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Novel anti-tumor mechanism of galanin receptor type 2 in head and neck squamous cell carcinoma cells

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Key words

Adeno-associated virus vector, Bim, extracellular signal-regulated kinases 1/2, galanin receptor, head and neck squamous cell carcinoma

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Galanin and its receptors, GALR1 and GALR2, are known tumor suppressors and potential therapeutic targets in head and neck squamous cell carcinoma (HNSCC). Previously, we demonstrated that, in GALR1-expressing HNSCC cells, the addition of galanin suppressed tumor proliferation via upregulation of ERK1/2 and cyclin-dependent kinase inhibitors, whereas, in GALR2-expressing cells, the addition of galanin not only suppressed proliferation, but also induced apoptosis. In this study, we first transduced HEp-2 and KB cell lines using a recombinant adeno-associated virus (rAAV)-green fluorescent protein (GFP) vector and confirmed a high GFP expression rate (>90%) in both cell lines at the standard vector dose. Next, we demonstrated that GALR2 expression in the presence of galanin suppressed cell viability to 40–60% after 72 h in both cell lines. Additionally, the annexin V-positive rate and sub-G0/G1 phase population were significantly elevated in HEp-2 cells (mock *vs* GALR2: 12.3 *vs* 25.0% ($P < 0.01$) and 9.1 *vs* 32.0% ($P < 0.05$), respectively) after 48 h. These changes were also observed in KB cells, although to a lesser extent. Furthermore, in HEp-2 cells, GALR2-mediated apoptosis was caspase-independent, involving downregulation of ERK1/2, followed by induction of the pro-apoptotic Bcl-2 protein, Bim. These results illustrate that transient GALR2 expression in the presence of galanin induces apoptosis via diverse pathways and serves as a platform for suicide gene therapy against HNSCC.

Head and neck squamous cell carcinoma (HNSCC) is diagnosed in more than 500 000 patients worldwide each year, accounting for 5% of all malignancies.[1,2] Despite multidisciplinary therapy, the prognosis of patients with advanced HNSCC has remained poor over several decades.[3] Thus, the invention of new therapeutic approaches against HNSCC, such as gene therapy and molecular targeted therapies has received much attention.[4–7] However, these novel treatments remain inadequate, because various signal transduction systems are defective in typical HNSCC, and little therapeutic effect is achieved by therapies targeting single mechanisms. Thus, there is a need to develop an effective drug delivery system and a novel molecular targeted therapy with multiple therapeutic targets.

In general, tyrosine kinase receptors and cytokine receptors, and their signaling pathways are important in carcinogenesis[5,8] and they have been analyzed as molecular targets and prognostic factors in HNSCC. However, the roles of G-protein–coupled receptors (GPCRs), which are important signaling molecules, are not fully understood. GPCRs control various signaling pathways in all tissues of the body, and approximately 30% of all current pharmaceuticals exert their therapeutic effects by interacting with GPCRs. Therefore, the field of GPCRs holds promise for further drug discovery and therapeutic development.[9]

Previously, we investigated the function and signal transduction of galanin receptors, which are representative GPCRs in HNSCC.[10,11] Galanin is a 30-amino acid peptide, which has three specific receptors (GALR1–3). Galanin and its receptors are mainly expressed in the central and peripheral nervous systems and function in neurotransmission.[12–14] Recently, galanin has been shown to regulate cell proliferation and survival in many tumors.[15–17] With the development of functional analysis methods for each receptor, it became clear that GALR1 and GALR2 are likely to act as tumor suppressors. Our previous study showed that activation of the GALR1 signaling pathway suppressed tumor cell proliferation via phosphorylation of ERK1/2, and involves upregulation of the cyclin-dependent kinase inhibitors in HNSCC.[10,18] Other studies showed that GALR1 inhibits proliferation by inactivat-

ing the mitogen-activated protein kinase (MAPK) pathway in oral carcinoma.[19] Moreover, activation of the GALR2 signaling pathway suppresses tumor cell growth through induction of apoptosis in some tumors, including HNSCC.[11,20,21] The cytotoxic effects of the GALR2 signaling pathway are partially mediated by dephosphorylation of Akt and the Bcl-2 protein, Bad, in PC12 cells, a pheochromocytoma cell line.[21]

However, further analysis is required with respect to prognostic factors in HNSCC, applications of molecular targeted therapy, and clinical applications of gene therapy. The adeno-associated (AAV) vector is a promising system in gene therapy, holding various benefits, such as a lack of pathogenicity, low immunogenicity, long-term transgene expression, and broad tissue-specificity.[22,23] To date, AAV vectors have been used in clinical trials for the treatment of various diseases, such as Parkinson's disease, hemophilia B, and inherited retinal disorders.[24–27] Additionally, it is thought to be applicable to suicide-gene therapy against various cancers, including HNSCC.[28–30] rAAV-galanin is currently clinically applied in the suppression of limbic seizure activity,[31] and this vector is suitable for use in the galanin–GALR system. Given this background, we set out to evaluate the effects of GALR expression on apoptosis and analyzed the associated mechanism of action using HNSCC cells.

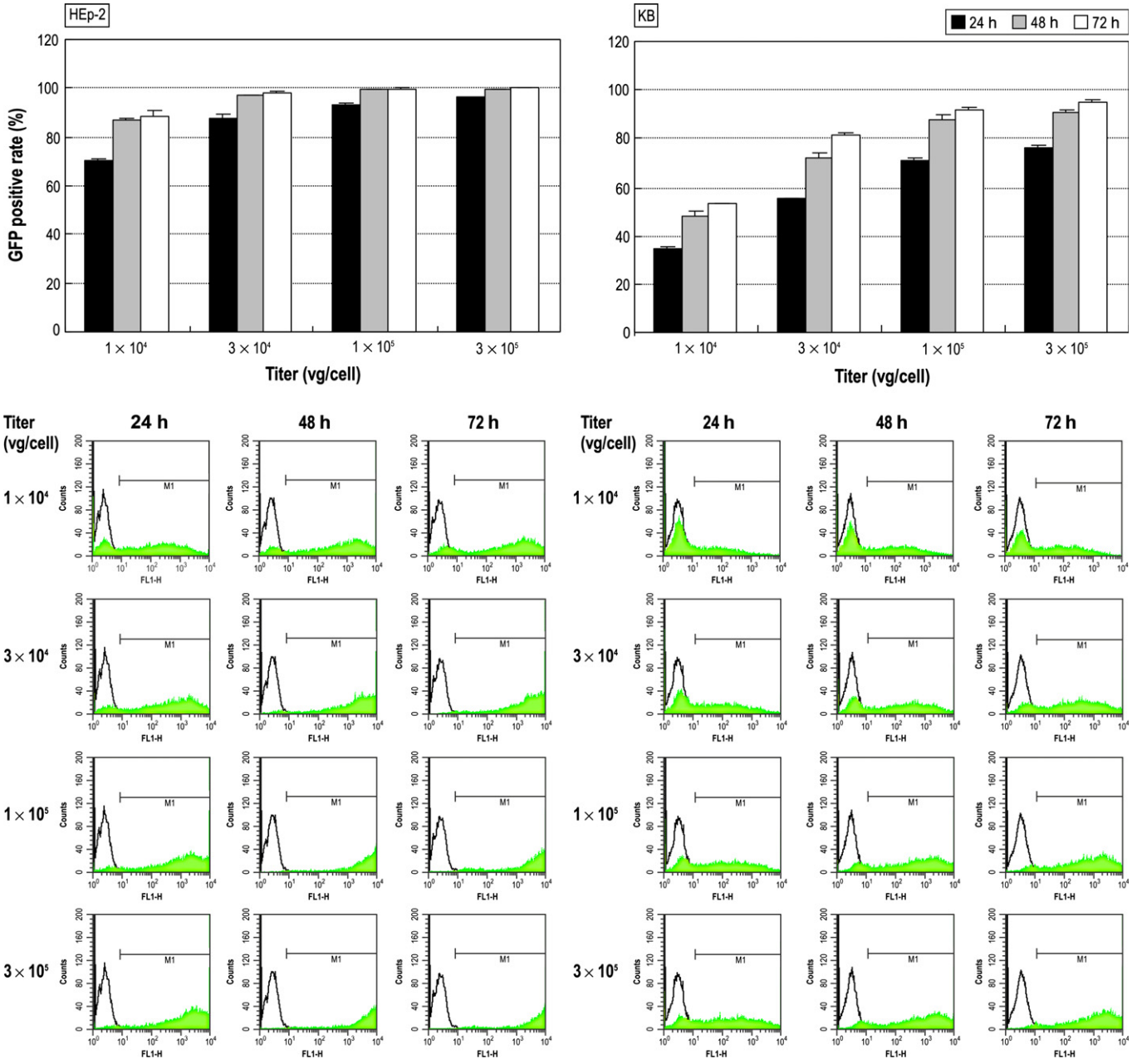


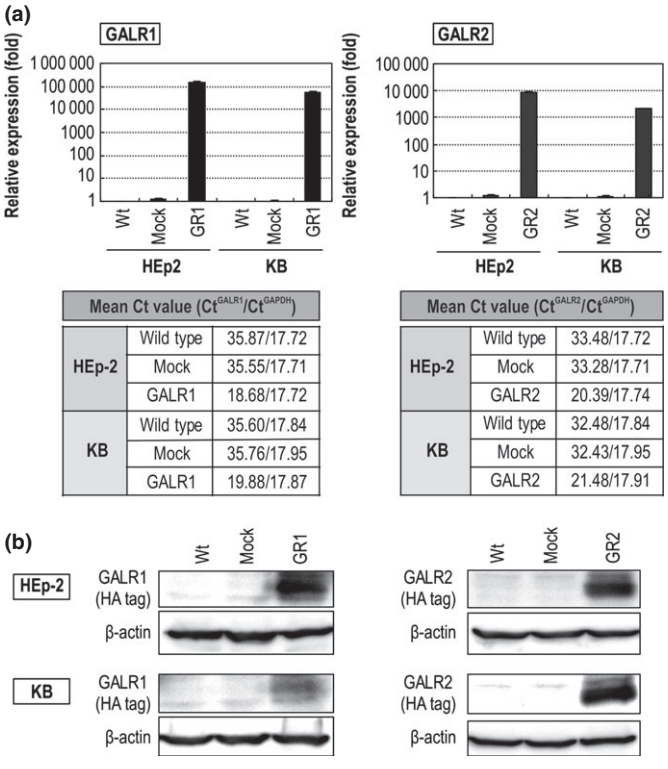
Fig. 1. Transduction efficiency using recombinant adeno-associated virus (rAAV)-enhanced green fluorescent protein (EGFP) in head and neck squamous cell carcinoma (HNSCC) cell lines. HEp-2 and KB cells were transduced by AAV-EGFP at various vector doses. *Upper*: GFP-positive cell rate under vector dose-dependent and time-course conditions in each cell line are indicated. *Lower*: Raw data expressing GFP intensity as a measure of gene expression.

Material and Methods

Cell culture and reagents. Human laryngeal carcinoma cell line HEp-2 and human oral carcinoma cell line KB were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in αMEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human galanin was obtained from ANASPEC (San Jose, CA, USA), PD98059 and staurosporine from Wako (Osaka, Japan), and z-VAD-fmk from Promega (Madison, WI, USA). Although KB cells were originally thought to be derived from an epidermal carcinoma of the mouth, HeLa cell contamination was found thereafter through isoenzyme and other analyses (http://www.atcc.org/Products/All/CCL-17.aspx). Therefore, KB cells may not be a pure HNSCC cell line. However, we used both cell lines to understand the universal function of GALR2, rather than its cell-specific function.

Plasmid and rAAV vector production. The GALR1HA-Ires-GFP and GALR2HA-Ires-GFP fragments were obtained from our previous experiments.[10] The sequences were subcloned into the pAAV-MCS vector (Agilent technologies, Palo Alto, CA, USA).

To produce recombinant AAV vectors, we used an AAV helper-free system (Agilent technologies). Each pAAV vector, pAAV-RC, and the pHelper plasmid were cotransfected into subconfluent HEK293 cells by the calcium phosphate precipitation method or by polyethylenimine. After 72-h incubation, cells were harvested and lysed using three freeze-thaw cycles. The crude viral lysate was purified by two rounds of CsCl density-gradient ultracentrifugation. The titer of rAAV vectors was subsequently determined by quantitative PCR analysis.



Mean Ct value (Ct^{GALR1}/Ct^{GAPDH})		
HEp-2	Wild type	35.87/17.72
	Mock	35.55/17.71
	GALR1	18.68/17.72
KB	Wild type	35.60/17.84
	Mock	35.76/17.95
	GALR1	19.88/17.87

Mean Ct value (Ct^{GALR2}/Ct^{GAPDH})		
HEp-2	Wild type	33.48/17.72
	Mock	33.28/17.71
	GALR2	20.39/17.74
KB	Wild type	32.48/17.84
	Mock	32.43/17.95
	GALR2	21.48/17.91

Fig. 2. Expression of GALR1 and GALR2 using individual recombinant AAV vectors in head and neck squamous cell carcinoma (HNSCC) cells. (a) Quantitative RT-PCR was performed to measure GALR1 and GALR2 expression in wild type (Wt) or HNSCC cells transduced by each of the rAAV vectors. (b) Western blot results showing exogenous GALR1 and GALR2 expression in Wt or HNSCC cells transduced by each of the rAAV vectors, as detected by an HA-antibody.

Quantitative RT-PCR. Total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. cDNA was amplified using TaqMan Expression Assays (Applied Biosystems): GalR1 (Hs00175668 m1), GalR2 (Hs00605839 m1), and GAPDH (Hs99999905 m1). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles each consisting of 95°C for 15 s and 60°C for 1 min. The expression levels of the target genes were analyzed using the comparative C_t method, and GAPDH was used as a control gene.

Immunoblotting. Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH6.8), 2% SDS, 10% glycerol, 6% mercaptoethanol, and 0.01% bromophenol blue. To detect GALRs expression, cell lysates were treated with N-Glycosidase F (New England BioLabs, Beverley, MA, USA) without boiling, as described previously.[10,11] Mitochondrial and cytosolic fractions were obtained using Mitochondrial Isolation Kit (ThermoFisher Scientific, Logan, UT, USA).

Samples were gel electrophoresed and transferred to polyvinylidene difluoride membranes, which were probed with specific antibodies. Mouse monoclonal antibodies to hemagglutinin (HA)-tag, phospho-Bad (Ser112), and β-actin, rabbit monoclonal antibodies to p44/42 MAPK (Erk1/2), phospho-p44/42 (Thr202/Tyr204), Akt, phospho-Akt, caspase-3, cleaved caspase-3, Bax, Bcl-2, Bcl-xL, Mcl-1, Bim, Bad, survivin, antibodies to Cox IV and XIAP, polyclonal antibodies to AIF, Endonuclease G, Bit1, HtrA2 and cytochrome c were purchased from Cell Signaling Technology (Beverly, MA, USA). Bands were visualized using an Enhanced Chemiluminescence Kit (GE Healthcare, Buckinghamshire, UK) and an ImageQuant LAS 4000 (Fujifilm, Tokyo, Japan).

Immunocytochemistry. Cells were seeded onto coverslips and incubated overnight, and transduced with each recombinant AAV vector. After 48 h of incubation, cells were fixed, and stained with mouse monoclonal anti-HA-tag antibody (Cell Signaling). Subsequently, cells were stained with Alexa Fluor 555-labeled goat anti-mouse IgG (Cell Signaling) and the nuclei counterstained with DAPI. Furthermore, to determine the transport potential of HA-tagged GALRs proteins to the cytoplasmic membrane, HEp-2 cells stably expressing these proteins were established by transfecting the AAV plasmid into HEp-2 cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA). Green fluorescent protein-positive cells were selected by flow cytometry using a FACS Vantage SE at 4 weeks after transfection (BD Biosciences, San Diego, CA, USA). Exogenous GALR1 or GALR2 were localized using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan) and an Olympus FV-500 Confocal Microscope (Olympus Corporation, Tokyo, Japan).

Cell viability assay. The effect of activation of the GALR signaling pathways on cell viability was examined using WST-1 (Roche Diagnostics, East Sussex, UK). Cells were seeded in a 96-well microculture plate, incubated overnight, and then cultured in serum-free media (SFM) containing 0.1% bovine serum albumin for 2 h. Subsequently, cells were exposed to the individual rAAV vectors and galanin for 24–72 h. WST-1 reagent was added for the last 1–2 h of the incubation, and the absorbance at 450 nm was measured

using a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Apoptosis and cell cycle analysis. Apoptotic cells were detected by staining with phycoerythrin (PE)-conjugated annexin V protein (BD Bioscience, San Jose, CA, USA) and flow cytometry. Cell cycle analysis was performed with the CycleTEST PLUS DNA Reagent Kit (BD Biosciences) and flow cytometry. Apoptosis-induced DNA fragmentation was measured for the sub-G0/G1 cell cycle phase populations.

Statistical analysis. Statistical analysis was performed using Student's *t*-test and Kruskal–Wallis test.

Results

Transduction efficiency of HNSCC cell lines using rAAV-EGFP. To determine transduction efficiency in HNSCC cell lines using rAAV vectors, HEp-2 and KB cells were infected with a rAAV-EGFP vector at various vector doses. After incubation for 24–72 h, GFP-positive cells were counted by flow cytometry. We confirmed a high GFP expression (>90%) in both HNSCC cell lines transduced by vector doses above 1×10^5 vector genomes (vg)/cell for 48 h (Fig. 1). Consequently, a titer of 1×10^5 vg/cell was used as standard vector dose.

GALR gene expression after transduction of rAAV-GALR vectors. We determined both the endogenous and exogenous mRNA expression levels of each GALR gene in HEp-2 and KB cells transduced by rAAV-GALR1 or rAAV-GALR2 by quantitative RT-PCR. After 48 h of transduction, elevated mRNA expression levels of exogenous GALR1 and GALR2 were observed in both cell lines (Fig. 2a).

The expression levels of exogenous GALR1 and GALR2 proteins were also assessed by western blot analysis and immunocytochemistry, using a mouse monoclonal anti-HA tag antibody, and confirmed robust expression of GALR1 and GALR2 proteins (Figs 2b,3). Immunofluorescence revealed that GFP-positive cells showed exogenous expression of GALRs, which was localized to both the cytoplasm and plasma membrane in cells transiently transduced with AAV vectors (Fig. 3a). However, in stable GALRs-expressing HEp-2 cells, exogenous GALRs located only to the plasma membrane (Fig. 3b). These results suggested that transient-transduction impelled movement of a large quantity of these proteins to the cytoplasm, although these proteins themselves have the potential to be transported to the plasma membrane, as expected for a G protein-coupled receptor.

Co-administration of rAAV-GALR2 and galanin inhibits cell proliferation and induces apoptosis. We examined the ability of each GALR signaling pathway to inhibit HNSCC growth. Culture of cells in the presence of both rAAV-GALR2 and varying doses of galanin in SFM for 24–72 h resulted in cell growth suppression in a time- and dose-dependent manner in both HNSCC lines, as assessed by WST-1 assay. After 72 h of stimulation, the cell growth rate was significantly decreased to 40% in HEp-2 and 60% in KB cells (Fig. 4a). In contrast,

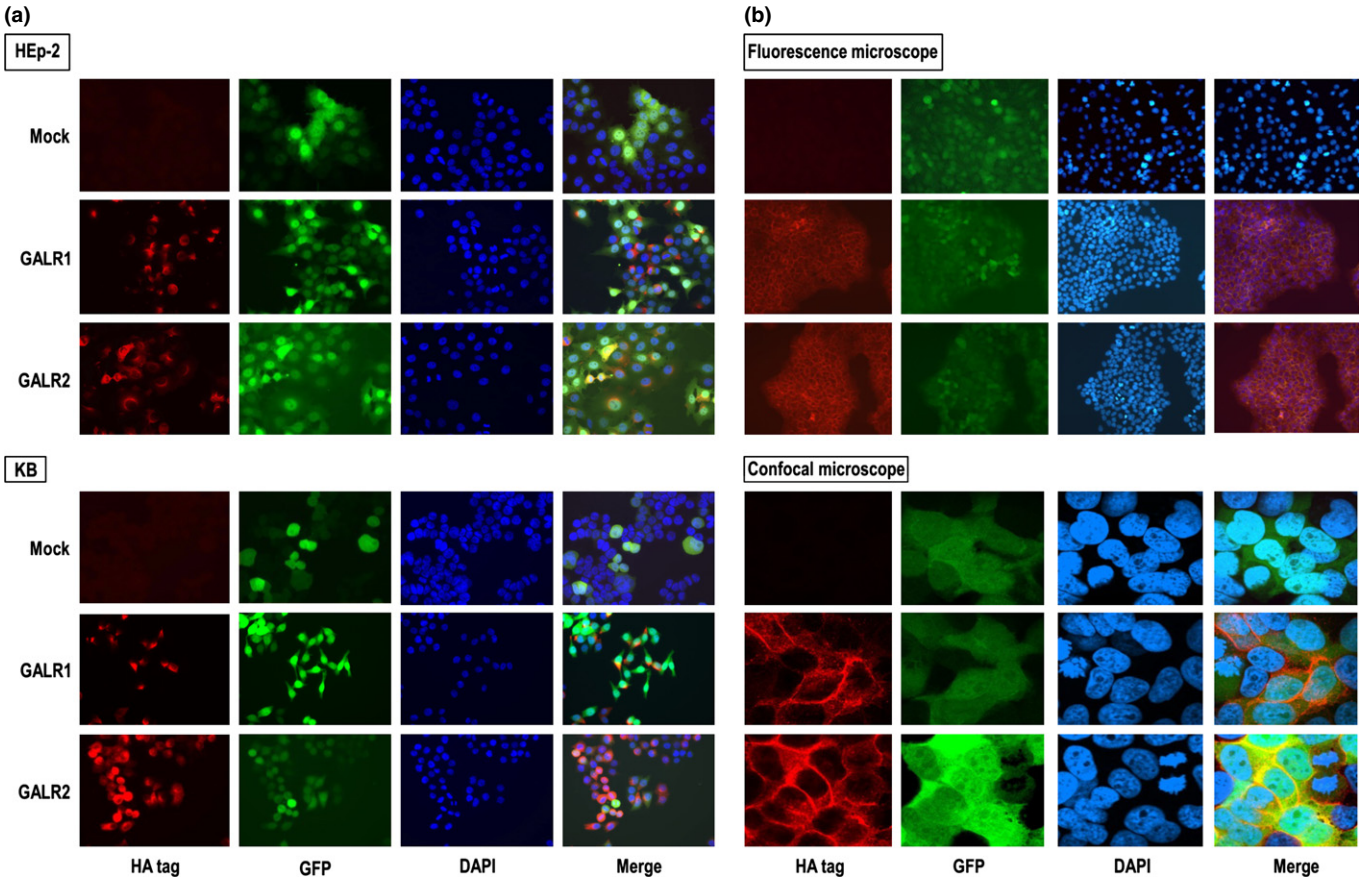


Fig. 3. (a) Exogenous GALR1 and GALR2 localization in head and neck squamous cell carcinoma (HNSCC) cells transiently transduced with each of the recombinant adeno-associated virus (rAAV) vectors. (b) Exogenous GALR1 and GALR2 localization in HEp-2 cells stably transfected with each of the AAV plasmids. Images show cells stained with the anti-HA antibody (left-most), green fluorescent protein (GFP) (second from the left), DAPI (4′6′-diamidino-2-phenylindole dihydrochloride) (second from the right), and merged images (right-most).

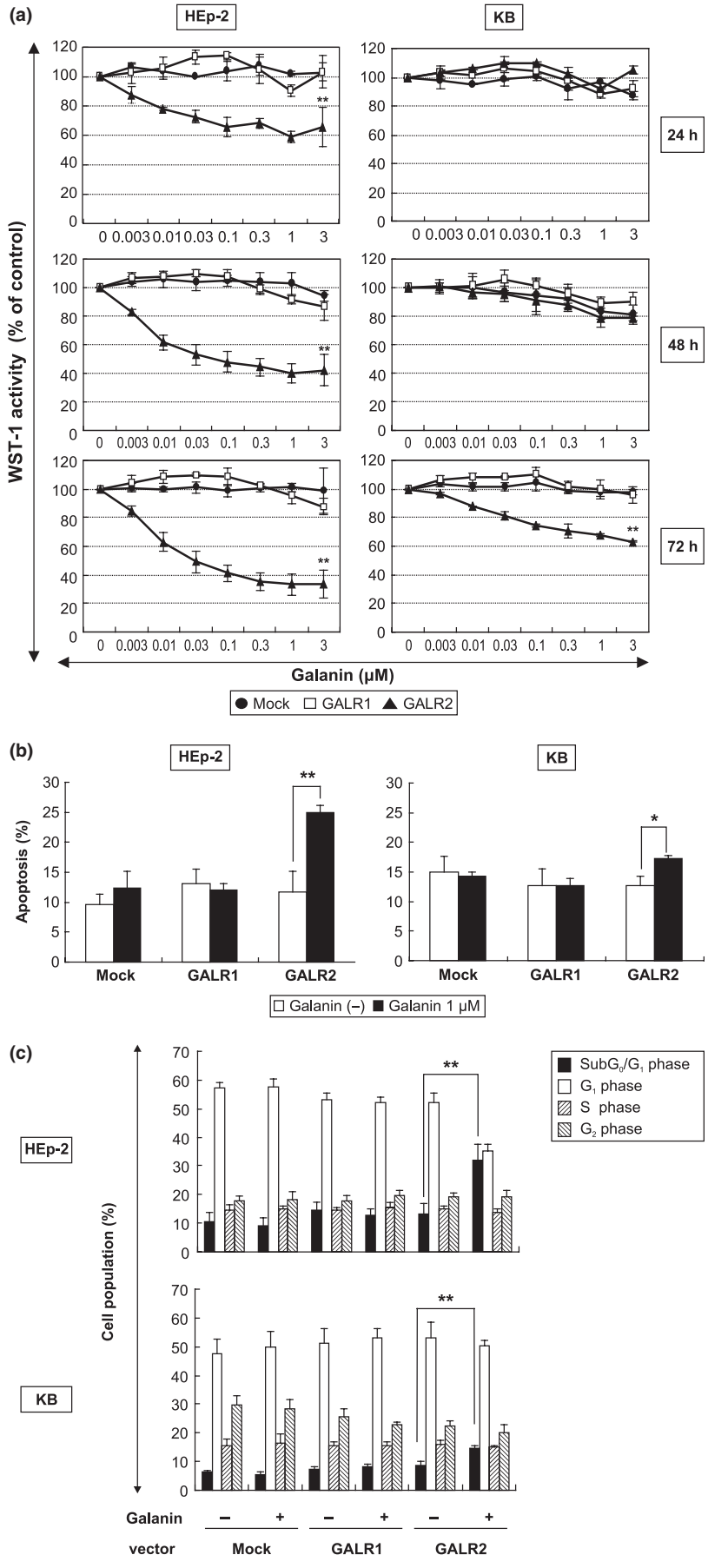


Fig. 4. Co-administration of galanin and recombinant adeno-associated virus (rAAV)-GALR2 during cell culture inhibited cell proliferation and induced cell death in head and neck squamous cell carcinoma (HNSCC) cells. (a) Inhibitory effects of galanin on growth of HNSCC cells transduced with the respective rAAV vectors. (b) Flow cytometric analysis of cell apoptosis using the annexin V. (c) Cell cycle analysis by flow cytometry. $*P < 0.05$; $**P < 0.01$.

co-administration of rAAV-GALR1 and galanin did not affect cell proliferation in either cell line (Fig. 4a).

The ability of GALR signaling to induce apoptosis was assessed by measuring annexin V staining in both cell lines. Co-treatment of cells with rAAV-GALR2 and galanin (1 μM) for 48 h significantly induced apoptosis in 25% of HEp-2 cells, and less markedly induced apoptosis in 16% of KB cells (Fig. 4b).

Furthermore, changes in the cell cycle distribution after activation of either GALR pathway were evaluated by flow cytometry. Co-administration of rAAV-GALR2 vector and galanin (1 μM) for 48 h significantly increased the sub-G0/G1 phase population, to 32% in HEp-2, and to 16.6% in KB cells (Fig. 4c), suggesting that DNA fragmentation was induced by activation of the GALR2 signaling pathway, along with apoptosis. No other effects on cell cycle distribution were observed (Fig. 4c). Additionally, GALR1 activation had no effects on induction of apoptosis or cell cycle distribution (Fig. 4b,c).

Stimulation of GALR2 signaling downregulates ERK1/2, and upregulates Bim. As the GALR2-mediated cytotoxic effects were mainly due to apoptosis induction, we examined whether stimulation of the GALR2 signaling pathway affected the phosphorylation states of ERK1/2 and Akt by immunoblotting. Sustained dephosphorylation of ERK1/2 was induced by stimulation of GALR2 signaling in both HNSCC cell lines (Fig. 5a), but no effect on Akt phosphorylation was observed (Fig. 5b).

Moreover, we examined the influence of the pathway on key apoptosis regulators, viz., the Bcl-2 protein and inhibitor of apoptosis protein (IAP) families. The proapoptotic "BH-3–only" Bcl-2 protein Bim was upregulated by activation of GALR2 signaling in HEp-2, but not in KB cells (Fig. 5a,c). No other apoptosis-related proteins investigated were affected by GALR2 activation in either cell line (Fig. 5c). Additionally, activation of GALR1 signaling did not affect the phosphorylation state of ERK or the other apoptotic regulators (Fig. 5c).

PD98059 inhibits cell proliferation and induces apoptosis via inactivation of the MEK/ERK pathway in HNSCC cells. To determine whether dephosphorylation of ERK1/2 results in cell growth inhibition and apoptosis induction in HNSCC cells, we examined the reproducibility of GALR2-mediated cytotoxicity using a specific ERK (MEK1) inhibitor, PD98059. As expected, dephosphorylation of ERK1/2 was induced by treatment of both HNSCC cell lines with PD98059 at 20–100 μM for 48 h (Fig. 6a). When cells were cultured in SFM in the presence of PD98059 for 48 h, dose-dependent cell growth suppression (Fig. 6b) and significant apoptosis induction (Fig. 6c) were observed; these effects were more marked in HEp-2 cells. In addition, dose-dependent upregulation of Bim was observed in HEp-2, but not in KB cells, after incubation with PD98059 for 48 h (Fig. 6a). Thus, the GALR2-mediated cytotoxic effects involved at least downregulation of ERK1/2, while Bim may play a role in modulation of GALR2-mediated apoptotic sensitivity. However, despite apoptosis induction in KB cells, Bim activation was not observed, suggesting the existence of multiple signaling pathways for apoptosis induction.

GALR2-mediated apoptosis is mainly induced via a caspase-independent pathway. We further investigated the role of the caspase signaling pathway in GALR2-mediated apoptosis by

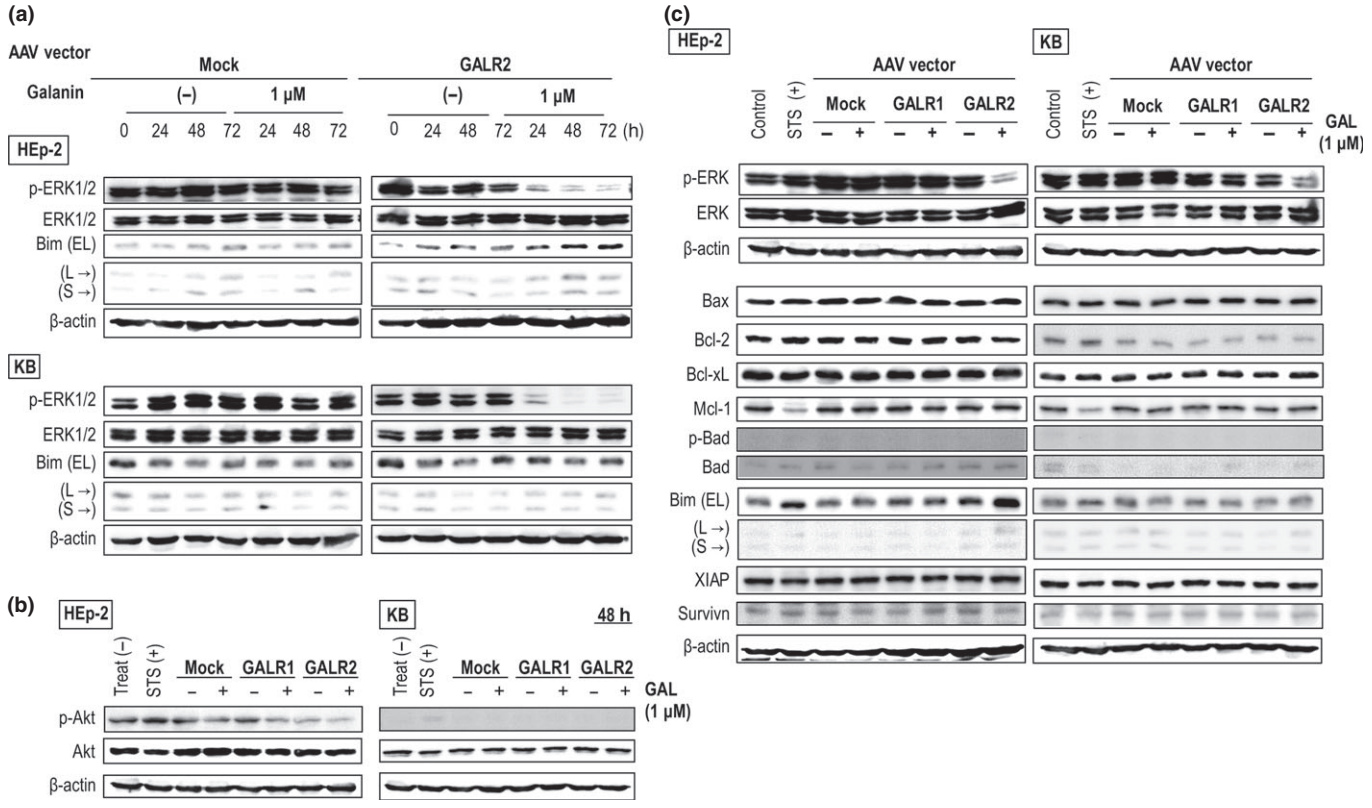


Fig. 5. Immunoblotting analysis of the phosphorylation of ERK1/2 and Akt and regulation of key apoptosis regulators by co-administration of recombinant adeno-associated virus (rAAV)-GALR2 vector and galanin. (a) Effect of galanin on ERK1/2 activation and Bim expression in rAAV-GALR2 vector-transduced head and neck squamous cell carcinoma (HNSCC) cells. (b) Effect of galanin on Akt activation in GALR-transduced HNSCC cells. (c) Effects of treatment of cells with galanin and transduction with individual rAAV vectors on the phosphorylation state of ERK1/2 and expression of proteins belonging to the Bcl-2 or IAP families.

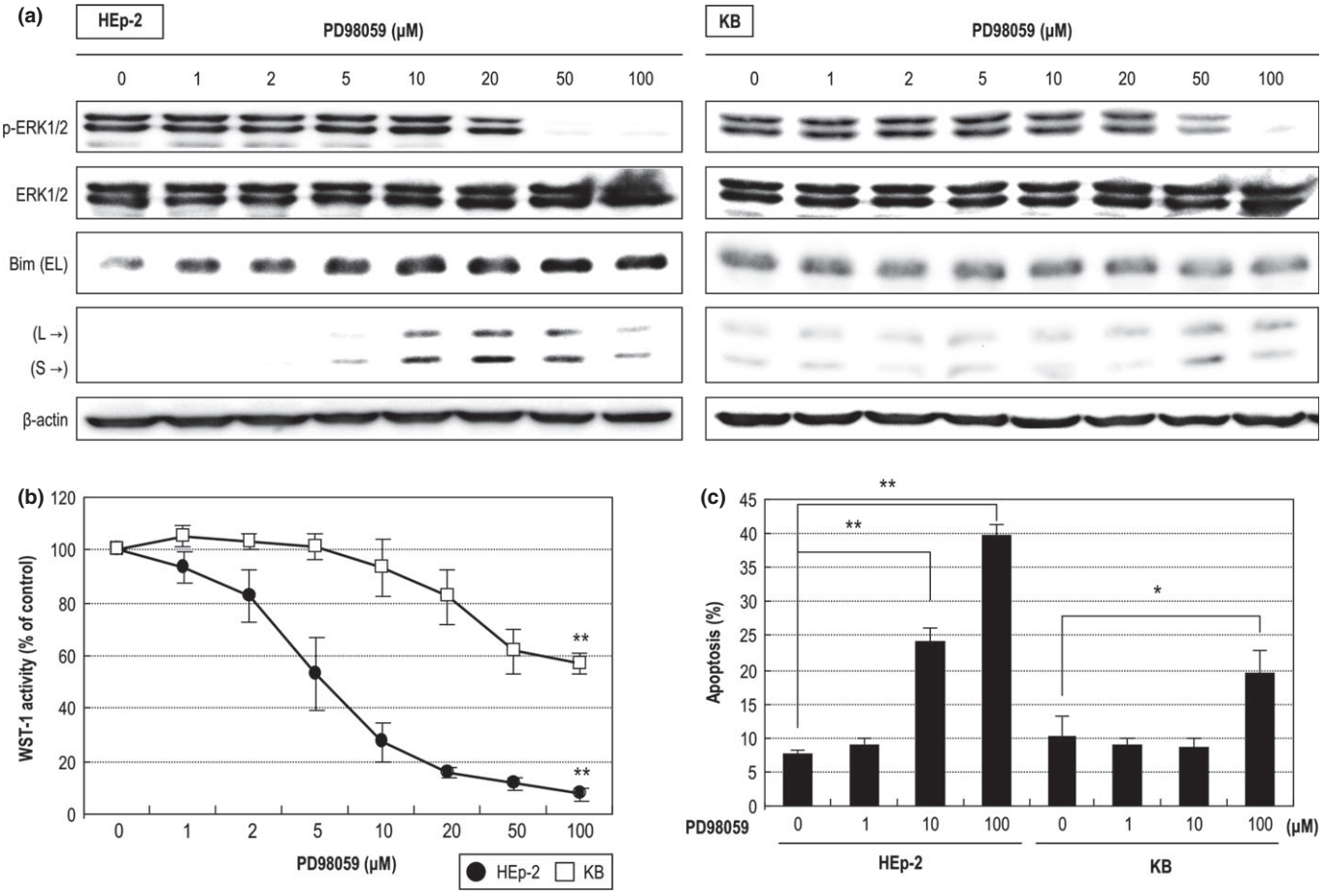


Fig. 6. Effect of the MEK inhibitor PD98059 on head and neck squamous cell carcinoma (HNSCC) cells. (a) Phosphorylation states of ERK1/2 after treatment with PD98059. (b) Effect of PD98059 on proliferation of HNSCC cells. (c) Apoptosis analysis by an Annexin V in HNSCC cells. $*P < 0.05$, $**P < 0.01$.

measuring the cleavage of caspase-3 by immunoblotting. Compared to cells treated with 1 μM staurosporine (STS), minimal cleavage of caspase-3 was observed after stimulation of GALR2 signaling pathway in either HNSCC line (Fig. 7a). Additionally, GALR2-mediated inhibition of cell growth was not abrogated by pretreatment with the pan-caspase inhibitor z-VAD-FMK (50 μM) in HEp-2 cells (Fig. 7b).

We further examined whether activation of mitochondrial apoptosis-related factors were involved in initiating a caspase-independent pathway. Apoptosis inducers, such as apoptosis inducing factor (AIF), endonuclease G, HtrA2, and Bit1, are known to be released from the mitochondria to the cytosol and the nucleus by caspase-independent apoptotic signals. No translocation of these proteins in response to GALR2 activation (Fig. 8) was seen in mitochondrial and cytosolic fractions from transduced HEp-2 cells cultured with/without galanin (1 μM) for 48 h.

Discussion

We previously demonstrated the function of the GALR1 and GALR2 signaling pathways. In HNSCC cell lines, we showed that stably re-expressed GALR1 suppressed tumor cell proliferation via ERK1/2-mediated effects on cyclin-dependent kinase inhibitors, whereas, in GALR2-transduced HNSCC cells, galanin suppressed cell proliferation and induced apoptosis. Thus, we deduced that GALR1 and GALR2 function as tumor suppressors and therefore focused on these proteins as therapeutic targets for HNSCC.

Here, we showed that transduction of GALR2 using rAAV vectors makes HNSCC cells susceptible to galanin-induced growth inhibition and apoptosis; however, we could not show anti-tumor effects of rAAV-mediated GALR1 activation in HNSCC cells. It is unclear why GALR1 and GALR2 have such different effects. We considered that, because of constitutive phosphorylation of ERK1/2 even under serum-free culture conditions in the HNSCC cells used in our experiments, further activation of ERK1/2 by GALR1 activation may not be critical to cell proliferation and survival in these cells.

Although these mechanisms are thought to be partially responsible for tumor cell growth inhibition, the apoptosis induction mechanisms mediated in HNSCC cells by GALR2 activation remain unclear. Our results indicated that downregulation of ERK1/2 is important as a novel apoptotic mechanism triggered by GALR2 activation.

Dephosphorylation of ERK1/2 via stimulation of other GPCRs has previously been reported. Yamada *et al.* reported that angiotensin I type 2 receptor (AT2R) mediated apoptosis followed by dephosphorylation of MAPK in growth factor-supported PC12 cells.[32] Others have reported that inhibition of MAPKs via AT2R was mediated by stimulation of serine/threonine phosphatase 2A (PP2A).[33] It has been reported that phosphotyrosine phosphatase DEP-1/PTPη-dependent dephosphorylation of ERK1/2 is required for inhibition of basic

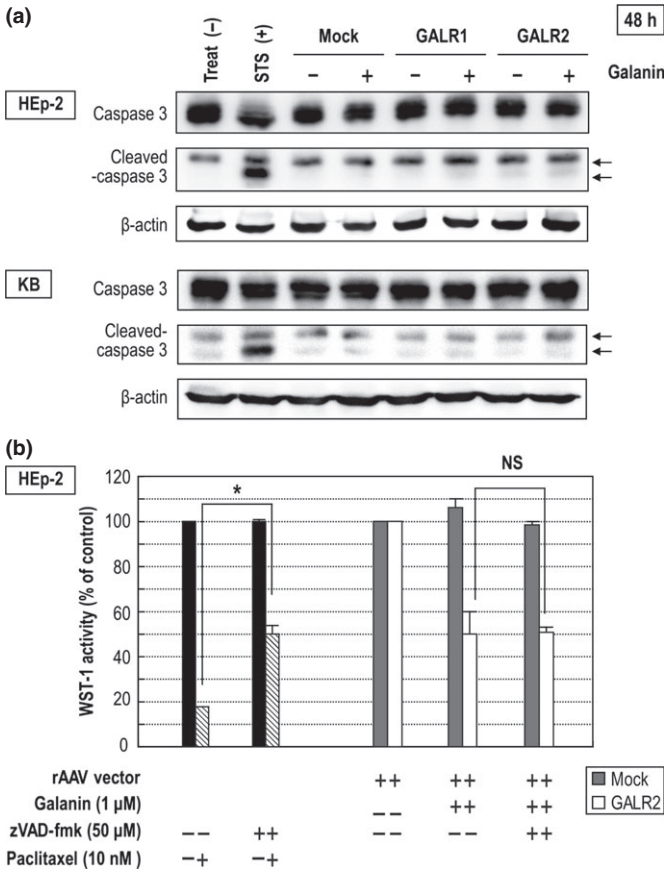


Fig. 7. Effect of galanin on the caspase signaling pathway in recombinant adeno-associated virus (rAAV)-GALR2 vector-transduced head and neck squamous cell carcinoma (HNSCC) cells. (a) Assessment of caspase-3 activation by immunoblotting. Staurosporine (STS; 1 μM) for 3 h was used as a positive control for caspase activation. (b) Effect of a pan-caspase inhibitor (z-VAD-fmk) on GALR2-mediated cytotoxic effects in HEp-2 cells. As positive control, to indicate the inhibitory effect of z-VAD-fmk, we used cells exposed to paclitaxel (10 nM) for 48 h.

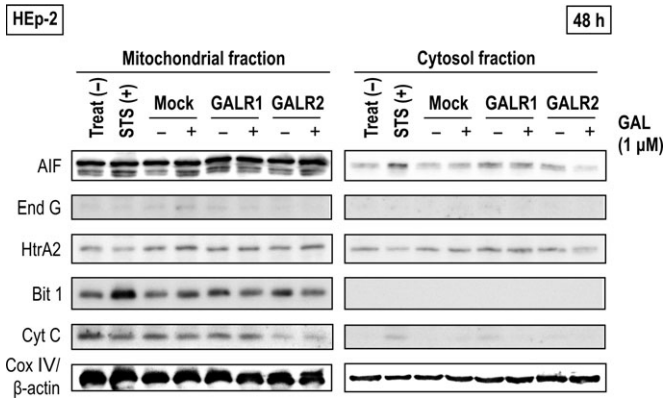


Fig. 8. Effects of mitochondrial apoptosis-associated proteins in HEp-2 cell co-administered with each of the recombinant adeno-associated virus (rAAV)-GALR vectors and galanin. Cox IV and β-actin were used as protein-loading controls for the mitochondrial and cytosolic fractions, respectively.

growth factor-dependent cell proliferation mediated by the stimulating somatostatin receptors in glioma cells.[34,35] Therefore, we anticipated that some protein phosphatase related to the dephosphorylation of MAPK cascades may be involved. As an alternative to protein phosphatase activation, Fushimi *et al.* reported that prostaglandin E2 downregulated TNF-α-induced production of matrix metalloproteinase-1 by inhibiting the Raf-1/MEK/ERK cascade through activation of the EP4 prostanoid receptor.[36] Tumor necrosis factor-α–mediated Raf activation, involving phosphorylation at Ser338, was suppressed by stimulation of the EP4 receptor; in contrast, phosphorylation of the inhibitory site on Raf, at Ser259, was stimulated via the EP4 receptor. EP4-mediated Raf inactivation was followed by dephosphorylation of MEK1/2 and ERK1/2. Hence, we examined whether GALR2 activation affects the phosphorylation state of MEK1/2, immediately upstream of ERK1/2. We confirmed that at least dephosphorylation of MEK1/2 was induced by GALR2 activation in HEp-2 cells (data not shown). A comprehensive analysis of the influence of the Ras/Raf/MEK/ERK cascades following activation of the GALR2 signaling pathway requires further study. In addition to the apoptotic mechanisms mediated by MAPK inhibition via specific inhibitors, Eisenmann *et al.* reported the importance of the MAPK pathway-downstream effector protein, RSK, and the BH-3-only proapoptotic protein Bad in melanoma cell survival.[37] Moreover, other groups reported that induction of apoptosis in melanoma cells following inhibition of MEK is mediated by the Bcl-2 family members PUMA, Bim, and Mcl-1, as well as through caspase-independent and AIF-dependent mechanism.[38,39] Therefore, we examined whether these regulators, including those belonging to the Bcl-2 and the IAP family of proteins, and caspase-dependent as well as caspase-independent apoptosis-related proteins, may be associated with the apoptotic mechanisms triggered by GALR2 activation. We found that upregulation of Bim is induced by GALR2 activation. Future studies involving downregulation of Bim expression, for instance by using siRNA, would verify this relationship further.

Although dephosphorylation of ERK1/2 by GALR2 activation was observed in both HNSCC cell lines, significant upregulation of Bim via GALR2 signaling was only induced in HEp-2, but not in KB cells. These results imply that there are both Bim-dependent and Bim-independent unknown pathways that induce apoptosis, and suggest that the type of pathways followed downstream of GALR2 depends on the cell line.

We hypothesize that the reason why the therapeutic effect of AAV-GALR2 in KB cells was less marked than in HEp-2 cells may be that KB cells do not possess the Bim-dependent pathway, whereas HEp-2 cells possess both pathways. Wittau *et al.*[40] demonstrated that there are multiple signaling pathways downstream of GALR2 in small cell lung cancer cells, resulting from coupling of GALR2 to various G-proteins. Their results supported our hypothesis that GALR2 has multiple signaling pathways by which apoptosis is induced, and that ERK inactivation, followed by upregulation of Bim, is associated with the intensity of apoptosis induction following activation of GALR2 signaling.

These findings about differences between in HEp-2 and in KB cells indicate that the pathways involved in GALR2-induced apoptosis diverge, and the details of these signaling pathways remain unclear. For further investigation, we divided GALR2-induced apoptosis pathways into caspase-dependent and -independent pathways in order to investigate this phenomenon further. When investigating the caspase-dependent pathway, we found that GALR2-inhibition of cell growth was not abrogated by the pan-caspase inhibitor z-VAD-FMK. Moreover, none of the apoptosis inducers of the caspase-independent pathway that we examined were translocated to the cytosol from the mitochondria, and activation of members of the IAP and the

Bcl-2 protein families, except for Bim, were unchanged by GALR2 signaling. These results suggested that the apoptotic pathway induced by GALR2 signaling encompasses other unknown pathways. Because carcinogenesis of HNSCC is mainly triggered by extrinsic factors, such as smoking or alcohol, which induce various genetic defects, this distinctive feature makes GALR2 advantageous as a therapeutic gene.

In conclusion, we have shown that GALR2 expression in the presence of galanin induced apoptosis via diverse pathways; thus, gene therapy using the GALR2 gene holds promise as a novel suicide-gene therapy against HNSCC.

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Disclosure Statement

The authors have no conflict of interest.

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