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Original article

Title: Enzootic nasal tumor virus type 2 envelope of goats acts as a retroviral oncogene in cell transformation

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Abstract

Enzootic nasal tumor virus type 1 (ENTV-1) (ovine nasal tumor virus) and ENTV-2 (caprine nasal tumor virus) are known to be causative agents of enzootic nasal adenocarcinoma (ENA) in sheep and goats, respectively. Although the nucleotide and amino acid sequences of ENTV-1 and ENTV-2 are quite similar, they are recognized as phylogenetically distinct viruses. The envelope protein of ENTV-1 functions as an oncoprotein in the *in vitro* transformation of epithelial cells and fibroblasts. Thus, it is the primary determinant of *in vivo* tumorigenesis in ENA. As per our knowledge, no previous studies have reported in detail the role of ENTV-2 in ENA tumorigenesis. Here, in order to investigate the molecular mechanism of caprine ENA oncogenesis by ENTV-2, we have attempted to identify the transforming potential of ENTV-2 envelope, and investigated the activation of cell signaling pathways in oncogenic transformation. Our findings confirmed that ENTV-2 envelope was capable of inducing oncogenic transformation of rat cell lines *in vitro*. Further, we found that MAPK, Akt, and p38 were constitutively activated in ENTV-2 envelope-transformed clone cells. In addition, inhibitor experiments revealed that MEK-MAPK and PI3K-Akt signaling pathways are involved in the ENTV-2 envelope-induced cell transformation. These data indicate that ENTV-2 envelope could induce oncogenic transformation by signaling pathways that are also utilized by ENTV-1 envelope.

Introduction

Enzootic nasal adenocarcinoma (ENA), previously known as enzootic intranasal tumor, is a contagious neoplasia in sheep and goats [1]. It has been reported worldwide [2-9], with several cases being reported in China in recent times [10-13]. The etiological agent of ENA has been identified as retroviruses [14-17]. Experimental infection was successful in inducing ENA by cell-free tumor filtrates in sheep [18-20], and by concentrated ENA nasal exudate in goats [21]. The sequences of ovine nasal tumor virus (ENTV-1 in sheep) and caprine nasal tumor virus (ENTV-2 in goats) were identified as similar but phylogenetically distinct viruses [22-26]. The target tissues in ENA were the secretory epithelial cells of the ethmoid turbinate of the nasal cavity; however, the tissue distribution of the two viruses was different; while ENTV-1 was detected in tumors, lymph nodes, kidneys, and lungs, ENTV-2 was additionally detected in peripheral blood mononuclear cells (PBMCs) and bone marrow [26].

Both ENTV-1 and ENTV-2 are classified as simple ovine β-retroviruses. This category also includes jaagsiekte sheep retrovirus (JSRV), which is an etiological agent for naturally occurring and experimentally induced ovine pulmonary adenocarcinoma (OPA) in sheep [27]. These three retroviruses consist of 5′-long terminal repeat (LTR)-*gag*-*pro*-*pol*-*env*-3′-LTR, without any viral oncogenes [23, 26, 27]. However, they encode another open reading frame x, designated as *orf-x*, which was initially in focus for its role in oncogenesis by JSRV [28]; but it was found that it was not required for the *in vitro* transformation and induction of OPA *in vivo* [29, 30]. In the classical transformation assay, the full-length envelope glycoprotein of ENTV-1, as well as that of JSRV, was identified as an oncoprotein contributing to the transformation of fibroblasts and epithelial cells *in vitro* [29, 31-33]. The JSRV envelope itself induced lung tumors not only in sheep [34] but also in mice *in vivo* [35-38]. Interestingly, ENTV-1 envelope also induced lung tumors in mice [39]. In addition to the envelopes, the LTR region is considered to be an important factor in determining their tissue tropism [37, 40]. A recent study has further revealed that the expression of a glycosylphosphatidylinositol-anchored cell surface protein hyaluronidase-2 (Hyal-2), an entry receptor

for JSRV and ENTV [31], influences the entry of ENTV into nasal target cells, but not JSRV [41].

The JSRV envelope transmembrane (TM) domain is required for the transformation of fibroblasts [42]. Further mutational analysis revealed that the cytoplasmic tail (CT) region of JSRV envelope TM, in particular, is required for transformation *in vitro*. Certain tyrosine residues of the CT region, including Tyr590 of JSRV and Tyr592 and Tyr596 of ENTV-1, were initially regarded as critical for transformation in the context of signal transduction [43-47]; phosphatidylinositol 3-kinase (PI3K) to Akt and mitogen extracellular regulated kinase (MEK) to mitogen-activated protein kinase (MAPK) pathways are involved in transformation *in vitro* [43-49]. Besides the molecules involved in the above signaling pathways, other molecules including Ras, Rac1, p38, Hsp90, and Sprouty2 have also been reported to play a role in cell transformation [48, 50, 51]. Signaling pathways utilized by ENTV-1 resemble those used by JSRV *in vitro* and *in vivo* [52, 53].

While a lot of research has been conducted on cell transformation induced by ENTV-1 and JSRV, no previous studies have reported on the molecular mechanism of caprine ENA oncogenesis induced by ENTV-2. The present study aims to address this by focusing on the identification of the transforming potential of ENTV-2 envelope *in vitro*, and further investigation on the activation of signaling pathways associated with oncogenic transformation.

Materials and methods

Constructs

ENA tumors isolated from goats were collected and embedded in paraffin. Integrated genomic ENTV-2 genes were recovered from a paraffin block using TaKaRa DEXPAT (TaKaRa Bio, Shiga, Japan). PCR was performed using the recovered genomes as templates, forward and reverse primers designed according to the ENTV-2 sequence (GenBank accession number: AY197548) [26], dNTP mixture, *TaKaRa Ex Taq* DNA polymerase, and *TaKaRa Ex Taq* Buffer (TaKaRa Bio). PCR conditions were as follows: 95°C for 2 min,

30 cycles of 95°C for 30 sec, 45°C for 30 sec, and 68°C for 1 min. The PCR products were purified with a Gel Extraction Kit and PCR Purification Kit (QIAGEN, CA, USA), cloned into T-Vector pMD20 (TaKaRa Bio), and sequenced. Each PCR product was ligated using a DNA Ligation Kit (Mighty Mix) (TaKaRa Bio), and inserted into the HA-tagged JSRV envelope expression plasmid ΔGP(JSRV-HA) [48], designated as ΔGP(ENTV-2). New ENTV-2 sequence has been deposited as NAOM-HU3118124 strain in DNA Data Bank of Japan with accession number LC570918. The JSRV envelope expression plasmid was provided by Dr. Hung Fan (University of California, Irvine, CA, USA). The ENTV-2 envelope expression construct with 6 histidines (His)-tagged version was also developed, designated as ΔGP(ENTV-2-His).

Cell culture

Rat embryonic fibroblasts 208F cells and rat kidney epithelial RK3E cells were purchased from the European Collection of Authenticated Cell Cultures and the American Type Culture Collection, respectively. The two cell lines, along with human kidney epithelial 293T cells, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Transformation assay and establishment of transformed clone cells

208F and RK3E cells were seeded at 4×10^5 cells/plate in 6 cm dishes, 24 h prior to transfection. Five micrograms of ΔGP(ENTV-2), ΔGP(ENTV-2-His), or ΔGP(JSRV) were transfected into the cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). To inhibit MAPK, PI3K, and p38 signaling pathways, cells after transfection were treated with inhibitors, PD98059, LY294002, and SB203580 (Calbiochem, CA, USA), respectively, or DMSO (WAKO, Osaka, Japan) as a control vehicle. Transformed foci were counted on days 11-20 after transfection. To establish transformed clone cells, ΔGP(ENTV-2-His) was transfected into 208F and RK3E cells. Transformed foci were picked up 3-4 weeks

after transfection, and single clone cells were isolated by a limiting dilution. The cells were lysed with lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100) supplemented with Complete protease inhibitor cocktail (Roche).

Treatment with epidermal growth factor (EGF)

Parental and transformed 208F cells were seeded at 5×10^5 cells/plate in 6 cm dishes. They were serum-starved for 20-24 h, and the parental 208F cells were subsequently stimulated with 100 ng/mL recombinant human EGF (BioAcademia, Osaka, Japan) for 30 min. The cells were lysed with lysis buffer supplemented with Complete protease inhibitor cocktail.

Immunoblot

The cell lysate was mixed with 5X sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris (pH 6.8), 10% glycerol, 1% SDS, and 0.05% bromophenol blue) with or without reducing agent 1% β-mercaptoethanol, and boiled at 95°C for 5 min. The total proteins were separated on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (GE Healthcare, USA). The membranes were incubated with Tris-buffered saline with Tween 20 (TBS-T) having 5% nonfat dry milk or bovine serum albumin at room temperature for 1 h. The membranes were incubated with specific antibodies overnight at 4°C. Monoclonal antibodies (mAbs) against p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Akt (pan), phospho-Akt (Ser473), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, MA, USA), and His-tag (MBL, Nagoya, Japan) were used as primary antibodies. After washing the membranes three times with TBS-T, the membranes were incubated with anti-mouse or anti-rabbit IgG labeled with Alexa Fluor 680 (Thermo Fisher Scientific, MA, USA) at room temperature for 1 h. After washing the membranes three times with TBS-T, signals were detected using an Odyssey CLx Infrared Imaging System (LI-COR Biosciences, NE, USA).

141

142 **Immunohistochemistry**

143 Naturally occurring ENA tumors and normal goat ethmoid turbinate were embedded in paraffin. For antigen
144 retrieval, the sections were microwaved at 95°C for 5 min. in 10 mM citrate buffer (pH 6.0). Endogenous
145 peroxidase was quenched with 0.3% hydrogen peroxidase in methanol for 30 min. The sliced sections were
146 stained with hematoxylin and eosin (H&E), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb,
147 and anti-phospho-Akt (Ser473) mAb. Immunohistochemistry was performed by using a commercially
148 available EnVision^{TM}+ (Dako, CA, USA) according to instructions by the manufacturer.

149

150 **Statistical analysis**

151 Statistically significant differences were calculated using Student's *t*-test and are indicated as *p* values.
152 Differences of $p < 0.05$ were considered to be statistically significant.

153

154 **Results**

155 **Transformation of rat cells by ENTV-2 envelope**

156 In order to make a construct for the expression of full-length ENTV-2 envelope, we performed PCR to
157 amplify proviral ENTV-2 *env* DNA derived from the ENA tumor genome. The nucleotide sequence of the
158 recovered ENTV-2 *env* gene had 98% identity (1,805 out of 1,848 nucleotides) with AY197548, which had
159 been previously identified in Spain [26]. The deduced amino acid sequence of the new ENTV-2 was
160 identical to that of the AY197548 strain, though Ser at position 111 was mutated to Arg. For efficient
161 expression in mammalian cells, the JSRV *env* gene in the expression plasmid ΔGP(JSRV-HA) was replaced
162 with the full-length ENTV-2 *env* gene for its expression under the cytomegalovirus (CMV) promoter,
163 designated as ΔGP(ENTV-2) (Fig. 1). In addition, for the easy detection of ENTV-2 envelope, an expression
164 construct was developed for ENTV-2 envelope with His-tag (ΔGP(ENTV-2-His)) (Fig. 1).

To investigate the transforming potential of the ENTV-2 envelope, we transfected rat fibroblast 208F cells with the ENTV-2 envelope expression plasmid ΔGP(ENTV-2), JSRV envelope expression plasmid ΔGP(JSRV) or pcDNA3.1(-), with the latter two plasmids as positive and negative plasmid controls, respectively. In the transformation assay, we found that the ENTV-2 and JSRV envelopes induced foci of transformed cells in 208F cells (Fig. 2a and c), as did the ENTV-2 envelope with His-tagged construct ΔGP(ENTV-2-His) (Fig. 2b). Moreover, we found that the ENTV-2 envelope induced foci of transformed cells in rat epithelia RK3E cells (Fig. 2e-g). The negative plasmid control did not induce any foci of transformed cells in either cell line (Fig. 2d and h).

Signaling pathways in ENTV-2 envelope-induced transformation *in vitro*

In order to investigate the molecular mechanisms involved in ENTV-2 envelope-induced transformation, we utilized the chemical inhibitors PD98059 for MEK, LY294002 for PI3K, and SB203580 for p38 in the transformation assay. The number of foci of transformed cells treated with MEK and PI3K inhibitors at day 20 after transfection was lower compared to that in cells treated with DMSO as a control vehicle (Fig. 3a). Moreover, the size of each focus on cells treated with MEK and PI3K inhibitors was smaller than that in cells treated with DMSO (Fig. 3b-j). These results suggested that MEK and PI3K pathways are involved in the ENTV-2 envelope-induced transformation. On the other hand, the number of foci of transformed cells treated with p38 inhibitor at day 13 after transfection showed an increase, compared to that in cells treated with DMSO (Fig. 4a). The foci on cells treated with p38 inhibitor were larger in size, compared to those in cells treated with DMSO (Fig. 4b-g). Similar results were also observed in the experiments conducted using RK3E cells (data not shown). These results suggest that the ENTV-2 envelope triggers MEK and PI3K signaling pathways to induce transformation, while the p38 signaling pathway may negatively regulate the transformation.

To further investigate the signaling pathways involved in ENTV-2 envelope-induced

189 transformation, we established 208F clone cells transformed with ENTV-2 envelope having a His-tag (Fig.
190 5a). The transformed cells (Fig. 5a) showed differences in morphology compared to the parental 208F cells
191 (Fig.5b). Full-length and TM domain of ENTV-2 envelope were detected by western blot analysis (Fig. 5c).
192 In the ENTV-2 envelope-transformed clone cells, we found constitutive activation of MAPK (also called
193 p44/42 or Erk1/2), Akt, and p38 (Fig. 5d-f).
194
195 **MAPK and Akt activation in naturally occurring ENA tumors *in vivo***
196 Immunohistochemical staining of naturally occurring caprine ENA tumors, from which the
197 ENTV-2 *env* gene was recovered, showed the typical histological features of adenocarcinoma (Fig. 6a and
198 d). In serial sections of the same sample, we detected phosphorylation of p44/42 MAPK at Thr202/Tyr204
199 (Fig. 6b and e) and Akt at Ser 473 (Fig. 6c and f). Normal goat ethmoid turbinate did not show any
200 phosphorylation of these molecules (Fig. 6g-l). These results, together with those of the *in vitro* experiments,
201 strongly suggest that the MEK-MAPK and PI3K-Akt signaling pathways are involved in ENTV-2
202 envelope-induced ENA oncogenesis *in vivo*.
203
204 **Discussion**
205 ENA and OPA caused by ENTV-1 and JSRV, respectively, are interesting and unusual examples
206 of neoplasms induced by virus envelopes having transforming potential [54]. While the mechanisms of
207 ENTV-1 and JSRV transformation have been widely studied, there's been a lack of in-depth investigation
208 into caprine ENA oncogenesis by ENTV-2. Thus, in order to understand the molecular mechanisms of
209 caprine ENA, this study focused on identifying the transforming potential of ENTV-2 envelope *in vitro*.
210 Our findings confirmed that ENTV-2 envelope was indeed capable of inducing oncogenic transformation
211 of rat cell lines *in vitro*. Further, we found that MAPK, Akt, and p38 were constitutively activated in ENTV-
212 2 envelope-transformed clone cells. In addition, inhibitor experiments revealed that MEK-MAPK and

PI3K-Akt signaling pathways are involved in the ENTV-2 envelope-induced cell transformation. Collectively, these results revealed that the transforming potential of ENTV-2 envelope seems to be quite similar to that of JSRV *in vitro*. In previous studies, activation of MAPK phosphorylated on Thr202/Tyr204 was observed not only in naturally occurring ENA, but also in naturally occurring OPA as well as experimentally induced OPA *in vivo* [48, 53]. However, while the staining of ENA tumors from sheep and goats with anti-phosphorylated Akt mAb was positive, the staining of OPA tumor section with that was not positive in all cases, although phosphorylation of Akt at Ser473 was observed in JSRV envelope-transformed cells *in vitro* [44]. This led us to postulate that although the signaling pathways leading to oncogenic transformation by the ENTV-1 and JSRV envelopes shared an overall similarity, there were slight differences in the mechanism. Additionally, we found that the caprine ENA tumor used in this study also exhibited phosphorylation of MAPK and Akt. Taken together, we concluded that ENTV-2 envelope could induce oncogenic transformation by signaling pathways that are also utilized by the ENTV-1 envelope.

The molecular mechanism through which the ENTVs and JSRV envelopes activate these signaling pathways has not yet been fully understood. In particular, the elucidation of the cellular molecules that interact with these envelopes should be a key factor towards understanding their oncogenic potential. One of the interacting partners identified as an entry receptor for JSRV as well as ENTV-1is Hyal-2, which was initially considered to be a tumor suppressor located at human chromosome 3p21.3 [55]. However, the involvement of Hyal-2 in transformation is controversial; mouse fibroblast NIH-3T3 cells are successfully transformed, although Hyal-2 of the murine homolog does not function as a receptor for the JSRV and ENTV-1 envelopes [56]. Two studies independently reported that RalA binding protein 1 (RALBP1) and zinc finger protein 111 (Zfp111) directly interacted with the JSRV envelope CT region [57, 58]. RALBP1 was involved in JSRV transformation, via the formation of a complex with CDC42 to induce the activation of downstream effectors such as the mammalian target of rapamycin and p70S6 kinase in canine kidney epithelial MDCK cells [57]. On the other hand, Zfp111 was found to interact with a nuclear form of JSRV

envelope in rat fibroblast 208F cells, suggesting that JSRV transformation involves events in the nuclear region [58]. These studies indicated that JSRV envelope interacts with multiple cellular or nuclear proteins to induce cellular transformation *in vitro*. Nevertheless, the mechanism of activation of MAPK and other molecules, including p38, Ras, and Rac1, in the JSRV envelope-induced transformation, as well as their involvement in OPA oncogenesis, remains unclear. Further research is needed on the identification of proteins interacting with the ENTV-1 and ENTV-2 envelopes. The amino acid sequence of variable region 3 (VR3), especially in the CT region, in the ENTV and JSRV envelopes, is not conserved; thus, the proteins interacting with ENTVs may be different from those interacting with JSRV. This fact could help distinguish between the similar yet slightly different signaling pathways leading to oncogenic transformation by the ENTV and JSRV envelopes. In addition to the transforming potential of CT regions, they have also been reported to contribute to the infectivity and fusogenicity of viruses; thus, the CT regions play a critical role in exhibiting multiple functions in the viral life cycle [59-65]. It has been observed that the Moloney murine leukemia virus envelope CT region possesses an amphipathic α-helix that facilitates membrane fusion [66, 67]. Previous studies have predicted the presence of a putative amphipathic α-helical wheel structure in the CT region of JSRV envelope [47]; this possibility is backed by multiple structural analyses (Maeda *et al*., unpublished observations). Further investigation is also needed to elucidate the structure-function and relationship of ENTV-2 envelope, especially the CT region, in ENA tumorigenesis.

So far, it is not yet unclear why ENTV-1 and ENTV-2 cause identical diseases but could be detected only in sheep and goats, respectively. JSRV could experimentally induce lung tumors in goats that differ from those induced in sheep, suggesting that there are several restricting factors which determine species specificity [68]. In addition to the main target cells, such as type II pneumocytes and club (Clara) cells, JSRV can infect other cell types, including undifferentiated cells in the respiratory tract [69], indicating that its envelope may be able to induce different neoplastic diseases in tissues other than those of the lungs. For instance, JSRV, but not ENTV-1, infection was detected in ovine ENA [70]. Thus, tissue

specificity is believed to be influenced by other viral factors apart from the envelope. The viral LTR could be a critical factor in determining retrovirus tropism. The JSRV LTR is preferentially active in lung epithelial cells [40]. Rosales *et al*. recently reported that nasal turbinate chondrocytes could be potential target cells for ENTV-1 infection *in vivo*, in cells in which ENTV-1 LTR is significantly active [41]. It is notable that the LTR sequence of ENTV-2, especially the U3 region, is highly divergent from that of ENTV-1 and JSRV [11, 26]. As mentioned above, the envelope VR3 sequence of ENTV-2 is also significantly different from that of ENTV-1 and JSRV. These facts collectively may account for the yet unexplained goat tropism of ENTV-2, which would be an interesting point of focus in future studies.

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Author Contributions

NM conceived and designed the experiments. MDLH and YI provided the materials. NM and MDLH carried out the experiments. NM wrote the manuscript. NM, YI, MDLH, and KM reviewed and edited the manuscript. NM, YI, MDLH, and KM read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of Interest

The authors declare no conflict of interest.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Informed consent

Informed consent was obtained from all individual participants included in this study.

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Figure legends

Figure 1 Expression constructs used in this study. ΔGP(JSRV) was previously described [29]. The JSRV *env* gene in the expression plasmid (ΔGP(JSRV-HA)) [47] was replaced with the full-length ENTV-2 *env* gene, designated as ΔGP(ENTV-2). The His-tag (black box) was inserted into ΔGP(ENTV-2) at the C-terminus of the ENTV-2 *env* reading frame. All *env* genes are expressed in rodent cells driven by the CMV promoter. SD: *env* splice donor, SA: *env* splice acceptor

Figure 2 Transformation of rat 208F and RK3E cells. 208F (a-d) and RK3E (e-h) cells were transfected with 5 μg of ΔGP(ENTV-2) (a, e), ΔGP(ENTV-2-His) (b, f), ΔGP(JSRV) (c, g), and pcDNA3.1(-) (d, h). Foci of transformed 208F cells or RK3E cells at day 11 after transfection are shown (Scale bar = 100 μm).

Figure 3 Effects of MEK and PI3K inhibitors on the transformation of 208F cells. 208F cells were transfected with 5 μg of ΔGP(ENTV-2) (b, e, h), ΔGP(JSRV) (c, f, i), and pcDNA3.1(-) (d, g, j), respectively. The number of foci of transformed 208F cells at day 20 after transfection was scored (Scale bar = 200 μm). The MEK and PI3K inhibitors at a final concentration of 20 μM were added daily to the cells. For (a), bars indicate mean values ± standard deviations of three independent experiments. Statistically significant differences are shown as *P* values (****; $P < 0.0001$).

Figure 4 Effects of p38 inhibitor on the transformation of 208F cells. 208F cells were transfected with 5 μg of ΔGP(ENTV-2) (b, e), ΔGP(JSRV) (c, f), and pcDNA3.1(-) (d, g), respectively. The number of foci of transformed 208F cells at day 13 after transfection was scored (Scale bar = 200 μm). The p38 inhibitor at a final concentration of 5 μM was added daily to the cells. For (a), bars indicate mean values ± standard deviations of three independent experiments. Statistically significant differences are shown as *P* values (***; $P < 0.001$, ****; $P < 0.0001$).

Figure 5 Activation of signaling pathways in His-tagged ENTV-2 envelope-transformed clone cells. Transformed clone cells (a) with a different morphology as parental 208F cells (b) were established (Scale bar = 100 μm). Expression of ENTV-2 envelope with His-tag (c), p44/42 MAPK (Erk1/2), and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (d), Akt (pan), phospho-Akt (Ser473) (e), p38 MAPK, and phospho-p38 MAPK (Thr180/tyr182) (f), in the transformed clone cells was detected by western blot analysis. For positive controls for detecting phosphorylated proteins, parental 208F cells were stimulated with 100 ng/mL recombinant human EGF to detect phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (d). T: transformed, P: parental, kDa: kilodalton

Figure 6 Immunohistochemical staining of the caprine ENA tumor and normal goat ethmoid turbinate. Lower magnification micrographs of naturally occurring ENA tumor and normal goat ethmoid turbinate sections immunohistochemically stained with H&E (a, g), phospho-p44/42 MAPK (Thr202/Tyr204) mAb (b, h), and phospho-Akt (Ser473) mAb (c, i) are shown, respectively, (Scale bar = 100 μm). Higher magnification micrographs of each section are also shown in (d) to (f) for the caprine ENA tumor, and (j) to (l) for normal goat ethmoid turbinate, respectively, (Scale bar = 50 μm).

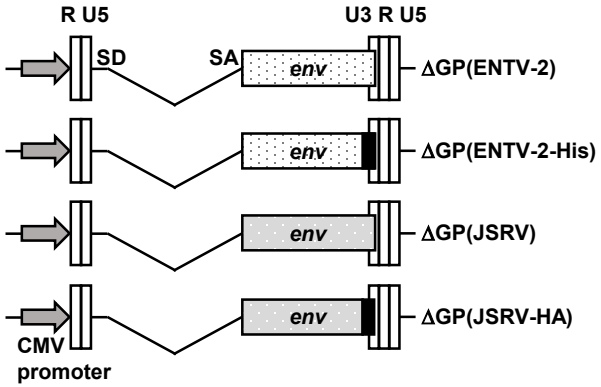
R U5
U3 R U5
SD
SA
env
ΔGP(ENTV-2)
env
ΔGP(ENTV-2-His)
env
ΔGP(JSRV)
env
ΔGP(JSRV-HA)
CMV
promoter

Figure 1

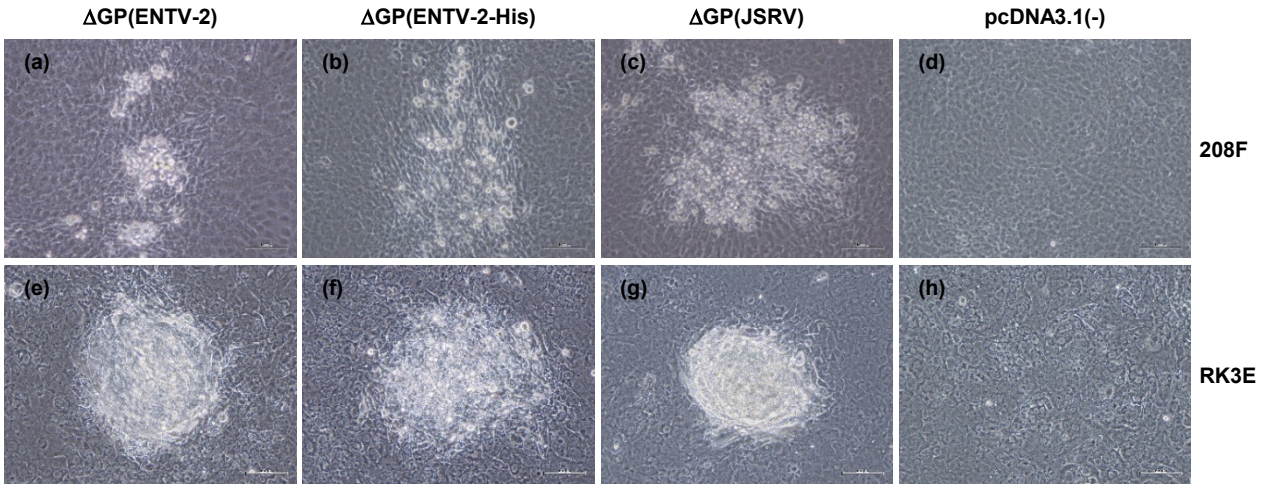

Figure 2

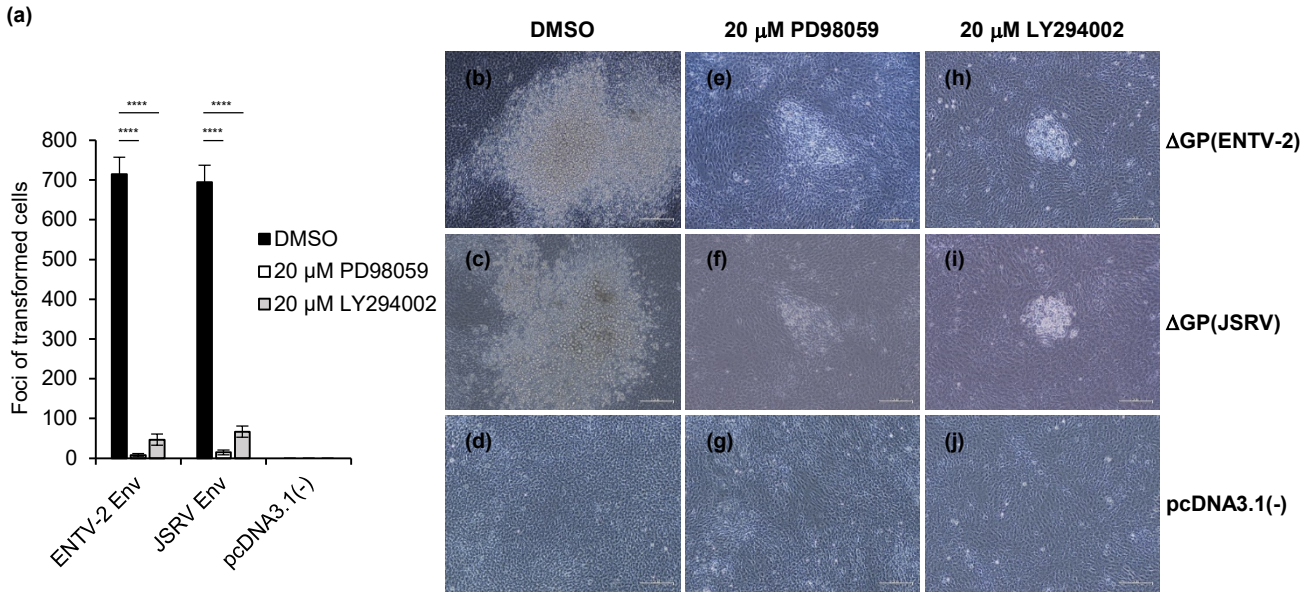
(a)
Foci of transformed cells
800
700
600
500
400
300
200
100
0
****
****
****
****
DMSO
20 μM PD98059
20 μM LY294002
ENTV-2 Env
JSRV Env
pcDNA3.1(-)
DMSO
20 μM PD98059
20 μM LY294002
(b)
(e)
(h)
(c)
(f)
(i)
(d)
(g)
(j)
ΔGP(ENTV-2)
ΔGP(JSRV)
pcDNA3.1(-)

Figure 3

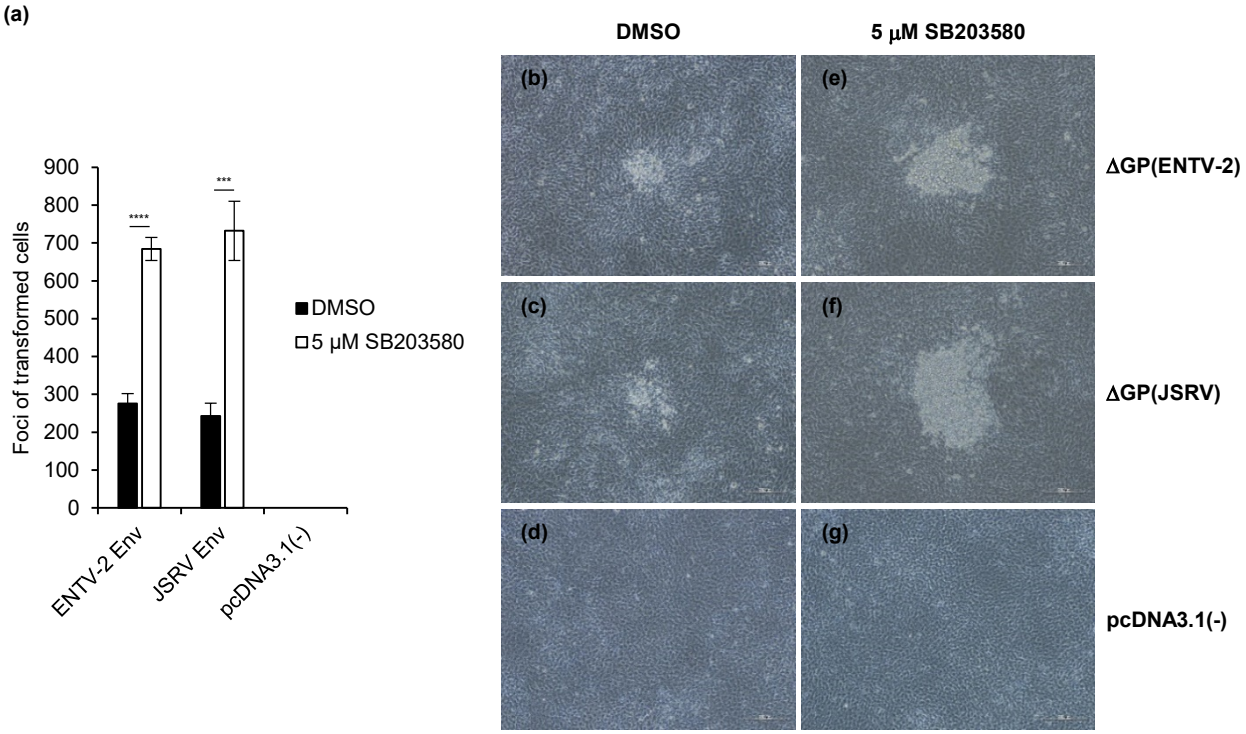
(a)
Foci of transformed cells
900
800
700
600
500
400
300
200
100
0
****
***
DMSO
5 μM SB203580
ENTV-2 Env
JSRV Env
pcDNA3.1(-)
DMSO
5 μM SB203580
(b)
(e)
(c)
(f)
(d)
(g)
ΔGP(ENTV-2)
ΔGP(JSRV)
pcDNA3.1(-)

Figure 4

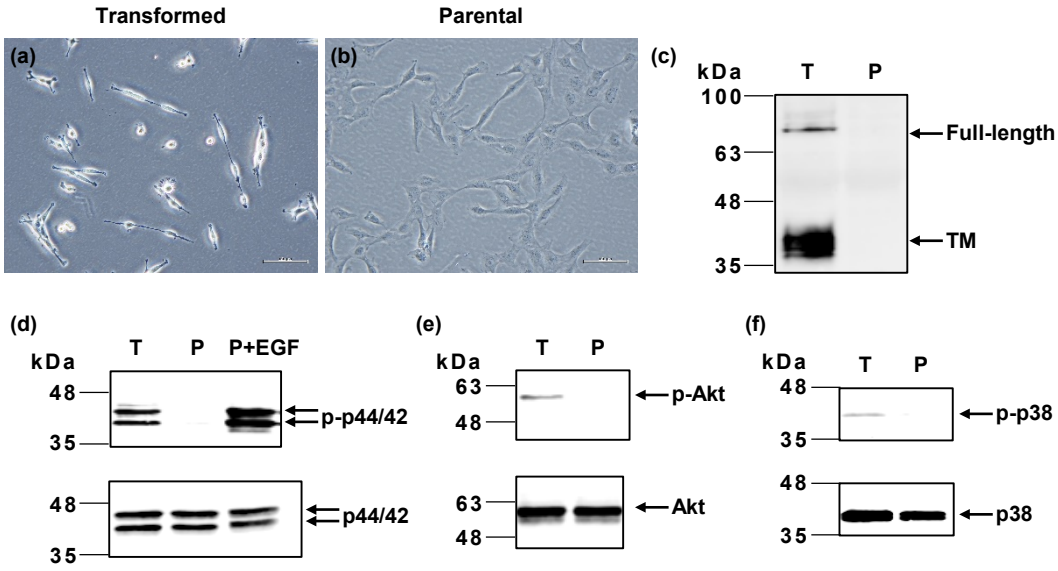
Transformed
Parental
(a)
(b)
(c)
kDa
T
P
100
63
48
35
Full-length
TM
(d)
kDa
T
P
P+EGF
48
35
p-p44/42
48
35
p44/42
(e)
kDa
T
P
63
48
p-Akt
63
48
Akt
(f)
kDa
T
P
48
35
p-p38
48
35
p38

Figure 5

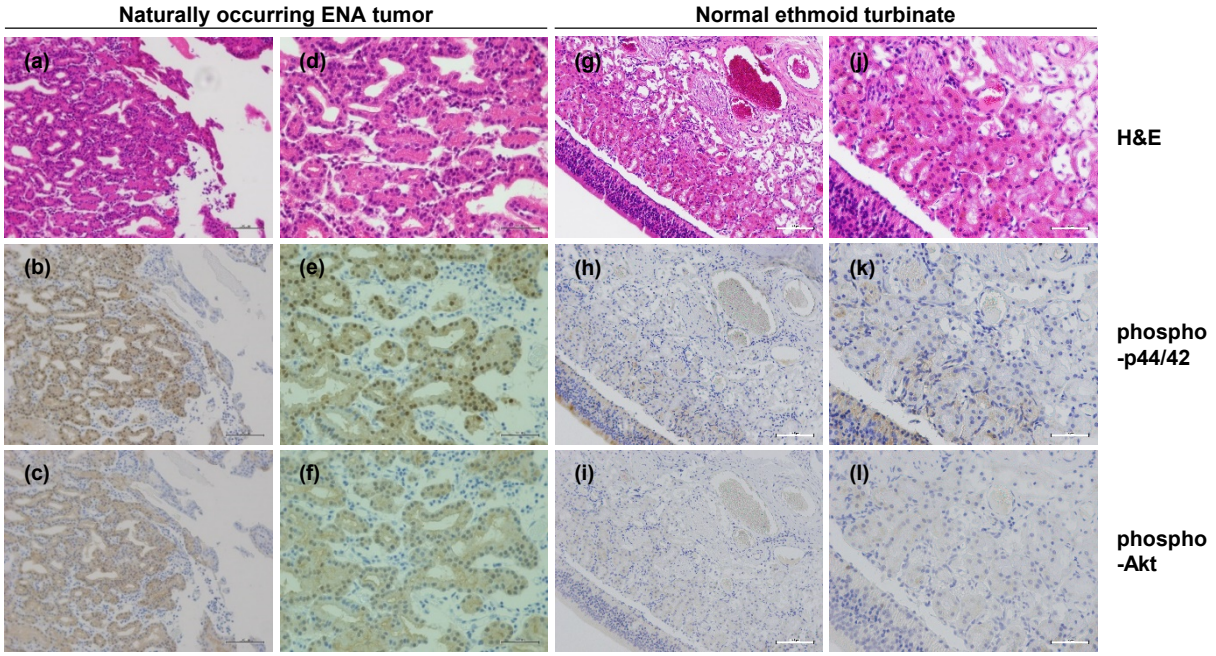
Naturally occurring ENA tumor
Normal ethmoid turbinate
(a)
(d)
(g)
(j)
H&E
(b)
(e)
(h)
(k)
phospho
-p44/42
(c)
(f)
(i)
(l)
phospho
-Akt

Figure 6