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Original Articles

Human telomerase reverse transcriptase (hTERT) promotes cancer invasion by modulating cathepsin D *via* early growth response (EGR)-1

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ABSTRACT

Human telomerase reverse transcriptase (hTERT) contributes to tumor progression as well as maintaining telomere length, however, the mechanism by which hTERT promotes invasiveness is not yet completely understood. This study aims to unravel the precise mechanism through which hTERT promotes cancer invasion. We established an hTERT-overexpressed immortalized cell line (IHOK/hTERT). In orthotopic xenograft models, IHOK/hTERT harbors higher tumorigenicity than IHOK/Control. IHOK/hTERT showed much higher migration and invasion activities compared to IHOK/Control. IHOK/hTERT co-cultured with fibroblasts displayed increased invasion compared to IHOK/hTERT without fibroblasts. We screened for genes that play an important role in intermodulation between cancer cells and fibroblasts using a microarray and identified fibroblast activation protein (FAP). hTERT knockdown showed decreased expression of FAP and early growth response (EGR)-1, one of the transcriptional regulators of FAP in IHOK/hTERT and oral cancer cell line YD10B. Furthermore, EGR-1 knockdown in IHOK/hTERT and YD10B showed reduced invasion and reduced cathepsin D expression compared to Control-siRNA cells. Taken together, this study provides evidence that hTERT overexpression is responsible for the upregulation of the cysteine protease cathepsin D by regulating EGR-1 to activate invasiveness in cancer progression.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy in the world. Oral squamous cell carcinoma (OSCC) comprises approximately 10% of HNSCC, in general, but almost one-third in specific geographic areas [1,2]. Despite the advances in therapeutic approaches, an overall 5-year survival rate of OSCC patients has been estimated for the lowest 25% [2].

Infection with human papilloma virus (HPV) is one of the leading causes of HNSCC, especially cancer in the oropharynx and base of the tongue. OSCC is the second most common high risk-HPV (hr-HPV)-related cancer, and its incidence is gradually increasing [2,3]. hr-HPV causes human cancers by expressing two viral oncoproteins, E6 and E7. The expressions of E6 and E7 alone are not sufficient for cellular transformation, and additional genetic alterations are necessary for malignant progression [4]. E6 and E7 have multiple binding partners that exert oncogenic effects beyond the degradation of p53 and pRb. For example, E6, in concert with E6AP, induces telomerase activity through the activation of human telomerase reverse transcriptase (hTERT) *via* degradation of NFX1, a transcription repressor of hTERT, thus contributing to cellular immortalization [5].

The catalytic subunit of telomerase, hTERT, is overexpressed in approximately 90% of human cancer cells, suggesting that hTERT is indispensable for cancer progression [6]. In addition to maintaining the telomere length for cellular immortalization, hTERT has been shown to play an active role in tumor progression by inducing mobility, invasion, and anti-apoptosis of cancer cells, supporting independent roles for telomerase beyond telomere lengthening [7–9]. A recent finding provided evidence that hTERT is involved in invasion through the modulation of matrix metalloproteinase (MMPs) expression [10]. In our previous study, we also found that knockdown of hTERT reduced invasiveness in both hr-HPV- and non-infected OSCC through down-regulation of MMP2 and MMP9 expression [11]. MMPs play an important role in various physiological and pathological processes. In particular, MMPs are prime candidates for invasion and metastasis activities. Although several MMP inhibitors have been investigated in clinical trials for various cancers, none of these trials have demonstrated satisfactory efficacy, likely due to the lack of inhibitor specificity and unclear scientific mechanism [12–14]. Accordingly, it is necessary to discover an effective molecular target and supporting mechanism as a substitute for MMPs for current anticancer therapy.

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A recent finding showed that cathepsin, but not MMPs, is significantly associated with poor survival [15]. However, there are fewer studies that connect cathepsins with carcinogenesis compared with MMPs. Cathepsins are lysosomal cysteine proteases that degrade the extracellular matrix (ECM) and thus play active roles in the invasion and metastasis of cancer cells. Among the cathepsins, cathepsin D is an invasion promoter and plays a critical role in various cancers including OSCC [16,17]. According to our previous study [18], cathepsin D expression in cancer cells was increased by fibroblast stimulation in collagen gel-based co-cultured models, suggesting that cathepsin D expression is related to cancer invasion *via* crosstalk between cancer cells and stromal fibroblasts.

Here, this study aims at investigating the role of hTERT in invasion related to crosstalk between cancer cells and stromal fibroblasts. We demonstrate that hTERT induces invasion by modulating cathepsin D *via* early growth response (EGR)-1 in HPV-16 E6/E7-transfected immortalized human oral keratinocytes (IHOK) and HPV-not related OSCC cells. This new discovery will contribute to the development of a novel chemotherapeutic approach targeting hTERT.

Materials and methods

Cell culture

IHOK was established by transfecting the pLXSN vector containing the E6/E7 open reading frame of HPV-16 as previously described [19]. IHOK/hTERT and IHOK/Control were constructed by using plpc-hTERT and pLXRN (Clontech, USA) vectors, respectively. Each vector was transfected into a GP2-293 packaging cell line to produce retrovirus particles that were subsequently used to infect IHOK. Four types of OSCC cells (YD9, YD10B, YD32, and YD38) [20,21] and immortalized human gingival fibroblasts (hTERT-hNOF) were used for this study [22]. Details about the cell culture procedures are described in the Supplementary Materials and Methods.

Co-culture

IHOK/hTERT, YD10B, and siRNA-transfected cells were co-cultured with hTERT-hNOF to observe whether fibroblasts have an impact on protein expression. The Supplementary Materials and Methods are described in details.

Mouse orthotopic xenograft model

The animal studies were approved by the animal ethics committee at Yonsei University College of Dentistry. BALB/c male mice (16 ± 2 g, 4 weeks of age) were provided from Orient Bio Incorporation (South Korea). Cells (5×10^5) were injected into the dorsal tongue of 15 mice in each group. Three mice in the IHOK/Control-injected group died during the procedure. The mice were then sacrificed after 6 weeks. The tongues of the mice were fixed in 10% neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for morphologic analysis. The tumor volume was assessed by two-dimensional measurements [23].

Immunofluorescence observation

Immunofluorescence staining was performed to detect protein expression of hTERT between IHOK/Control and IHOK/hTERT. The details were described in the Supplementary Materials and Methods.

BrdU incorporation assay

Cell proliferation was measured by the BrdU Flow Kit (BD Pharmingen™, UK) according to the manufacturer's instructions.

Telomerase repeat amplification protocol (TRAP) assay

Telomerase activity was measured by the Telomerase TeloTAGGG PCR ELISA (Roche, Switzerland) according to the manufacturer's instructions using 0.05 and 0.5 μg total protein.

Wound-healing migration assay

Cells (3×10^5) were seeded on 6-well plates and allowed to adhere overnight in growth media containing 1% FBS to achieve up to 90% confluence. The monolayer was scratched using a sterile 200 μL pipette tip. After 24 h, wound closure was evaluated by light microscopy (Olympus, Japan).

Invasion assay

Invasion assay was performed to compare the invasive activity between IHOK/Control and IHOK/hTERT. The Supplementary Materials and Methods are described in details.

Organotypic culture

Organotypic culture was performed to confirm the invasive activity between IHOK/Control and IHOK/hTERT. The Supplementary Materials and Methods are described in details.

Polymerase chain reaction (PCR)

Total DNA was extracted from each cell line using QIAamp DNA minikit (Qiagen, Germany). The primer sequences are listed in Table S1. DNA was amplified by using Accu Power Hot Start PCR Pre Mix (Bioneer, South Korea) with the following conditions: 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 40 s at 72 °C. The amplified products were separated on 1.0% agarose gel stained with 0.1 μg/mL of ethidium bromide, and photographed under UV light (Bio-Rad, USA).

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted from each cell line using a RNeasy plus mini kit (Qiagen, Germany), and complementary DNA was synthesized using the 2.5× RT-&GO™ Mastermix (MP Biomedicals, USA) according to the manufacturer's instructions. The primer sequences are listed in Table S1. Details about the procedures of RT–PCR are described in the Supplementary Materials and Methods.

siRNA transfection

Cells (1.5×10^5) were seeded in a 6-well plate for 24 h before transfection. hTERT-specific siRNA, EGR-1-specific siRNA, and control siRNA were the products of Bioneer Corporation (South Korea). Transfection of siRNA was performed using Lipofectamine RNAi MAX (Invitrogen, USA) according to the manufacturer's instruction. Total RNA and proteins were extracted 48 h after transfection. The sequences of siRNA are listed in Table S2.

Western blotting and zymography

Cells were lysed using a lysis buffer (Cell Signaling, USA) and used for western blotting. Collected conditioned medium was used for zymography. Details about procedures are described in the Supplementary Material and Methods.

Microarray data analysis

Microarray was performed to find altered gene expressions between IHOK/Control and IHOK/hTERT. The Supplementary Materials and Methods are described in details.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U* test to determine the statistical significance. All of the variables were tested in three independent experiments, and each experiment was performed at least in triplicate. The results are reported as the mean ± standard deviation (SD). The value of $p < 0.05$ was considered statistically significant.

Results

Construction of the hTERT-overexpressed cell line

To evaluate the functional significance and mechanism of hTERT on cancer invasion, we established hTERT-overexpressed immortalized cells (IHOK/hTERT). No significant change in morphology was observed between IHOK/Control and IHOK/hTERT (Fig. S1A). HPV-16 *E6* and *E7* DNA infection was confirmed in IHOK/Control and IHOK/hTERT by PCR (Fig. 1A). The mRNA expression levels of hTERT exhibited much higher in IHOK/hTERT than IHOK/Control, confirming exogenous hTERT was successfully expressed in IHOK/hTERT (Fig. 1B). The expression of hTERT was also confirmed by immunofluorescence staining (Fig. 1C and D). Significant difference in telomerase activity was observed between IHOK/Control and IHOK/hTERT when TRAP assay was performed using 0.05 μg total protein (Fig. 1E). No significant difference in proliferative activity was observed between the two cell lines when a BrdU incorporation assay

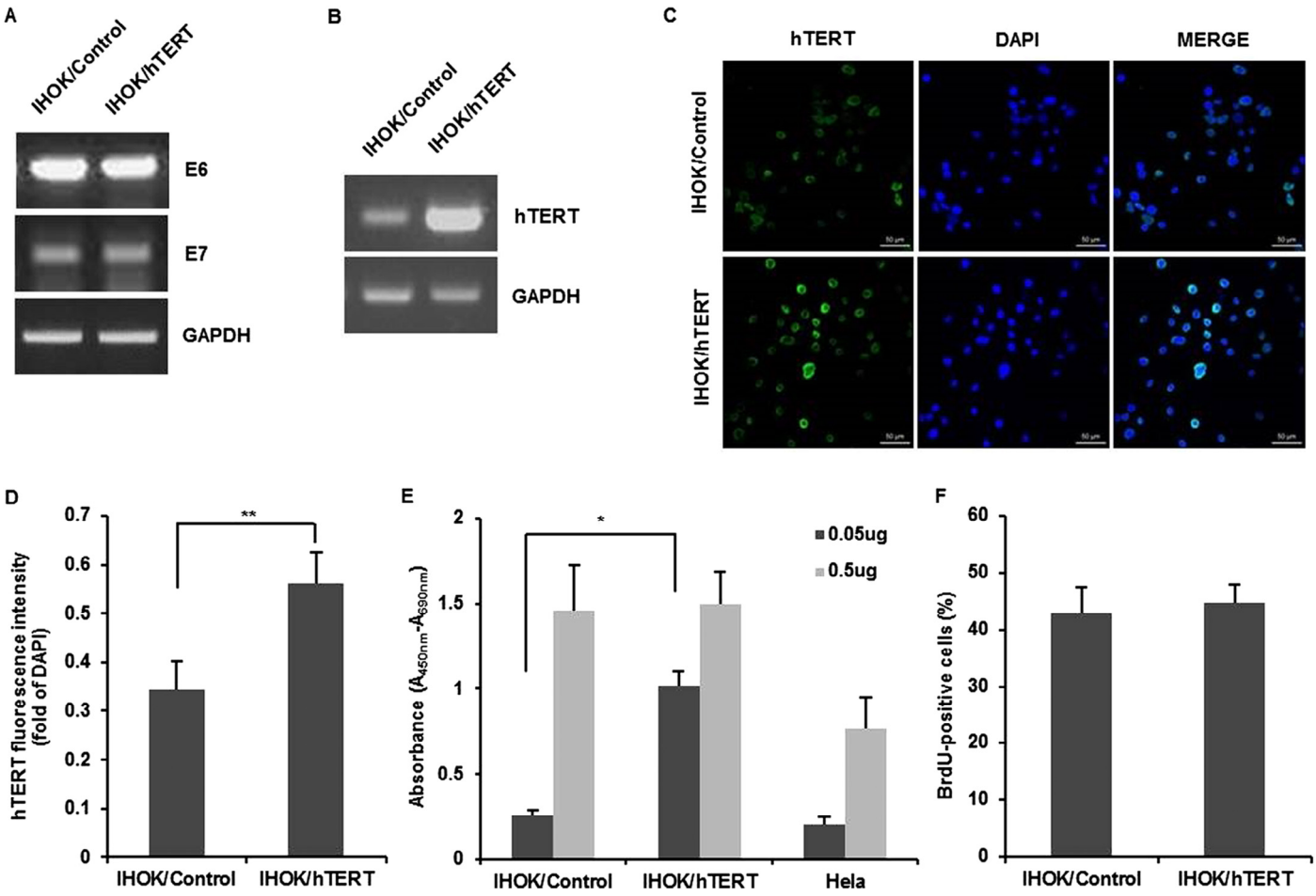

Fig. 1. Construction of the hTERT-overexpressed cell line. (A) HPV-16 *E6* and *E7* DNA expressions in IHOK/Control and IHOK/hTERT. (B) The mRNA expression of hTERT in IHOK/Control and IHOK/hTERT was measured by RT–PCR. GAPDH was used as a loading control. (C) Immunofluorescence examination of hTERT expression in IHOK/Control (upper) and IHOK/hTERT (lower). hTERT (green), nucleus (DAPI; blue) (magnification: 200×, Scale bar: 50 μm). (D) Fluorescence intensity of hTERT in IHOK/Control and IHOK/hTERT. hTERT expression was normalized to each DAPI intensity. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (**$p < 0.01$). (E) Telomerase activity of IHOK/Control and IHOK/hTERT. Telomerase activity was measured by the TRAP assay using 0.05 and 0.5 μg total protein. Hela is a positive control of telomerase activity. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test. (F) BrdU assay of IHOK/Control and IHOK/hTERT. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was performed (Fig. 1F). We confirmed that IHOK/Control and IHOK/hTERT were derived from IHOK by autosomal short tandem repeats (STR) profiling (Table S3).

Comparison of in vivo *tumorigenesis between IHOK/Control and IHOK/hTERT*

To investigate the effect of hTERT on *in vivo* tumorigenesis, IHOK/Control and IHOK/hTERT were injected into the dorsal tongues of nude mice. After 6 weeks, 3 out of 12 mice (25%) in the IHOK/Control-injected group showed tumor formation, while 12 out of 15 mice (80%) formed tumors in the IHOK/hTERT-injected group (Fig. 2A and B). The tumor volume in IHOK/hTERT-injected mice was significantly higher than in IHOK/Control-injected mice (Fig. 2C). These results indicate that IHOK/hTERT harbors higher tumorigenicity than IHOK/Control.

Difference of migration and invasion activities between IHOK/Control and IHOK/hTERT

To investigate whether hTERT induces migration and invasion to enhance tumorigenicity, we examined the effect of hTERT on cell migration by a wound healing assay. IHOK/hTERT displayed a 2.5-fold increase in migratory activity compared to IHOK/Control (Fig. 3A and B). To determine whether hTERT is involved in the induction of cell invasion, we performed an invasion assay in the presence and absence of fibroblasts in the lower layer. IHOK/hTERT showed a 2-fold increase in invasive activity compared to IHOK/Control in the absence of fibroblasts in the lower well (Fig. 3C and D). When epithelial cells were stimulated by fibroblasts, IHOK/hTERT showed a 4-fold increase in invasiveness compared to IHOK/Control (Fig. 3C and D). IHOK/hTERT co-cultured with fibroblasts showed higher invasive activity than IHOK/hTERT cultured without fibroblasts (Fig. 3D). In addition, we confirmed the invasive activity of IHOK/hTERT using an organotypic culture. IHOK/hTERT displayed a 1.8-fold increase in invasive activity compared to IHOK/Control (Fig. 3E and F). Taken together, IHOK/hTERT showed higher invasiveness than IHOK/Control. The co-cultured condition with fibroblasts facilitated the invasiveness of IHOK/hTERT.

Overexpressed genes in IHOK/hTERT compared to IHOK/Control

We performed a large-scale genomic screening three times to identify altered gene expression patterns between IHOK/Control and

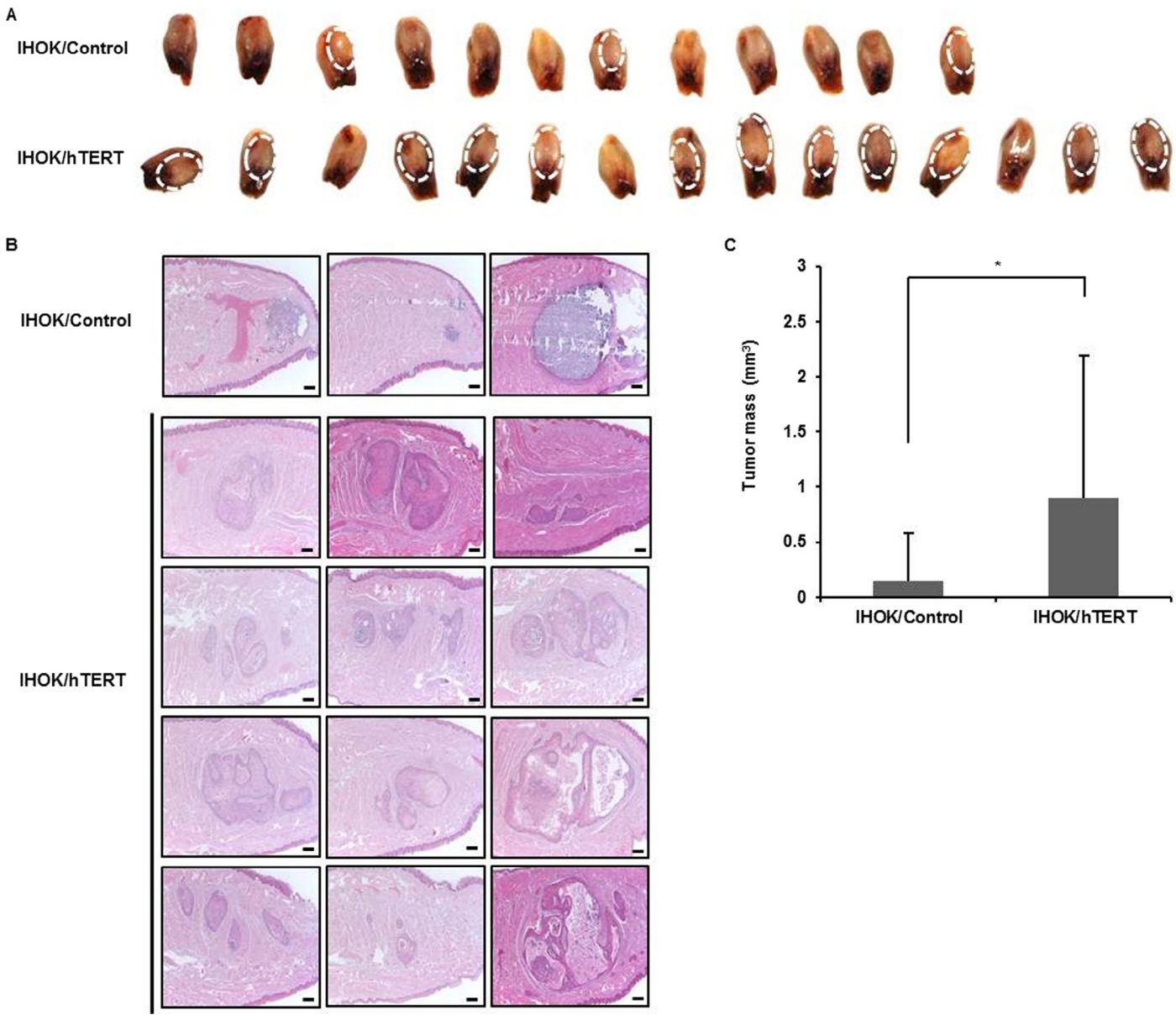

Fig. 2. Comparison of *in vivo* tumorigenesis between IHOK/Control and IHOK/hTERT. (A) Representative pictures of tumor masses from IHOK/Control-injected mice (upper) and IHOK/hTERT-injected mice (lower). (B) Representative microscopic pictures of IHOK/Control and IHOK/hTERT *in vivo* tumorigenicity. Cells (5×10^5) were injected into dorsal tongues for each cell line. After 6 weeks, the mice were sacrificed (magnification: 40×, scale bar: 200 μm). (C) Tumor volume of IHOK/Control-injected mice and IHOK/hTERT-injected mice. Tumor volume was assessed by two-dimensional measurements. The results are shown as the mean value ± SD (n = 15) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$).

IHOK/hTERT. We first screened invasion-related genes because the invasion activity of IHOK/hTERT was higher than that of IHOK/Control. MMP2 and MMP9 showed a 1.36-fold and 1.79-fold increase in IHOK/hTERT compared with IHOK/Control, respectively (Table 1). Although these microarray data were not confirmed at the mRNA levels, gelatin zymography showed higher MMP2 and MMP9 activities in IHOK/hTERT than in IHOK/Control (Fig. S1). Based on the observation that the difference in invasion activity between the two cell lines was much higher when co-cultured with fibroblast compared to mono-cultured conditions, we analyzed the genomic screen to identify the genes that are associated with functions modulating fibroblasts. Fibroblast growth factor 2 (FGF2), FGF11, fibroblast growth factor receptor 3 (FGFR3), and fibroblast activation protein (FAP) were up-regulated in IHOK/hTERT compared with IHOK/Control (Table 2). To confirm the microarray data, mRNA expression of FAP, FGF2, FGF11, and FGFR3 was measured in the two cell lines. Among them, FAP showed the highest expression level in IHOK/hTERT compared to IHOK/Control (Fig. 4A and B).

FAP and EGR-1 expression regulated by hTERT

To evaluate whether hTERT regulates FAP expression, we used siRNA to knock down the expression of hTERT in IHOK/hTERT. Expression of hTERT mRNA was reduced up to 59% in IHOK/hTERT transfected with hTERT-siRNA (Fig. 4C and D). Increased FAP expression with hTERT overexpression was reduced up to 50% following hTERT knockdown, confirming that hTERT regulates FAP (Fig. 4C–E). To investigate the mechanism underlying hTERT-mediated regulation of FAP, the levels of transcription factors that modulate FAP expression were screened [24]. Only the mRNA and protein

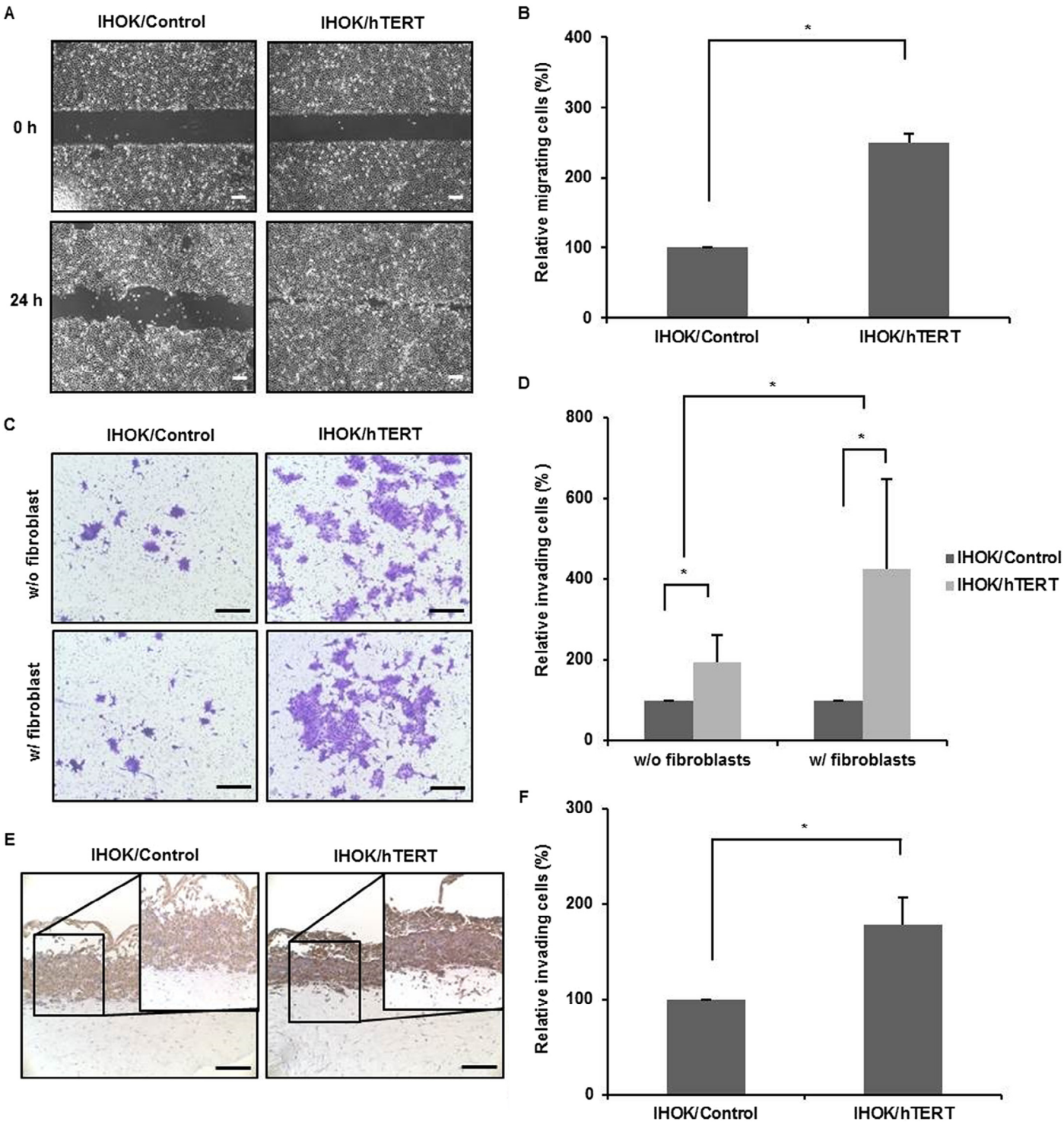

Fig. 3. Difference of migratory and invasive activities between IHOK/Control and IHOK/hTERT. (A) The wound-healing migration assay of IHOK/Control and IHOK/hTERT (magnification: 40×, scale bar: 200 μm). The confluent monolayer was scratched. After 24 h, wound closure was evaluated. (B) The number of migrated cells was normalized by dividing by the number of total cells to rule out the effect of cell proliferation and presented as the % of migration. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (C) The invasion assay of IHOK/Control and IHOK/hTERT in the absence (w/o fibroblasts) or presence (w/fibroblasts) of fibroblasts in the lower layer (magnification: 100×, scale bar: 200 μm). After 48 h, invaded cells were counted by light microscopy. (D) The number of invaded cells was normalized by dividing by the number of total cells to rule out the effect of cell proliferation and presented as the % of invasion. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (E) The organotypic culture of IHOK/Control and IHOK/hTERT. Samples were stained with cytokeratin to confirm invaded cells (magnification: 100×, scale bar: 200 μm). (F) All invaded cells were counted and presented as the % of invasion. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$).

expression of EGR-1 were significantly down-regulated by transfection of IHOK/hTERT with hTERT-siRNA (Fig. 4C, D, and F). No significant difference in mRNA expression of specificity protein 1 (Sp1), E2F transcription factor 1 (E2F1), and homeobox A4 (HOXA4) was observed between control cells and hTERT-siRNA transfected cells (Fig. S2). In addition, we verified that FAP was regulated *via* EGR-1 (Fig. S4A, B, E, and F). To confirm the results obtained in IHOK/hTERT, we screened mRNA and the protein expression of hTERT, FAP,

Table 1
Invasion-related overexpressed genes in IHOK/hTERT compared with IHOK/Control.

Description	Gene symbol	IHOK/hTERT/IHOK/Control intensity
Matrix metalloproteinase 9	MMP9	1.791236099
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	1.435109233
TNF receptor-associated factor 4	TRAF4	1.407743952
Vascular endothelial growth factor A, transcript variant 6	VEGFA	1.381553311
Matrix metalloproteinase 2	MMP2	1.366809303
TNF receptor-associated factor 4	TRAF4	1.237399983
Apoptotic peptidase activating factor 1, transcript variant 3	APAF1	1.183889472
Vascular endothelial growth factor A, transcript variant 1	VEGFA	1.168283047
v-akt murine thymoma viral oncogene homolog 1, transcript variant 1	AKT1	1.147317564
Bone morphogenetic protein 2	BMP2	1.136742457
SATB homeobox 1, transcript variant 1	SATB1	1.130307847
Adrenergic, beta-2	ADRB2	1.104408898
Tumor necrosis factor	TNF	1.10148696
Forkhead box K1	FOXK1	1.045099253
Vestigial like 4, transcript variant 2	VGLL4	1.038220356
Snail homolog 2	SNAI2	1.023804641
Nucleolin	NCL	1.015369219
Golgi reassembly stacking protein 1	GORASP1	1.015262462
PIN2/TERF1 interacting, telomerase inhibitor 1	PINX1	1.011766675
p53 and DNA-damage regulated 1	PDRG1	1.007545115
Cadherin 1	CDH1	1.000712434

and EGR-1 in several OSCC cells. We selected OSCC cell line YD10B because this cell line showed high levels of hTERT, FAP, and EGR-1 expression (Fig. S3A and B). hTERT depletion resulted in significantly reduced protein expression of FAP and EGR-1 compared with control cells (Fig. S3C–E). In addition, we also verified that FAP was regulated *via* EGR-1 in YD10B (Fig. S4C, D, G, and H), confirming the mechanism underlying hTERT-mediated regulation of FAP *via* EGR-1 in OSCC.

Invasion activity modulated by cathepsin D via *EGR-1*

To evaluate the importance of hTERT and EGR-1 in cell invasion, we used siRNA to knock down the expression of hTERT and EGR-1 in IHOK/hTERT and YD10B. The invasive activity was markedly decreased both in hTERT-siRNA and EGR-1-siRNA transfected cells compared with control cells, indicating that hTERT and EGR-1 regulated by hTERT play a critical role in upregulating cell invasion in OSCC (Figs. 5A–C and 6A–C).

To better understand the molecular mechanism by which EGR-1 promotes invasive activity, the activities of MMP2 and MMP9 were measured following EGR-1 depletion. No significant change in MMP2 and MMP9 activities was observed between control cells and EGR-1-siRNA transfected cells (Fig. 5D–F). Cathepsin D, another candidate protease, was measured following EGR-1 depletion. The expression of cathepsin D showed statistically significant decrease in EGR-1-siRNA transfected cells compared with control cells (Fig. 5G and H). To search whether the regulation of cathepsin D expression by EGR-1 is related to fibroblasts stimulation, we examined cathepsin D expression in EGR-1-siRNA transfected cells in co-cultured condition with fibroblasts. In the presence of fibroblasts, no significant difference in cathepsin D expression was observed between monocultured (Fig. 5G and H) and co-cultured conditions (Fig. 5I and J). To confirm the effects of EGR-1 on the induction of cathepsin D in OSCC cells, YD10B was transfected with EGR-1-siRNA. Cathepsin D expression was significantly decreased following EGR-1 depletion (Fig. 6D–G), indicating that cathepsin D is regulated by EGR-1 to modulate invasive activity. The presence of fibroblasts did not potentiate down-regulation of cathepsin D in YD10B.

Table 2
Fibroblast-related overexpressed genes in IHOK/hTERT compared with IHOK/Control.

Description	Gene symbol	IHOK/hTERT/IHOK/Control intensity		
		1st	2nd	3rd
Fibroblast growth factor 2	FGF2	1.932276	2.00641	2.446
Fibroblast growth factor 11	FGF11	6.05235	3.24537	2.76532
Fibroblast growth factor receptor 3	FGFR3	7.36015	4.76887	1.712476
Fibroblast activation protein	FAP	3.35802	4.00645	4.68621

Discussion

hTERT has been reported to have various functions in the induction of cancer development and progression [7,8,25]. Despite these observations, the precise molecular mechanisms of these hTERT functions have not been fully elucidated yet. In this study, we elucidated, for the first time, the mechanism underlying hTERT-mediated regulation of the cysteine protease cathepsin D, which is mediated by EGR-1.

To determine the molecular mechanism driving hTERT-mediated tumor progression, we established an artificially manipulated OSCC cell line (IHOK/hTERT) by overexpressing hTERT in IHOK. IHOK/hTERT harbored higher *in vivo* tumorigenesis than IHOK/Control. Accumulating evidence shows that hTERT is correlated with tumor cell migration and invasion, independent of telomere-lengthening function [7,26,27]. Thus, we assumed that the main driving force leading to higher *in vivo* tumorigenesis in IHOK/hTERT may be attributed to the high migratory or invasive potential, because no difference in proliferating activity was found between IHOK/Control and IHOK/hTERT. Providing evidence of a causal relationship between proliferating activity and hTERT *in vitro* is diverse according to cell types. Stable overexpression of hTERT in a hepatocellular carcinoma did not affect cell proliferation [8]. In contrast, hTERT transfection in osteosarcoma cells promoted cell proliferation [25]. The proliferation of hTERT-knockdown colorectal cancer cells was inhibited after a prolonged period of time, suggesting that telomerase activity remained after knockdown may be sufficient to maintain telomere length and sustained growth [7]. Considering that BrdU assay performed in our study did not reflect long-term proliferating effect of hTERT, we could focus on evaluating invasive activity of hTERT.

To identify genes that could contribute to and promote cell invasion by hTERT, microarray analysis was performed by comparing gene expression between IHOK/Control and IHOK/hTERT. Among the numerous invasion-related genes, cancer studies focused on the gelatinases, MMPs, because they degrade type IV collagen, the main component of the basement membrane. Specifically, MMP2 and MMP9 are shown to play a crucial role in the invasion and metastasis of OSCC. In our study, IHOK/hTERT showed higher expression and activity of MMP2 and MMP9 compared with IHOK/Control, which is consistent with our previous results [11].

Interestingly, the difference in invasive activity between two cell lines in co-cultured conditions was enhanced compared with monocultured condition (Fig. 3C and D). Cancer studies have acknowledged that the stromal microenvironment plays a pivotal role in carcinogenesis through crosstalk between cancer cells and stromal fibroblasts [28,29]. Previously, we reported a reciprocal interaction between cancer cells and stromal fibroblasts: IL1α, released from the carcinoma, induced cytokine secretion in fibroblasts, inducing cancer cell invasion. Collectively, we hypothesized that hTERT may

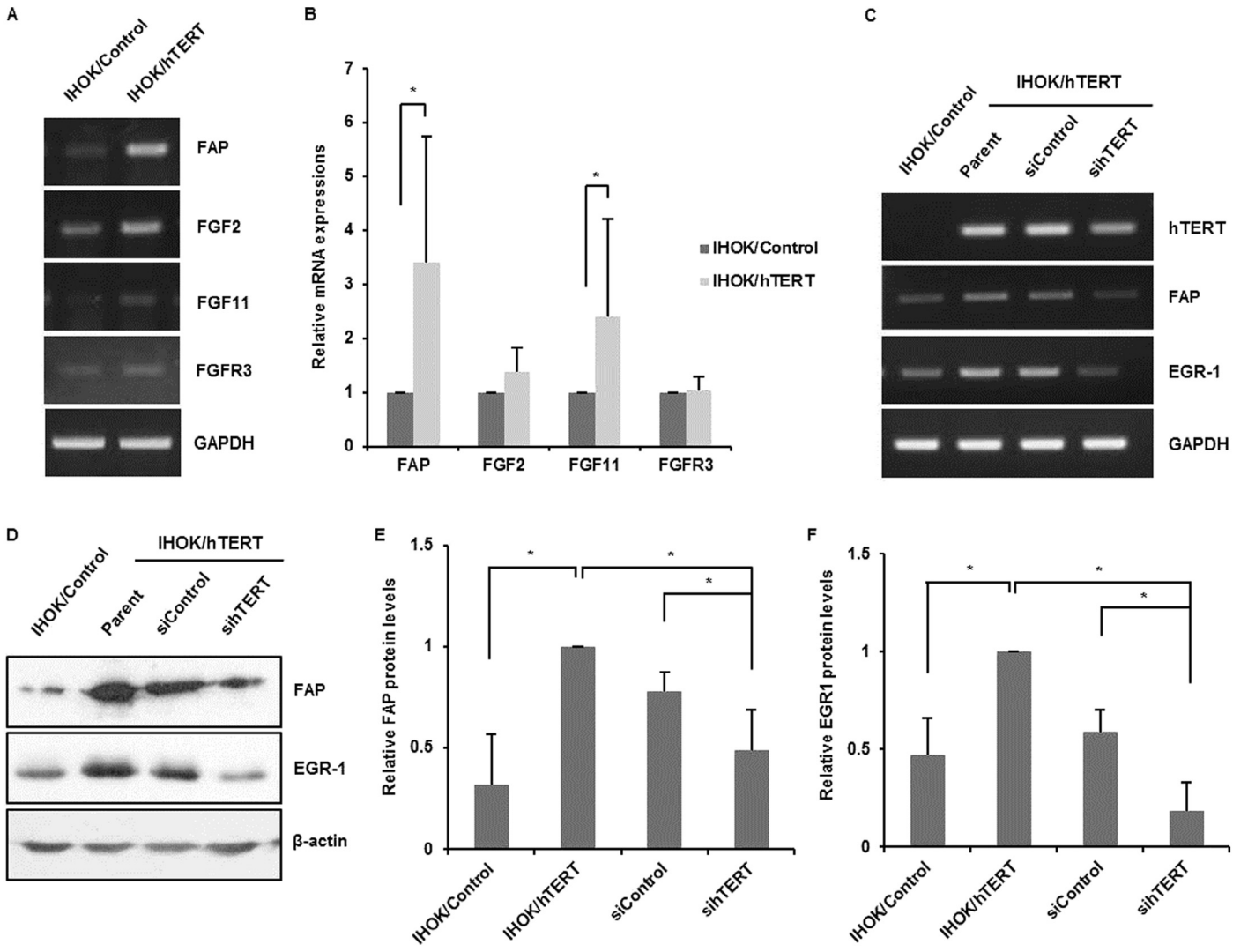

Fig. 4. FAP and EGR-1 regulation by hTERT. (A) mRNA expression for FAP, FGF2, FGF11, and FGFR3 in IHOK/Control and IHOK/hTERT. mRNA expressions for FAP, FGF2, FGF11, and FGFR3 were measured by RT–PCR and GAPDH was used as a loading control. (B) Densitometric analysis of FAP, FGF2, FGF11, FGFR3 mRNA expression in IHOK/Control and IHOK/hTERT. mRNA expression was normalized to each GAPDH mRNA expression. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (C) mRNA expression of hTERT, FAP, and EGR-1 after transfection with hTERT-siRNA in IHOK/hTERT. GAPDH was used as a loading control. (D) Protein expressions of FAP and EGR-1 after transfection with hTERT-siRNA in IHOK/hTERT. β-actin was used as a loading control. (E, F) Densitometric analysis of FAP and EGR-1 protein expression after transfection with hTERT-siRNA in IHOK/hTERT. Protein expression was normalized to each β-actin expression. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$).

stimulate the stromal microenvironments with tumor-associated proteolytic enzymes and thus facilitate cancer cell invasion.

To confirm our hypothesis, we collected microarray data to compare gene expression between IHOK/Control and IHOK/hTERT. Among the genes that were increased in IHOK/hTERT, we focused on overexpressed fibroblast-related genes, such as FGF2, FGF11, FGFR3, and FAP (Table 2). Among these genes, FAP showed the greatest difference between the two cell lines. FAP, a membrane gelatinase of the serine protease family, is one of the controlling factors in ECM. FAP itself induces degradation of localized ECM and cell invasion in epithelial tumors [30–33]. It is commonly known that FAP is mainly expressed in activated fibroblasts during wound healing or within tumor stromal fibroblasts [34]. However, its expression has also been shown in some types of malignant epithelial cells [32,33,35]. It is also involved in the control of fibroblast growth and epithelial–mesenchymal interactions during carcinogenesis [30–32,36–39]. In our study, we showed that FAP expression was regulated by hTERT, as evidenced by reduced FAP expression following hTERT depletion.

To better understand the precise molecular mechanism of hTERT-mediated invasive activity related to stromal interaction, mRNA expressions of transcription factors that regulate FAP expression were assessed. Among these transcription factors, only mRNA expression of EGR-1 was reduced by transfection with hTERT-siRNA in IHOK/hTERT, indicating that hTERT could regulate FAP *via* EGR-1, a regulatory factor of FAP. Consistent with these results, knockdown of hTERT in YD10B OSCC cells reduced the expression of FAP and EGR-1. The knockdown of hTERT also showed reduced invasiveness in IHOK/hTERT and YD10B. To evaluate the importance of EGR-1 in the induction of invasiveness, an invasion assay was performed after EGR-1 knockdown in IHOK/hTERT and YD10B. EGR-1 depletion resulted in a significant reduction of invasion in both cell lines, indicating that EGR-1 plays a crucial role in the induction of invasiveness in OSCC. The present study shows that EGR-1 acts as a positive regulator of tumor progression in OSCC. However, there are contradictory findings of the roles of EGR-1, depending on the cell type. EGR-1 has been considered to be a tumor suppressor in breast, lung, and brain tumors, and a tumor promoter in kidney and

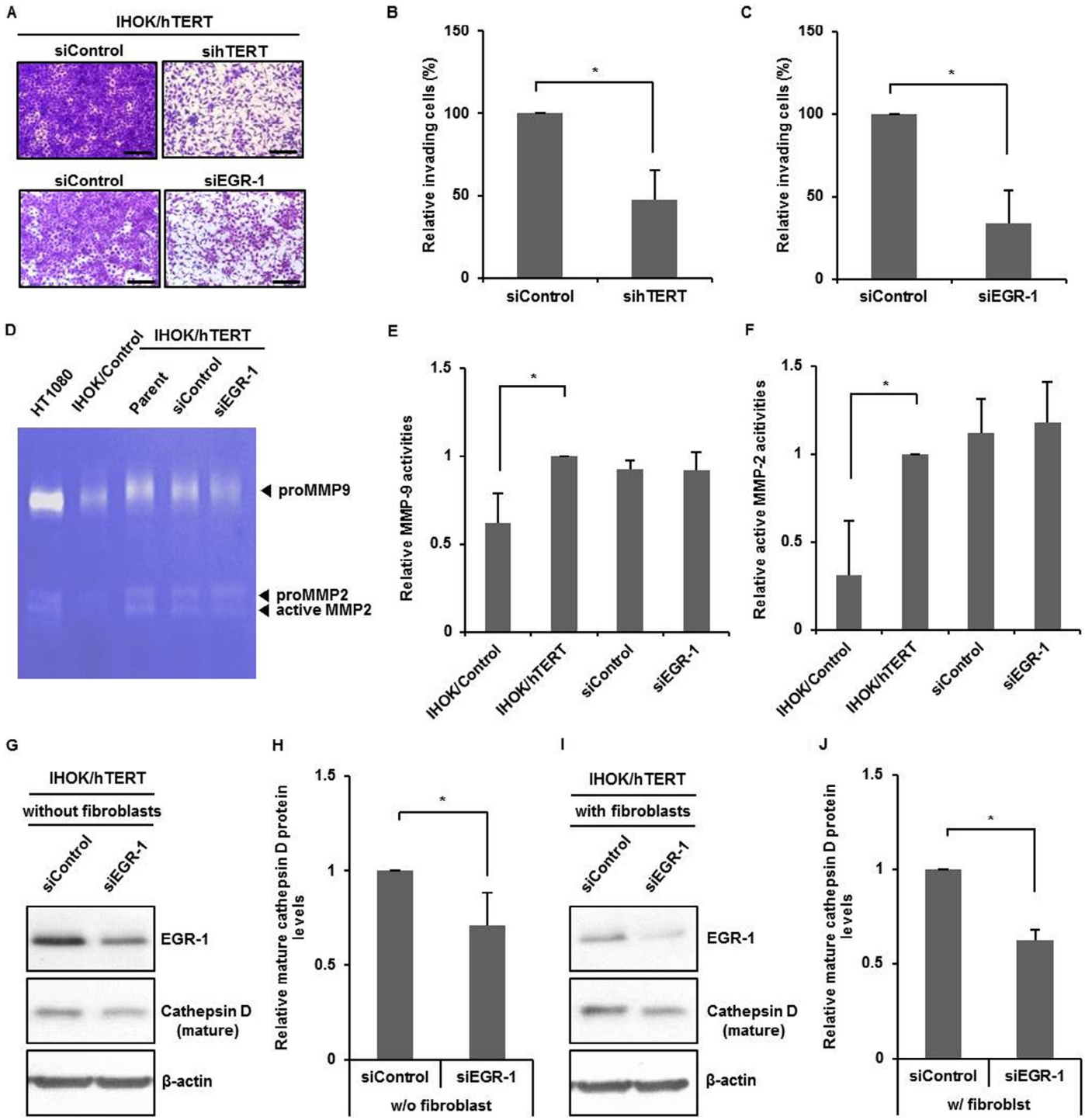

Fig. 5. Invasive activity modulated by cathepsin D *via* EGR-1 in IHOK/hTERT. (A) The invasion assay of IHOK/hTERT transfected with hTERT-siRNA and EGR-1-siRNA (magnification: 100×, scale bar: 200 μm). After 48 h, invaded cells were counted by light microscopy. (B, C) The number of invaded cells was normalized by dividing by the number of total cells to rule out the effect of cell proliferation and presented as the % of invasion. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (D) MMP2 and MMP9 activities were examined by gelatin zymography in IHOK/Control, IHOK/hTERT, and EGR-1-siRNA transfected IHOK/hTERT. HT1080 was used as a positive control. (E, F) Densitometric analysis of MMP2 and MMP9 activities in IHOK/Control, IHOK/hTERT, and EGR-1-siRNA transfected IHOK/hTERT. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (G) Protein expression of EGR-1 and cathepsin D of EGR-1-siRNA transfected IHOK/hTERT in the absence of fibroblasts (w/o fibroblasts). β-actin was used as a loading control. (H) Densitometric analysis of cathepsin D protein expression in EGR-1-siRNA transfected IHOK/hTERT. Cathepsin D protein expression was normalized to each β-actin expression. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (I) EGR-1 and cathepsin D protein expression of EGR-1-siRNA transfected IHOK/hTERT in the presence of fibroblasts (w/fibroblasts). β-actin was used as a loading control. (J) Densitometric analysis of cathepsin D protein expression in EGR-1-siRNA transfected IHOK/hTERT co-cultured with fibroblasts. Cathepsin D protein expression was normalized to each β-actin expression. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$).

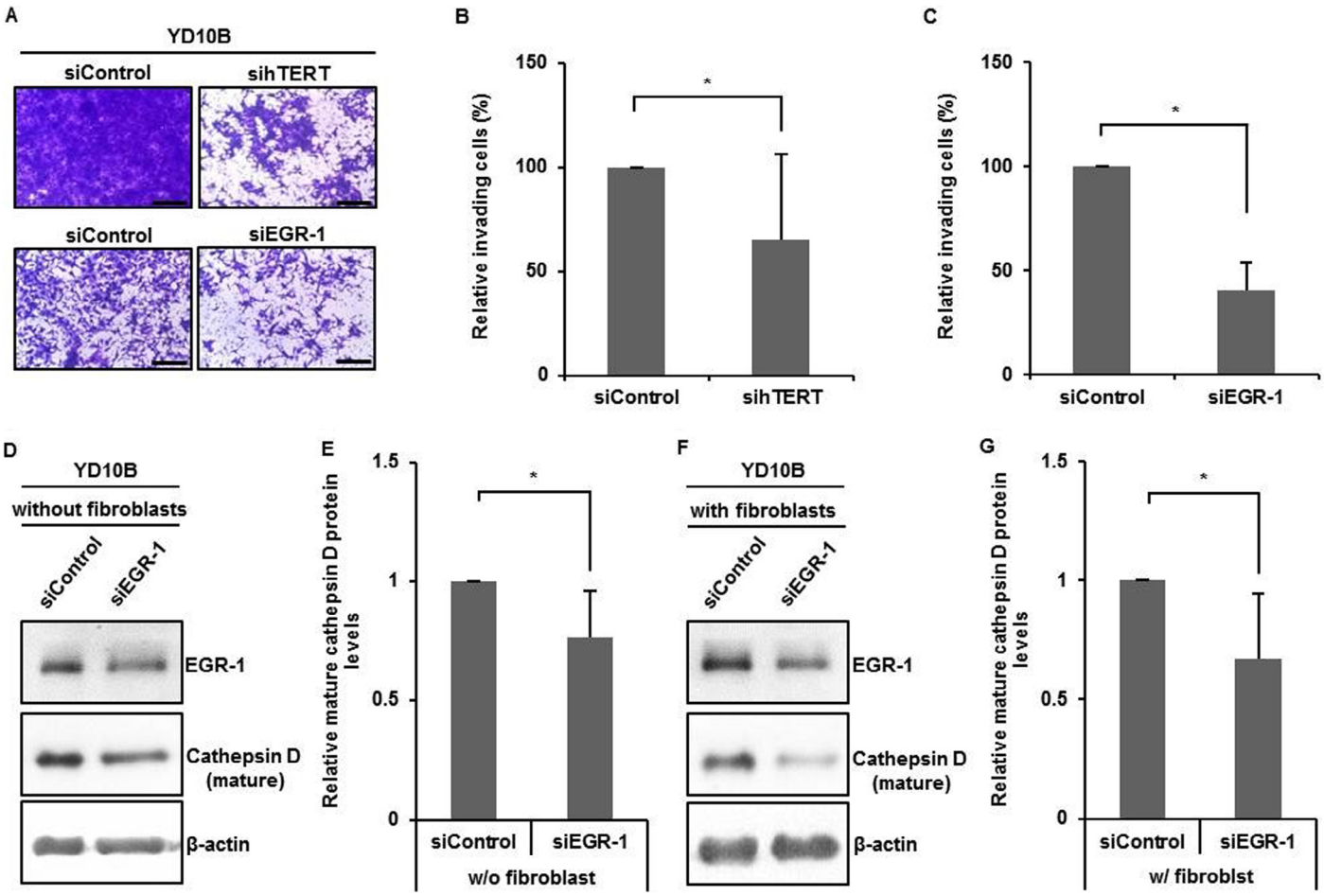

Fig. 6. Invasive activity modulated by cathepsin D *via* EGR-1 in YD10B. (A) The invasion assay of YD10B transfected with hTERT-siRNA and EGR-1-siRNA (magnification: 100×, scale bar: 200 μm). After 48 h, the invaded cells were counted by light microscopy. (B, C) The number of invaded cells was normalized by dividing by the number of total cells to rule out the effect of cell proliferation and presented as the % of invasion. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (D) Protein expression of EGR-1 and cathepsin D in EGR-1-siRNA transfected YD10B in the absence of fibroblasts (w/o fibroblasts). β-actin was used as a loading control. (E) Densitometric analysis of cathepsin D protein expressions in EGR-1-siRNA transfected YD10B. Protein expression of Cathepsin D was normalized to each β-actin expression. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$). (F) Protein expression of EGR-1 and cathepsin D in EGR-1-siRNA transfected YD10B in the presence of fibroblasts (w/fibroblasts). β-actin was used as a loading control. (G) Densitometric analysis of cathepsin D protein expression in EGR-1-siRNA transfected YD10B co-cultured with fibroblasts. Protein expression of Cathepsin D was normalized to each β-actin expression. The results are shown as the mean value ± SD (n = 3) and were analyzed by the Mann–Whitney *U* test (*$p < 0.05$).

endothelial cell tumors [40–42]. One possible explanation for such differences in the roles of EGR-1 is that its signal transduction pathway is different in different cancer cells. We assessed whether EGR-1 induces MMP2 and MMP9 expression based on previous reports [43,44]. However, the activities of MMP2 and MMP9 were not reduced after EGR-1 knockdown in IHOK/hTERT in our study.

Based on our previous study that cathepsin D expression was increased by fibroblast stimulation in collagen gel-based co-cultured models [18], cathepsin D expression was assessed following EGR-1 depletion. Cathepsin D expression was significantly reduced by transfection with EGR-1-siRNA in IHOK/hTERT and YD10B, indicating that EGR-1 modulates the expression of cathepsin D to induce invasive activity.

In our study, co-culture with fibroblasts did not potentiate down-regulation of cathepsin D in EGR-1-siRNA transfected cells, suggesting that fibroblasts cannot directly stimulate cancer cells to induce cathepsin D expression. Further study will be required to elucidate whether hTERT plays a direct role in cancer invasion by interaction between cancer cells and fibroblasts. According to a previous study with breast cancer cells, fibroblasts could not influence production of cathepsin D [45]. The discrepancy from our previous results that cathepsin D expression increased in OSCC cells by co-culture with fibroblasts could be attributed to the use of collagen-gel based co-cultured models [18].

In summary, our study showed that hTERT induces cancer invasion *via* MMP2, MMP9 as well as cathepsin D. We also demonstrated that hTERT down-regulation could inhibit cancer invasion through modulating cathepsin D expression *via* EGR-1. This study could contribute to, and provide insight for, the development of potential therapeutic targets for cancer treatment.

Authors' contributions

Conception and design: Young-Jin Park, Eun Kyoung Kim, Jin Kim.

Development of methodology: Young-Jin Park, Eun Kyoung Kim.

Acquisition of data: Young-Jin Park, Eun Kyoung Kim, Sook Moon.

Analysis and interpretation of data: Young-Jin Park, Eun Kyoung Kim, Jung Yoon Bae.

Writing, review, and/or revision of the manuscript: Young-Jin Park, Eun Kyoung Kim, Jung Yoon Bae, Jin Kim.

Administrative, technical, or material support: Young-Jin Park, Eun Kyoung Kim.

Study supervision: Jin Kim.

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Conflict of interest

The authors disclose no potential conflicts of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.10.021.

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