was induced as determined by SABG assays (Fig. 5A and B). Cell proliferation was suppressed greatly (Fig. 5C) along with the upregulation of p21 (Fig. 5D). This supported the notion that mutant p53 may alleviate EGFR-induced senescence by suppressing p21 as observed in EPC2-hTERT-EGFR-p53R175H cells (Supplementary Fig. S4A and B), thus contributing to the expansion of the EMT-competent cells during EGFR transduction. By contrast, senescence was minimally induced in EPC2-hTERT-EGFR-p53R175H cells (Fig. 5), corroborating that p53R175H does not have wild-type p53 activity.

When stimulated by TGF-β, EPC2-hTERT-EGFR-p53V143A cells were prone to undergo EMT at 37°C (Fig. 6A and B). When senescence was induced fully, however, EPC2-hTERT-EGFR-p53V143A cells no longer underwent EMT upon TGF-β treatment, as indicated by lack of cadherin class switch at 32°C (Fig. 6B). Despite p53 activation, apoptosis was not induced with or without TGF-β treatment (data not shown; Supplementary Fig. S3A), excluding apoptosis as a potential mechanism preventing EMT. Interestingly, TGF-β stimulation neither augmented ZEB1 and ZEB2 levels nor induced TWIST1, SNAI1, and SNAI2 in senescent EPC2-hTERT-EGFR-p53V143A cells (Supplementary Fig. S3B; Fig. 6C), indicating that senescence abates the induction of downstream transcription factors crucial for EMT. Nonetheless, senescence per se did not block TGF-β receptor activation in EPC2-hTERT-EGFR-p53V143A cells (Supplementary Fig. S3C). Thus, activation of cellular senescence program seemed to prevent TGF-β from inducing transcription factors essential in EMT.

In aggregate, our data indicate that EGFR overexpression and p53 mutation in nontransformed human esophageal cells may lead to the enrichment of an EMT-competent subpopulation of cells with ZEB upregulation. ZEB1 and ZEB2 may negatively regulate p15INK4B and p16INK4A to facilitate cells overcoming EGFR-induced senescence. Mutant p53 may also alleviate EGFR-induced senescence by suppressing p21. In the EMT-competent cells with suppressed senescence checkpoint functions, TGF-β induces ZEB and other factors to promote EMT.

**Discussion**

TGF-β is a potent inducer of EMT. However, EMT is not necessarily a common outcome of TGF-β treatment, especially in human cell lines (35). However, there are carcinoma cell lines with mesenchymal traits suggestive of EMT. Such cell lines have been attributed to specific molecular states, such as acquisition of K-Ras independence (36) and ZEB1 and ZEB2 upregulation through the suppression of the miR-200 family of miRs (29). We now show that EGFR and mutant p53, essential for malignant transformation of human esophageal cells (22) may promote selective expansion of an EMT-competent subpopulation of cells expressing ZEB1 and ZEB2 (Figs. 1 and 2). Our data also suggest that EMT-competent cells may be capable of negating onco-gene-activated senescence checkpoint functions through ZEB1 and/or ZEB2 (Figs. 3 and 4), whereas cellular senescence may prevent TGF-β from inducing ZEB and other transcription factors to activate the EMT program (Figs. 5 and 6). In our proposed model, p53 as well as the INK4C

![Figure 5. Activation of the wild-type p53 functions of temperature-sensitive mutant p53V143A results in senescence. The wild-type p53 activity of p53V143A was induced by exposing EPC2-hTERT-EGFR-p53V143A or EPC2-hTERT-EGFR-p53R175H (control) cells to 32°C for 72 h. A, phase-contrast and bright-field images were taken for SABG-stained cells. Arrowheads, SABG-positive cells. Scale bar, 100 μm. B, histogram represents SABG-positive rates (%) in A. **, P < 0.001 versus 37°C (n = 6). C, cell proliferation assessed by BrdUrd uptake in A. **, P < 0.001 versus 37°C (n = 3). D, relative p21 mRNA levels in A. **, P < 0.001 versus 37°C (n = 3).](image-url)
locus-encoded CDKI p15^{INK4B} and p16^{INK4A} serve as barrier functions against EGFR oncogene-mediated cellular stress (Supplementary Fig. S6). Interestingly, ZEB1 and ZEB2 expression was associated with EGFR overexpression (Fig. 2) and was implicated in the suppression of p15^{INK4B} and p16^{INK4A} (Fig. 4).

EMT confers cancer cell resistance to EGFR inhibitors (37). ZEB1 knockdown resulted in mesenchymal to epithelial transition and increased sensitivity to Erlotinib, an EGFR inhibitor in head and neck squamous cell carcinoma cell lines (38). Thus, EMT influences EGFR activities in transformed cells. However, the EGFR kinase activity did not seem to be required for ZEB expression or TGF-β-induced EMT in established EPC2-hTERT cell derivatives with EGFR overexpression (Supplementary Fig. S2). Nonetheless, ZEB1 and ZEB2 expression was increased in the EGFR-overexpressing cells without TGF-β stimulation (Fig. 2). We speculate that a small subset of parental EPC2-hTERT cells expressing ZEB1 and ZEB2 were selected as a result of EGFR-induced senescence (Fig. 3), eliminating cells without ZEB expression. Alternatively, ZEB may be induced through a cellular reprogramming event in a unique subset of cells, acquiring an EGFR-independent status. In agreement with such a notion, ZEB1 has been implicated in stemness maintenance through miR-200 family-mediated regulation of Sox2, Klf4, and Bmi1 (39). Given the downregulation of p15^{INK4B} and p16^{INK4A} following EGFR-induced senescence (Fig. 3), it is tempting to speculate that EGFR triggered an epigenetic reprogramming event involving miRs such as miR-200b and miR-141 (Supplementary Fig. S3), resulting in the induction of ZEB as well as Bmi1, a Polycomb factor essential in the transcriptional repression of p16^{INK4A}, leading to the repression of these CDKIs. Thus, cellular reprogramming events may take place during the malignant transformation of EPC2-hTERT cells selecting EMT-competent cells with ZEB expression.

Induction of senescence by wild-type human EGFR is a novel finding. However, EGFR activation is known to trigger cell cycle arrest (40, 41), which is antagonized by human papilloma virus E6 and E7 proteins (42), implicating the pRB and

Figure 6. Senescence prevents TGF-β from inducing EMT. Senescence was induced in EPC2-hTERT-EGFR-p53^{V143A} as shown in Fig. 5. Cells were further stimulated with or without TGF-β for 14 d at either 32°C or 37°C. A, phase-contrast images. Arrows, spindle-shaped cells. Arrowheads, enlarged cells consistent with senescence. Scale bar, 100 μm. Histogram represents spindle-shaped cell rate (%). **, P < 0.001 versus 37°C (n = 6). B, E-cadherin and N-cadherin levels determined by Western blotting in A. C, relative mRNA levels for ZEB1, ZEB2, and SNAI1 in EPC2-hTERT-EGFR-p53^{V143A} cells. **, P < 0.001 versus TGF-β (−) at 37°C; ns, not significant versus TGF-β (−) at 32°C (n = 3).
p53 pathways. EGFR overexpression led to the upregulation of p15\textsuperscript{INK4B} and p16\textsuperscript{INK4A}, and p21 in EPC2-hTERT cells (Supplementary Fig. S4B). ZEB-mediated suppression of CDKI in our cells (Fig. 4B) is reinforced by premature replicative senescence associated with the upregulation of p15\textsuperscript{INK4B} and p21 in Zeb1 knockout mouse embryonic fibroblasts (15), although ZEB knockdown did not result in the derepression of p21 in our cell systems (data not shown). Twist was not upregulated in EGFR-transduced EPC2-hTERT cells without TGF-\beta stimulation (Fig. 2), raising the possibility that ZEB may have a function independent of TGF-\beta. In addition, \beta\textsuperscript{TGF-EPC2-hTERT} cell derivatives (Supplementary Fig. S1A).

In conclusion, our novel data underscore the role of EGFR overexpression and p53 mutations in the enrichment of a subset of esophageal cells that is capable of undergoing EMT in response to TGF-\beta through ZEB transcription factors, shedding new insights on invasive cell growth and inactivation of senescence checkpoint functions during malignant transformation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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