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Relation	

Eldecalcitol (ED-71)-induced exosomal miR-6887-5p suppresses squamous cell carcinoma cell growth by targeting heparin-binding protein 17/fibroblast growth factor binding protein-1 (HBp17/FGFBP-1)

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Keywords: miR-6887-5p; Eldecalcitol (ED-71); HBp17/FGFBP-1; Oral squamous cell carcinoma; Exosome

ABSTRACT

Heparin-binding protein 17/fibroblast growth factor binding protein-1 (HBp17/FGFBP-1) was purified from A431 cell-conditioned media based on its capacity to bind to fibroblast growth factor 1 and 2 (FGF-1 and FGF-2). HBp17/FGFBP-1 has been observed to induce the tumorigenic potential of epithelial cells and is highly expressed in oral cancer cell lines and tissues. HBp17/FGFBP-1 is also recognized as a pro-angiogenic molecule as a consequence of its interaction with FGF-2. We have previously reported that Eldecalcitol (ED-71), an analog of $1\alpha,25(OH)_2D_3$, downregulated the expression of HBp17/FGFBP-1 and inhibited the proliferation of squamous cell carcinoma (SCC) cells *in vitro* and *in vivo* through NF-κb inhibition. To explore the possibility of microRNA (miRNA) control of HBp17/FGFBP-1, we analyzed exosomal miRNAs from medium conditioned by A431 cells treated with ED-71. Microarray analysis revealed that 12 exosomal miRNAs were upregulated in ED-71-treated A431 cells. Among them, miR-6887-5p was identified to

have a predicted mRNA target matching the 3' un-translated region (3'-UTR) of HBp17/FGFBP-1. The 3'-UTR of HBp17/FGFBP-1 was confirmed to be a direct target of miR-6887-5p in SCC/OSCC cells, as assessed with a luciferase reporter assay. Functional assessment revealed that overexpression of miR-6887-5p in SCC/OSCC cells inhibited cell proliferation and colony formation *in vitro,* and inhibited tumor growth *in vivo* compared with control. In conclusion, our present study supports a novel anti-cancer mechanism involving the regulation of HBp17/FGFBP-1 function by exosomal miR-6887-5p in SCC/OSCC cells, which has potential utility as a miRNA-based cancer therapy.

INTRODUCTION

Heparin-binding protein 17 (HBp17) was first purified in 1991 from the conditioned medium of A431 cells (Wu et al. 1991), and later renamed fibroblast growth factor binding protein 1 (FGFBP-1) (Liu et al. 2002). This 17 kDa molecule was isolated based on its ability to reversibly bind to and act as a chaperone for fibroblast growth factor (FGF) -1 and -2 (Wu et al. 1991). This novel binding protein has since been shown to reversibly bind other members of the FGF family (Beer et al. 2005).

HBp17/FGFBP-1 mobilizes both FGF-1 and -2 in the extracellular matrix such that they can bind to their cognate FGF receptors. Interaction of FGF-1 or -2 with their receptors elicits a signaling cascade that promotes the survival of cancer cells through growth stimulation and angiogenesis (Wu et al. 1991). As HBp17/FGFBP-1 is highly expressed in a number of cancers, HBp17/FGFBP-1 has been proposed as a diagnostic marker for early cancer detection (Begum et al. 2007; Okamoto et al. 1996). The overexpression of FGF-2 in oral squamous cell carcinoma (OSCC) cells promotes cell growth and angiogenesis through autocrine and paracrine mechanisms, respectively (Myoken et al. 1994a, b).

1α,25 dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$; $1,25D_3$) is a multifunctional hormone that exerts its actions through vitamin D receptor. Vitamin D and its analogs have also been long recognized as compounds with anti-cancer potential (Giovannucci et al. 2006; Holick 2011). In a number of clinical studies, the risk of oral cancer, esophageal cancer, pancreatic cancer and leukemia decreased in association with increased $1,25D_3$ levels (Giovannucci et al. 2006; Holick 2011). In addition to being well-known as an anti-inflammatory agent, $1,25D_3$ has also been reported to downregulate the levels of NF-κB protein in human lymphocytes (Gilmore 1999). Additionally, Janjetovic *et al.* (Janjetovic et al. 2010) reported on the inhibition of

keratinocyte growth through suppression of the NF-κB signaling pathway. Similarly, we reported the efficacy of $1,25D_3$ in suppressing the expression of HBp17/FGFBP-1 in OSCC cell lines via the NF-κB pathway (Rosli et al. 2013). The downregulation of HBp17/FGFBP-1 results in a reduction of FGF-2 released into the conditioned medium (Rosli et al. 2014).

Eldecalcitol (1α,25-dihydroxy-2β-[3-hydroxypropyloxy] vitamin D3) (ED-71) is a $1,25D_3$ analogue that is used as an orally administered treatment for osteoporosis (Sanford and McCormack 2011). Like its parental compound calcitriol ($1,25D_3$), ED-71 has been reported to have chemo-preventive activity against tumors, as well as an ability to induce differentiation of tumor cells (Hatakeyama et al. 2010). The presence of a hydroxypropoxy group gives ED-71 a higher affinity towards Vitamin D Binding Protein, thus bestowing it with a longer half-life in serum in comparison to $1,25D_3$ (Nishii and Okano 2001). The longer half-life of ED-71 could also stem from a greater resistance towards the 24-hydroxylase, an enzyme encoded by Cyp24A1 that hydroxylates vitamin D3. We characterized ED-71 resistance to 24-hydroxylase by demonstrating that ED-71 antitumor activity was not affected by a large induction of Cyp24A1 *in vitro* and *in vivo* (Shintani et al. 2016). In addition, we demonstrated that

ED-71 exhibited anti-tumor activity for squamous cell carcinoma (SCC) both *in vivo* and *in vitro* by downregulating expression of HBp17/FGFBP-1 (Shintani et al. 2017).

Exosomes are 30-100 nm diameter nano- and micro-sized lipid-bilayered membrane vesicles that encapsulate cytosolic proteins, mRNAs and microRNAs (miRNAs) derived from producing cells (Colombo et al. 2014; Melo et al. 2015; Tkach and Thery 2016; Trams et al. 1981). Recent studies have shown that exosomes act as messengers in intracellular communication networks through protein and RNA transfer between cells, utilizing both paracrine and autocrine mechanisms (Kramer-Albers and Hill 2016; Tkach and Thery 2016). Sporn has designated growth factors, protein factors, hormones, lipids, exosomes and miRNAs, as a regulatory chemical messengers (RCMs) (Sporn 2006).

Exosomes are thought to be potentially useful as therapeutics as well as cancer biomarkers (Pucci et al. 2016; Andaloussi et al. 2013). miRNAs are a new class of endogenous, small (19~22 nt) non-coding single-stranded RNA molecules that post-transcriptionally regulate the expression of protein-coding genes by recognizing specific mRNAs with complementary sequences (Bartel 2004; He and Hannon 2004). miRNAs match imperfectly with the 3' untranslated regions (UTR) of mRNA to inhibit translation and/or promote degradation (Ambros 2004; Lee et al. 1993) (Liu et al.

2019) , and are associated with many types of cancers as either oncogenes or tumor suppressor genes (Esquela-Kerscher and Slack 2006; Zhang et al. 2007)(Kia et al. 2018) . For instance, it has been shown that miR-655 and miR-1294 inhibit OSCC cell growth by regulating the PTEN/AKT pathway and c-myc pathways, respectively (Wang et al. 2018; Wang et al. 2018).

In the present study, miRNA microarray analysis of exosomal miRNAs revealed 12 upregulated and 1 downregulated miRNAs in ED-71-treated-A431 cells. The 3'-UTR of HBp17/FGFBP-1 mRNA was verified as a direct target gene of miR-6887-5p using a luciferase assay. We then investigated the potential role of miR-6887-5p in SCC/OSCC cells by studying its effect on the expression of HBp17/FGFBP-1 and on tumorigenicity of SCC/OSCC cells.

MATERIALS AND METHODS

Cell culture

The cell lines used in this study were the epidermoid carcinoma cell line A431 (RRID:CVCL_0037) (Fabricant et al. 1977), and OSCC cell line (Ca9-22

(RRID:CVCL_1102) (Kimura 1978)) those were purchased from the Cell Bank, RIKEN BioResource Center (Tsukuba, Japan). Other OSCC cell lines (HO-1-N-1 (RRID:CVCL_1284) (Miyauchi et al. 1988) and KO (Michimukai et al. 2001)) were established in our lab from a patient with oral cancer. All cell lines were cultured in serum-free medium as described previously (Okamoto et al. 1996; Sato et al. 1987). Cells were grown and sub-cultured in 60 mm culture dishes (BD Falcon, San Jose, CA) in DF6F medium. DF6F medium was composed of a 1:1 mixture (by volume) of DMEM and Ham F-12 medium (DF) supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), 2-aminoethanol (10 μM), sodium selenite (10 nM), 2-mercaptoethanol (10 μM), and oleic acid conjugated with fatty acid-free bovine serum albumin (9.4 μg/ml). All chemicals were from Sigma-Aldrich (St. Louis, MO). All reagents used for cell culture were free of Mycoplasma and viral pathogens.

Cells were cultured at 37°C in a humidified 95% air/5% CO_2 atmosphere in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA) until they were 60-70% confluent. Subsequently, 0.4 nM ED-71 (kindly provided from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) or 99.5% ethanol (EtOH) (Sigma-Aldrich) as control was added and the cells were further cultured for 48 hours.

All human cell lines have been authenticated using short tandem repeat (STR) profiling (BEX CO., LTD. Tokyo, Japan) within the last three months. The STR profiles of A431, HO-1-N-1, and Ca9-22 matched with the publicly available reference profiles. Although the KO cell line hasn't yet deposited to the cell bank, the STR profile of the KO cell line matched with the STR profile of cryopreserved primary KO cells in our laboratory (see Table S1).

Preparation of SCC/OSCC-derived exosomes from conditioned medium

SCC/OSCC cells were used for the preparation of exosomes. Cells were cultured in 10 mL of DF6F medium in 100 mm dishes (BD Falcon). DF6F medium was changed to the nutrient DF medium when the cells grown at 80% confluency. Then the cells were further cultured for 24 hours and the conditioned medium (CM) was collected. The CM was centrifuged at 10,000 x *g* for 30 min at 4ºC to remove cells and debris, and concentrated 50 times using an Amicon Ultra-15 Ultracel-100K (Merck KGaA, Darmstadt, Germany). Exosomes were then purified using an immunoaffinity method based on the interaction with the phosphatidylserine on the exosome surface using the MagCapture™ Exosome Isolation Kit PS (Wako Pure Chemical Industries, Ltd., Osaka,

Japan), according to manufacturer's instructions. We observed exosomes with Transmission Electron Microscopy (Hanaichi Ultra Structure Research Institute. Aichi, Japan).

SDS PAGE and Western Blotting

Cells were cultured at 2.5 x 10^4 cells/well in 6-well plates (BD Falcon) in DF6F medium for 48 hours and then transfected with miRNA or miR-negative control (NC).

The cells were collected at the designated times by scrapping the cells from the surface of the plates in the presence of RIPA buffer (10 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.4 [Nacalai Tesque Inc., Kyoto, Japan]). The cell suspension was then sonicated on ice for 30 seconds (Taitec Ultra S Homogenizer VP-5S, Taitec, Tokyo, Japan). Protein content was quantified with the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific).

Purified exosomes (0.2 μg) or cell extract (5 μg) were lysed with 5 x SDS sample buffer (625 mM Tris-HCl (pH 6.8), 10 mM EDTA, 15% glycerol, 0.1% BPB (bromophenol blue)) and electrophoresed under non-reducing conditions on 15% or 11% polyacrylamide gels.

The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) using a semi-dry blotting system (Bio-Rad Laboratories) (90 mA/gel). After incubating with anti-CD9 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome c (Becton Dickinson and company, Franklin Lakes, NJ), biotinylated anti-HBp17/FGFBP-1 (Sigma-Aldrich), anti-FGF-2 (Santa Cruz Biotechnology) or anti-β-actin antibody (Sigma-Aldrich), the membrane was incubated with peroxidase-labeled secondary antibody (Cell Signaling Technology, Danvers, Massachusetts, USA). Bound antibody was detected with the Clarity Western ECL Substrate (Bio-Rad Laboratories), and chemiluminescence was captured using a ChemiDoc XRS Imaging System (Bio-Rad Laboratories).

miRNA microarray analysis of A431-derived exosomes

We examined the effect of ED-71 on the miRNA profile of A431-derived exosomes. Total RNA was extracted from purified exosomes using miRNeasy Serum/Plasma Kit (QIAGEN) according to manufacturer's instructions. Samples were labeled using a miRCURYHy3/Hy5 Power Labeling Kit and were hybridized to a human miRNA Oligo chip (Toray Industries, Tokyo, Japan). The chips were scanned

with a 3D-Gene Scanner 3000 (Toray Industries). The results were analyzed using 3D-Gene extraction software (Toray Industries). Differences in miRNA expression between ED-71-treated and control cells were assessed using GeneSpring GX (Agilent Technologies, Santa Clara, CA). Quantile normalization was performed on the raw data that were above the background level.

Prediction of miR-6887-5p target gene and dual-luciferase reporter assay

For the dual-luciferase assay, the 3'-UTR of HBp17/FGFBP-1 containing the predicted miR-6887-5p seed-matching sites and corresponding mutant sites were amplified by PCR using a human cDNA template. The 3'-UTR target sites of HBp17/FGFBP-1 and a mutant variant were synthesized and cloned in the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, Madison, WI). The new vectors were designated as an HBp17/FGFBP-1 3'-UTR WT/Mut.

The oligonucleotide sequences of human miR-6887-5p mimics (5'-UGGGGGGACAGAUGGAGAGGACA-3') or miR-451a mimics (5'-AAACCGUUACCAUUACUGAGUU-3') were synthesized. A scrambled oligomer sequence (5'-UUCUCCGAACGUUCACGUTT-3') was used as miR-NC.

Luciferase activity was assessed in 4 groups for each cell line evaluated (A431, HO-1-N-1, KO and Ca9-22 cells): ① Wild type (WT) + miR- NC mimics, ② WT + miR-6887-5p mimics, ③ Mutant (Mut) + miR-NC mimics, and ④ Mut + miR-6887-5p mimics. Cells were seeded in 48-well plates (Becton Dickinson and company) and co-transfected 12 hours later with WT or Mut 3'-UTR of HBp17/FGFBP-1 reporter plasmids (0.4 μg), *mir*Vana miR-6887-5p/miR-451a mimics (10 pmol) (Life Technologies, Waltham, MA) or *mir*Vana miRNA mimics, as the miR-NC (10 pmol) (Life Technologies) using Lipofectamine 3000 (Life Technologies). Forty-eight hours after transfection, the cells were harvested, and firefly and renilla luciferase activities were analyzed with the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions.

Transfection of miRNA mimics

Transfections of miR-6887-5p/miR-451a mimics or miR-NC mimics were performed with GenomONE-Si (Ishihara Techno Corporation, Osaka, Japan) for A431 and with siPORT NeoFX Transfection Agent (Thermo Fisher Scientific) for HO-1-N-1, KO and Ca9-22, according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR (qRT-PCR) for miR-6887-5p and miR-451a

Total RNA was isolated from the cells with TRIzol reagent (Thermo Fisher Scientific). Synthesis of complementary DNA (cDNA) and real-time polymerase chain reaction (RT-PCR) for miR-6887-5p and miR-451a were performed using a Universal cDNA synthesis kit 2 (QIAGEN) according to the manufacturer's protocol. Real-time PCR was performed using ExiLENT SYBR Green master mix (QIAGEN) and miRCURY LNA^{TM} Universal RT microRNA PCR Primer set (QIAGEN). UniSp6, RNA Spike-in template (QIAGEN) was used as the internal control. The expression of each gene was quantified by measuring cycle threshold (Ct) values and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6 snRNA.

RNA extraction and qRT-PCR for HBp17/FGFBP-1 and FGF-2 mRNA

Total cellular RNA was isolated as described above. Reverse transcription (RT) for HBp17/FGFBP-1 and FGF-2 mRNA was performed using the Super Script

first-strand synthesis system (Life Technologies). qRT-PCR analyses for HBp17/FGFBP-1 and FGF-2 were performed using the Stratagene Mx3000P^{TM} system (Agilent Technologies) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. The sequences of primers and TaqMan^{TM} fluorogenic probes were designed according to the ProbeFinder software of Roche Universal Probe Library system (Roche Applied Science, Nurley, NJ). The primers used and their sequences are as follows: HBp17/FGFBP-1: (NM_005130) 5'-CGTGTGCTCAGAACAAGGTG-3', 5'-GAGCAGGGTGAGGCTACAGA-3' #46 fluorescent probe (Roche Applied Science); FGF-2: (NM_002006.4) 5'-TTCTTCCTGCGCATCCAC-3', 5'-TGCTTGAAGTTGTAGCTTGATGT-3', #7 fluorescent probe (Roche Applied Science); GAPDH (glyceraldehyde 3-phosphate dehydrogenase): (NM_020529) 5'-GCTCTCTGCTCCTCCTGTTC-3', 5'- ACGACCAAATCCGTTGACTC-3', #60 fluorescent probe (Roche Applied Science).

Cell growth and colony formation assays

We examined the effect of miR-6887-5p and miR-451a on the growth of the cells in serum-free culture. Briefly, A431, HO-1-N-1, KO and Ca9-22 cells transfected

with miRNA mimics or miR-NC mimics were cultured at a density of 5×10^3 cell/well in 24-well plates (BD Falcon). Cells were counted with a Coulter Counter (Coulter Electronics Inc. Hialeah, FL) daily for 6 days.

For the colony formation assay, tumor cells transfected with miR-6887-5p mimics or miR-NC mimics were transplanted in 6-well plates (BD Falcon) at 200 cells per well. The cells were cultured for 14 days and culture medium was replaced every 5 days. Colonies were counted only if they contained more than 50 cells. Colony formation was visualized with Giemsa staining solution (Wako Pure Chemical Corporation).

Animal experiments

The study used 4-week-old male athymic Balb/c nude mice (Charles River Japan, Tokyo, Japan) for A431 or 4-week-old male athymic Balb/c SCID mice (Charles River Japan) for Ca9-22. The animals were maintained under specific pathogen-free (SPF) conditions. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Hiroshima University (Permission #A18-14). Experiments were conducted after the animals were allowed to acclimated to

their surroundings for one week. A431 cells (1 x 10^6) or Ca9-22 (5 x 10^6) cells transfected with miR-6887-5p mimics or miR-NC were re-suspended in 0.2 mL of DF nutrient medium prior to being injected subcutaneously in the dorsal surface of the mouse. Tumor volume [1/2 x (the major axis) x (the minor axis)^2] was measured twice a week. Mice bearing A431 and Ca9-22 cells were sacrificed 39 and 60 days after injection, respectively. The tumor tissues removed from the mice were placed in 10% PBS-buffered formalin (Sigma), embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and rapidly frozen in liquid nitrogen for subsequent immunohistochemical analysis.

Immunohistochemistry

Paraffin-embedded tissues were cut at 4-6 μm thickness and immuno-stained to evaluate the expression of Ki-67. The sections were deparaffinized in xylene, dehydrated with alcohol and rehydrated in PBS. To detect CD31/PECAM-1 (rat monoclonal anti-mouse CD31/PECAM-11:100; Becton Dickinson and Company) expression, OCT compound-embedded frozen sections (10 μm thick) were fixed in cold acetone for 10 minutes. Endogenous peroxidase was blocked with 3% hydrogen

peroxide in methanol. The sections were exposed to protein block (5% normal horse serum (Thermo Fisher Scientific) and 2% normal goat serum (Thermo Fisher Scientific)) and incubated overnight at 4°C with mouse monoclonal anti-Ki-67 (1:100; DAKO A/S, Copenhagen, Denmark). The sections were then exposed to peroxidase-conjugated secondary antibody for 1 hour, and positive staining was detected with 3,3-diaminobenzidine (DAKO). Slides were counterstained with hematoxylin (Sigma) and mounted in Mount-Quick (Fisher Scientific, Houston, TX).

Statistical analysis

Statistical analysis was performed using Bell Curve for Excel (Social Survey Research Information Co., Ltd. Tokyo, Japan). All data were presented as the mean ± SD (standard deviation) of at least three independent experiments. A student's *t*-test was used to compare the difference between two groups. χ^2 test was used for tumor incidence. The differences were considered significant with $p < 0.05$.

Data availability

The data will be made available upon reasonable request.

RESULTS

Evaluation of exosomes purified from A431-CM

We evaluated the quality of A431-derived exosomes through morphological observation under the scanning electron microscope (SEM), and by western blotting analysis of CD9 as an exosome biomarker and cytochrome c as the NC for exosomes (Lobb et al. 2015; Yoshioka et al. 2013). SEM imaging indicated that exosomes appeared as flattened spheres with diameters ranging between 30 to 150 nm. In addition, the expression of CD9 and the absence of cytochrome c were confirmed by western blotting analysis (Fig. 1A).

Identification of the A431-exosomal miRNA by microarray analysis

Exosomal miRNA profiles were obtained by microarray analysis (Toray Industries). Statistical analysis comparing miRNA profiles of ED-71-treated and control cells revealed 13 differentially expressed miRNA, of which 12 of were upregulated (Fig. 1B). The top three highly expressed miRs were miR-451a,

miR-3162-5p and miR-6887-5p. miR-452 was the only miRNA downregulated in ED-71-treated A431 cells (Fig. 1B).

MiR-6887-5p targets HBp17/FGFBP-1

HBp17/FGFBP-1 was selected as a putative miR-6887-5p target, based on a sequence of eight nucleotides (GUCCCCCC), by the bioinformatic database TargetScan (Fig. 2A) (Kehl et al. 2017). To explore whether miR-6887-5p targets the 3'-UTR region of HBp17/FGFBP-1 mRNA, SCC/OSCC cells were co-transfected with WT or Mut HBp17/FGFBP-1 luciferase reporter plasmid and miR-6887-5p or miR-NC mimics. The wild-type 3'-UTR reporter of HBp17/FGFBP-1 exhibited a significant reduction in luciferase activity in miR-6887-5p-transfected cells as compared to the miR-NC-transfected cells ($p < 0.05$) (Fig. 2B). Similar observations were made in all cell lines used in the experiment (A431, HO-1-N-1, KO and Ca9-22). The same procedure was repeated for miR-451a-transfected cells. The results indicated that there was no interaction between the 3'-UTR of HBp17/FGFBP-1 and miR-451a (Fig. S1). Taken together, these results strongly support the conclusion that miR-6887-5p directly interacts with HBp17/FGFBP-1 mRNA.

ED-71 upregulates miR-6887-5p expression in exosomes derived from SCC/OSCC

The effect of ED-71 on the induction of miR-6887-5p in exosomes derived from SCC/OSCC cell lines was examined. Fig. 3A shows that treatment of SCC/OSCC cells with 0.4 nM ED-71 for 48 hours induced miR-6887-5p expression in exosomes. Conversely, there was no difference in miR-6887-5p expression in the cell lysate (Fig. 3B).

MiR-6887-5p regulates HBp17/FGFBP-1 expression in SCC/OSCC cells

We investigated the role of miR-6887-5p in regulating the expression of HBp17/FGFBP-1 in various SCC/OSCC cell lines. For this study, four cell lines over-expressing miR-6887-5p were produced by transient transfection with synthetic miR-6887-5p mimics. The overexpression of miR-6887-5p in A431, HO-1-N-1, KO and Ca9-22 cells transfected with miR-6887-5p mimics was verified (Fig. 4A). Subsequently, HBp17/FGFBP-1 expression in these cells was determined. We found that HBp17/FGFBP-1 mRNA expression was suppressed in all miR-6887-5p

mimic-transfected SCC/OSCC cells (Fig. 4B). Furthermore, the HBp17/FGFBP-1 protein expression was also suppressed in miR-6887-5p-transfected SCC/OSCC cells, suggesting that miR-6887-5p mimics introduced into the cells had inhibited HBp17/FGFBP-1 mRNA levels, thus disrupting the synthesis of HBp17/FGFBP-1 protein (Fig. 4C). On the other hand, transfection of A431 or HO-1-N-1 cells with miR-451a did not affect HBp17/FGFBP-1 mRNA expression (Fig. S2). In addition, there was no difference in FGF-2 mRNA and protein expression between miR-6887-5p and NC mimic-transfected cells (Fig. S3).

Overexpression of miR-6887-5p inhibits proliferation of SCC/OSCC cells *in vitro*

We examined the biological role of miR-6887-5p on the proliferation of SCC/OSCC cells. The proliferation rate of miR-6887-5p-overexpressing SCC/OSCC cells was lower than that of miR-NC-expressing cells (Fig. 5A). Furthermore, overexpression of miR-451a in SCC/OSCC cells did not alter cell proliferation (Fig. S4).

The effect of miR-6887-5p on cell proliferation was also evaluated using a colony formation assay. miR-6887-5p-transfected cells formed fewer colonies than the miR-NC transfectants (Fig. 5B).

MiR-6887-5p inhibits tumor growth in athymic nude mice

To examine the effect of miR-6887-5p on SCC/OSCC tumor growth *in vivo*, A431 or Ca9-22 cells transfected with miR-6887-5p mimics or miR-NC mimics were transplanted into athymic nude mice. A431 and Ca9-22 tumor sizes were measured twice a week, and the animals were sacrificed on days 39 and 60, respectively. The tumor growth of miR-6887-5p-transfected A431 cells was reduced than that of miR-NC-transfected A431 cells (Fig. 6A). The tumor volumes of miR-6887-5p-transfected-A431 tumors were less than those of miR-NC-transfected tumors (Fig. 6B). The reduced tumor weight of the miR-6887-5p group was clearly apparent (Fig. 6C). Moreover, a remarkable effect of miR-6887-5p on tumor growth was observed in the Ca9-22 tumors. Tumor formation was not observed in the mice transplanted with miR-6887-5p-transfected Ca9-22 cells, whereas tumor incidence of miR-NC group was 100% ($p < 0.05$) (Table 1).

These results indicate that miR-6887-5p inhibits tumor growth *in vivo*. In addition, the tumors derived from miR-6887-5p-transfected A431 cells were less positive for Ki-67 and exhibited lower microvessel density compare to the control tumors (Fig. 6D and 6E).

DISCUSSION

The expression of FGF-1 and -2 was reported to be increased during the malignant transformation of epithelial cells (Myoken et al. 1994). Furthermore, it was reported that the expression of HBp17/FGFBP-1 and FGF-2 was proportional to the severity of epithelial dysplasia (Begum et al. 2007; Okamoto et al. 1996). Together, these observations suggested that HBp17/FGFBP-1 plays an important role in the stabilization of FGFs as well as in promoting the release of FGFs lacking a signal sequence (Wu et al. 1991).

The anti-cancer effect of 1,25-D_3 has been established since the 1980s. Unfortunately, its translational application was hindered by several side effects resulting from the high doses required for cancer treatment. In cancer studies, 1,25-D_3 has provided us with considerable understanding of cancer progression and suggested

possible means to combat it with molecules that mediate 1,25-D_3 anti-cancer action (Maleklou et al. 2016). One such molecule that is currently receiving considerable attention is miRNA. A number of studies have examined the effect of 1,25-D_3 on miRNA profiles in several types of cancer, such as colon cancer (Alvarez-Diaz et al. 2012), human myeloid leukemia (Gocek et al. 2011), prostate cancer (Ting et al. 2013), colorectal cancer (Padi et al. 2013) and others. In the present study, we report on the effect of ED-71, an analog of 1,25-D_3, on miRNA profiles in SCC cells. To date, there have been no such reports examining the effects of ED-71 on miRNA profiles in cancer cells.

Hatakeyama and colleagues first reported the chemopreventive activity of ED-71 against tumors, with reduced hypercalcemic effect (Hatakeyama et al. 2010). Thereafter, we reported the anti-cancer capacity of ED-71 toward SCC/OSCC cells *in vitro* as well as *in vivo* (Shintani et al. 2016). Our previous animal study showed that ED-71 inhibited microvessel formation and cell proliferation in A431-derived tumors (Shintani et al. 2017). We demonstrated the chemopreventive efficacy of ED-71 at an exceptionally low concentration (0.4 nM) in comparison with 1,25-D_3, which required 40 nM to produce similar effects (Shintani et al. 2016). However, it is impossible to completely eliminate the risk of developing hypercalcemia. We are proposing a cancer

treatment based on miRNA instead of ED-71 or vitamin D to circumvent their attendant hypercalcemic side effects.

In this study we have used miRNA profiling to identify a potential therapeutic miRNA for SCC/OSCC that acts by inhibiting the expression of HBp17/FGFBP-1. Exosomal miRNA expression in response to ED-71 in A431 cells was screened. Microarray data revealed 13 differentially regulated exosomal miRNA in ED-71-treated A431 cells. We focused on miR-6887-5p because of its predicted target mRNAs, which include HBp17/FGFBP-1 among three-thousand others. Using a luciferase reporter assay, we confirmed that miR-6887-5p targets HBp17/FGFBP-1 expression via the 3'-UTR of HBp17/FGFBP-1. This experiment resulted in the downregulation of HBp17/FGFBP-1 mRNA expression and protein levels in SCC/OSCC cell lines. These results confirmed the binding of miR-6887-5p with a putative target mRNA encoding HBp17/FGFBP-1. Interestingly, miR-6887-5p expression was observed to be upregulated in exosomes from ED-71 treated-SCC/OSCC cells, but not in cells. Previous reports showed that miRNAs are released selectively and don't correlate with the quantity of cytosolic miRNAs (Huang et al. 2018; Pigati et al. 2010). Additionally, miRNA profiles differed depending on the type of tumor or malignancy. It has been suggested that certain miRNAs might be selectively incorporated in exosomes (Li et al.

2018). However, a detailed mechanism underlying this process has not been described to date. Based on our results, we surmise that miR-6887-5p is selectively packaged into exosomes following treatment with ED-71.

Cells over-expressing miR-6887-5p exhibited lower growth rates and a lower capacity for colony formation. In an *in vivo* assay using immunodeficient mice, we further demonstrated that two different cell lines (A431 and Ca9-22) over-expressing miR-6887-5p produced much smaller tumors with reduced expression of Ki-67 and CD-31. This is consistent with the hypothesis that miR-6887-5p suppresses tumor growth by inhibiting angiogenesis in A431-derived tumors. Interestingly, no tumors formed in mice implanted with miR-6887-5p-transfected Ca9-22 cells. Among the SCC/OSCC cells tested, the greatest degree of suppression of HBp17/FGFBP-1 mRNA and protein expression was observed in miR-6887-5p transfected Ca9-22 cells. Czubayko *et al.* reported that HBp17/FGFBP-1 was an angiogenic switch molecule in human cancer (Czubayko et al. 1997). Our results indicate that angiogenesis was potently inhibited by suppressing HBp17/FGFBP-1 in miR-6887-5p-transfected Ca9-22-derived tumors.

Notably, among the 13 miRNAs that were induced by ED-71 (0.4 nM), miR-451a was induced to the highest degree. We ruled out a role of miR-451a in

regulating HBp17/FGFBP-1 by showing that there was no binding between miR-451a mimics and the HBp17/FGFBP-1 3'UTR. In addition, miR-451a did not inhibit SCC/OSCC proliferation, thus eliminating its potential for anti-cancer activity in head and neck cancers.

CONCLUSION

We report that ED-71 stimulates exosomal miR-6887-5p in SCC/OSCC cells, and that miR-6887-5p suppresses tumor growth *in vitro* and *in vivo* as well as colony formation of SCC/OSCC cells via direct targeting of HBp17/FGFBP-1. Our findings suggest that exosomal miR-6887-5p as a therapeutic agent for SCC tumors that would target HBp17/FGFBP-1 while avoiding the hypercalcemic effects of $1\alpha,25(OH)_2D_3$ and its analogs.

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Table 1. MiR-6887-5p inhibits tumorigenesis of SCC/OSCC cell lines in immunodeficient mice.

Figure 1. ED-71 induces miR-6887-5p expression in the conditioned media (CM) of A431 cells.

Exosomes were extracted from A431-CM using the phosphatidylsine (PS) affinity method. (A) Western blotting verified the expression of CD9 and the absence of cytochrome c in exosomes (i). SEM analysis indicated that the size of vesicles ranged from 30-150 nm, further confirming the presence of exosomes in the eluent (ii). (B) The purified exosomes were subjected to miRNA microarray profiling analysis. Statistical analysis comparing miRNA profiles of ED-71-treated (0.4 nM) and control cells revealed 13 differentially expressed miRNA, 12 of which were upregulated. The top three highly expressed miRs were miR-451a, miR-3162-5p, and miR-6887-5p. MiR-452 was the only miRNA downregulated in ED-71-treated A431 cells.

Figure 2. HBp17/FGFBP-1 is directly suppressed by miR-6887-5p in SCC/OSCC cells.

(A) The complementary pairings of miR-6887-5p with HBp17/FGFBP-1 wild-type (WT) and mutant (Mut) 3'-UTR reporter constructs. (B) Relative luciferase activity in SCC/OSCC cells co-transfected with WT or Mut 3'-UTR HBp17/FGFBP-1 reporter plasmids and miR-6887-5p or miR-NC. Assays were performed in triplicate with the data presented as means $\pm$ SD, $*p < 0.05$.

Figure 3. **ED-71 upregulates the expression of miR-6887-5p in exosomes purified from SCC/OSCC cells**.

SCC/OSCC cells were cultured for 48 h after ED-71 (0.4 nM) or EtOH was added. Exosomes were purified from CM of each cell line. Exosomal or cytosolic RNA was extracted. Relative miR-6887-5p expressions were upregulated in exosomes purified from ED-71 treated-SCC/OSCC cells compared with control (A), but not in cell extracts (B). Experiments were performed in triplicate with the data presented as means $\pm$ SD, $*p < 0.05$.

Figure 4. **Overexpression of miR-6887-5p inhibits HBp17/FGFBP-1 expression in SCC/OSCC cell lines.**

(A) Relative miR-6887-5p levels were measured using qRT-PCR after transient transfection of miR-6887-5p mimics or miR-NC mimics in SCC/OSCC cells. U6 RNA levels were used as an internal control. (B) Downregulation of HBp17/FGFBP-1 mRNA in the miR-6887-5p-overexpressing cells was evaluated by qRT-PCR. GAPDH levels were used as an internal control. (C) Reduced expression of HBp17/FGFBP-1 protein in miR-6887-5p overexpressing cells was evaluated by western blotting. Band intensities were quantified by Image Master software and presented as % of adjusted volume relative to β-actin (HBp17/FGFBP-1/ β-actin). Experiments were performed in triplicate with the data presented as means $\pm$ SD, $*p < 0.05$.

Figure 5. **MiR-6887-5p inhibits cell proliferation and colony formation of SCC/OSCC cells.**

(A) Cell proliferation and (B) colony formation were determined in SCC/OSCC cells transfected with miR-6887-5p mimics or miR-NC mimics. Experiments were performed in triplicate with the data presented as means $\pm$ SD, $*p < 0.05$.

Figure 6. **MiR-6887-5p suppresses SCC/OSCC tumor growth in immunodeficient mice.**

(A) Growth curves (tumor volumes) of xenografts expressing either miR-6887-5p of miR-NC in immunodeficient mice. (B) Weights of tumors from the A431/miR-6887-5p and A431/miR-NC groups. (C) Representative images of tumors from the A431/miR-6887-5p and A431/miR-NC groups. (D, E) Immunohistochemical analysis was used to detect Ki-67 and CD-31/PECAM-1 expression in tumor tissues excised from the A431/miR-6887-5p and A431/miR-NC groups. Experiments were performed in 5 mice with the data presented as means $\pm$ SD, $*p < 0.05$.

Supplementary Figure 1. HBp17/FGFBP-1 is not a target of miR-451a in SCC/OSCC cell lines.

There is no change in luciferase activity in any of the cells generated, which confirmed that miR-451a mimics do not bind to the putative 3'-UTR of HBp17/FGFBP-1. Experiments were performed in triplicate with the data presented as means $\pm$ SD.

Supplementary Figure 2. Overexpression of miR-451a did not modulate HBp17/FGFBP-1 expression in SCC/OSCC cells.

Relative HBp17/FGFBP-1 mRNA levels were measured in A431 and NA cells transfected miR-451a mimics and miR-NC mimics. Experiments were performed in triplicate with the data presented as means ± SD.

Supplementary Figure 3. FGF-2 is not suppressed by miR-6887-5p in SCC/OSCC cells.

Relative FGF-2 mRNA (A) and protein expression (B) levels in SCC/OSCC cells transfected miR-6887-5p mimics or miR-NC mimics were not changed. Experiments were performed in triplicate with the data presented as means ± SD.

Supplementary Figure 4. MiR-451a exhibits no effect on cell proliferation of SCC/OSCC cell lines.

Cell proliferation was determined in A431 and NA cells transfected with miR-451a mimics or miR-NC mimics. Experiments were performed in triplicate with the data presented as means ± SD.

Figure 1 Higaki M. et al.

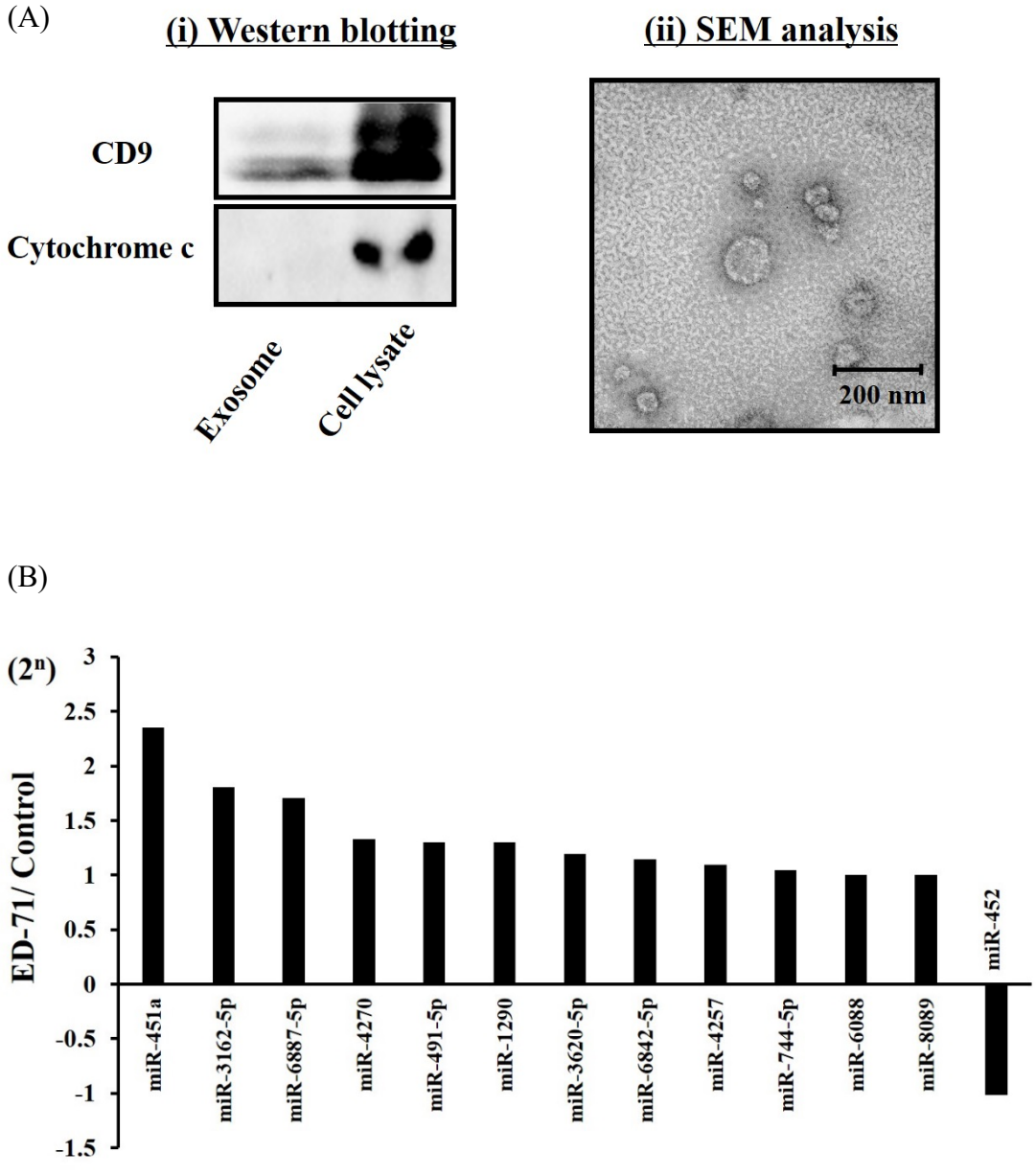

Figure 2. Higaki M. et al.

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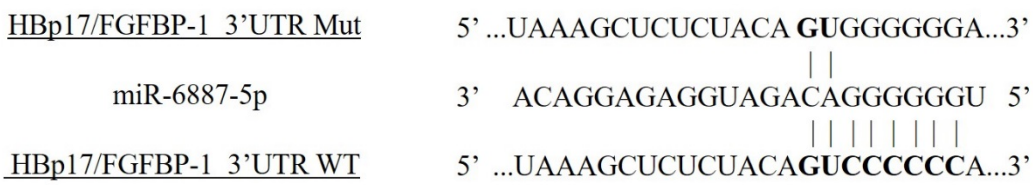

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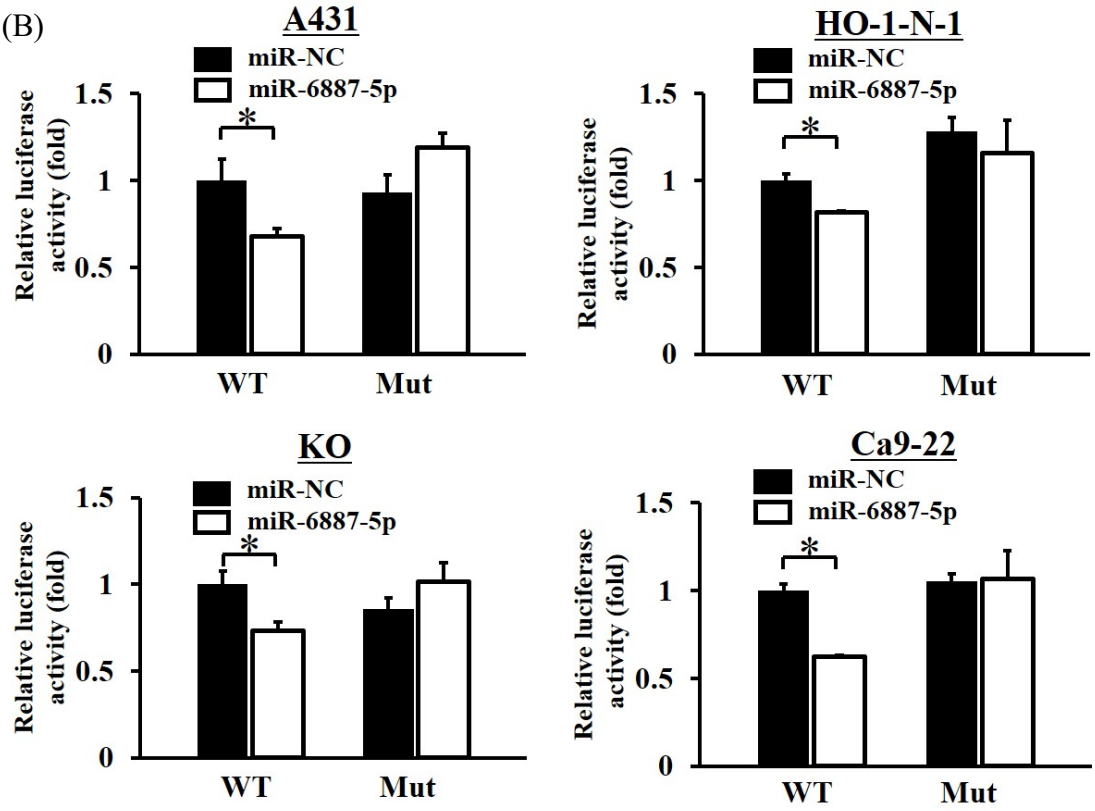
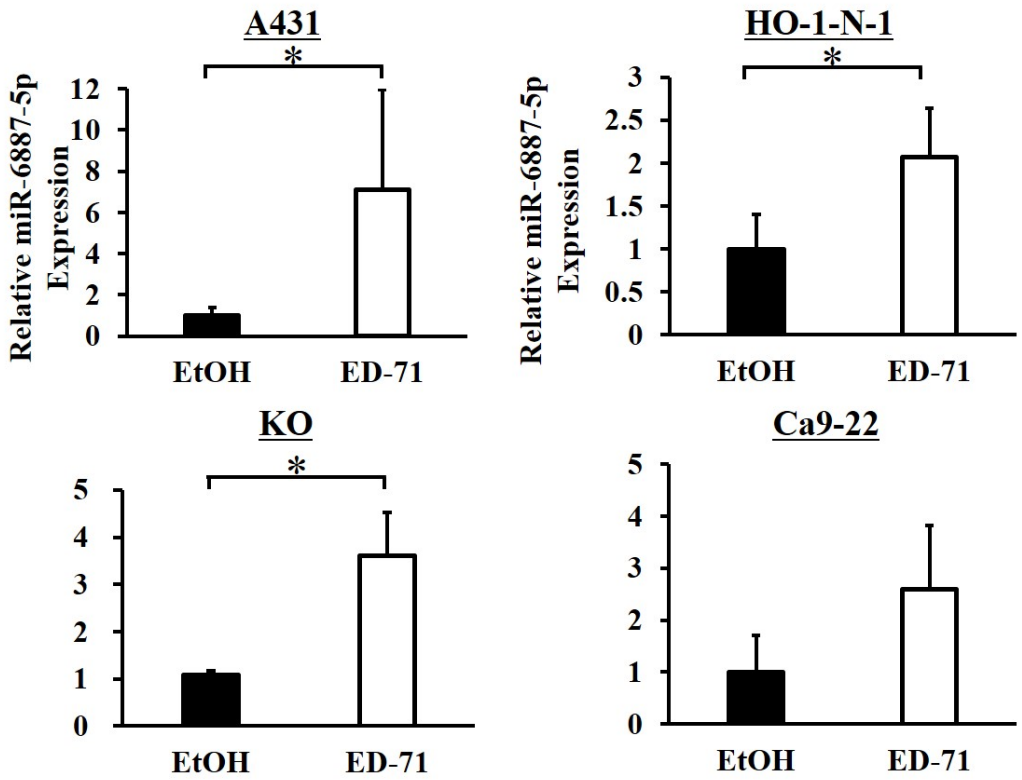


Figure 3. Higaki M. et al.

(A)



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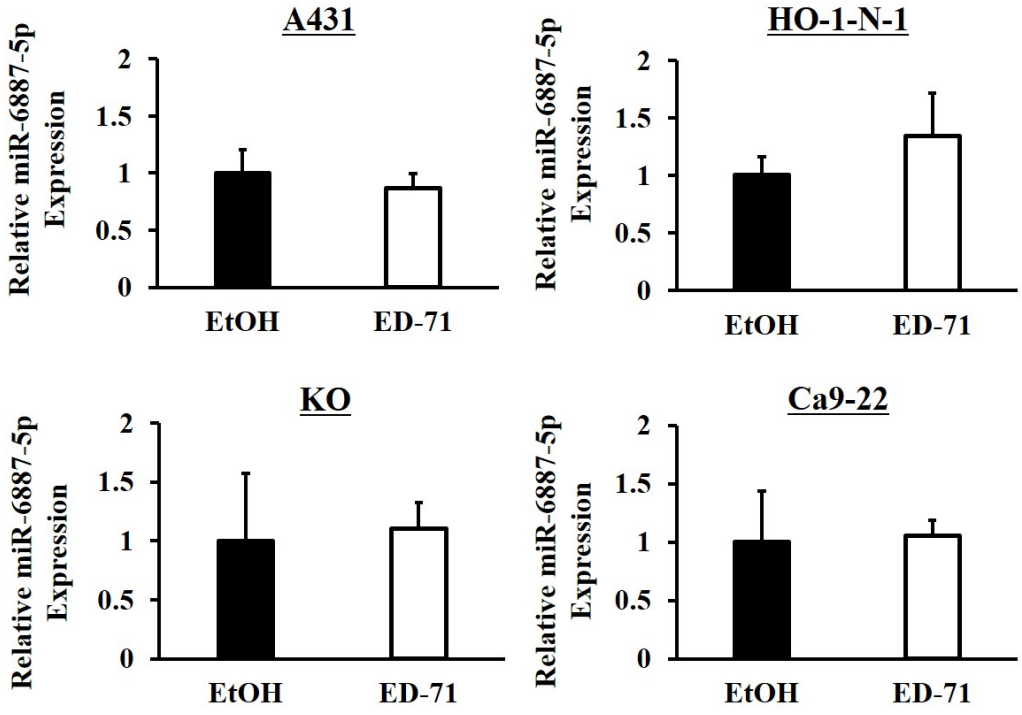

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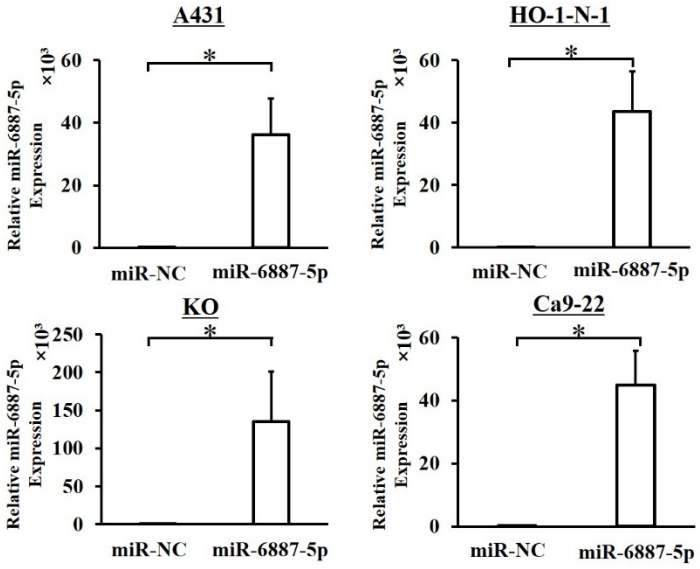
(A)



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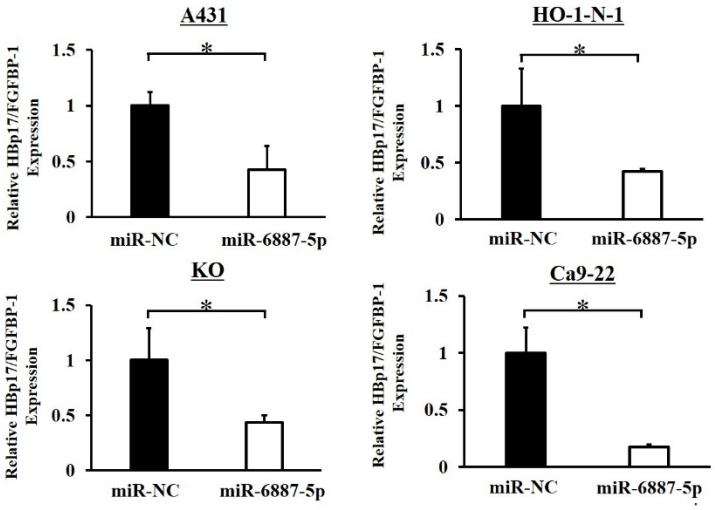

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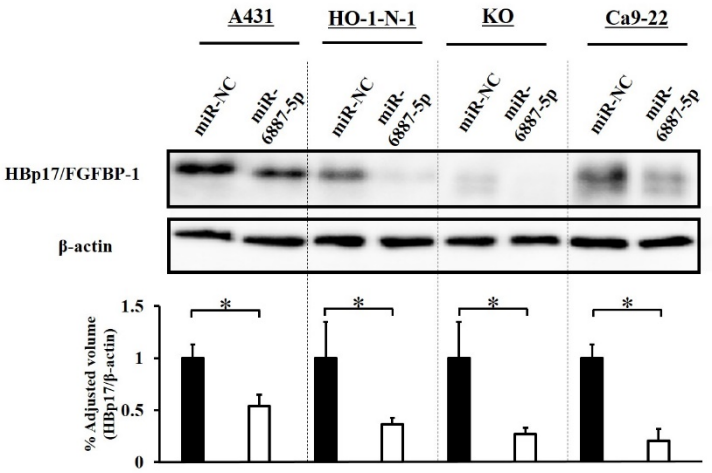

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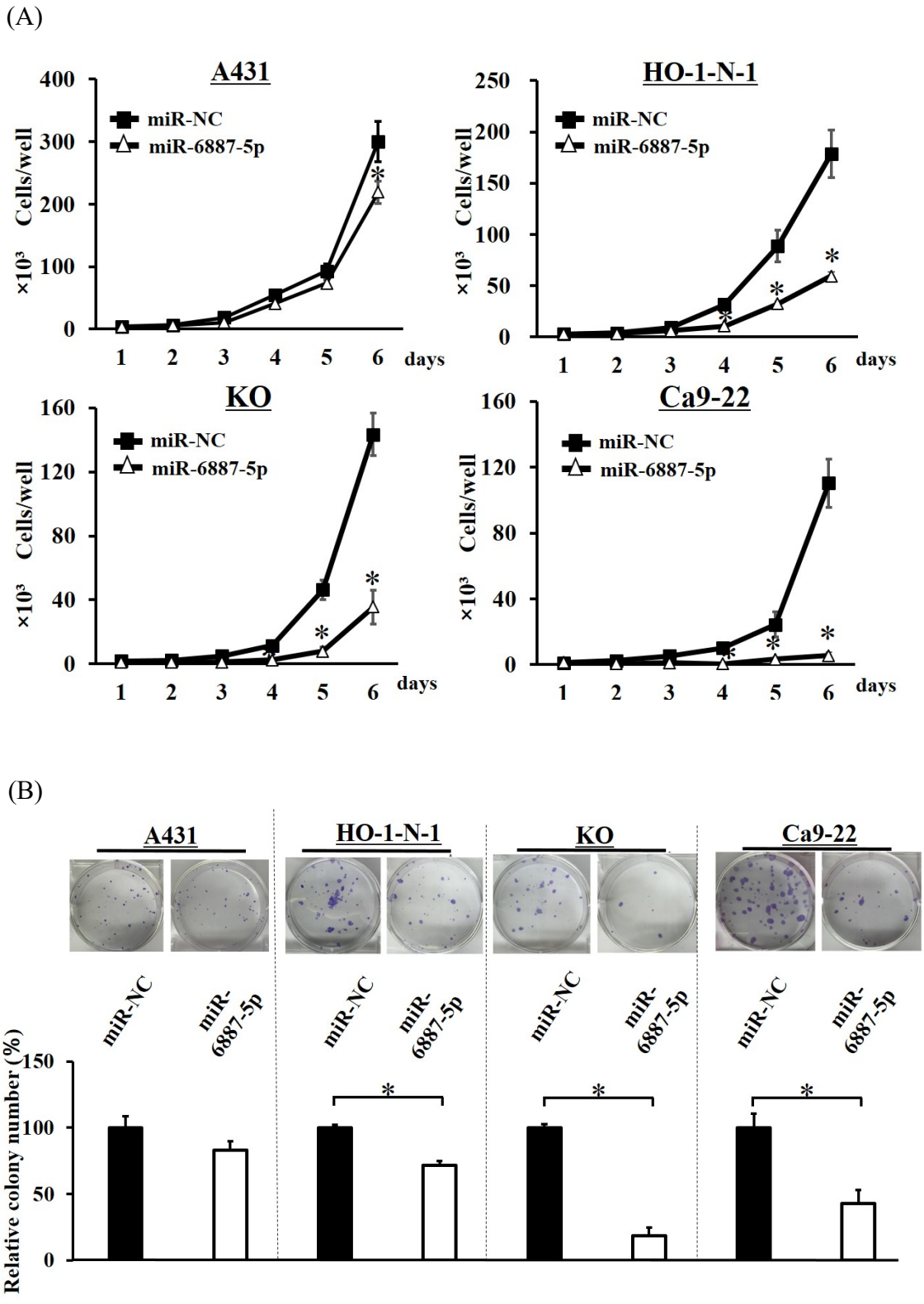

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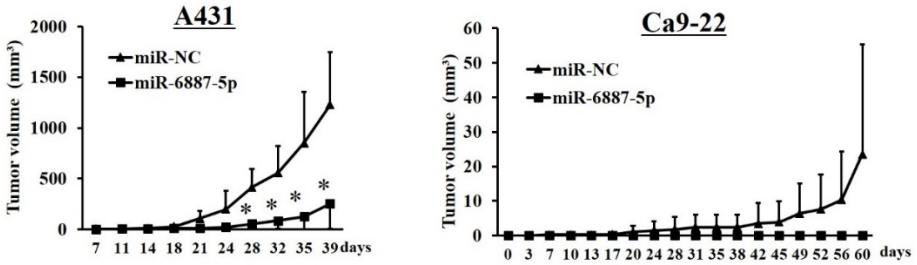
(A)



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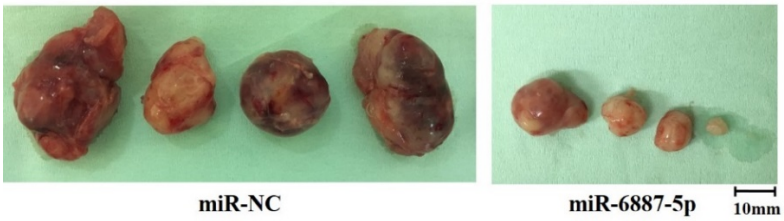

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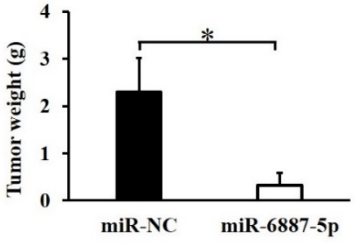

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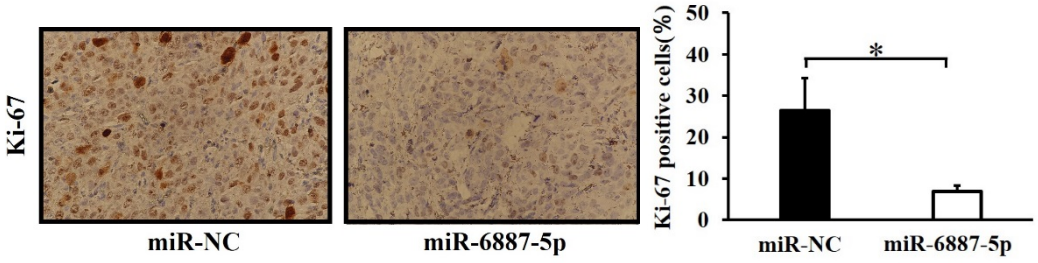

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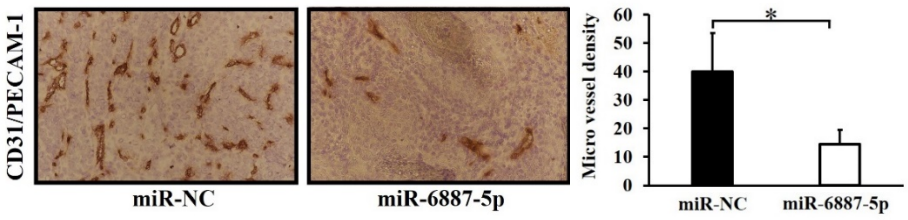
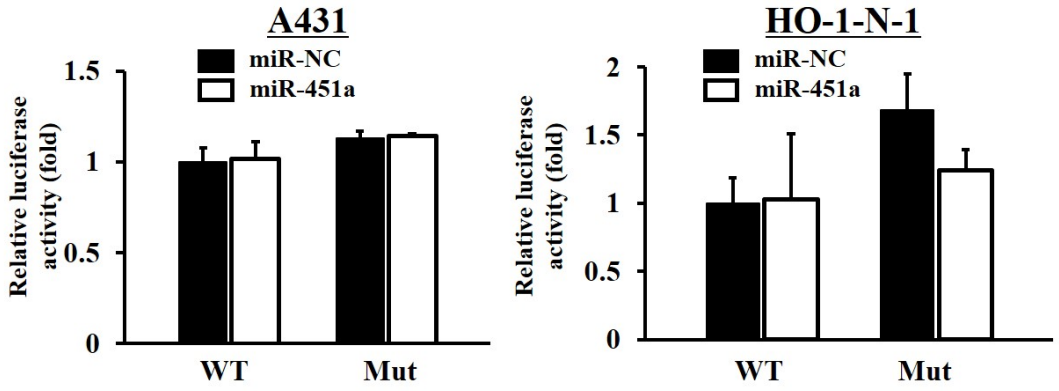


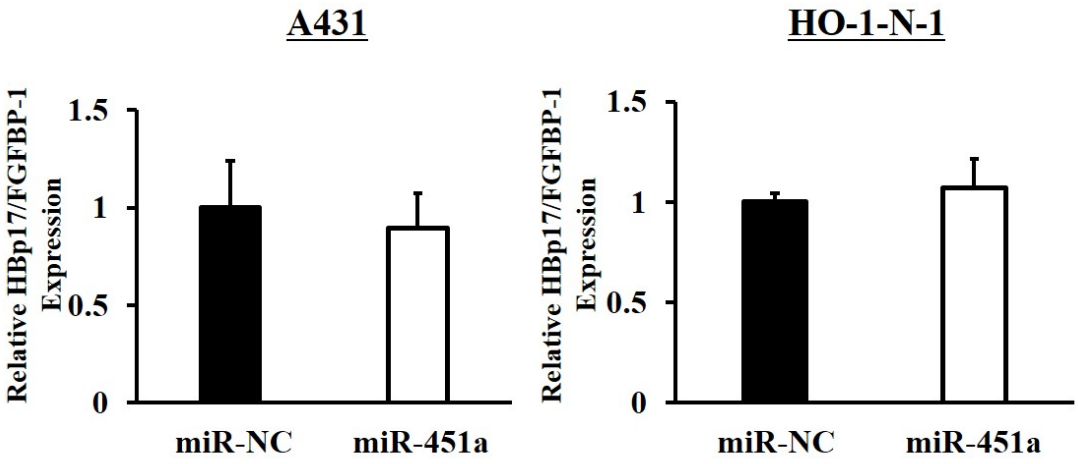
Supplementary Table 1. STR profile of each cell line

	A431			HO-1-N-1		KO			Ca9-22	
D3S1358	14			16		15	16		20	
TH01	9			9		9			6	
D21S11	28			28	30	31	32.2		28	
D18S51	13	17		15	16	14			15	
Penta_E	12	13		11		15			10	17
D5S818	13			9		11	13		12	
D13S317	9	13	14	10	11	12	13	14	11	
D7S820	10			10	13	10			11	13
D16S539	12	14		9	11	10	11		9	10
CSF1PO	12			12		10	11		12	
Penta_D	11			9	13	12	13		9	
vWA	15	17		16	18	19			16	
D8S1179	13	14		10	15	10	14		14	
TPOX	11			11		8	9		11	
FGA	20			19	22	23			22	
AMEL	X			X	Y	X			X	Y

Figure S1. Higaki M. et al.

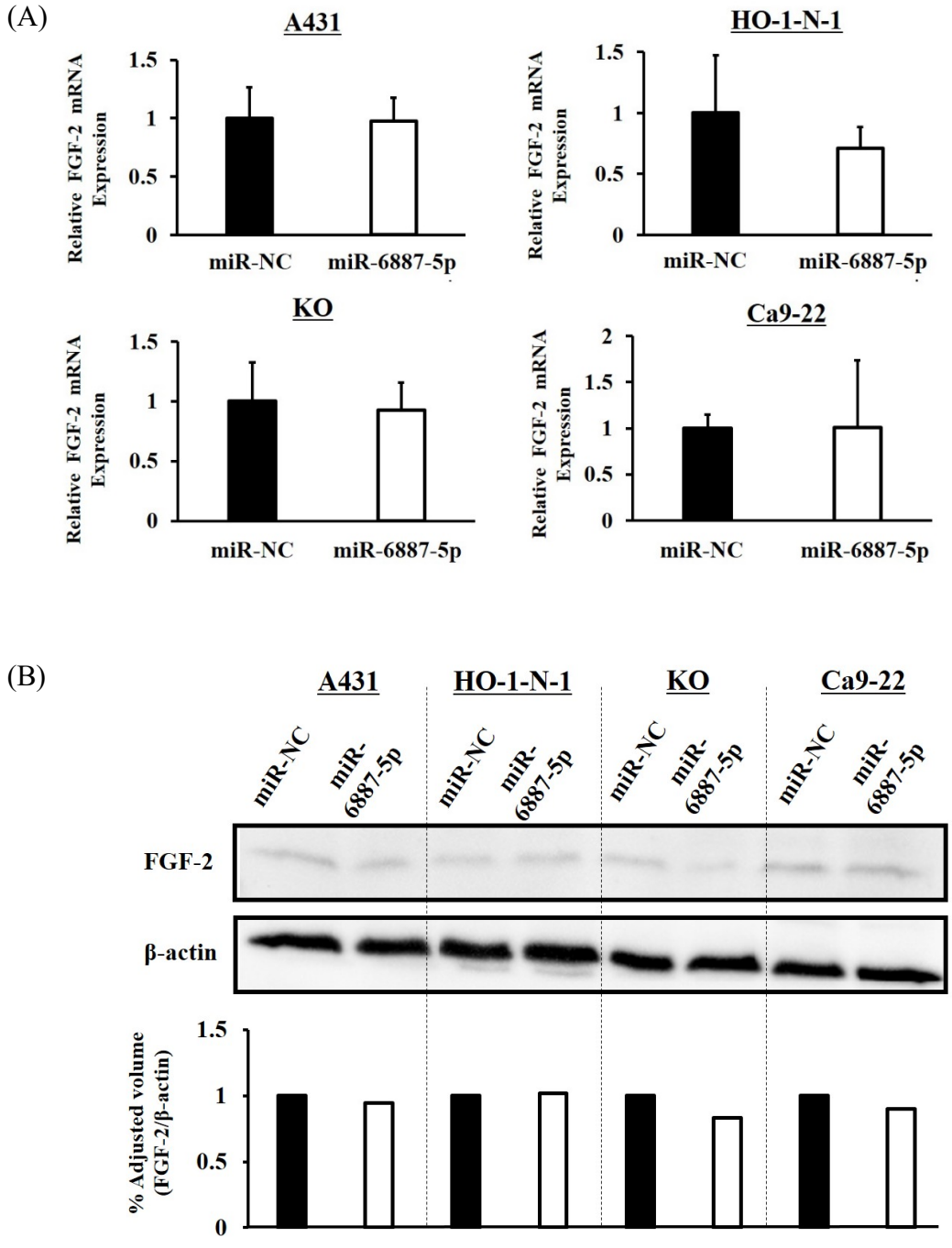


Supplementary Figure 1. HBp17/FGFBP-1 is not a target of miR-451a in SCC/OSCC cell lines. There is no change in luciferase activity in any of the cells generated, which confirmed that miR-451a mimics do not bind to the putative 3'-UTR of HBp17/FGFBP-1.



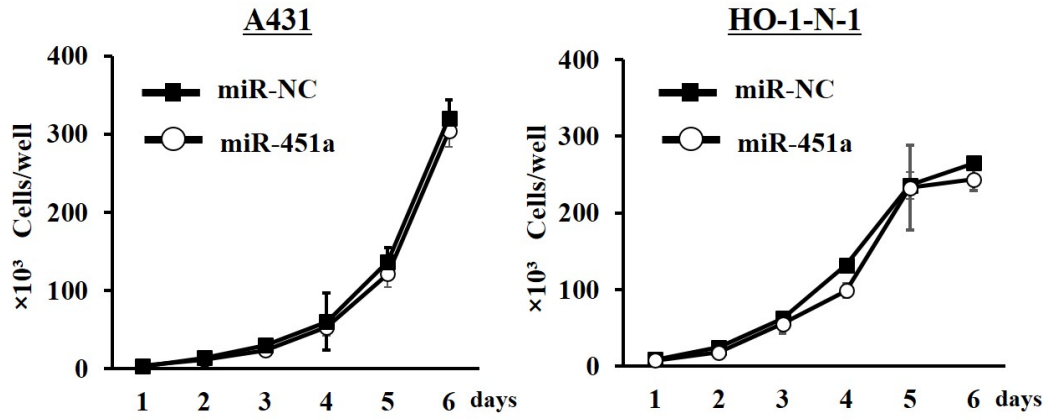
Supplementary Figure 2. Overexpression of miR-451a did not modulate HBp17/FGFBP-1 expression in SCC/OSCC cells. Relative HBp17/FGFBP-1 mRNA levels were measured in A431 and NA cells transfected miR-451a mimics and miR-NC mimics.

Figure S3. Higaki M. et al.



Supplementary Figure 3. FGF-2 is not suppressed by miR-6887-5p in SCC/OSCC cells. Relative FGF-2 mRNA (A) and protein expression (B) levels in SCC/OSCC cells transfected miR-6887-5p mimics or miR-NC mimics were not changed.

Figure S4. Higaki M . et al.



Supplementary Figure 4. MiR-451a exhibits no effect on cell proliferation of SCC/OSCC cell lines. Cell proliferation was determined in A431 and NA cells transfected with miR-451a mimics or miR-NC mimics.