Received: 25 March 2018 | Revised: 24 August 2018 | Accepted: 1 September 2018

DOI: 10.1111/cas.13791

ORIGINAL ARTICLE

WILEY Cancer Science

Hypoxia-inducible transgelin 2 selects epithelial-to-mesenchymal transition and γ-radiation-resistant subtypes by focal adhesion kinase-associated insulin-like growth factor 1 receptor activation in non-small-cell lung cancer cells

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Funding information
Ministry of Science and ICT (Nuclear Research and Development Program, Grant/Award Number: NRF-2013M2A2A7043660

Microenvironment, such as hypoxia common to cancer, plays a critical role in the epithelial-to-mesenchymal transition (EMT) program, which is a major route of cancer metastasis and confers γ-radiation resistance to cells. Herein, we showed that transgelin 2 (TAGLN2), an actin-binding protein, is significantly induced in hypoxic lung cancer cells and that Snail1 is simultaneously increased, which induces EMT by downregulating *E-cadherin* expression. Forced *TAGLN2* expression induced severe cell death; however, a small population of cells surviving after forced *TAGLN2* overexpression showed γ-radiation resistance, which might promote tumor relapse and recurrence. These surviving cells showed high metastatic activity with an increase of EMT markers including Snail1. In these cells, TAGLN2 activated the insulin-like growth factor 1 receptor β (IGF1Rβ)/PI3K/AKT pathway by recruitment of focal adhesion kinase to the IGF1R signaling complex. Activation of the IGF1Rβ/PI3K/AKT pathway also induced inactivation of glycogen synthase kinase 3β (GSK3β), which is involved in Snail1 stabilization. Therefore, both the IGF1Rβ inhibitor (AG1024) and the PI3K inhibitor (LY294002) or AKT inactivation with MK2206 lower the cellular level of Snail1. Involvement of GSK3β was also confirmed by treatment with lithium chloride, the inducer of GSK3β phosphorylation, or MG132, the 26S proteasomal inhibitor, which also stabilized Snail1. In conclusion, the present study provides important evidence that hypoxia-inducible TAGLN2 is involved in the selection of cancer cells with enhanced EMT properties to overcome the detrimental environment of cancer cells.

KEYWORDS
γ-radiation resistance, hypoxia, IGF1Rβ, Snail1, transgelin 2

1 | INTRODUCTION

Epithelial-to-mesenchymal transition (EMT), found in the process of gastrulation during embryogenesis, is involved in the development of tissue or organs. [1] Recent studies have shown that EMT is also critical for wound healing and tissue regeneration.[2] More importantly, EMT not only induces the motility and invasiveness of cancer cells but also allows them to avoid anoikis, apoptosis, and cellular

senescence, which is necessary for cancer cells to travel long distances from the original tumor site.[3,4] Thus, the EMT process plays a major role in conferring cancer stem cell (CSC) properties or therapeutic resistance to cells and is consequently associated with tumor recurrence.[5,6] Human non-small-cell lung cancer (NSCLC) cell lines with WT epidermal growth factor receptor (EGFR) show a range of sensitivity to EGFR inhibitors, such as gefitinib and erlotinib, depending on the extent to which they have undergone EMT.[7] There is also emerging evidence for a stimulating effect of therapeutic drugs and γ-radiation on phenotypic changes consistent with EMT or CSC characteristics.[8-10]

Epithelial-to-mesenchymal transition is a multistep process involving disruption of intercellular adherence and increased motility, which is mainly dependent on the loss of the cell surface marker E-cadherin and the increase in mesenchymal marker proteins such as N-cadherin and vimentin.[11-13] Furthermore, several transcription factors, including zinc-finger E-box-binding homeobox (Zeb), Twist, and Snail1 family members and their associated signaling pathways, are implicated in the phenotypic switch during the EMT process by regulating *cadherin* expression.[14] Thus, there is a lot to be explained regarding the molecular signal mechanism by which the expression or stabilization of these various EMT-related proteins is regulated at the beginning of EMT.

Hypoxia, a reduction in tissue oxygen tension, is a common microenvironmental stimulus that plays a critical role in embryonic development and malignant progression of tumors.[15,16] Limited availability of oxygen determines whether cells initiate cell death or adapt to hypoxia. Small subtypes of cells can adapt to this environmental stress so that after repeated periods of hypoxia, selection for resistance to radiation therapy or chemotherapy can occur.[17,18] Recent advances in understanding the molecular signal pathways that govern the association of hypoxia with malignant tumors also point to the importance of EMT.[19,20] Microenvironmental conditions, such as hypoxia or inflammation in tumors, are important factors in the induction of a pathological EMT.[21,22] Indeed, the hypoxic status of various solid tumors mediates the progression of malignant tumors by selecting cells with diminished apoptotic potential and activating genes involved in metastasis, angiogenesis, and metabolism.[23,24] Therefore, identifying the intrinsic and extrinsic factors that induce EMT under a hypoxic environment and characterizing their signal networks will be important in overcoming the limitation of cancer therapy in several tumor types.

To date, only hypoxia-inducible factor 1 (HIF-1) and hypoxia-inducible microRNA have been identified as intrinsic inducers of EMT-associated CSC properties under hypoxic microenvironmental conditions.[25,26] HIF-1α induces Snail1, which is a central transcription regulator in EMT.[27,28] In the present study, we showed that with an increase in Snail1, the cellular level of transgelin 2 (TAGLN2), an actin-binding protein with an ambiguous function, is also significantly induced in hypoxic conditions (0.5%~1%). In addition, hypoxia-inducible TAGLN2 is involved in the selection and survival of reinforced EMT and the radiation-resistant small population of cells by enhancing stabilization of Snail1 by phosphorylation of glycogen synthase kinase 3β (GSK3β) through the focal adhesion kinase (FAK)-mediated insulin-like growth factor 1 receptor β (IGF1Rβ)/PI3K/AKT activation pathway.

2 | MATERIALS AND METHODS

2.1 | Cell culture, hypoxic exposure of non-small-cell lung cancer cells, and chemicals

Human NSCLC cell lines (A549, H460, H358, H23, H1299, and Calu-3) and human fibroblast-like fetal lung cells (WI38) were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea) and cultured with RPMI-1640 media (Hyclone, Logan, UT, USA) containing 10% (v/v) FBS (FBS; Hyclone), 2 mmol/L glutamine and antibiotics (Hyclone) in a 5% CO_2 humidified incubator. For cell culture in hypoxic conditions, cells were plated in 100-mm dishes until 80% confluence and were subjected to hypoxia by placing them in a hypoxia incubator (Innova Co-48; New Brunswick Scientific, Edison, NJ, USA) for up to 16 hours with final oxygen concentration of 0.5% or 1%. For glucose deprivation experiments, confluent cells were cultured in glucose-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with antibiotics, glutamine, and 10% FBS. Lithium chloride (LiCl) was purchased from Sigma-Aldrich (St Louis, MO, USA), and AG1024, MG132, and LY294002 were purchased from Calbiochem (La Jolla, CA, USA), and cells were treated as indicated. The cytotoxic and DNA-damaging reagents methyl methanesulfonate (MMS) and cis-platinum (II)-diamine dichloride (cisplatin) were purchased from Sigma-Aldrich, and cells were treated as indicated. For γ-irradiation, cells were plated in a T25 flask (1×10^6 cells/flask) and, after 24 hours, irradiated with a single dose of 20 Gy (^{60}Co γ-ray source; dose rate, 2 Gy/minutes). Then, the cells were cultured in a 5% CO_2 humidified incubator for the indicated time period.

2.2 | Construction of *TAGLN2* expression vector and transfection

To construct the *TAGLN2* expression vector, a 691-bp insert of human TAGLN2 was cloned from human lung carcinoma cells poly (A) mRNA by RT-PCR using the following primers: *Hin*dIII (forward): 5′-ATTCAAAGCTTTTGAGTCAGTGCGCT-3′ and *Eco*RI (reverse): 5′-TTGAATTCGGGTGGGATCAGAGG-3′. TAGLN2 cDNA was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). TAGLN2-expression vector was transfected into the cells using Lipofectamine 2000 (Invitrogen). For preparation of a cell population of TAGLN2-overexpressing NSCLC cells, cells transfected with TAGLN2-expression vector were treated with G418 (500 μg/mL; Calbiochem) for 10-20 days and used for cellular analysis without further clonal selection. For the establishment of a TAGLN2-overexpressing A549 or H450 cell line, cells transfected with TAGLN2 expression vector were treated with G418 for approximately 21 days and cell clones overexpressing TAGLN2 were selected by RT-PCR and western blot analysis.

2.3 | Reverse transcription-polymerase chain reaction

Total RNA was isolated from H460 and A549 cells with the use of TRIzol reagent (Invitrogen). To generate first-strand complementary DNA, we used a cDNA synthesis kit (Intron Biotechnology, Kyunggido, Korea). Resultant cDNAs served as templates for PCR amplification with the following primers: TAGLN2 (forward), 5′-ATTCAA AGCTTTTGAGTCAGTGCGCT-3′; TAGLN2 (reverse), 5′-TTGAATTCG GGTGGGATCAGAGG-3′; Snail1 (forward), 5′-GCTGCCAATGCTCAT CTGGGACTCT-3′; Snail1 (reverse), 5′-TTGAAGGGCTTTCGAGCCTG GAGAT-3′; β-actin (forward), 5′-ATGTGCAAGGCCCGCTTCG-3′; β-actin (reverse), 5′-TTAATGTCACGCACGATTTCC-3′.

2.4 | siRNAs and transfection

Chemically synthesized siRNAs were purchased from Bioneer (Daejeon, Korea) and annealed according to the manufacturer's protocol. RNA sequences for siTAGLN2, siHIF-1α, and siAKT were as follows: siTAGLN2 (sense): 5′-CUCUGUGUCCUCCGUUCAU-3′, siTAGLN2 (antisense): 5′-AUGAACGGAGGACACAGA-3′; siHIF-1α (sense): 5′-G UGGUUGGAUCUAACACUA-3′, siHIF-1α (antisense): 5′-UAGUGU UAGAUCCAACCAC-3′; siAKT (sense): 5′-GACAACCGCCAUCCA-GACU-3′, siAKT (antisense): 5′-AGUCUGGAUGGCGGUUGUC-3′. Cells were transfected with either siRNA oligomers (Bioneer) or Stealth RNAi Negative Control Medium GC (Invitrogen) at a concentration of 100 nmol/L using the Lipofectamine RNAi MAX reagent (Invitrogen). The cells were incubated for 48 or 72 hours post-transfection, after which target gene expression was determined.

2.5 | Flow cytometric analysis and sorting

For cell death analysis using propidium iodide (PI) staining, cells (1×10^6 cells in 200 μL PBS) were suspended in 1 mL ice-cold 70% ethanol and left for 30 min at −20°C. Cells were harvested by centrifugation and resuspended in 500 μL PBS. Then, 100 μL RNase (50 U/mL) and 100 μL PI (50 μg/mL) were added and the cells were incubated at 4°C for 1 hour. After incubation, the cells were analyzed using flow cytometry (FC500; Beckman Coulter, Brea, CA, USA). For apoptotic cell analysis, cells stained with Annexin V-FITC/PI (FITC Annexin V Apoptosis Detection Kit I; BD Biosciences, San Jose, CA, USA) were analyzed using flow cytometry, and the percentage of apoptotic cell death was measured. Aldehyde dehydrogenase (ALDH)1^{low} and $ALDH1^{high}$ cells were isolated using ALDEFLUOR reagent (STEMCELL Technologies, Vancouver, BC, Canada) and MoFlo XDP cell sorter (Beckman Coulter, Fullerton, CA, USA) in A549 cells.

2.6 | Immunofluorescence staining

Cells (2×10^5) were seeded on glass coverslips in six-well plates. After fixing with 4% paraformaldehyde followed by permeabilization with 1% Triton X-100, cells were stained with Alexa Fluor 594 phalloidin (Invitrogen) to label F-actin. Stained cells were observed under a fluorescence microscope (Olympus IX71; Olympus, Tokyo, Japan).

2.7 | Western blot analysis and immunoprecipitation

Cell lysates were prepared in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.25% sodium deoxycholate, 1% NP-40, 1 mmol/L EDTA) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany) and, then, equal amounts of protein were separated on SDS-PAGE. The proteins were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) and probed with primary antibodies. Antibodies used in the present study were as follows: antibody against TAGLN2 (10234-2-AP; ProteinTech Group, Inc., Rosemont, IL, USA); TAGLN2 (166697), PI3-kinase p110 (8010), phosphatase and tensin homolog (PTEN; 7974), Twist (15393), Snai1 (10432), and Slug (166476) were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p-AKT (9271), AKT (9272), p-EGFR (2236), EGFR (2232), p-IGF1Rβ (101703), IGF1Rβ (3018), p-GSK3β (9322), GSK3β (9315), E-cadherin (3195), and FAK (3285) purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA); N-cadherin (610921) and HIF-1α (610958) were purchased from BD Biosciences; vimentin (9120) was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Fremont, CA, USA); ALDH1A1 (52492) and ALDH1A3 (80176) were purchased from Abcam (Abcam, Cambridge, UK) and the corresponding secondary antibodies (antimouse IgG HRP-linked antibody and antirabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology). Protein concentration was determined by the Lowry method. For protein concentration normalization for equal loading, each blot was probed with anti-β-actin antibody (Cell Signaling Technology). For coimmunoprecipitation assay, cells were lysed either in NP-40 lysis buffer (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 10% glycerol) containing protease and phosphatase inhibitors. Cell lysates containing equal amounts of proteins were incubated with anti-TAGLN2, IGF1Rβ or FAK antibodies overnight at 4°C. The antibody–protein complexes were captured with protein A/G plus agarose beads by incubation for 2 hours at 4°C, and antibody–protein complexes bound to the beads were pelleted at $2000 \times g$ for 2 minutes. The beads were washed three times with lysis buffer and resuspended in reducing SDS sample buffer. The samples were resolved on 12% SDS-PAGE and analyzed by western blotting. Cell lysates containing 10 μg protein were analyzed as input in parallel experiments.

2.8 | Cellular assays

Cell migration/invasion assay, sphere formation assay and colony forming assay were carried out as described in a previous study.[29]

3 | RESULTS

3.1 | Hypoxia-inducible factor 1α-independent induction of TAGLN2 and Snail1 under hypoxic and glucose-deficient conditions

Hypoxia promotes poor prognosis of tumor through the induction of EMT and therapeutic resistance.[30] Transcription factors regulated by HIF-1α, such as Zeb, Twist and Snail1, drive pathological EMT and thus promote malignant tumor progression and metastasis.[31,32] We have found that hypoxia (1% or 0.5%) or glucose deprivation induces cellular TAGLN2 and Snail1 in NSCLC cell lines (Figure 1A, D). Gene Expression across Normal and Tumor tissue (GENT) analysis (http://medical-genome.kribb.re.kr/GENT/search/search.php) also shows that *TAGLN2* expression is elevated in lung cancer compared to normal lung tissues. Interestingly, several lung cancer tissues show highly elevated expression of *TAGLN2* (Figure S1; indicated by dashed line box). When lung cancer cells were treated with the anticancer drug cisplatin or cytotoxic agents such as H_2O_2 and the DNA-damaging agent MMS, cellular levels of TAGLN2 were also increased with AKT activation, suggesting that these factors may be related to poor prognosis through induction of EMT or therapeutic resistance of lung cancer (Figure 1B). Furthermore, TAGLN2 and Snail1 levels were significantly upregulated in ALDH1^{high} cells which show EMT-associated CSC-like properties and are involved in the selection of radiation-resistant tumor cells[33] (Figure 1C, Figure S2).

Under hypoxia conditions (0.5%), with cellular level induction of HIF-1α and TAGLN2, the cellular level of Snail1, a key transcription regulator in the induction of a phenotypic switch in EMT, was significantly increased in NSCLC A549 and H460 cells. PI3K/AKT and IGF1Rβ, one of the upstream receptor tyrosine kinases (RTK) of the PI3K/AKT pathway, were also highly activated. More importantly, in spite of *HIF-1α* suppression with siRNA in A549 or H460 cells, hypoxia significantly increased the cellular level of Snail1 (Figure 1D). In these cells, IGF1Rβ and the AKT pathway were also activated irrespective of *HIF-1α* expression. TAGLN2 was also significantly induced in an HIF-1α-independent way under hypoxic conditions. However, when *TAGLN2* expression was knocked down with siRNA, the hypoxic state did not increase the cellular levels of Snail1 and did not activate IGF1Rβ as much as in control cells (Figure 1E). AKT activation was also slightly but clearly decreased by forced *TAGLN2* suppression. These results strongly suggest that TAGLN2 can be associated with hypoxia-induced EMT through activation of the IGF1R pathway and upregulation of Snail1, irrespective of HIF-1α-mediated mesenchymal switch.

3.2 | Transgelin 2 is associated with the EMT process and resistance to γ-radiation or cytotoxic agents

We investigated whether hypoxia-inducible TAGLN2 is involved in resistance to γ-radiation and EMT, which has been recognized as a contributor to the metastatic progression of cancer. To elucidate the biological function of TAGLN2 in cancer survival, a *TAGLN2* expression vector (pcDNA3.1) was constructed and transiently transfected with A549 or H460 lung cancer cells. Forced *TAGLN2* suppression with siRNA significantly reduced cancer cell growth (Figure 2A). However, unexpectedly, forced *TAGLN2* overexpression also significantly induced cell death and cell growth inhibition (Figure 2B).

Necrosis has been implicated in tumor progression and aggressiveness by releasing a variety of intact cellular components including nuclear factors and cytokines into the extracellular space.[34,35] Necrosis with late apoptosis was the most prevalent type of cell death induced by *TAGLN2* overexpression (Figure 2C, Figure S3). However, Trypan blue staining showed that a small portion of cells survived after transfection of *TAGLN2* overexpression vector (Figure 2D). Subsequently, we examined the live TAGLN2-expression vector transfected cells which were alive after repeated G418 treatment on transfected cells to eliminate non-transfected cells, and verified that the cell growth rate of remaining cells was upregulated (Figure 2E; Transient Tg2+). Cell growth was also upregulated in *TAGLN2*-expressing stable NSCLC cell lines (Figure 2E; Tg2 (+) stable). Surviving *TAGLN2*-overexpressing cells also showed changes in cellular morphology from cobblestone-like cells to spindle-shaped mesenchymal cells and phalloidin staining, which are typical biological phenomena of EMT (Figure 2F). Sphere-forming capacity was also significantly increased in *TAGLN2*-overexpressing cells compared with control cells (Figure 2G). Collectively, we found that TAGLN2 overexpression in NSCLC cells induced changes to mesenchymal cell types and CSC-like properties that enhance radiation resistance.

3.3 | Surviving cells selected after forced *TAGLN* overexpression show enhanced EMT and γ-radiation-resistant properties

With the increase in EMT-associated CSC-like properties, we confirmed that after forced *TAGLN2* overexpression, surviving cells show increased cell invasion and migration capacity. In contrast, *TAGLN2* suppression with siRNA reduced the cell invasive and migratory behavior of lung cancer cells (Figure 3A,B, Figure S4). EMT is closely related to increased CSC properties, which endow cancer cells with drug and γ-radiation therapeutic resistance. When cells surviving after *TAGLN2* overexpression were exposed to cisplatin or γ-radiation (a single dose of 20 Gy), cell death was diminished when compared to that of the counterpart control cells. In contrast, *TAGLN2* suppression with siRNA treatment significantly alleviated the resistance to cisplatin or γ-radiation (Figure 3C). This result strongly indicates that forced *TAGLN2* overexpression or TAGLN2 induction by cytotoxic-damaging agents may also be closely related to the cell protection mechanism against anticancer agents including γ-radiation and cisplatin. In contrast, *TAGLN2* suppression with siRNA treatment sensitized cells to a variety of cell-damaging agents including γ-radiation (Figure 3C).

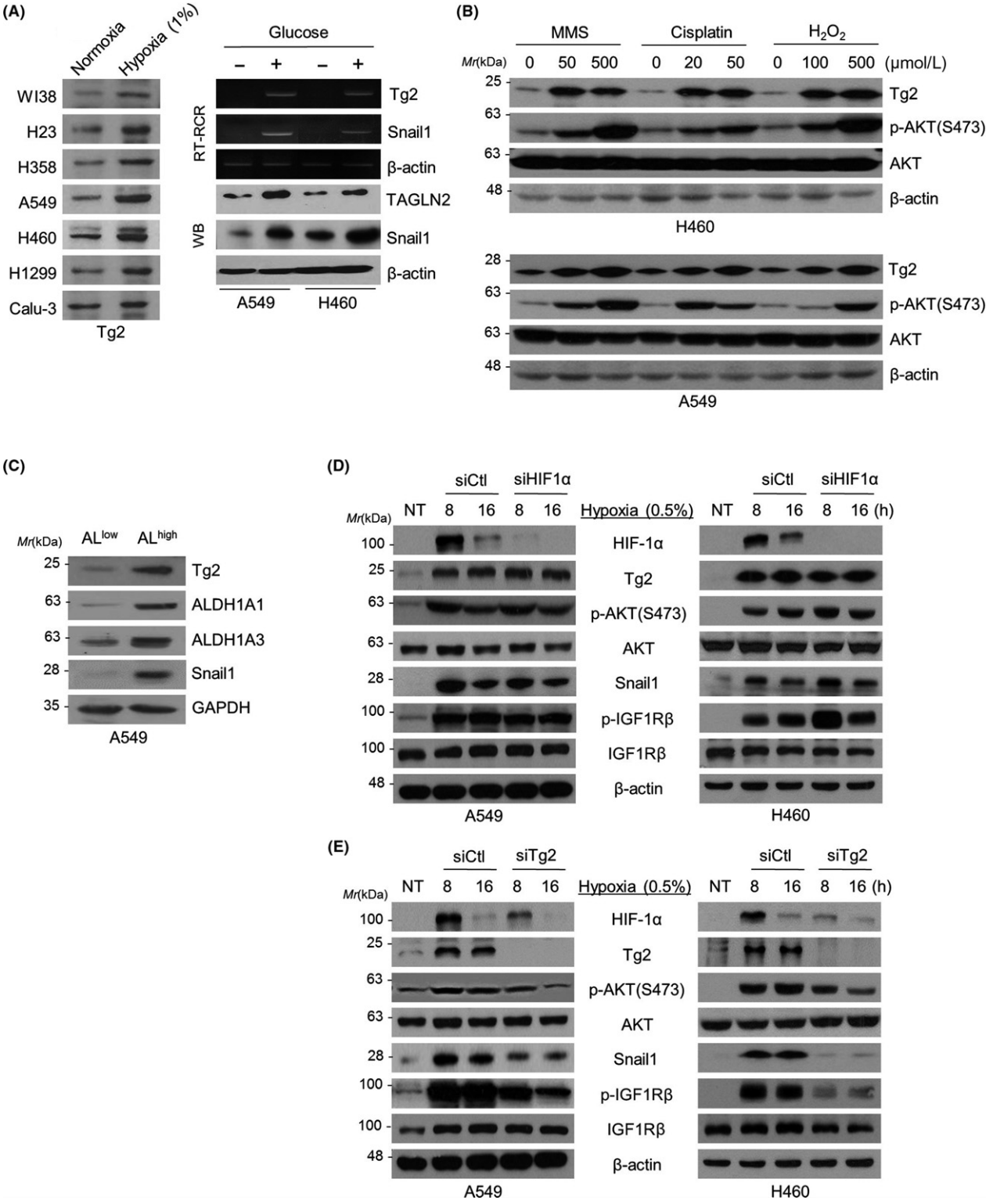


FIGURE 1 Hypoxia inducible factor (HIF)-1α-independent induction of transgelin 2 (TAGLN2) and Snail1 under hypoxic and glucose-deficient microenvironment. A, Western blot analysis and RT-PCR of cellular TAGLN2 (designated Tg2) and Snail1, induction in non-small-cell lung cancer (NSCLC) cell lines under hypoxic (1%, 6 h) or glucose-deficient conditions (6 h). B, Western blot analysis of cellular TAGLN2 induction by cytotoxic agents including methanesulfonate (MMS), cisplatin, and H_2O_2. Cells were treated with the indicated conditions for 6 h and analyzed. C, Western blot analysis of TAGLN2 and Snail1 in aldehyde dehydrogenase (ALDH)1 low and high cells (AL^{low} and AL^{high}) sorted from the A549 cell line (Figure S2). D,E, Changes in TAGLN2, insulin-like growth factor 1 receptor (IGF1R) signaling, and representative epithelial-to-mesenchymal transition markers including E-cadherin and Snail1 under hypoxic stress (0.5%) in NSCLC cells (A549 and H460) transfected with siRNA against HIF-1α (D) or TAGLN2 (E)

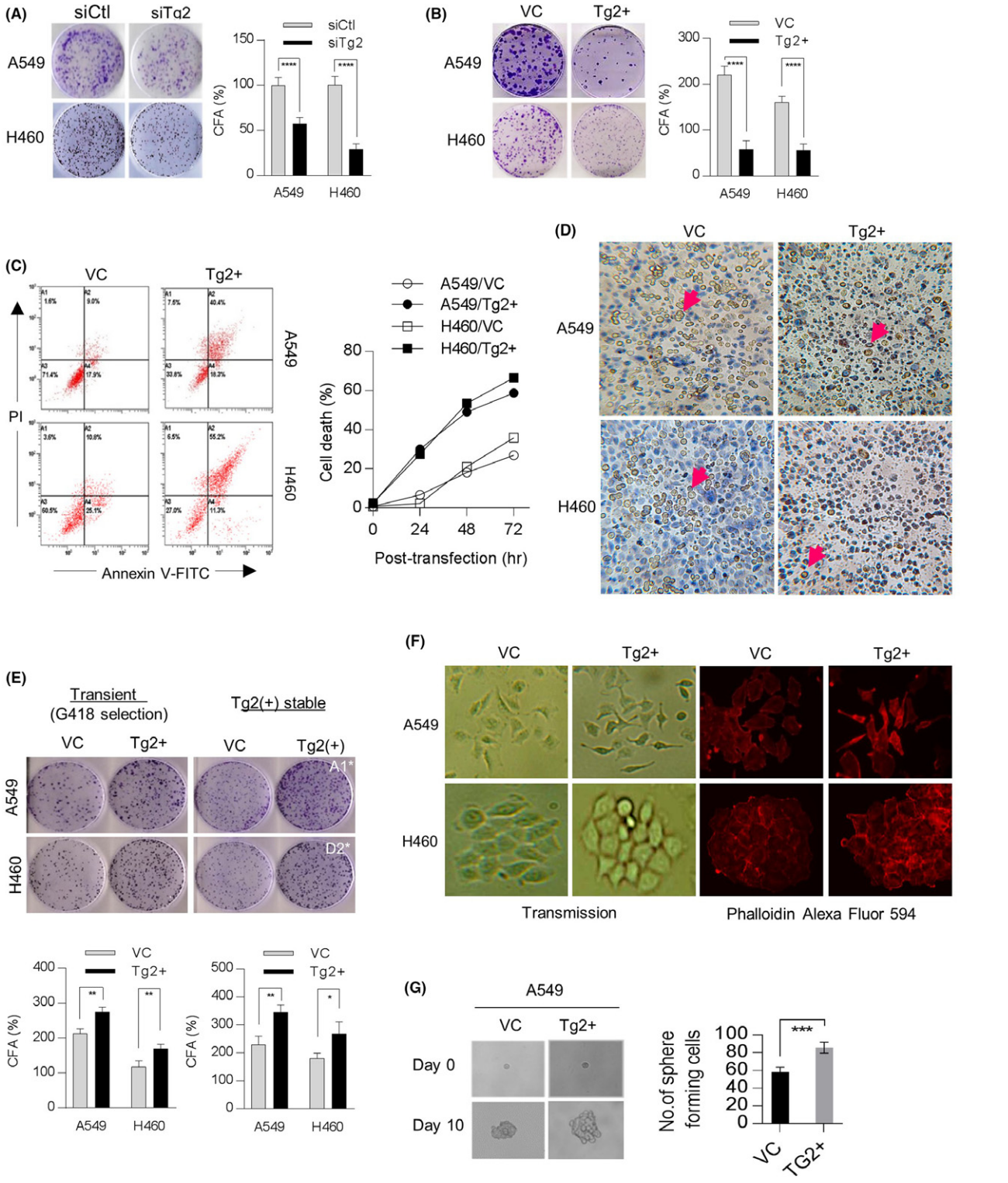


FIGURE 2 Transgelin 2 (TAGLN2) is associated with the epithelial-to-mesenchymal transition process and resistance to γ-radiation or cytotoxic agents. A, Changes in colony-forming activity by *TAGLN2* suppression with siRNA in A549 and H460 cells. B, Changes in colony-forming activity by *TAGLN2* overexpression with pcDNA3.1-*TAGLN2* expression vector in A549 and H460 cells. C, Cell death analysis by annexin V-propidium iodide (PI) staining in non-small-cell lung cancer cells transfected with *TAGLN2* expression vector (see more detail in Figure S3). D, Identification of surviving (arrowhead) or dead cells using Trypan blue staining after *TAGLN2* overexpression with pcDNA3.1-*TAGLN2* expression vector in A549 or H460 cells. E, Increase in colony-forming activity in A549 and H460 cells after transfection with pcDNA3.1-*TAGLN2* expression vector. F, Morphological changes and phalloidin staining of A549 and H460 cells that survived after TAGLN2 overexpression. G, Sphere-forming capacity of *TAGLN2*-overexpressing A549 cells. Quantified results are presented as mean ± SD (three independent experiments) using two-tailed *t* test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ were considered significant. ns, not significant; VC, vector control; Tg2, TAGLN2

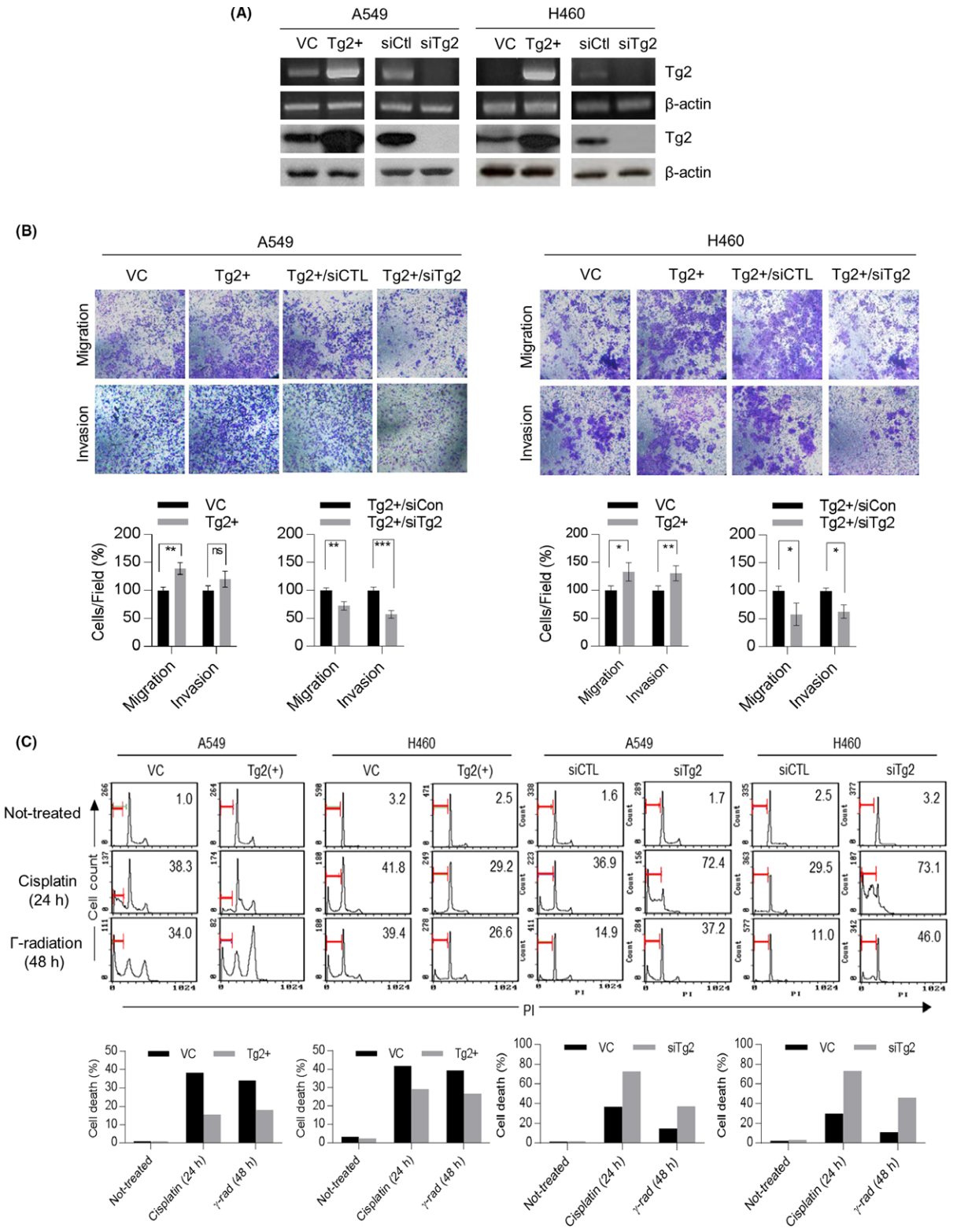
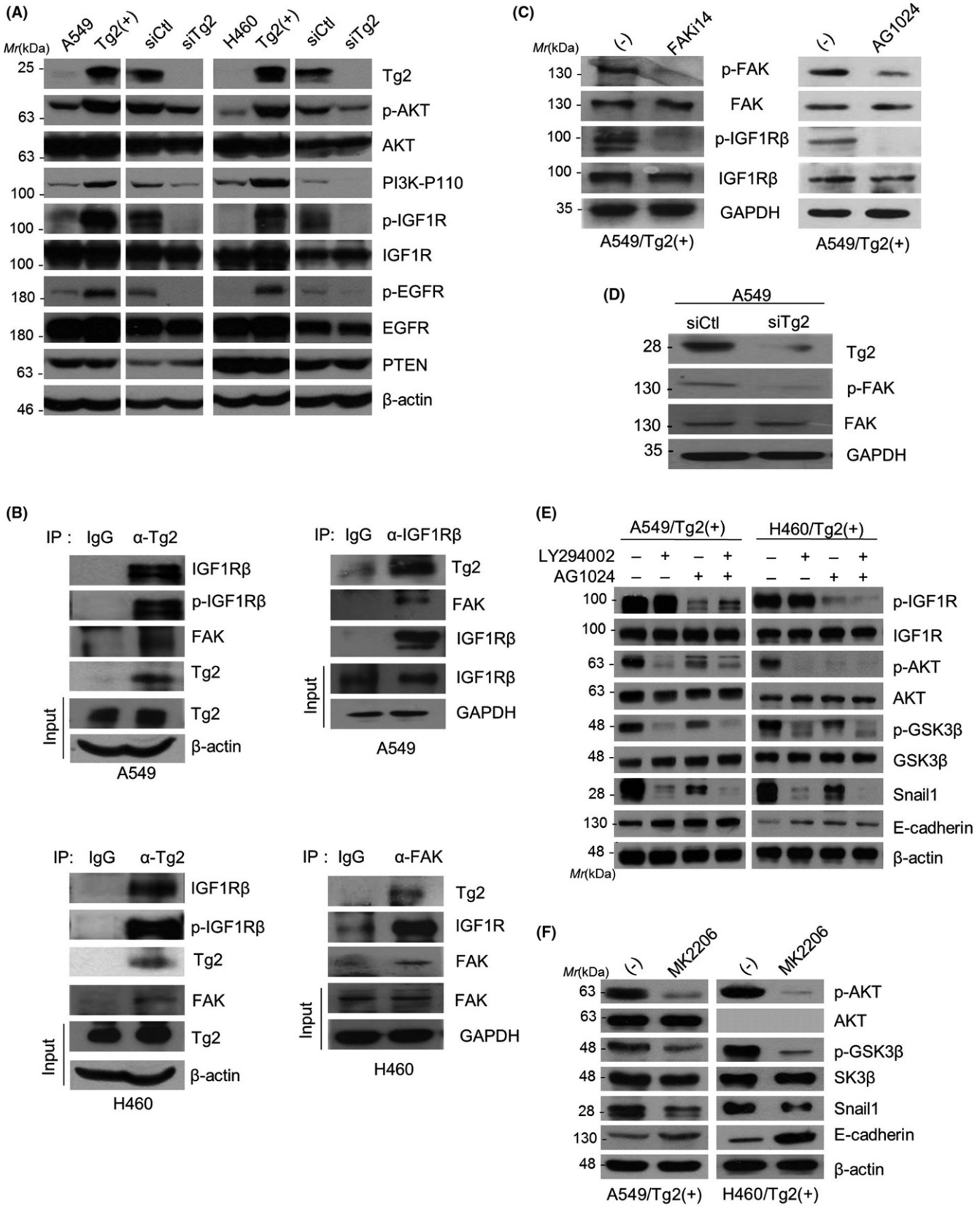


FIGURE 3 Surviving cells selected by forced transgelin 2 (*TAGLN2*) overexpression show epithelial-to-mesenchymal transition and γ-radiation-resistant properties. A, Expression level of *TAGLN2* in A549 or H460 cells transfected with pcDNA3.1-*TAGLN2* expression vector or siRNA. B, Changes in migration and invasion capacity of *TAGLN2*-overexpressing or suppressing A549 and H460 cells. Matrigel-coated invasion and migration chambers were used as described in Materials and Methods. C, Changes in resistance against cisplatin (20 μM) and γ-radiation (exposure to a single dose of 20 Gy using a ^{60}Co γ-ray source; dose rate, 2 Gy/min) in *TAGLN2*-overexpressing cells or *TAGLN2*-suppressing A549 and H460 cells. Cell death was assayed using propidium iodide staining of ethanol-fixed cells. Quantified results are presented as mean ± SD (three independent experiments) using two-tailed *t* test. **P* < .05, ***P* < .01, ****P* < 0.001 were considered significant. ns, not significant; VC, vector control; Tg2, TAGLN2


(A)
A549
Tg2(+)
siCtl
siTg2
H460
Tg2(+)
siCtl
siTg2
Mr(kDa)
25
Tg2
63
p-AKT
63
AKT
100
PI3K-P110
100
p-IGF1R
100
IGF1R
180
p-EGFR
180
EGFR
63
PTEN
46
β-actin
(C)
Mr(kDa)
(-)
FAKi14
130
p-FAK
130
FAK
100
p-IGF1Rβ
100
IGF1Rβ
35
GAPDH
A549/Tg2(+)
(-)
AG1024
A549/Tg2(+)
(D)
A549
siCtl
siTg2
28
Tg2
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p-FAK
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FAK
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GAPDH
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FAK
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Tg2
β-actin
H460
IP : IgG α-FAK
Tg2
IGF1R
FAK
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FAK
GAPDH
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A549/Tg2(+)
H460/Tg2(+)
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100
p-IGF1R
100
IGF1R
63
p-AKT
63
AKT
48
p-GSK3β
48
GSK3β
28
Snail1
130
E-cadherin
48
β-actin
Mr(kDa)
(F)
Mr(kDa)
(-)
MK2206
(-)
MK2206
63
p-AKT
63
AKT
48
p-GSK3β
48
SK3β
28
Snail1
130
E-cadherin
48
β-actin
A549/Tg2(+)
H460/Tg2(+)

FIGURE 4 Transgelin 2 (TAGLN2)-induced activation of insulin growth factor 1 receptor (IGF1R)β/P13K/AKT pathway stabilizes Snail1 protein by glycogen synthase kinase 3β (GSK3β) inactivation: phosphatase and tensin homolog (PTEN) independent AKT activation. A, Western blot analysis of cellular p-AKT, PI3K-P110, p-IGF1Rβ, and p-epidermal growth factor receptor (EGFR) levels in *TAGLN2*-overexpressing or *TAGLN2*-suppressing A549 and H460 cells. B, Interactions between TAGLN2, IGF1Rβ and focal adhesion kinase (FAK) were determined using immunoprecipitation assay in *TAGLN2*-overexpressing A549 or H460 cells. C, FAK inactivation inhibited the phosphorylation of IGF1Rβ in *TAGLN2*-overexpressing A549 cells (left). Inactivation of IGF1Rβ also inhibited the phosphorylation of FAK (right). A549 cells were treated with 5 μmol/L FAK inhibitor 14 for 24 h or with 5 μmol/L AG1024 (IGF1R inhibitor) for 12 h. D, Western blot analysis of cellular p-FAK and FAK in *TAGLN2*-suppressing A549 cells. E, Western blot analysis of IGF1R signaling pathway and Snail1 in TAGLN2-overexpressing non-small-cell lung cancer (NSCLC) cells by treatment of kinase inhibitors (AG1024, LY294002 : PI3K inhibitor). F, Western blot analysis of cellular p-AKT, p-GSK3β, Snail1, and E-cadherin levels in MK2206 (AKT inhibitor)-treated *TAGLN2*- overexpressing NSCLC cells. Tg2, TAGLN2

3.4 | Transgelin 2 recruits FAK to IGF1R to activate IGF1Rβ and TAGLN2-induced activation of IGF1Rβ/PI3K/AKT pathway stabilizes Snail1 protein by GSK3β inactivation

We then investigated how TAGLN2 affects tumor metastasis by EMT. With forced *TAGLN2* overexpression, PI3K/AKT was significantly phosphorylated and activated in selected surviving cells (Figure 4A). Previous studies showed that AKT activation is related to tumor metastasis as well as chemo- or radioresistance and cell growth.[36-38] The IGF1Rβ or EGFR pathway, a typical receptor tyrosine kinase (RTK), serves as a hub in the cellular PI3K/AKT activation signaling pathways that control tumor cell progression and survival. Therefore, we investigated whether *TAGLN2* overexpression is associated with IGF1Rβ activation in relation to cell survival and tumor metastasis. Forced *TAGLN2* overexpression apparently increased IGF1Rβ activation (Figure 4A). Furthermore, TAGLN2 also partially activated EGFR, which is another upstream target of PI3K/AKT activation (Figure 4A). However, PTEN, which can also affect PI3K/AKT activation, was not significantly changed. By immunoprecipitation assay, we showed that direct physical interaction between TAGLN2 and IGF1Rβ can occur in vitro (Figure 4B). Subsequently, we investigated whether TAGLN2 interacted with SRC or FAK, a non-receptor tyrosine kinase protein (non-RTK), which are both known to activate RTK reciprocally.[39,40] Interestingly, immunoprecipitation analysis showed that TAGLN2 directly interacted with FAK, but did not interact with SRC or JAK2, other non-RTK (Figure 4B, Figure S5). Moreover, it was shown that FAK also interacts with IGF1Rβ. These results strongly indicate that FAK is recruited to IGF1Rβ by hypoxia-inducible TAGLN2 to activate IGF1Rβ, and activated TAGLN2-FAK-IGF1Rβ complexes lead to subsequent PI3K and AKT activation, promoting EMT-associated self-renewal. Thus, the treatment of FAK inhibitor 14 in *TAGLN2*-overexpressing cells inhibited IGF1Rβ activation. Conversely, the treatment of IGF1Rβ inhibitor AG1024 inhibited FAK activation, meaning that reciprocal activation is carried out between FAK and IGF1Rβ (Figure 4C). Therefore, suppression of *TAGLN2* expression by siRNA induced inactivation of FAK as well as inactivation of IGF1Rβ (Figure 4D).

We also investigated whether the IGF1Rβ/PI3K/AKT activation pathway is related to inactivation of GSK3β (phosphorylation), which inhibits phosphorylation of Snail1 and induces stabilization of this protein in EMT control.[41] We showed that treatment with the IGF1Rβ inhibitor AG1024 in turn inhibited AKT activation, activated GSK3β and decreased the cellular level of Snail1. Moreover, the PI3K inhibitor LY294002 also decreased the cellular level of Snail1 with inhibition of GSK3β phosphorylation (Figure 4E). With inactivation of the IGF1Rβ/AKT/GSK3β pathway, cellular level of Snail1 was significantly reduced and E-cadherin was increased in surviving cells. Furthermore, treatment with the AKT inhibitor MK2206 significantly inhibited cellular GSK3β phosphorylation and thus suppressed the cellular level of Snail1 (Figure 4F). These results mean that TAGLN2 induced by hypoxia or cytotoxic-damaging agents partially increases the cellular level of Snail1 by GSK3β inactivation by FAK/IGF1Rβ (or EGFR)/PI3K/AKT activation pathway and makes lung adenocarcinoma cells more invasive/migratory and resistant to γ-radiation. Collectively, TAGLN2 is closely associated with pathological EMT induction in NSCLC cells and, thus, poor tumor prognosis.

3.5 | Transgelin 2 induces EMT through stabilization of Snail1, a transcription repressor of *E-cadherin*

Hypoxia is a critical microenvironment in tumor pathogenesis, and the hypoxic responses of tumor cells are mainly regulated by HIF1, a transcription factor composed of two subunits, HIF-1α and HIF-1β. Hypoxia induces EMT by stabilization of Snail1 by HIF-1α in a variety of tumor cells.[27,28,42] As shown earlier, forced *TAGLN2* overexpression, which is induced by adverse environmental conditions such as hypoxia, also stabilized cellular Snail1 by inactivation of GSK3β. In addition, Slug or Twist levels were also influenced by TAGLN2 (Figure 5A). In addition, TAGLN2 downregulated the cell surface marker E-cadherin and upregulated mesenchymal markers, such as N-cadherin and vimentin. In contrast, *TAGLN2* knockdown significantly downregulated representative EMT markers (Figure 5A). Immunocytochemistry staining of TAGLN2 and EMT markers such as Snail1, Slug and Twist confirmed these results (Figure S6). A hallmark of EMT is the loss of *E-cadherin* expression, and Snail1 is closely involved in *E-cadherin* regulation. Snail1 binds to the E-box, which is the promoter region of *E-cadherin*, to inhibit the gene expression of this adhesion molecule.[43] Therefore, with TAGLN2-mediated upregulation of Snail1, the cellular E-cadherin level was significantly decreased.

Snail1 activity is regulated by various signaling pathways at multiple levels. GSK3β was shown to regulate the Snail1 level by

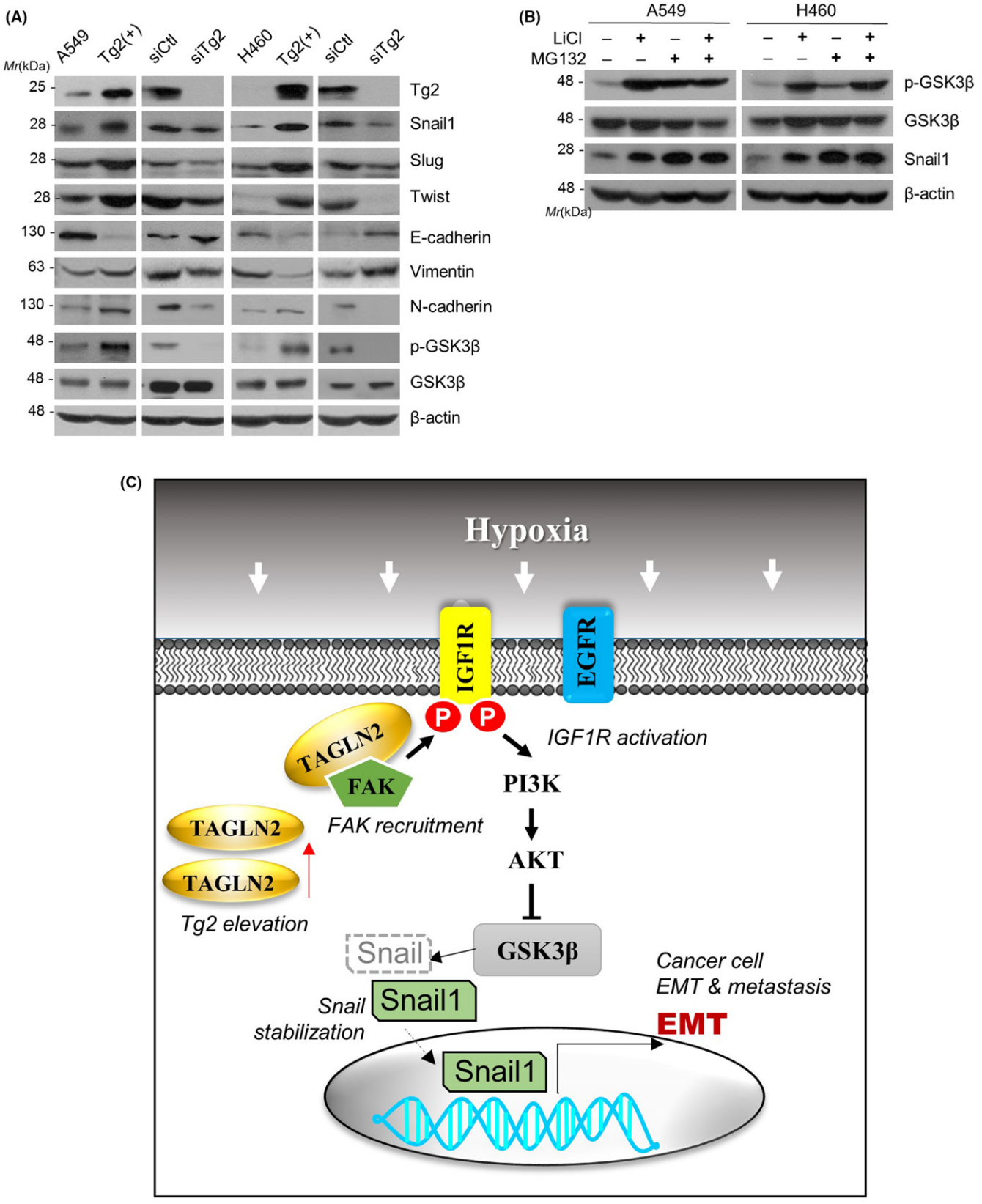

FIGURE 5 Transgelin 2 (TAGLN2) induces epithelial-to-mesenchymal transition (EMT) by stabilization of EMT-involved transcription factor Snail1. A, Western blot analysis of glycogen synthase kinase 3β (GSK3β) and EMT-related factors, including Snail1, Slug, Twist, E-cadherin, N-cadherin, and Vimentin in *TAGLN2*-overexpressing or *TAGLN2*-suppressed non-small-cell lung cancer (NSCLC) cells. B, Western blot analysis of GSK3β and Snail1 in LiCl-treated (inducer of GSK3β phosphorylation) or MG132-treated (proteasome inhibitor) NSCLC cells. C, Schematic model showing EMT characteristics of NSCLC cells induced by hypoxia-inducible TAGLN2 through insulin growth factor 1 receptor (IGF1R) activation and stabilization of Snail1. EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; Tg2, TAGLN2

GSK3β phosphorylation, transducin repeats-containing proteins-directed ubiquitination, and proteasomal degradation.[41,44] We investigated whether TAGLN2 regulates GSK3β phosphorylation to control the stabilization and degradation of cellular Snail1. Forced *TAGLN2* overexpression significantly induced GSK3β inactivation (phosphorylation) in surviving A549 and H460 cells and subsequently upregulated cellular Snail1 and downregulated expression of *E-cadherin* (Figure 5A). In contrast, forced *TAGLN2* suppression led to dephosphorylation of GSK3β and downregulation of Snail1 level. Treatment with either LiCl, a GSK3β inhibitor, or MG132, a cell-permeable 26S proteasome inhibitor, increased the cellular level of Snail1 in A549 and H460 cells (Figure 5B). These results strongly confirmed that TAGLN2 is associated with Snail1 stabilization and degradation through the IGF1Rβ/PI3K/AKT/GSK3β signaling pathway (Figure 5C).

TAGLN2 has been thought to be a tumor suppressor or a tumor promoter, although the specific functions and signal pathways involved in TAGLN2 are still unclear. In the present study, we identified for the first time that hypoxia-inducible TAGLN2 is involved in the EMT program that provides stemness and γ-radiation resistance to cancer cells by mediating the selection and survival of small subpopulations among heterogeneous cell populations of cancer cells.

4 | DISCUSSION

Actin is a major cytoskeleton component that participates in a variety of cellular programs, such as cell growth, cell death, migration, and cell signaling.[45] Thus, proteins binding to actin can participate in the modulation of various cellular processes.[46] Like SM22α (TAGLN) protein, TAGLN2 is also an actin-binding protein.[45,47] cDNA-encoding TAGLN2 was first isolated from a cDNA library derived from a human immature myeloid cell line.[48] TAGLN2 is expressed in a wide variety of human tissues, including lung, liver, kidney, spleen, and thymus.[49] TAGLN2 was also identified on the membrane of human embryonic stem cells.[50] Although its localization differs among cell types, it has been reported as a membrane-associated or cytoplasmic protein. Several proteome studies reported that *TAGLN2* is overexpressed in a variety of tumors and the deregulation of TAGLN2 is associated with the development of malignant tumors.[51-53] Therefore, it has been considered an important diagnostic marker of tumors. Recent studies have investigated TAGLN2-targeting microRNAs for tumor suppression.[54-56] However, despite many proteomic data, it is not fully understood how TAGLN2 acts on tumor progression and signaling pathways. In the present study, we investigated the biological roles of TAGLN2 and its underlying mechanism in depth.

The PI3K/AKT pathway regulates various cellular functions, the most important of which is the mechanism of radiation resistance in cells with EMT or CSC characteristics. PTEN is the central negative regulator of PI3K/AKT signal transduction.[57] However, PTEN cellular level was not changed by *TAGLN2* suppression or overexpression, as shown in the results. Activation of IGF1Rβ, a RTK, is closely related to resistance to chemotherapy and radiotherapy in various tumor types.[58-60] We showed that IGF1Rβ, which is one of the major upstream targets of PI3K/AKT activation, undergoes phosphorylation in *TAGLN2*-overexpressing cells. With these critical findings, it is not difficult to predict that TAGLN2-induced IGF1Rβ/PI3K/AKT activation can enhance the stabilization of Snail1 protein through GSK3β phosphorylation. We showed here that TAGLN2 recruits FAK, a non-receptor tyrosine kinase, to the IGF1R signaling complex and consequently activates IGF1R/PI3K/AKT pathway.

Cancer stem cells, a rare subpopulation of cancer cells, are found in all tumors including hematological cancers and have characteristics associated with normal stem cells. EMT is a crucial morphological event contributing to the metastasis of epithelial tumors, and it seems to play a critical role in the generation and maintenance of CSC. In short, a cell undergoing EMT is a migrating CSC that has properties with chemo- and radioresistance.[30] CSC persistently generate tumors through the stem cell processes of self-renewal and differentiation into multipotent cell types (heterogeneity). Therefore, intrinsic to the CSC hypothesis is the heterogeneity of tumor cells. There is a growing body of evidence that patient tumors contain various heterogeneous cell populations.[61,62] Such heterogeneity would be beneficial to survival under harsh conditions such as extreme hypoxia (<1%). If cancer cells are all essentially identical with only natural variability among them, such a harsh condition may kill most of a cell population. However, in heterogeneous cell populations, some cells have random genetic backgrounds (or random fluctuation of protein expression levels), allowing them to survive despite extreme environmental conditions. If such conditions occur repeatedly or over the long term, cells may be transitioned or consequently selected by some genes, which did not uniquely function in other cell populations. Such cells are proposed to persist in tumors as distinct populations and cause relapse and metastasis. In the present study, we showed that despite the presence of hypoxia-inducible proteins of TAGLN2, its enforced overexpression kills most of the cells in vitro. However, small numbers of cells survive and transit to mesenchymal phenotype cells which favor metastasis and have more resistant, invasive, and motile properties. TAGLN2 may also trigger the activation of proteins and expression of genes essential for a pathological EMT process in a HIF-1α-independent method and thus assist the selection for poor prognosis. In conclusion, TAGLN2 plays an important role in overcoming harsh environments, such as extreme hypoxia, and selecting new resistant cell populations through the EMT process.

ACKNOWLEDGMENTS

This research was supported by grants from the Ministry of Science and ICT (Nuclear Research and Development Program: NRF-2013M2A2A7043660) of the Republic of Korea.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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How to cite this article: Kim I-G, Lee J-H, Kim S-Y, Hwang H-M, Kim T-R, Cho E-W. Hypoxia-inducible transgelin 2 selects epithelial-to-mesenchymal transition and γ-radiation-resistant subtypes by focal adhesion kinase-associated insulin-like growth factor 1 receptor activation in non-small-cell lung cancer cells. *Cancer Sci*. 2018;109:3519–3531. https://doi.org/10.1111/cas.13791