口腔癌の浸潤・転移に関与する上皮・間葉移行機構におけるp63の役割

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＜はしがき＞

口腔癌の浸潤・転移の機構を解明する目的の下，上皮・間葉移行のマスターレギュレーターであるSnailが癌抑制遺伝子p53のファミリー遺伝子であるp63の発現を抑制すること，そしてそのp63の発現消失が口腔癌細胞の浸潤をエンハンスすることを見いだした．

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東川晃一郎他，EMTを介した扁平上皮癌の高度悪性化におけるp63の発現の役割，第50回日本口腔外科学会総会，2005年10月24日

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Abstract

p63 is a member of the p53 family and regulates crucial events in the formation of epithelial structures, but the role of p63 in tumor is unclear. We found that Snail-induced epithelial-to-mesenchymal transition (EMT) is accompanied by downregulation of p63 in human squamous cell carcinomas (SCCs). ΔNp63α is the predominantly expressed p63 isoform in SCC cells. *ΔNp63* promoter activity required a C/EBP binding element and was reduced remarkably by Snail. Downregulation of ΔNp63α and reduction of C/EBPα were observed in EMT-phenotype cells, which exhibited invasive activity *in vitro*. p63 knockdown in cells enhanced invasive activity in the presence of E-cadherin. Conversely, forced expression of ΔNp63α blocked invasive activity of cells with the EMT phenotype. These findings indicate that Snail downregulates ΔNp63α, leading to acquisition of the invasive phenotype by SCC. The invasive activity caused by downregulation of ΔNp63α does not require downregulation of E-cadherin.

Introduction

In embryonic development, epithelial-to-mesenchymal transition (EMT) is the process of disaggregating structured epithelial units to enable cell motility and morphogenesis (1, 2). Wound healing and progression of carcinomas to invasive and metastatic phenotypes also involve localized EMT (3, 4). The term 'EMT' comprises a wide spectrum of changes in epithelial plasticity. Among these EMT subtypes, 'complete EMT', defined by a fibroblastoid phenotype, loss of E-cadherin and gain of vimentin, a mesenchymal marker, was most closely correlated with local invasion (5). E-cadherin is a cell-cell adhesion molecule expressed on the cell membrane of epithelial cells. Loss of E-cadherin expression is a primal molecular event that contributes to tumor invasion and metastasis (6). Snail, a zinc-finger transcription factor, triggers EMT through direct repression of E-cadherin (7, 8). The correlation of Snail and E-cadherin has been reported for various human cancers, including squamous cell carcinoma (SCC) (7, 9-11). Other repressors of E-cadherin and inducer of EMT are the zinc-finger transcription factor SIP1 (ZEB-2, ZFHX1B), Slug and Twist (12-14). In this context, EMT has attracted attention in studies of tumor progression.

p63 (TP73L/TP63) is a member of the *p53* gene family (15, 16) and has two different promoter usage generating proteins containing (TA) or lacking (ΔN) an amino terminus, which is homologous to the transactivation domain of *p53*. ΔNp63 isoforms act transcription repressors in a dominant-negative fashion to oppose p53- or TAp63-mediated transactivation *in vitro* and *in vivo*

(17). However, ΔNp63 isoforms also display transcriptional activity that is independent of the presence of the transactivating domain (18). p63 plays essential roles during development in the formation of epithelial structures. *p63*$^{-/-}$ mice have striking developmental defects including complete lack of all stratified squamous epithelia, epidermal appendages, mammary, lacrimal and salivary glands (19, 20). Heterozygous germline mutations in *p63* are the cause of ectrodactyly, ectodermal dysplasia and facial clefts syndrome (EEC syndrome) (21). In contrast to *p53*, *p63* is rarely mutated in human cancers, and the role of p63 in tumors is still unclear (22), although some links to the DNA damage response pathway have been reported (15, 23). Upstream transcriptional regulators of p63, in particular of ΔNp63, are poorly understood in contrast to the many downstream target genes of p63 that have recently been reported (22). Here, we report a novel mechanism whereby downregulation of ΔNp63α by Snail triggers invasion of SCC cells parallel with downregulation of E-cadherin.

Materials and Methods

Cell lines and cell culture. The human vulval epidermal cell line, A431, and four human oral SCC cell lines, OM-1, HOC719, HOC313 and TSU, have been described previously (9). HOC719-PE (positive E-cadherin) and HOC719-NE (negative E-cadherin) cells were isolated from HOC719 cells which express E-cadherin heterogeneously (9). All SCC cell lines have a $p53^{-/-}$ phenotype (24). A431-SNA1 and OM-1-SNA1 cells were generated by transfection with pcDNA3-mm SnailHA (GenBank Accession Number: BC034857), a kindly provided by Dr. de Herreros (Universitat Pompeu Fabra, Barcelona, Spain) as described previously (25). GT-1 cells are immortalized fibroblasts derived from human gingiva by transfection with an hTERT expression vector (26). All cell lines were cultured at 37°C in a humidified atmosphere of 5 % CO_2 in air and maintained with DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma).

RNA extraction and first-strand cDNA synthesis. Total RNAs were isolated from the cells in 70-80% confluence with Trizol (Invitrogen, Carlsbad, CA). First-strand synthesis was performed with First-strand cDNA synthesis kit (Roche, Bromma, Sweden).

Semi-quantitative RT-PCR. RT-PCR reactions (20 μl) were amplified with 30 cycles of denaturing at 95°C for 30 sec, annealing for 30 sec and extension at 72°C for 1 min. For

amplification of specific regions of *TAp63* and *ΔNp63*, the primers and annealing temperatures used were described previously (15). For other amplifications, primers and annealing temperatures were follows:

p63, 5'-TCCTCAGGGAGCTGTTATCC-3' and 5'-ACATACTGGGCATGGCTGTT-3', 56°C; *ΔNp63α*, 5'-ATGTTGTACCTGGAAAACAATG-3' and 5'-ATCTGATAGATGGTGGTCAGCC-3', 56°C; *p63αβ*, 5'-GGCCGTTGAGACTTATGAAATGC-3' and 5'-GCTCAGGGATTTTCAGACTT GC-3', 56°C; *p63γ*, 5'-GGCCGTTGAGACTTATGAAATGC-3' and 5'-CTCTATGGGTACACTGA TCGGTTT-3', 56°C; *C/EBPα*, 5'-CAGACCACCATGCACCTG-3' and 5'-TTGTCACTGGTCAG CTCCAG-3', 58°C; *Snail*, 5'-AATCGGAAGCCTAACTACAG-3' and 5'-GGAAGAGACTGAAG TAGAG-3', 60°C; *G3PDH*, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTG CTGTA-3', 52°C. PCR products were analyzed by 1.8% agarose gel electrophoresis and sequenced to verify their identity.

Cell lysates and immunoblotting. Cells were harvested and lysates prepared according to standard methods. Immunoblotting was also performed according to a standard method. Antibodies were anti-p63 which are specific for ΔNp63 isoforms (Ab-1, Oncogene Research Products, San Diego, CA), anti-E-cadherin (H-108, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α-tubulin (Zymed Laboratories, South San Francisco, CA).

Luciferase reporter assay. The *ΔNp63* promoter region of nucleotide (nt) -558 to +262 (construct 1) was amplified with *Pfx* polymerase (Invitrogen) from genomic DNA of normal human fibroblasts with primers as described previously (27). Other fragments of the *ΔNp63* promoter region were also amplified by PCR. The sense primer sequences for each fragment were follows:

nt -203 (construct 2), 5'-GGTACCGAAATGCCTTCTGTAAATCG-3';

nt -167 (construct 3), 5'-GGTACCTGTTTGGGGAGATTTGTTTTGTTTT-3';

nt -160 (construct 4), 5'-GGTACCGGAGATTTGTTTTGTTTTTAAAAGACAGTGCA-3';

nt -115 (construct 5), 5'-GGTACCGAGACAGGGAAAGTTTTACC-3';

nt -44 (construct 6); 5'-GGTACCGATTGGTGATAAGGAATTC-3'.

Each PCR product was cloned into the *Kpn* I and *Xho* I sites of pGL3-basic vector (Promega, Madison, WI). The mutant C/EBP binding element clone (pGL3-ΔNp63_C/EBP mt) was also generated by changing AGATTT (underline: nt -158 to -155) to GCTAGC in the fragment of nt -558 to +262. A431 cells were co-transfected with 4 μg of the reporter construct containing the *ΔNp63* promoter sequence, empty pcDNA3 or pcDNA3-mm SnailHA and 1ng of pRL-CMV as an inner control for transfection efficiency with Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were lysed with passive lysis buffer, and the promoter activity was measured with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The results correspond to the mean of at least three independent experiments.

RNA interference (RNAi). Small DNA fragment encoding a short hairpin RNA (shRNA), targeting all of the p63 isoforms was cloned into pRNA-U6.1 (GeneScript, Piscataway, NJ). The sequences of the short interfering RNAs (siRNAs) were follows: p63 siRNA, GGUACCAGCACACUCUGUCUU; Control siRNA, GUCGAUCCGAACACUCUCUGU. Vectors were transfected into A431 and OM-1 cells, and stable cell clones were established by hygromycin selection.

Vector construction and cloning of forced ΔNp63α-expressing cells. ΔNp63α full-length cDNA (GenBank Accession Number: AB042841) was amplified by RT-PCR with *Pfx* polymerase (Invitrogen) and cloned into the *Nhe* I and *Xba* I sites of pcDNA6-V5/His-tagged expression vector (Invitrogen). Primers for amplification were 5'-GCTAGCAACATGTTGTACC TGGAAAACAATGCCC-3' and 5'-TCTAGAGGAACTCCCCCTCCTCTTTGATGC-3'. The sequence of the PCR product was verified by sequencing. The ΔNp63α expression vector or the empty pcDNA6-V5/His vector as control was transfected into HOC313 cells, and stable cell clones were established by blasticidin selection.

Immunofluorescent staining. To detect localization of F-actin and E-cadherin in cells, Alexa Fluor 488 phalloidin (Invitrogen) and rabbit anti-E-cadherin antibody (H-108, Santa Cruz Biotechnology) were used for *immunofluorescent staining* with standard method. Briefly, cells

cultured on Lab-Tek II Chamber Slides (Nalge Nunc, Tokyo, Japan) were fixed with 4% paraformaldehyde and treated with 0.1% Triton X-100 for 5 min, 1% bovine serum albumin (BSA) in PBS for 30 min, a 1:50 dilution of anti-E-cadherin anti-body in PBS containing 1% BSA for 1 h, and a 1:50 dilution of Alexa Fluor 568-labeled goat anti-rabbit IgG (Invitrogen) for 45min. Vectashield (Vector Laboratories, Burlingame, CA) was used as mounting media.

MatriGel™ cell invasion assay. Cell invasion activity was measured with BioCoat MatriGel™ Invasion Chamber (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, cells were suspended in DMEM medium containing $5x10^5$ cells/ml. Cell suspension (500 μl) was added to each chambers containing an 8 μm pore size PET membrane with MatriGel™ basement membrane and incubated for 12-48 h at 37°C and 5% CO_2 atmosphere. Cells on the bottom surface of the PET membrane were fixed with 4% paraformaldehyde, and stained with Trypan blue and counted as the invading cells.

***In vitro* three-dimensional (3D) culture.** Three-dimensional cultures of epithelial cells with contracted collagen gel containing fibroblasts were prepared as described (28). GT-1 fibroblasts were suspended in a mixture of type I collagen (Koken, Tokyo, Japan) and DMEM medium containing 10% Fetal bovine serum and seeded in 12-well culture dishes. The collagen was allowed to solidify by incubating at 37°C for 1 h. The final concentrations of collagen and

GT-1 fibroblasts were 1 mg/ml and 1×10^6 cell/ml, respectively. SCC cells (1×10^6) suspended in 1 ml of culture medium were seeded on the collagen gel. After incubation at 37°C for 1 h, the gels were removed from the sides and bottoms of dishes and floated in the medium. After 1 week of incubation, the contracted gel was placed on a nylon mesh, and culture medium was added until the fluid level reached the upper edge of the gel. The gels were incubated under air-liquid interface culture for 1 more week. Culture medium was changed every second day. The gel was fixed with Mildform® (Wako, Osaka, Japan), embedded in paraffin and stained with hematoxylin-eosin (HE) as previously described (29).

Results

Downregulation of p63 in SCC cells with the Snail-induced EMT phenotype. The A431 and OM-1 cell lines derived from human SCCs have an epithelial manner, which includes cuboidal cell shape, cell-cell adhesion and strong expression of E-cadherin. We previously established the A431-SNA1 and OM-1-SNA1 cell lines that express exogenous Snail, and these cells underwent EMT and acquired invasive activity naturally *in vitro* (25). A431-pcD1 and OM-1-pcD1 cells, which were transfected with empty vector, were prepared as control cells.

First, we examined expression of *p63* and its isoforms by semi-quantitative RT-PCR. Primers specific for p63 isoforms are shown in Fig. 1A. *p63* was expressed in A431 and OM-1 cells (Fig. 1B). Expression of TA isoforms was reduced and expression of ΔN isoforms was elevated, which indicates that *ΔNp63* is the predominant isoform (Fig. 1B). In addition, *p63α* is predominant form of the C-terminus variants (Fig. 1C). Expression of *p63β* was also detected, but at lower levels than expression of *p63α* (Fig. 1C). Therefore, *ΔNp63α* is the *p63* isoform expressed at highest levels in SCC with epithelial phenotype, which is consistent with previous reports (30).

A431 and OM-1 cells showed complete reduction of *ΔNp63α* expression in response to exogenous expression of Snail (Fig. 1B). The downregulation of p63 in Snail-expression cells was confirmed by immnoblotting (Fig. 1D).

C/EBP-independent suppression of ΔNp63 promoter activity by Snail. To confirm that ΔNp63α expression is influenced by Snail, the *ΔNp63* promoter activity in response to Snail was monitored by luciferase reporter assay. Various lengths of the *ΔNp63* promoter region were amplified and cloned into pGL3-basic vector (construct 1 to 6) (Fig. 2A). A431 cells were transiently co-transfected with the ΔNp63 promoter constructs, and Snail-expression vector (Snail (+)) or empty vector (Snail (-)) (Fig. 2A). Snail consistently reduced the basal activities of the *ΔNp63* reporter constructs by ~50% (Fig. 2A). The basal activity of the reporter construct containing nt -160 to +262 of the *ΔNp63* promoter region (construct 4), was significantly lower than the activity of the -167 reporter construct (construct 3) (Fig. 2A). These data indicate that Snail inhibits promoter activity of the *ΔNp63* promoter, and that the sequence of nt -167 to -161 was important for positive regulation of the *ΔNp63* promoter.

Putative RREB-1, C/EBP and GATA-1 binding elements are located at nt -171 to -159, nt -167 to -155 and nt -160 to -151, respectively, in the *ΔNp63* promoter (MOTIF Search, http://motif.genome.jp/) (Fig. 2B). There is some overlap between these sequences (Fig. 2B). The construct 4 contains the full-length GATA-1 binding element and had low transcriptional activity (Fig. 2B). To determine whether transcription of *ΔNp63* requires binding to the C/EBP or RREB-1 element, we generated a reporter construct with a mutant C/EBP binding element (C/EBP mt reporter construct) (Fig. 2B). The C/EBP mt reporter construct has an intact RREB-1 binding element (Fig. 2B). The transcriptional activity of the construct with the mutant C/EBP binding

element was significantly lower than activity of wild-type promoter in A431 cells (Fig. 2C). The level of suppression of activity of the promoter with the mutant C/EBP binding element was similar to that caused by Snail (Fig. 2C). Moreover, the activity of the C/EBP mt reporter construct was the same as that of the construct 4, which lacks the C/EBP binding element (Fig. 2A and 2C). These data identify the C/EBP binding element as a positive regulatory region for transcription of *ΔNp63*. C/EBPα, a member of the C/EBP family of transcription factors, is abundantly expressed in keratinocytes and modulates squamous differentiation (31). *C/EBPα* was expressed in A431 and OM-1 cells in the presence or absence of Snail (Fig. 2D). Taken together, these data suggest that Snail inhibits the promoter activity of *ΔNp63* enhanced by C/EBPα.

Suppression of ΔNp63α and C/EBPα in SCC cells with EMT phenotype. Although forced expression of Snail induced EMT of SCC cells, HOC719-NE, HOC313 and TSU cells acquired EMT and invasive behavior naturally *in vitro* (9). Interestingly, HOC719-NE cells were isolated as E-cadherin-negative phenotype cells from HOC719 cells, which show heterogeneous E-cadherin expression (9). We next confirmed p63 expression in cells with the EMT phenotype by semi-quantitative RT-PCR and immunoblotting (Fig. 3A and 3B). Cells with EMT phenotype showed complete suppression of *ΔNp63α* (Fig. 3A and 3B). Intriguingly, reduction of *C/EBPα* and upregulation of *Snail* were also observed in these cells simultaneously (Fig. 3C). These data suggest that the loss of *ΔNp63α* also results from reduced expression of C/EBPα independent of

Snail.

p63-independent E-cadherin expression and morphology in p63 knockdown SCC cells without EMT. To confirm the functional contribution of ΔNp63α to tumor invasion, we generated p63 knockdown SCC cell lines (A431_p63 siRNA and OM-1_p63 siRNA) by transfecting vector expressing a p63-silencing siRNA. Control cells, A431_control siRNA and OM-1_control siRNA, were generated by transfection of vector expressing non-silencing siRNA. A431 and OM-1 cells displayed slight changes in cell shape and cell-cell adhesion after p63 knock-down (Supplementary information, Fig. S1A). To examine growth rate, cell growth assay were performed. p63 knockdown cells showed growth rates similar to those of corresponding control cells (Supplementary information, Fig. S1B). Under these conditions, E-cadherin expression was observed (Fig. 4A). To confirm the localization of E-cadherin, immunofluorescent staining was performed. E-cadherin was localized on the cell membrane (Fig. 4B). These findings indicate that loss of p63 did not result in EMT.

p63 knockdown SCC cells acquire an invasive phenotype. The invasion assay with an 8 μm pore size PET membrane with MatriGel™ basement membrane containing laminin and collagen IV allows assessment of migration and invasive property of SCC cells. The cells on the bottom surface of the membrane of each chamber were counted as invading cells after staining cells

with Trypan blue. As expected, the numbers of invading A431 and OM-1 cells increased remarkably (9.2 and 12.9 fold, respectively) after p63 knockdown (Fig. 4C). *In vitro* 3D culture reconstructs the squamous epithelial structure by overlaying SCC cells on collagen I gel containing immortalized fibroblasts. *Intriguingly, t*he p63 knockdown cells formed a single-layer surface on the gel and invade into the gel layer (Fig. 4D). In contrast, the corresponding control cells remained confined to the upper gel surface and stratified. ***These invasion assays provide evidence that loss of p63 leads to acquisition of high migration activity and invasive phenotype in vitro. The invasive activity acquired by loss of p63 does not require downregulation of E-cadherin.***

Suppression of invasive activity by forced expression of ΔNp63α in SCC cells with EMT phenotype. HOC313 cells acquired the EMT phenotype and invasive phenotype *in vitro*, which is characterized by fibroblastoid shape and scattered growth due to release of cell-cell adhesions (9). As a gain-of-function approach, we generated forced ΔNp63α-expressing HOC313 cells (HOC313_ΔNp63α) by transfecting a V5-tagged full-length ΔNp63α expression vector (pcDNA6-V5/His/ΔNp63α). Control HOC313_pcDNA6 cells were prepared by transfecting the empty vector. ΔNp63α did not affect cell morphology (Supplementary information, Fig. S2A), or growth rate (Supplementary information, Fig. S2B) of HOC313 cells. The ΔNp63α-V5 tag fusion protein was detected in the HOC313_ΔNp63α cells, but E-cadherin expression was not induced by ΔNp63α (Fig. 5A). Therefore, HOC313 cells still show the EMT phenotype even in the presence

of ΔNp63α.

In the MatriGel™ invasion assay, the number of invading HOC313 cells significantly decreased 0.42 fold in response to forced expression of ΔNp63α (Fig. 5B). Moreover, most forced ΔNp63α-expressing cells were unable to invade the gel layer and formed stratified layers on the collagen gel in *in vitro* 3D cultures (Fig. 5C). Taken together, these findings indicate that ΔNp63α blocks invasion of SCC cells with the EMT phenotype, confirming that loss of p63 leads to acquisition of invasive activity regardless of E-cadherin expression.

Discussion

In this study, we demonstrated that Snail inhibits expression of ΔNp63α and that forced depletion of ΔNp63α enhances invasiveness of SCC cell, whereas re-expression of ΔNp63α in ΔNp63α-deficient cells suppresses their invasive activity *in vitro*. Furthermore, the invasive activity caused by forced depletion of ΔNp63α does not require downregulation of E-cadherin. The SCC cells displayed slight change of their morphology by p63 knockdown (Supplementary information, Fig. S1A) or forced ΔNp63α-expression (Supplementary information, Fig. S2A). Expression of vimentin was not elevated remarkably by p63 knockdown in the SCC cells and was not eliminated by expression of ΔNp63α in the EMT phenotype cells (Supplementary information, Fig. S3). These findings suggest that loss of ΔNp63α acquires the invasive phenotype of SCC cell in parallel with 'complete EMT'.

The molecular mechanism of tumor invasion involves altered interactions between tumor cells and their environment as well as intra- and intercellular events such as cell proliferation, loss of cell-cell adhesion, acquisition of cell motility, and loss of cell polarity. How does downregulation of ΔNp63α enhanced the invasive activity in SCC? In our data, ΔNp63α-deficient SCC cells formed a single-layer on the collagen gel and invade into the gel layer in *in vitro* 3D cultures. During development of the epidermis, ΔNp63 is expressed during a late stage of the single-layered surface ectoderm and allows basal keratinocytes to commit to epidermal maturation and terminal differentiation (32). *p63*$^{-/-}$ mice have striking developmental defects,

including complete lack of all stratified squamous epithelia, epidermal appendages, mammary, lacrimal and salivary glands (19, 20), which suggests that p63 plays fundamental roles in formation of epithelial structures. The fact that ectoderm extends epithelial sheets to form buds into the mesoderm during development of epidermal appendages and derivative organs (33) seems to contradict our present results, however, extended epithelial sheets or buds maintain the stratified epithelial structure. The localized expression of ΔNp63α in basal keratinocytes strongly suggests its role in formation of stratified squamous epithelial structures by regulating asymmetric division (34). Polarity of epithelial cells directs the apical-basal and planar axes, and plays crucial roles for cell-cell adherens and tight junctions, and asymmetric cell division (35). We speculate downregulation of ΔNp63α occurs in SCC cells at the invasive front, which may cause loss of cell polarity, and loss of cell polarity then promotes invasion into the adjacent connective tissue.

Because p63 is a member of the p53 family, many studies of p63 functions have been reported, nonetheless, the role of p63 in tumors is not well understood (22). Recently, a genome-wide microarray analysis revealed that the 3q26-29 locus encompassing *p63* is frequently amplified in SCCs of the lung, suggesting that overexpression of p63 facilitates tumorigenesis (36). Barbieri et al. reported that microarray analysis identified genes associated with invasion and metastasis due to loss of p63 in keratinocytes (37). Loss of p63 caused downregulation of cell adhesion-associated genes, cell detachment and anoikis in mammary epithelial cells and keratinocytes (38). The present study provides enforced evidence that loss of p63 directly

involves tumor invasion. Furthermore, ΔNp63α expression is directly correlated with the clinical response to cisplatin in SCCs of head and neck (39). Taken together, these reports and our novel findings indicate that the overexpression of ΔNp63α might involve tumorigenesis of keratinocyte, and its suppression by Snail during progression of SCC leads to the tumor invasion. Various levels of ΔNp63α expression might decide fate of SCC cells in their growth or against clinical treatment.

In conclusion, during progression of SCC toward a more malignancy, Snail is expressed as an initial event, and then downregulates E-cadherin, resulting EMT and acquisition of the invasive phenotype. Figure 6 here shows the schematic representation of our novel findings, indicating that Snail downregulates ΔNp63α in parallel with E-cadherin, leading to acquisition of another invasive phenotype. Reduction of C/EBPα, a positive transcriptional regulator of ΔNp63, also results in loss of ΔNp63α. The precise mechanism by which loss of ΔNp63α exerts this function remains poorly defined and is currently under investigation. The modulation of ΔNp63α expression may provide a target for clinical treatment of progressive SCC.

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Figure legends

Figure 1. Downregulation of p63 in SCC cells with EMT phenotype induced by Snail. *A*, The *p63* gene consists of 15 exons and an extra exon 3' that contains the transcriptional start site for ΔN isoforms. Arrows indicate primers for RT-PCR amplification specific for isoforms/variants. All isoforms were amplified with primers located in exons 4 and 5 because all isoforms contain exons 4 to 10. For amplification of TA or ΔN isoforms, the sense primer was placed in exon 1 or 3', respectively. The C-terminal variants arise from alternative splicing. The full-length form is referred to as *p63α*, which was amplified specifically with an antisense primer located in exon 13. Transcription of the *α*, *β* and *γ* variants terminates in exons 14, 14 and 15, respectively. Antisense primers were positioned in exons 14 and 15 for amplification of these variants. *B*, To quantify expression of *p63* and its various isoforms, semi-quantitative RT-PCR was performed. *C*, The primers located in exons 10 and 14, distinguish isoforms *p63α* and *β*. The longer product containing exon 13 is *p63α*. The shorter is *p63β*. *p63γ* was amplified with primers located in exons 10 and 15. *D*, p63 and E-cadherin protein levels were examined by immunoblotting (IB). Anti-p63 antibody detected two bands: ΔNp63α and β.

Figure 2. C/EBP-independent suppression of *ΔNp63* promoter activity by Snail. *A*, Luciferase reporter assay was performed to identify the core promoter for *ΔNp63* transcription and to monitor activity of the *ΔNp63* promoter in response to Snail. Six area of the *ΔNp63* promoter

region were amplified and cloned into pGL3-basic vector (construct 1 to 6 include the *ΔNp63* promoter sequence between nt -558 and +262, nt -167 and +262, nt -160 and +262, nt -115 and +262, and nt -44 and +262, respectively). A431 cells were transiently co-transfected with the *ΔNp63* reporter constructs and a Snail-expression vector (Snail (+)) or empty vector (Snail (-)). The luciferase activities were normalized to *Renilla* activity and luciferase values are expressed as a percentage of the construct 1. *B*, Putative RREB-1, C/EBP and GATA-1 binding sites are located at nt -171 to -159, nt -167 to -155 and nt -160 to -151 in the *ΔNp63* promoter region, respectively. The construct 1, which contains a part of the C/EBP binding element, was mutated between nt -157 to 153 to generate pGL3-ΔNp63_C/EBP mt. *C*, A431 cells were also transiently co-transfected with the construct 1 (C/EBP wt) or the pGL3-ΔNp63_C/EBP mt (C/EBP mt) and Snail-expression vector (Snail (+)) or empty vector (Snail (-)). The luciferase activities were normalized to *Renilla* activity and luciferase values are expressed as a percentage of the construct 1. *D*, C/EBPα expression was examined by semi-quantitative RT-PCR.

Figure 3. Loss of p63 expression in SCC cells with EMT phenotype. *A*, To detect expression of *p63* and its isoforms, semi-quantitative RT-PCR was performed in SCC cell lines. HOC719-PE (positive E-cadherin) cells and HOC719-NE (negative E-cadherin) cells were isolated from HOC719 cells which express E-cadherin heterogeneously (9). SCC cell lines HOC719-NE, HOC313 and TSU express the EMT phenotype, which consists of strongly expression of vimentin,

naturally acquired invasive behavior *in vitro*, fibroblastoid morphology and reduced expression of E-cadherin (9). In contrast, A431, OM-1 and HOC719-PE cells display the epithelial phenotype, which includes expression of E-cadherin. *B*, p63 and E-cadherin protein levels were examined by immunoblotting (IB). Anti-p63 antibody detected proteins: ΔNp63α and β. *C*, Expression of *C/EBPα* and *Snail* were examined by semi-quantitative RT-PCR.

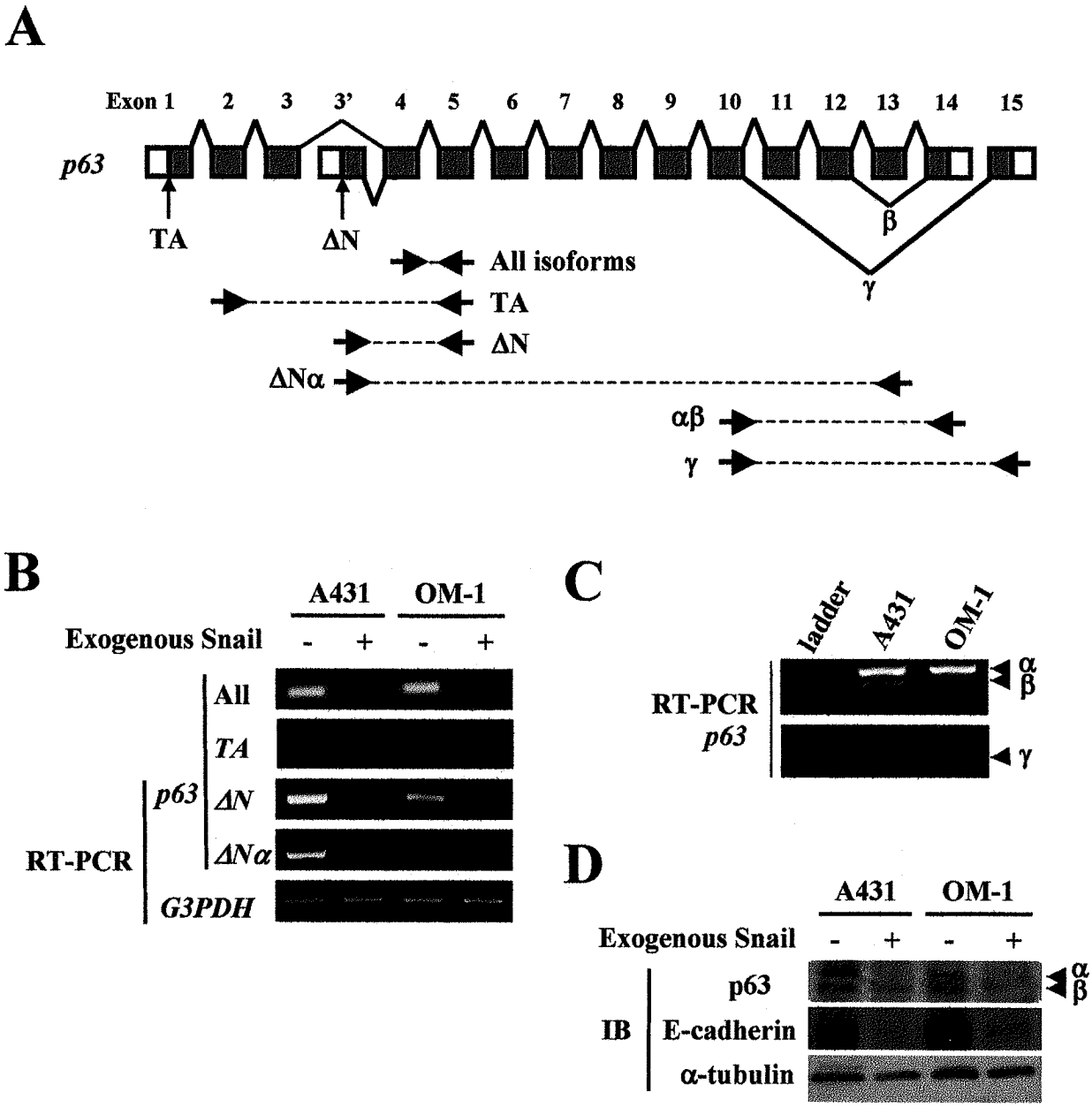
Figure 4. p63-independent E-cadherin expression, morphology and gain of invasive activity in p63 knockdown SCC cells without EMT. p63 knockdown SCC cell lines, A431_p63 siRNA and OM-1_p63 siRNA, were generated by transfecting the p63-silencing siRNA expression vector into A431 and OM-1 cells, respectively. The siRNA targets all p63 isoforms. Control cell lines A431_control siRNA and OM-1_control siRNA were generated by transfecting a vector encoding non-silencing siRNA into A431 and OM-1 cells, respectively. *A*, p63 and E-cadherin protein levels were examined by immunoblotting (IB). Anti-p63 antibody detected proteins: ΔNp63α and β. *B*, Localization of E-cadherin and F-actin was assessed by immunofluorescent staining. Alexa Fluor 568-labeled anti-rabbit IgG was used for staining E-cadherin (red). F-actin fibers were stained with Alexa Fluor 488 phalloidin (green). *C*, Invasion assay with an 8 μm pore size PET membrane with MatriGel™ basement membrane containing Laminin and collagen IV allows assessment of migration activity and invasive property. Cells on the bottom surface of each PET membrane were counted as the number of invading cells (vertical axis) per microscopic field

after staining with Trypan blue. Invading cell numbers represent the mean of six independent fields at x100 magnification. *D*, To investigate invasive growth into feeder layers and the mode of invasion of SCC cells, *in vitro* 3D culture was performed. The paraffin sections (4-6 μm) were stained with hematoxylin-eosin (HE).

Figure 5. Suppression of invasive activity of SCC cells with EMT phenotype by re-expression of ΔNp63α. HOC313_ΔNp63α cells were isolated by transfecting a vector encoding V5-tagged full-length ΔNp63α (pcDNA6.1/V5/ΔNp63α) into the naturally invasive HOC313 SCC cell line *in vitro*. *A*, Expression of p63-V5 tag fusion protein was confirmed, and the E-cadherin levels were examined by immunoblotting (IB). Anti-V5 and anti-p63 antibodies detected a single band, the ΔNp63α-V5 tag fusion protein. To investigate the invasive activities of HOC313_ΔNp63α and control cells, MatriGel™ invasion assay (*B*) and *in vitro* 3D cultures (*C*) were performed.

Figure 6. Downregulation of ΔNp63α modulated by Snail or C/EBPα acquires invasive phenotype of SCC independent of E-cadherin. During progression of SCC toward a more malignancy, Snail is expressed as an initial event, and then downregulates E-cadherin, resulting EMT and acquisition of the invasive phenotype. Snail downregulates ΔNp63α in parallel with E-cadherin, leading to acquisition of another invasive phenotype. Reduction of C/EBPα, a

positive transcriptional regulator of ΔNp63, also results in loss of ΔNp63α.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Exon 1 2 3 3' 4 5 6 7 8 9 10 11 12 13 14 15
p63
TA
ΔN
β
γ
All isoforms
TA
ΔN
ΔNα
αβ
γ
B
A431
OM-1
Exogenous Snail - + - +
All
TA
p63
ΔN
ΔNα
RT-PCR
G3PDH
C
ladder
A431
OM-1
RT-PCR
p63
α
β
γ
D
A431
OM-1
Exogenous Snail - + - +
p63
IB
E-cadherin
α-tubulin
α
β

Figure 1.

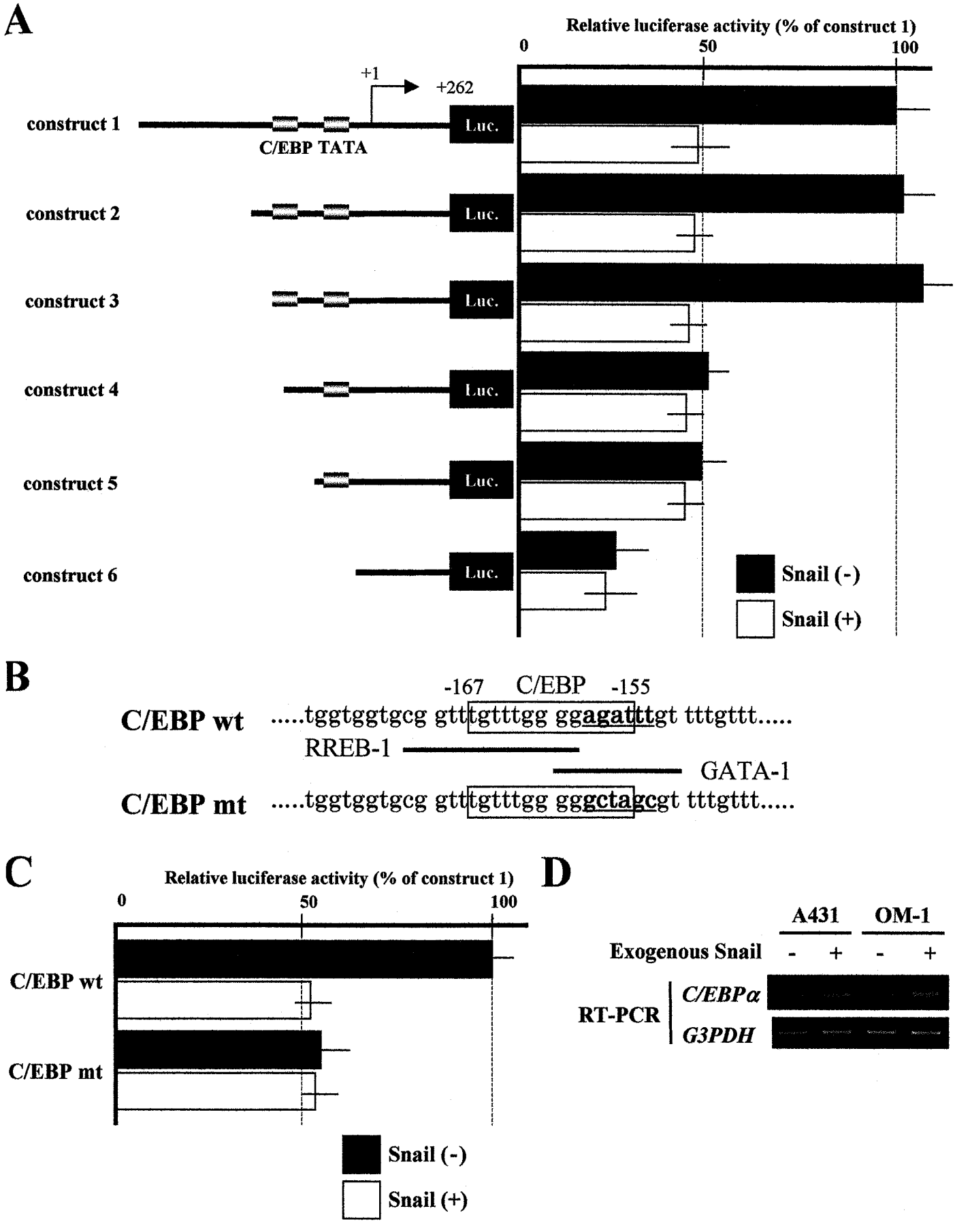

A
Relative luciferase activity (% of construct 1)
0
50
100
+1
+262
construct 1
C/EBP TATA
Luc.
construct 2
construct 3
construct 4
construct 5
construct 6
Snail (-)
Snail (+)
B
-167 C/EBP -155
C/EBP wt .....tggtggtgcg gtttgtttgg ggagatttgt tttgttt.....
RREB-1
GATA-1
C/EBP mt .....tggtggtgcg gtttgtttgg gggctagcgt tttgttt.....
C
Relative luciferase activity (% of construct 1)
0
50
100
C/EBP wt
C/EBP mt
Snail (-)
Snail (+)
D
A431
OM-1
Exogenous Snail - + - +
RT-PCR
C/EBPα
G3PDH

Figure 2.

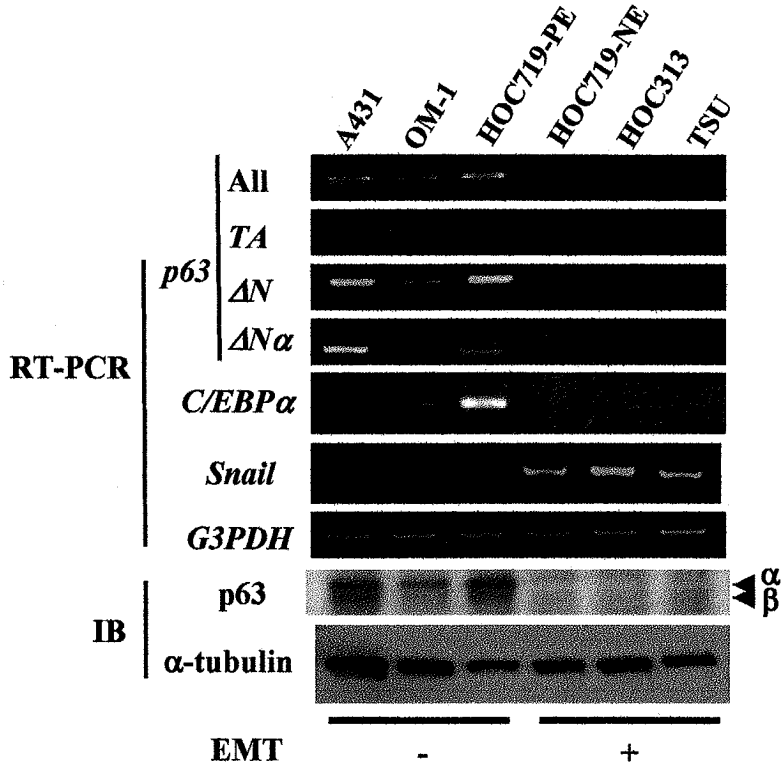

A431
OM-1
HOC719-PE
HOC719-NE
HOC313
TSU
RT-PCR
p63
All
TA
ΔN
ΔNα
C/EBPα
Snail
G3PDH
IB
p63
α
β
α-tubulin
EMT
-
+

Figure 3.

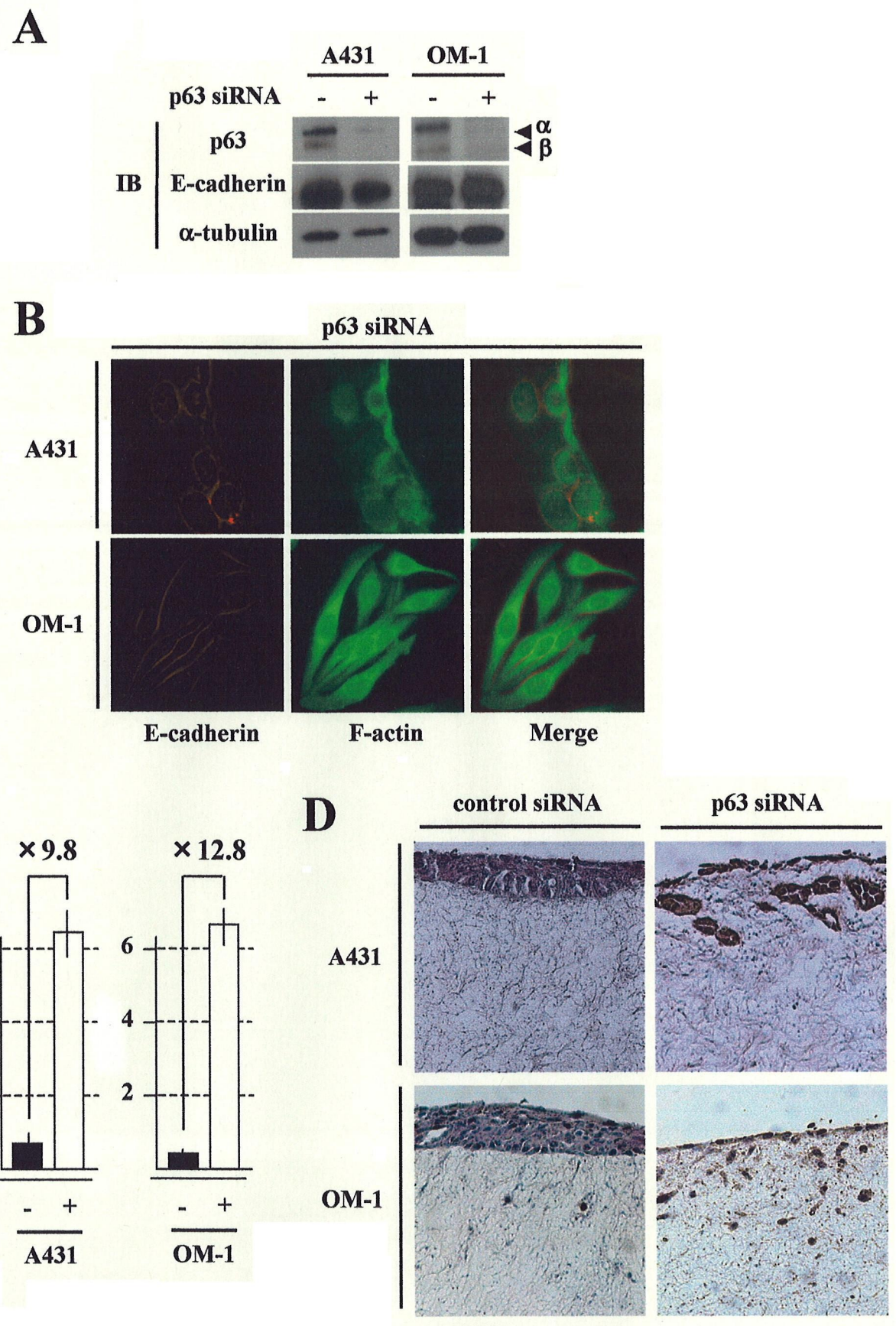
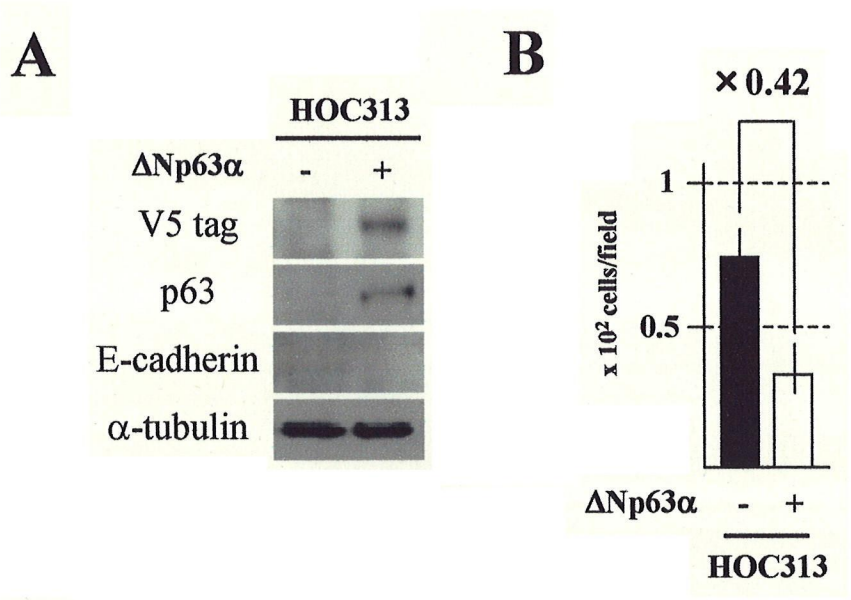

A
A431
OM-1
p63 siRNA
- + - +
IB
p63
E-cadherin
α-tubulin
α
β
B
p63 siRNA
A431
OM-1
E-cadherin
F-actin
Merge
C
×9.8
×12.8
x 10² cells/field
3
2
1
6
4
2
p63 siRNA
- + - +
A431
OM-1
D
control siRNA
p63 siRNA
A431
OM-1

Figure 4.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ΔNp63α - +
V5 tag
p63
E-cadherin
α-tubulin
B
× 0.42
1
0.5
x 10² cells/field
ΔNp63α - +
HOC313

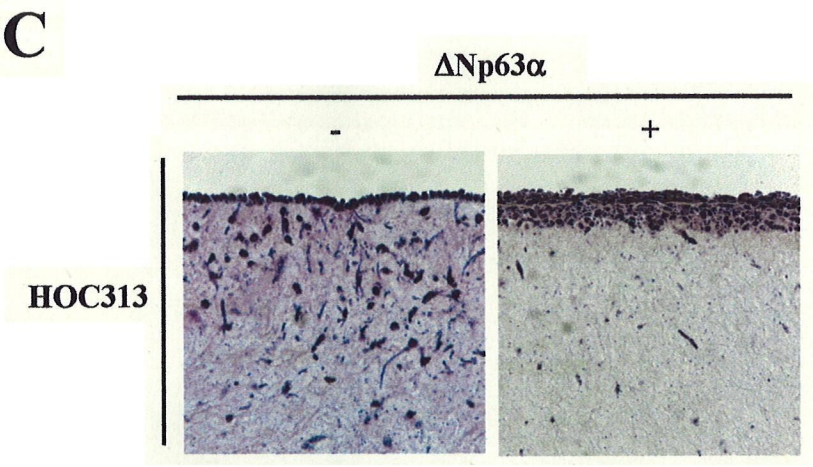

C
ΔNp63α
- +
HOC313

Figure 5.

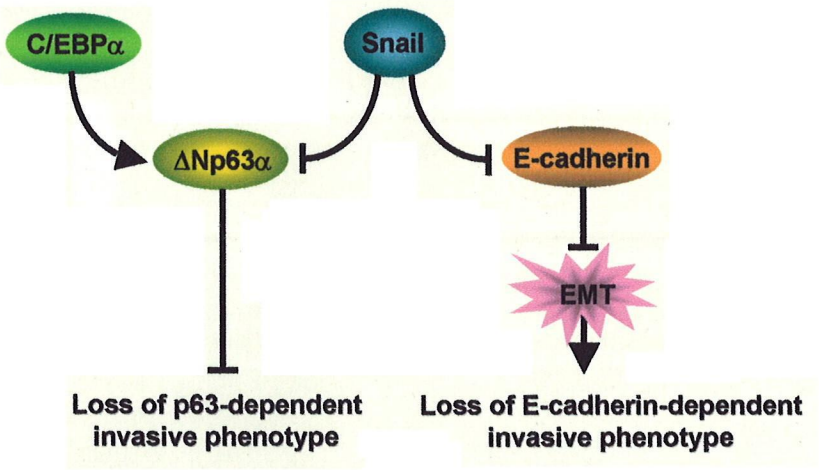
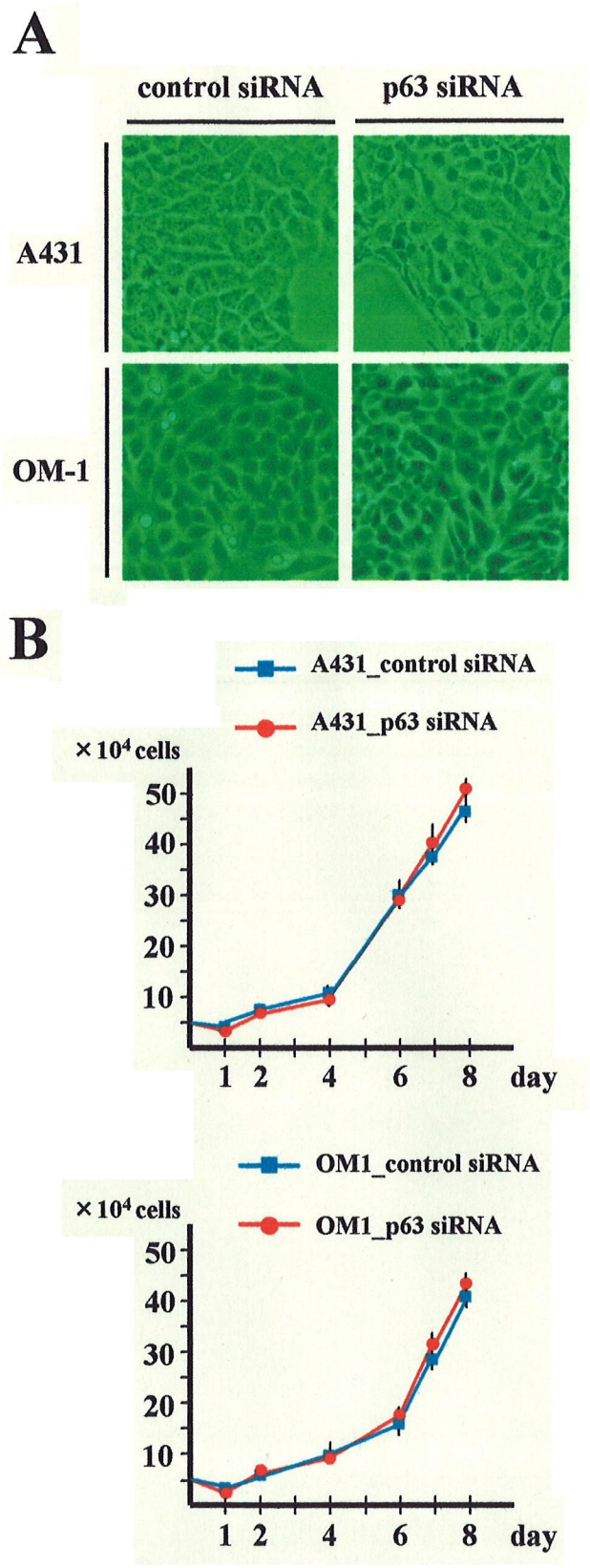
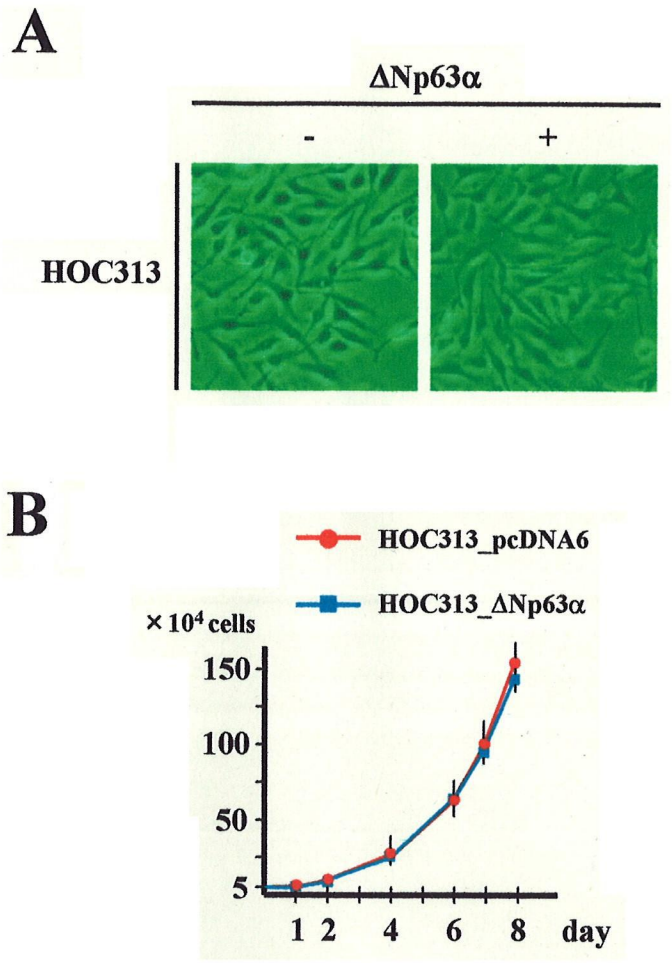

C/EBPα
Snail
ΔNp63α
E-cadherin
EMT
Loss of p63-dependent invasive phenotype
Loss of E-cadherin-dependent invasive phenotype

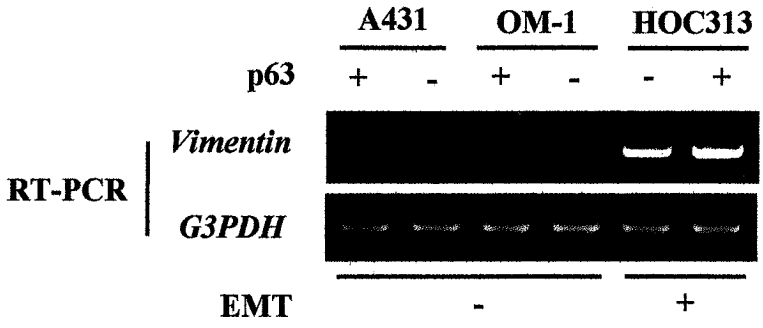
Figure 6.



Supplementary Figure 1.



Supplementaly Figure 2.



Supplementary Figure 3.