PLAG1 enhances the stemness profiles of acinar cells in normal human salivary glands in a cell type-specific manner.

Yuriko Goto [a], Miho Ibi [a], Hirotaka Sato [a], Junichi Tanaka [b], Rika Yasuhara [b], Keiko Aota [c], Masayuki Azuma [c], Toshiyuki Fukada [b, d], Kenji Mishima[b], Tarou Irié [a, b,*]

[a] Division of Anatomical and Cellular Pathology, Department of Pathology, Iwate Medical University, 1-1-1 Idaidori, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan

[b] Division of Pathology, Department of Oral Diagnostic Sciences, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

[c] Department of Oral Medicine, Tokushima University Graduate School of Biomedical Sciences, 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan

[d] Faculty of Pharmaceutical Sciences, Tokushima Bunri University, 180 Nishihamabouji, Yamashiro, Tokushima 770-8055, Japan

*Correspondence:

Tarou Irié

Division of Anatomical and Cellular Pathology, Department of Pathology, Iwate Medical University, 1-1-1 Idaidori, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan

Tel: +81-19-651-5111

Fax: +81-19-908-8018

E-mail address: tarou@iwate-med.ac.jp

Highlights

• PLAG1 enhances the stemness profile in acinar cells in normal human salivary glands.

• PLAG1 enhances the stemness profile in a cell-type-specific manner.

• PLAG1 up-regulates the expression of genes involved in the stemness-related pathway.

• PLAG1 may cause tumorigenesis by increasing the stemness character of the cells.

Abstract

Objectives: Details of the histogenesis of salivary gland tumors are largely unknown. The oncogenic role of PLAG1 in the salivary gland has been demonstrated in vivo. Herein, we demonstrate the roles of PLAG1 in the acinar and ductal cells of normal human salivary glands in an attempt to clarify the early events that occur during the histogenesis of salivary gland tumors.

Methods: Normal salivary gland cells with acinar- (NS-SV-AC) and ductal- (NS-SV-DC) phenotypes were transfected with PLAG1 plasmid DNA. Subsequently, the PLAG1 overexpressed and mock cells were examined by cell proliferation, transwell migration, and salisphere formation assays. The expression levels of salivary and pluripotent stem cell markers and differentiation markers were evaluated by quantitative real-time polymerase chain reaction and immunofluorescence. Alterations in transcriptional expressions were investigated via cap analysis of gene expression with gene-enrichment and functional annotation analysis.

Results: PLAG1 promoted cell proliferation and transwell migration in the acinar and ductal cells, and markedly enhanced the stemness profiles and luminal cell-like profiles in acinar cells; the stemness profiles were partially increased in the ductal cells.

Conclusion: PLAG1 enhanced the stemness profiles in the acinar cells of normal human salivary glands in a cell type-specific manner. Thus, it may be involved in salivary gland tumorigenesis by increasing the stemness character of the normal salivary gland cells.

Keywords:

Salivary gland neoplasm, Tumorigenesis, Pleomorphic adenoma gene 1, Stemness, Acinar cell

1. Introduction

Pleomorphic adenoma gene 1 (PLAG1) is a crucial oncogene involved in the development of pleomorphic adenomas in the salivary glands [1]. Tumorigenesis is caused by the swapping of promoters between PLAG1 and beta-catenin through chromosomal reciprocal translocations [2]. These events cause the ectopic overexpression of PLAG1, which is driven by the constitutively active beta-catenin promoter. The definite oncogenic capacity of PLAG1 has been demonstrated in vivo; moreover, PLAG1 overexpression in the salivary gland caused the development of a tumor in the salivary gland [3]. Human insulin-like growth factor 2 (IGF2) gene is the representative target gene of PLAG1 with regard to tumorigenesis [1]. PLAG1 binds to IGF2 promoter 3 and stimulates its activity; IGF2 transcripts derived from the P3 promoter are highly expressed in salivary gland pleomorphic adenomas that overexpress PLAG1 [4]. PLAG1 influences tumorigenesis, at least in part, via the mitogenic action of IGF2, presumably by the activation of IGF1R and the Ras/Raf/MAPK signaling pathway in NIH3T3 cells [5, 6].

The histogenesis of salivary gland tumors is not well understood because they are generally well-developed during surgical resection. Among the few hypothetical concepts of the histogenesis of salivary gland tumors, the most novel one is a multicellular theory that involves the differentiation of cells at all the levels of the gland; the acinar and basal cells were also thought to be capable of cell division, and were presented as the hypothetical cells of origin for salivary gland neoplasms [7]. This concept is based on the findings of an autoradiography study in adult female mouse submandibular glands, wherein basal, luminal, and acinar cells were found to be capable of DNA synthesis and mitosis [8]. These results overlap with recent evidence that suggests that differentiated acinar cells are self-duplicating and maintained without significant input from stem/progenitor cells in the intercalated duct in adult salivary glands [9, 10]. Furthermore, it has

been shown that there are more than one progenitor cell population in the adult salivary gland [11]. However, whether the stem/progenitor cells in normal salivary glands are directly transformed into cancer stem cells is poorly understood; moreover, the origin of cancer stem cells in normal salivary glands remains unknown [12, 13].

In this study, we report the roles of PLAG1 in acinar and ductal cells of normal human salivary glands in order to elucidate the early events of the histogenesis of salivary gland tumors.

2. Materials and methods

2.1. Plasmid construction for human PLAG1 overexpression

Full-length, human PLAG1 cDNAs subcloned into pCR4-TOPO were purchased from DNAFORM (Yokohama, Kanagawa, Japan). The adaptors of EcoRI and BglII sites were added to the 5’ and 3’ side ends of the full-length cDNAs by PCR amplification using the following primer sets: 5'-GAATTCCGCCACTGTCATTCCTGGT-3' (for the addition of the EcoRI site to the 5’ side end) and 5'-AGATCTCTACTGAAAAGCTTGATGGAA-3' (for the addition of the BglII site to the 3’ side end). The PCR product was subcloned by insertion into a TOPO TA vector (ThermoFisher Scientific, CA, USA). The EcoRI- BglII fragment, including the PLAG1 cDNA from the TOPO TA vector, was inserted into the multi-cloning site of the p3XFLAG-Myc-CMV-26 expression vector (Sigma-Aldrich Co, CA, USA). All gene recombination experiments were approved by the Gene Recombination Experiment Safety Committee of Iwate Medical University (Permit Number: 528).

2.2. Cell culture

Immortalized normal human salivary gland acinar (NS-SV-AC) and ductal (NS-SV-DC) cell lines were used in this study. Detailed characteristics of these cells have been described elsewhere [14]. The cells were maintained in Keratinocyte-serum-free medium supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epithelial growth factor (EGF; Gibco, Life Technologies, NY, USA), and antibiotics (Nacalai Tesque, Kyoto, Japan) at 37°C in a humidified atmosphere containing 5% CO_2. For plasmid DNA transfection, the cells were grown to 70%–80% confluency and transfected with the human PLAG1 construct or an empty vector (negative control) using FUGENE^{TM} HD transfection reagent (Promega Corporation, Madison, WI, USA). At 24 h after transfection, we observed and photographed the cell morphology using the

Keyence microscope BZ-X710 (Keyence, Tokyo, Japan). After confirming the overexpression of PLAG1 in the cells by immunoblotting, the transfected cells were used for subsequent analysis.

2.3. Western blot analysis

The cells were washed with cold phosphate-buffered saline (Takara, Shiga, Japan) and lysed in RIPA buffer (Nacarai Tesque, Kyoto, Japan). After centrifugation, the supernatant was collected and protein concentrations were determined by microplate assay (DC Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Five micrograms of protein were loaded and resolved in SDS-polyacrylamide gel and transferred to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in 2.5% ECL Blocking Agent (GE Healthcare, Fairfield, CT, USA) for 30 min, and then probed with mouse monoclonal anti-human PLAG1 primary antibody (clone; 3B7, H00005324-M02, Abnova, Taipei, Taiwan) and mouse monoclonal anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (clone; 0411, sc-47724, Santa Cruz Biotechnology, CA, USA) at room temperature for 1 h. After washing in Tris-buffered saline (Takara, Kyoto, Japan) containing 0.1% tween 20 (Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (NA931V, GE Healthcare, Buckinghamshire, UK) at room temperature for 1 h. The antibody used for GAPDH detection was diluted with Can Get Signal solution (TOYOBO CO., LTD., Osaka, Japan). The blots were visualized with ECL or ECL select Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) followed by scanning with the ChemiDoc XRS Plus System and analysis with Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Cell proliferation assay

Human PLAG1 or empty vector-transfected cells were plated into a 96-well plate (1.0×10^4 cells per well), and after 5 days of culture, assessed by addition of 10 μl of WST-8 solution (Cell Count Reagent SF; Nacalai Tesque, Kyoto, Japan) followed by further incubation for 2 h. The absorbance was measured at a wavelength of 450 nm using the CHROMATE 4300 microplate reader (Awareness Technology Inc., FL, USA); the absorption at 630 nm was used as a reference. All experiments were performed in quadruplicate (acinar cells) or septuplicate (ductal cells).

2.5. Matrigel transwell migration assay

The Matrigel transwell migration assay was performed using Falcon^R cell culture inserts (8 μm pore size; Coring, NY, USA). The inserts were coated with BD Matrigel Basement Membrane Matrix (Coring, NY, USA) following which, the transfected cells (5×10^4) were inoculated into the upper chamber of the cell culture insert. The growth medium was placed in the lower well of the 24-well companion plate and the cells were incubated for 24 h at 37°C in 5% CO_2. After 24 h, the cells on the upper surface of the membrane were removed with a cotton swab, and the membrane was fixed with 100% methanol followed by staining with hematoxylin (Muto Pure Chemicals, Tokyo, Japan). The cells that had migrated into the lower side of the membrane were observed under a microscope (OLYMPUS AX80, Tokyo, Japan) equipped with a CCD digital camera (OLYMPUS DP70, Tokyo, Japan) and counted.

2.6. Salisphere culture

The transfected cells were suspended in DMEM/F12 (Gibco, Life Technologies, NY, USA) containing antibiotic, N2 supplement (Gibco, Life Technologies, NY, USA), human recombinant fibroblast growth factor 2 (FGF2, 20 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), EGF (20 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), insulin (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA), and dexamethasone (1 μM; Sigma-Aldrich, St. Louis, MO, USA), and mixed with Corning Matrigel Basement Membrane Matrix (Corning, NY, USA) to 2:1 ratio. The cells were plated in a 24-well plate at a density of 1×10^4 cells per well and cultured for 12 to 14 days at 37°C in 5% CO_2. The medium was changed every three days. For salisphere collection, they were released from the Matrigel by Cell Recovery Solution (Corning, NY, USA) according to the manufactures' protocol.

2.7. Immunohistochemistry

The salispheres were embedded in ipGell (PG20-1, Genostaff, Tokyo, Japan) and fixed with 4% paraformaldehyde (PFA; Nacalai Tesque, Kyoto, Japan). After sucrose replacement, the fixed salispheres were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and sectioned (thickness, 6 μm). The sections were stained with hematoxylin and eosin (HE), or incubated with primary Aquaporin 5 (AQP5: PA5-36529, Invitrogen, Waltham, USA), cytokeratin 18 (CK18: 10830-1-AP, Protein Tech, IL, USA), cytokeratin 14 (CK14: clone:LL002, MCA890H, AbD Serotec Ltd., Oxford, UK), alpha-smooth muscle actin (αSMA: clone:GT445, GTX629702, GeneTex International Corporation, Hsinchu, Taiwan), POU5F1 (also known as Oct4, clone:EPR17929, ab181557, Abcam Cambridge, UK), NANOG (clone: EPR2027(2), ab109250, Abcam Cambridge, UK), leucine-rich repeat-containing G-protein-coupled protein 5 (LGR5, also known as GPR49, clone:OTI2A2, MA5-25644, Invitrogen, Waltham, USA) and THY1 (also known

as CD90: clone:EPR3133, ab133350, Abcam Cambridge, UK) antibodies. For the immunofluorescent staining, the sections were incubated with Alexa Flour anti-mouse IgG 568 (ab175472, Abcam Cambridge, UK) or anti-Rabbit IgG 594 (A11037, Invitrogen, Waltham, USA) and stained with 4'-6diamidino-2-phenylindole (DAPI**:** D9542, Sigma-Aldrich Japan, Tokyo, Japan). The stained images were observed and obtained using the Keyence microscope BZ-X710.

2.8. Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using the RNeasy Plus Mini kit (QIAGEN, Tokyo, Japan) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instruction. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in triplicate using a 7500 Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Nine μl of the diluted cDNA, 1 μl of PCR primer set, and 10 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) were used according to the manufacturer's instructions. The primers used for amplification were as follows: AQP5, forward: 5'-ACTGGGTTTTCTGGGTAGGG-3' and reverse: 5'-GTGGTCAGCTCCATGGTCTT -3'; CK18, forward: 5'-TGGTCACCACACAGTCTGCT -3' and reverse: 5'-CCAAGGCATCACCAAGATTA -3'; CK14, forward: 5'-TGAGCCGCATTCTGAACGAG-3' and reverse: 5'-GATGACTGCGATCCAGAGGA-3'; α-SMA, forward: 5'-CGATAGAACACGGCATCATC-3' and reverse: 5'-CATCAGGCAGTTCGTAGCTC -3'; POU5F1, forward: 5'-AAGCGATCAAGCAGCGACTA-3' and reverse: 5'-GAAGTGAGGGCTCCCATAGC-3'; NANOG, forward: 5'-CAGAAAAACAACTGGCCGAA -3' and reverse: 5'-GGCCTGATTGTTCCAGGATT-3'; LGR5, forward: 5'-AGGATCTGGTGAGCCTGAGAA-3'

and reverse: 5’-CATAAGTGATGCTGGAGCTGGTAA-3’; THY1, forward: 5’-GACAGCCTGAGAGGGTCTTG-3’ and reverse: 5’-CCCAGTGAAGATGCAGGTTT-3’; GAPDH, forward: 5’-ATGGGGAAGGTGAAGGTCG-3’ and reverse: 5’-TAAAAGCAAGCCCTGGTGACC-3’. The PCR conditions consisted of enzyme activation for 2 min at 50°C and 10 min at 95°C, followed by 35 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Cycle threshold (Ct) data were collected using the Sequence Detection Systems 1.4 software (Applied Biosystems, Foster, CA, USA). Quantitative amplification of GAPDH cDNA was performed and used as normalization control. The relative gene expression was calculated by transforming the difference in Ct values between the control and the target using the following equation: relative gene expression = $2^{-(\text{Ct target}-\text{Ct control})}$.

2.9. Cap analysis of gene expression (CAGE).

Cap analysis of gene expression (CAGE) library preparation, sequencing, mapping, and gene expression analysis were performed by DNAFORM (Yokohama, Kanagawa, Japan). In brief, RNA quality was assessed with a Bioanalyzer (Agilent) to ensure that the RIN (RNA integrity number) was above 7.0 and the A260/280 and 260/230 ratios are above 1.7. First-strand cDNAs were transcribed to the 5’ ends of capped RNAs and attached to CAGE "bar code" tags; the sequenced CAGE tags were mapped to the mouse mm9 genomes using the BWA software (v0.5.9) after discarding ribosomal or non-A/C/G/T base-containing RNAs. The CAGE-tag 5’ coordinates were input for CAGEr clustering [15] using the Paraclu algorithm [16] with default parameters. The up-regulated genes in PLAG1 were overexpressed in the acinar and ductal cells when compared with the mock cells and identified with a threshold log2 fold change of >2.0, whereas the

down-regulated genes in the cells were identified with a threshold log2 fold change of < -2.0.

2.10. Gene-enrichment and functional annotation analysis of CAGE data

To explore the function of PLAG1 in normal human salivary gland acinar and ductal cells, a gene ontology (GO) term enrichment analysis was performed using the DAVID database (https://david.ncifcrf.gov/). The GO term enrichment analysis was carried out using the annotation category of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Differences with $P < 0.05$ were regarded as statistically significant.

2.11. Statistical analysis

Data were analyzed and presented using GraphPad QuickCals (https://www.graphpad.com/quickcalcs/, GraphPad Software, San Diego, CA, USA). The two-tailed Student's t-test was used to analyze the differences between two groups. Data are presented as mean ± standard deviation (SD) with $P < 0.05$ considered as statistically significant.

3. Results

3.1. PLAG1 does not cause remarkable changes in the morphological features of acinar and ductal cells

To examine the function of PLAG1 in salivary gland cells, the human PLAG1 DNA plasmid was transfected with NS-SV-AC (acinar cells) and NS-SV-DC (ductal cells) [14]. After 24 h, no significant changes in the morphologies of both acinar and ductal cells were observed (Fig. 1A). The overexpression of PLAG1 in both cells at 24 and 48 h after PLAG1 transfection was confirmed by immunoblotting (Fig. 1B). Based on these results, the following experiments were performed using the cells that were transfected with PLAG1 for 24 h.

3.2. PLAG1 promotes cell proliferation and transwell migration of acinar and ductal cells

Cell proliferation assays revealed that PLAG1 significantly increased the proliferative capacity of both acinar and ductal cells after five days of culture (Fig. 2A). PLAG1 also increased transwell migration of both acinar and ductal cells (Figs. 2B, C).

3.3. PLAG1 enhances luminal cell-like profiles in acinar cells, but not in ductal cells

To investigate how PLAG1 influences the differentiation of acinar and ductal cells in a biological environment that partially mimics the very early stage of salivary gland tumor, we utilized a salisphere culture that has been used to grow adult salivary gland epithelial cells into 3D clusters for in vitro differentiation studies [17]. In salispheres composed of acinar cells, PLAG1 induced the mRNA expression of AQP5, an acinar cell marker, and CK18, a ductal cell marker, but not CK14, a basal cell marker, and α-SMA, a myoepithelial cell marker (Fig. 3B). These results

were compatible with those of immunofluorescence (Fig. 3C) indicating that PLAG1 enhances the luminal cell-like profiles in the salispheres of acinar cells. In the ductal cells, no remarkable changes in the differentiation of the ductal cell characters were noted, but a slight reduction in the basal cell-like character of the ductal cells was observed (Fig. 4B). Furthermore, PLAG1 attenuated the mRNA expression levels of CK14, but not AQP5, CK18, and α-SMA; these results were compatible with those of immunofluorescence (Fig. 4C). Interestingly, PLAG1 influenced the differentiation of acinar and ductal cells in a cell type-specific manner.

3.4. PLAG1 enhances the stemness profile in acinar cells

Next, we examined whether PLAG1 affects the expression of stemness markers in acinar and ductal cells. Several stem/progenitor cell markers have been reported in human salivary gland cells [18-20]. We analyzed the expression of pluripotent cell markers, POU5F1 (Oct4) and NANOG, and salivary gland stem cell markers, leucine-rich repeat-containing G-protein-coupled protein 5 (LGR5) and THY1 (CD90) by qRT-PCR and immunohistochemistry in the salispheres of the acinar and ductal cells. Interestingly, the mRNA and protein expression levels of NANOG, LGR5, and THY1 were markedly up-regulated in the acinar cells (Fig. 5), whereas in the ductal cells, only THY1 was significantly up-regulated (Fig. 6).

3.5. Pathways concerning stemness and cancer were enriched among differentially up-regulated genes in acinar cells with PLAG1 overexpression.

CAGE analysis revealed 849 up-regulated and 120 down-regulated genes in the acinar cells and 445 up-regulated and 80 down-regulated genes in the ductal cells that overexpressed PLAG1.

The functions of these genes in the cells were investigated by subjecting them to a GO term enrichment analysis (Table 1, 2). The results of the analysis indicated that the up-regulated genes in the acinar cells with PLAG1 overexpression predominantly participated in cancer-related pathways, including “Pathways in cancer”, “Ras signaling pathway”, “Basal cell carcinoma”, “PI3K-Akt signaling pathway” and “MAPK signaling pathway” and in stemness-related pathways, such as the “Signaling pathways regulating pluripotency of stem cells” (Table 1). Likewise, the up-regulated genes in the ductal cells with PLAG1 overexpression appeared to predominantly participate in the cancer-related pathways (out of the top 10), but not in the stemness-related pathway (Table 2).

4. Discussion

In the present study, we showed that PLAG1 enhanced the stemness profiles predominantly in acinar cells, but only to some extent in ductal cells in normal human salivary glands. PLAG1 enhanced the luminal cell-like characters of acinar cells but caused unremarkable changes in the ductal cells. PLAG1 influenced the stemness profiles and differentiation in a cell type-specific manner in the normal human salivary gland cells.

Little is known about the involvement of PLAG1 in stemness. It has been reported that PLAG1 and upstream stimulating factor-2 (USF2) co-regulate the expression of Musashi-2 and play a key role in the function of hematopoietic stem and progenitor cells [21]. PLAG family genes are expressed in the progenitor cells of the cerebellar lineage in the developing cerebellum during the early stages of the mouse embryo [22]. These facts suggest that PLAG1 plays a potential role in stemness in the hematopoietic and neuronal systems; however, the physiological role of PLAG1 with regard to stemness in normal human salivary gland cells remains unknown. The results of the present study demonstrated that the overexpression of PLAG1 in normal salivary gland acinar cells resulted in the transcriptional activation of pluripotent stem cells and salivary stem/progenitor cell markers. To date, the exact mechanism by which PLAG1 exerts the induction of stemness in normal human salivary gland cells remains unclear. Wingless/integrated (Wnt) is a key molecule in most types of stem cells in adult mammals [23]. Earlier studies demonstrated that the Wnt signaling pathway was activated through the upregulation of β-catenin in salivary gland tumors induced by the overexpression of PLAG1 in transgenic mice [24]. In addition, the three-dimensional spheroid culture of human salivary gland-resident stem cells in nanofibrous microwells has been shown to promote stem cell properties via Wnt signaling activation [20]. Therefore, it is suggested that the Wnt/β-catenin signaling pathway could play an important role in the enhancement of the stemness

profile by PLAG1 overexpression in normal human salivary gland cells. This is in accordance with the results of the GO term enrichment analysis in this study wherein, the up-regulated genes in the acinar cells that overexpressed PLAG1 (including WNT1, 3A, 5A, 7A, 7B, and 11) were found to be predominantly involved in the stemness-related pathway (Table 1). Moreover, the Rap1 signaling pathway was found to be involved in the acinar cells with PLAG1 overexpression. Recently, it was reported that the Rap1 activation status in hematopoietic stem/progenitor cells (HSPC) is important for maintaining HSPC at the bone marrow niche for long-term hematopoiesis in adults [25]. Thus, PLAG1 appeared to enhance the stemness profiles in the acinar cells via Wnt and other signaling pathways (such as the Rap1 signaling pathway). However, the mechanism by which PLAG1 enhances the stemness in salivary gland cells needs to be evaluated in the future.

Our results demonstrated that PLAG1 enhances luminal cell-like profiles in the acinar cells using the salisphere culture, which suggested that PLAG1 promotes differentiation in acinar cells. PLAG1 has been reported to act as a regulator of neuronal gene expression and neuronal differentiation in neural progenitor cells in the developing mouse neocortex [26]. These results may contradict with the findings of the current study wherein, PLAG1 was found to enhance the stemness profiles in the acinar cells, which suggests that PLAG1 promotes the dedifferentiation of these cells. These conflicting findings may be attributed to the potential role of PLAG1 in facilitating asymmetric stem cell division, progenitor division, and lineage differentiation in the acinar cells. This speculation may be supported by previous reports which show that PLAG1 is required for zygotic genome activation during early preimplantation embryo development [27]. Regulation of zygotic genome activation is thought to be closely involved with pluripotency control and lineage differentiation [27].

It is thought that the constitutive renewal and aberrant expansion of the stem cell pool caused by uncontrolled Wnt signaling are required for the development of cancerous tissues [28]. Thus, it is possible that PLAG1 aids in the development of salivary gland tumors by increasing the number of cells with stemness features in the progenitor cell pool in normal salivary glands by virtue of the uncontrolled Wnt signaling.

Our results showed that the overexpression of PLAG1 in normal human salivary gland cells caused different effects on stemness profiles and differentiation in a cell type-specific manner. The cell type- and context-specific functions of PLAG1 have been reported previously [29]. The roles of PLAG1 in insulin-like growth factor 2 (IGF2) regulation in Hep3B and JEG-3 cells are different [29]. It has been suggested that the binding of PLAG1 to the IGF2 P3 promoter resulting in IGF2 expression is cell type-specific; furthermore, the PLAG1 transcription factor acts as a transcriptional facilitator that partially overrides the insulation by the H19 imprinting control region [29]. Epigenetic control at the H19–IGF2 locus for the maintenance of adult stem cells has been demonstrated in hematopoietic cells [30]. Furthermore, DNA methylation is required for the control of stem cell differentiation in the digestive system [31]. Thus, these differences in the epigenetic status between different stem/progenitor acinar and ductal cells in normal salivary glands may be responsible for the cell type-specific reactions of PLAG1 overexpression. The results of the present study probably account for the histological diversity of salivary gland tumors caused by differences in the origin of tumor cells.

In a previous study, the proliferation rate of NIH3T3 cell lines overexpressing PLAG1 was significantly higher than that of mock-infected cells [6]. Moreover, NIH3T3 cell lines overexpressing PLAG1 displayed the typical markers of neoplastic transformation: loss of cell-cell

contact inhibition; anchorage-independent growth; and ability to induce tumors in nude mice [6]. PLAG1 has been reported to inhibit cell migration through miR-218-1 by directly targeting RET and PLAG1 [32]. The GO term enrichment analysis in the present study showed that signaling pathways, such as MAPK, PI3K/AKT, and Ras, were predominantly involved. The MAPK–Snai2 pathway was found to be involved in the migration and invasion of cells in salivary adenoid cystic carcinoma (ACC) [33]. In addition, almost all ACCs overexpressed c-Kit [34, 35], which is thought to be a therapeutic molecular target in ACCs [36-38]. The PI3K/AKT, MAPK, and Ras/Raf pathways are known to be the downstream effectors of c-Kit [37]. The Ras/RAF/MEK/MAPK and PIK3/AKT/mTOR pathways are highly conserved pathways necessary for growth, proliferation, differentiation [39], and tumorigenesis [40, 41]. Moreover, the PI3K and MAPK pathways mediate salivary gland branching [42]. It is suggested that PLAG1 induces cell proliferation, migration, and tumorigenesis through the MAPK, PI3K/AKT, and Ras signaling pathways in human normal salivary gland.

5. Conclusions

Our results demonstrated that PLAG1 enhances the stemness profiles of acinar cells in normal human salivary glands in a cell type-specific manner. Moreover, the up-regulated genes in cells with PLAG1 overexpression were found to be predominantly involved in the stemness-related pathway and the MAPK, PI3K/AKT, and Ras signaling pathways. This cell type-specific function of PLAG1 might indicate that the histological diversity of salivary gland tumors is caused by differences in the origin of tumor cells. Thus, it might be reasonable to conclude that PLAG1 aids in

the development of salivary gland tumors by increasing the stemness feature in normal salivary gland cells.

Conflict of interest

The authors declare that they have no potential conflicts of interest.

Author contributions

Y. G performed most of the experiments, prepared figures and contributed to the writing of the manuscript. M. I. analyzed the data, prepared figures and wrote the manuscript. H. S. provided technical support with histological analysis. J. T., R. Y., T. F., K. M. provided technical support with cell biological analysis. M. A. produced salivary gland cell lines. K. A. provided salivary gland cell lines. T. I. conceived and planned the project, analyzed the data and wrote the manuscript.

Ethical approval

All gene recombination experiments were reviewed and approved by the Gene Recombination Experiment Safety Committee of Iwate Medical University (Permit Number: 528).

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Figure Legends

Figure 1.

PLAG1 overexpression does not affect the cell morphology of acinar and ductal cells. Acinar (NS-SV-AC) and ductal (NS-SV-DC) cells were transfected with PLAG1 and empty vector using the lipofection method. (A) Cell morphology of acinar (upper) and ductal (lower) cells at 24 h after transfection. Scale bar = 100 μm. (B) Immunoblotting of acinar and ductal cells at 24 and 48 h after PLAG1 transfection. GAPDH was used as the control.

Figure 2.

PLAG1 promotes cell proliferation and migration of acinar and ductal cells. (A) Cell proliferation assay of acinar and ductal cells transfected with PLAG1. The absorbance of acinar (left) and ductal (right) cells at 450 nm were measured at five days after the seeding of the cells transfected with PLAG1 or an empty vector. (B, C) Transwell migration assay. PLAG1- or empty vector-transfected acinar and ductal cells were seeded onto Matrigel Basement Membrane Matrix-coated transwell chambers. After 24 h, the cells that had invaded into the lower sides of the membranes were counted under the microscope. All values shown are mean ± standard deviation (SD) of septuplicate (A) or quadruplicate (C) measurements. **P < 0.01.

Figure 3.

PLAG1 enhances luminal cell-like profiles in acinar cells. (A) Hematoxylin and Eosin (HE) staining of salispheres formed from PLAG1-transfected acinar cells. (B) qRT-PCR analysis on salispheres formed from PLAG1-transfected acinar cells. All values shown are mean ± standard deviation (SD; n = 3-6). *P < 0.05; **P < 0.01; n.d., not detected; n.s., not significant. (C)

Immunofluorescence staining of AQP5, CK18, CK14, and αSMA (red) in salispheres formed from PLAG1-transfected acinar cells. The nuclei were stained with DAPI (blue). The salispheres for all results were cultured for 12 or 13 days by salisphere culture. Scale bar = 50 μm.

Figure 4.

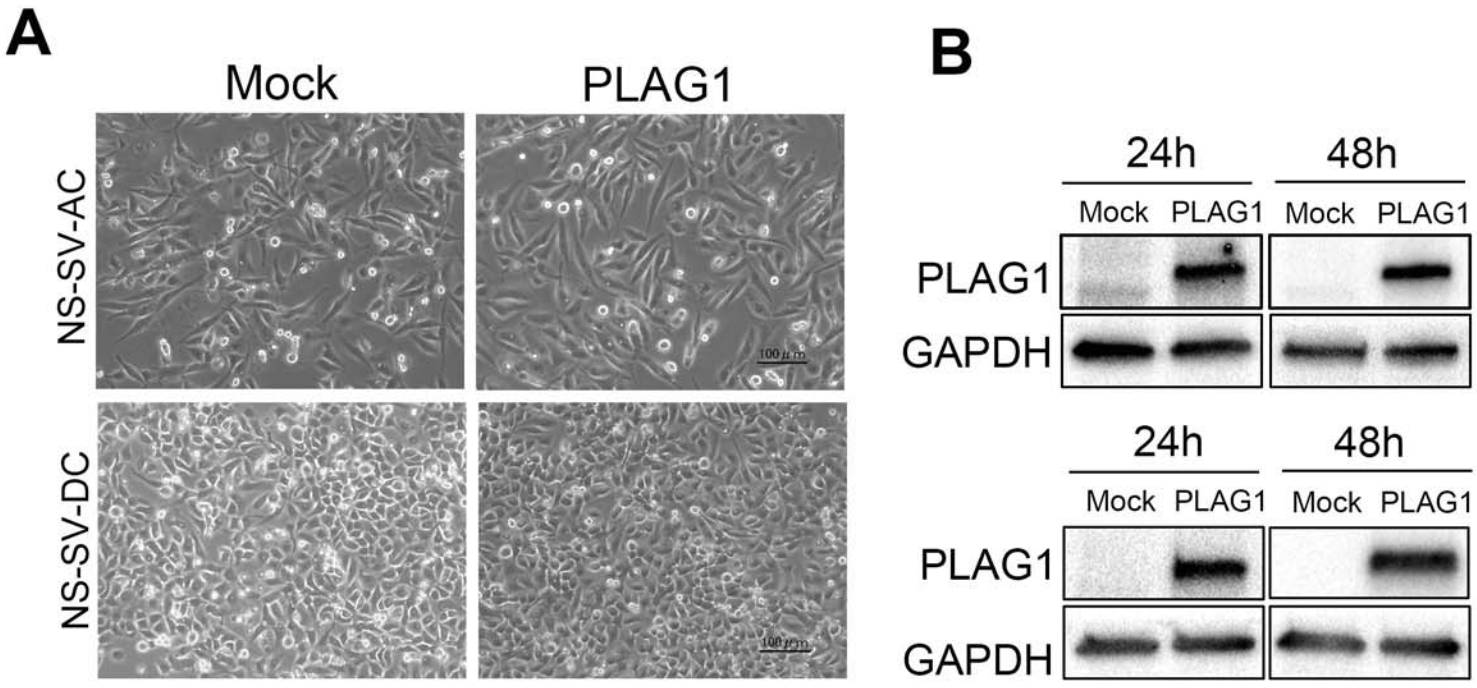
PLAG1 slightly reduces the basal cell-like character in ductal cells. (A) HE staining of salisphere formed from PLAG1-transfected ductal cells. (B) qRT-PCR analysis of salispheres formed by PLAG1-transfected ductal cells. All values shown are mean ± standard deviation (SD; n = 4-6). *P < 0.05; n.s., not significant. (C) Immunofluorescence staining of AQP5, CK18, CK14, and αSMA (red) in salispheres formed by PLAG1-transfected ductal cells. The nuclei were stained with DAPI (blue). The salispheres for all results were cultured for 13 days by salisphere culture. Scale bar = 50 μm.

Figure 5.

PLAG1 enhanced the stemness profiles in acinar cells. (A) qRT-PCR analysis of stem cell markers. All values shown are mean ± standard deviation (SD; n = 3-6). *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. (B) Immunofluorescence staining of POU5F1, NANOG, LGR5, and THY1 (red) in salisphere formed by PLAG1-transfected acinar cells. The nuclei were stained with DAPI (blue). The salispheres for all results were cultured for 12 or 13 days by salisphere culture. Scale bar = 50 μm.

Figure 6.

PLAG1 increases the expression of THY1 in ductal cells. (A) qRT-PCR analysis of stem cell markers. All values shown are mean ± standard deviation (SD; n = 4-6). *P < 0.05; n.s., not significant. (B) Immunofluorescence staining of POU5F1, NANOG, LGR5, and THY1 (red) in salispheres formed by PLAG1-transfected ductal cells. The nuclei were stained with DAPI (blue). The salispheres for all results were cultured for 13 days by salisphere culture. Scale bar = 50 μm.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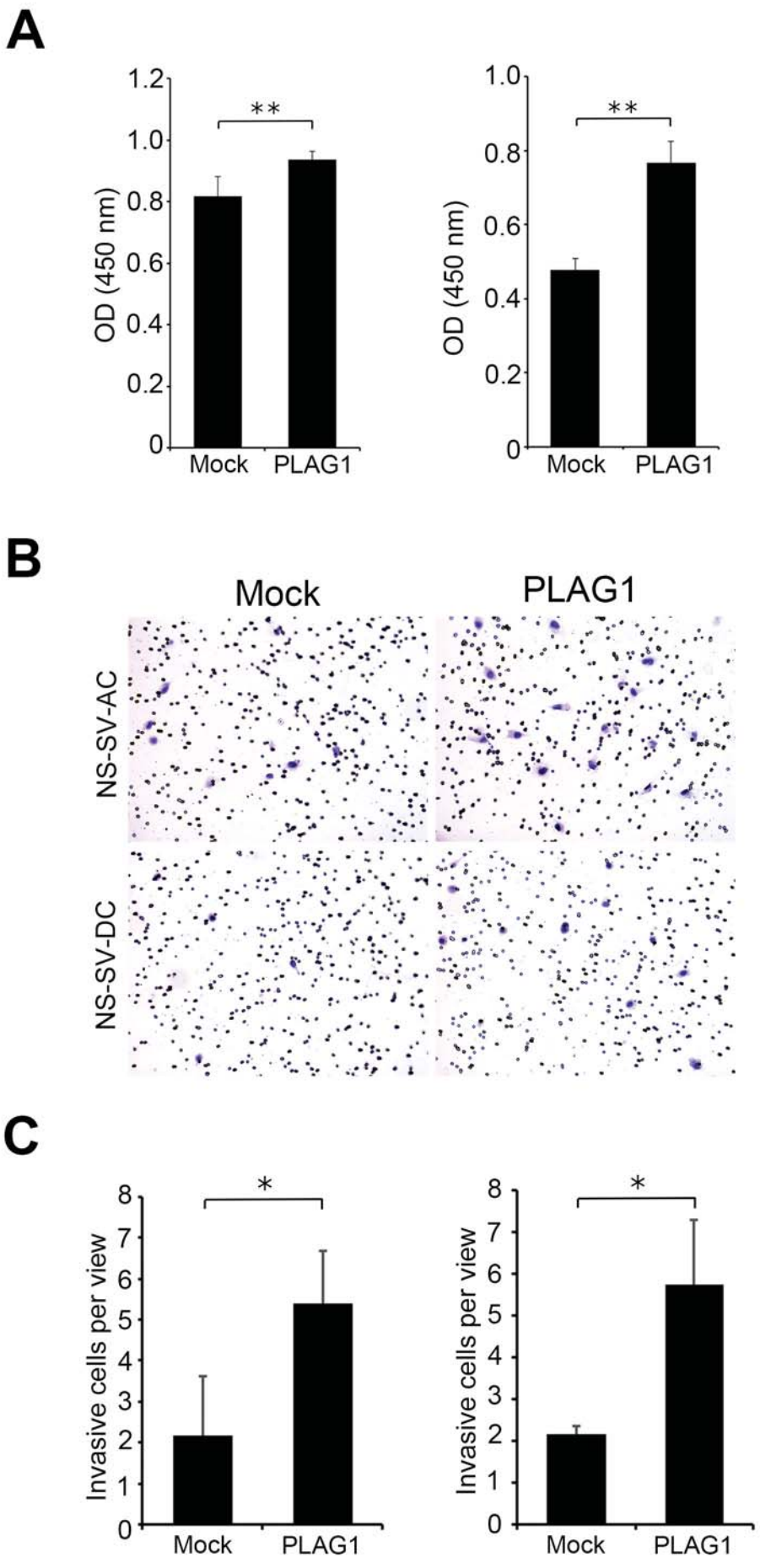
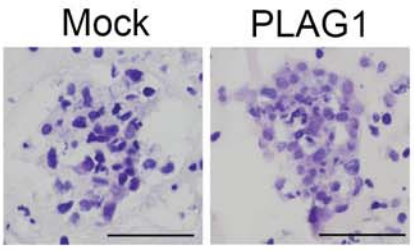
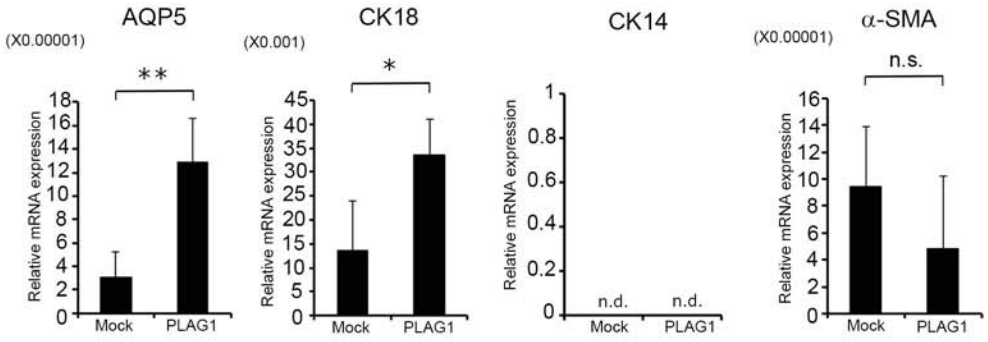
PLAG1
NS-SV-AC
NS-SV-DC
100 μm
100 μm
B
24h
48h
Mock
PLAG1
Mock
PLAG1
PLAG1
GAPDH
24h
48h
Mock
PLAG1
Mock
PLAG1
PLAG1
GAPDH


A
OD (450 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
Mock
PLAG1
OD (450 nm)
1.0
0.8
0.6
0.4
0.2
0
**
Mock
PLAG1
B
Mock
PLAG1
NS-SV-AC
NS-SV-DC
C
Invasive cells per view
8
7
6
5
4
3
2
1
0
*
Mock
PLAG1
Invasive cells per view
8
7
6
5
4
3
2
1
0
*
Mock
PLAG1

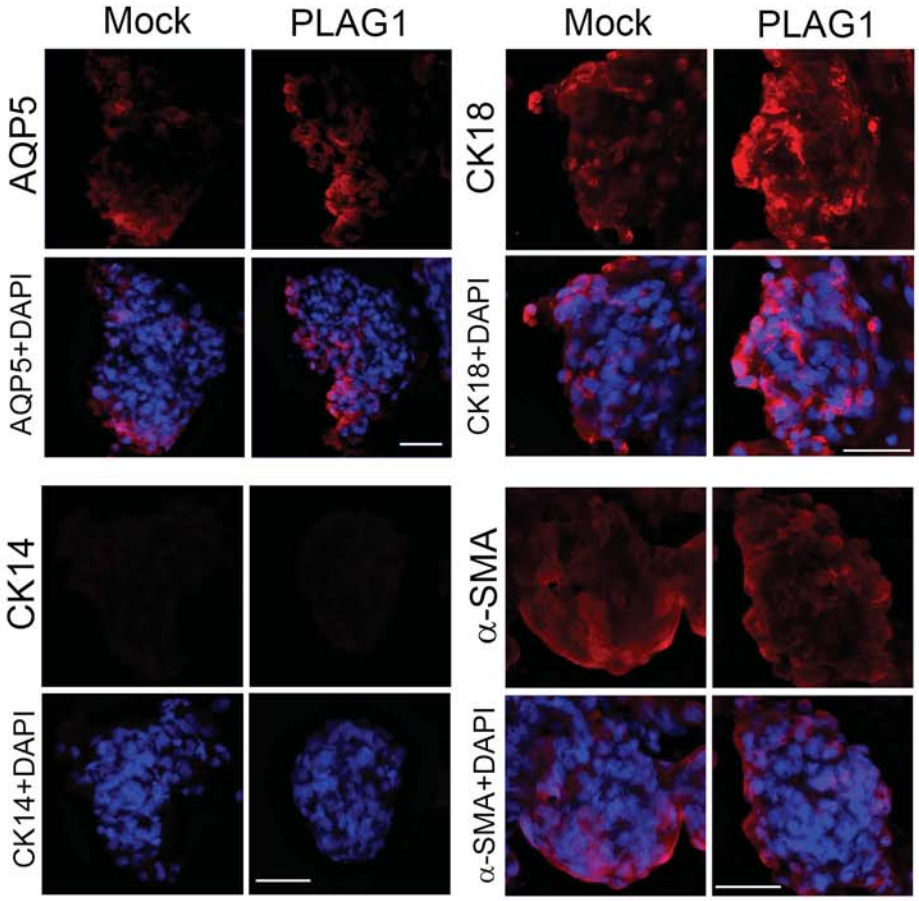
A


Mock
PLAG1

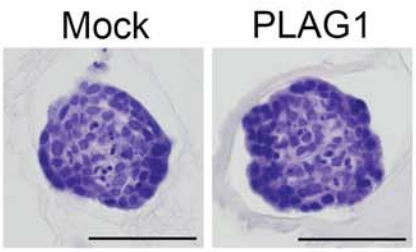
B


AQP5
(X0.00001)
**
Relative mRNA expression
Mock
PLAG1
CK18
(X0.001)
*
CK14
n.d.
n.d.
α-SMA
(X0.00001)
n.s.

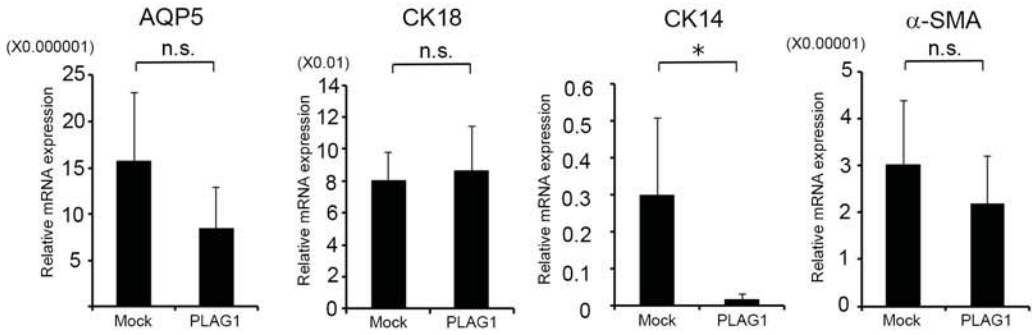
C


Mock
PLAG1
Mock
PLAG1
AQP5
CK18
AQP5+DAPI
CK18+DAPI
CK14
α-SMA
CK14+DAPI
α-SMA+DAPI

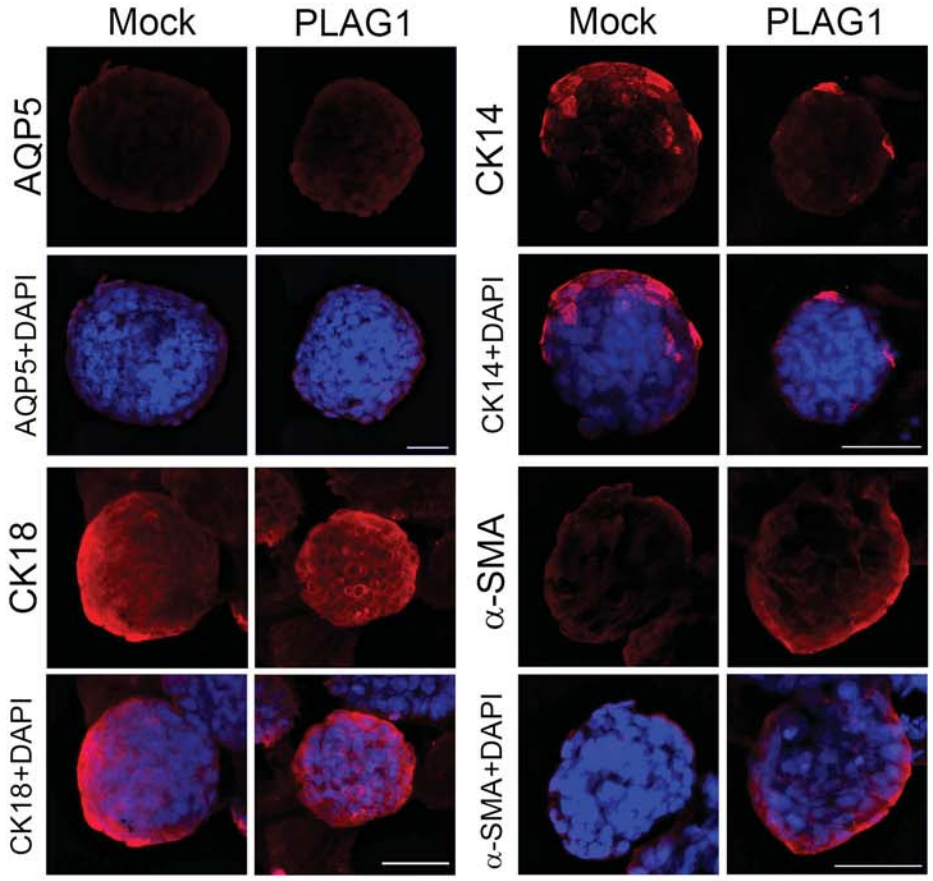
A


Mock
PLAG1

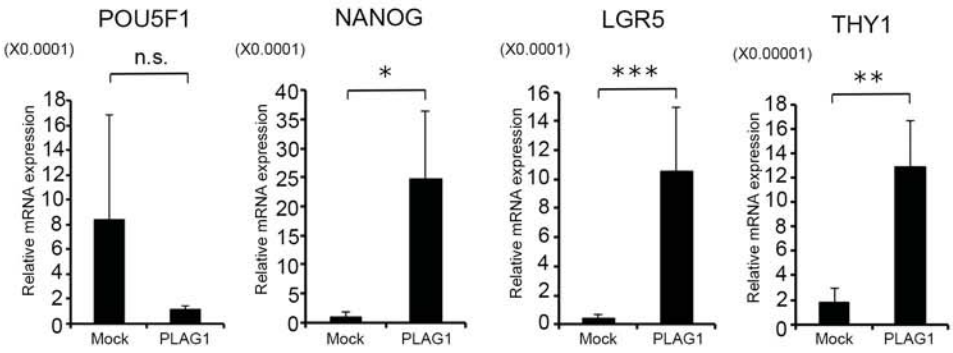
B


AQP5
(X0.000001)
n.s.
Relative mRNA expression
25
20
15
10
5
Mock
PLAG1
CK18
(X0.01)
n.s.
14
12
10
8
6
4
2
0
CK14
*
0.6
0.5
0.4
0.3
0.2
0.1
α-SMA
(X0.00001)
n.s.
5
4
3
2
1

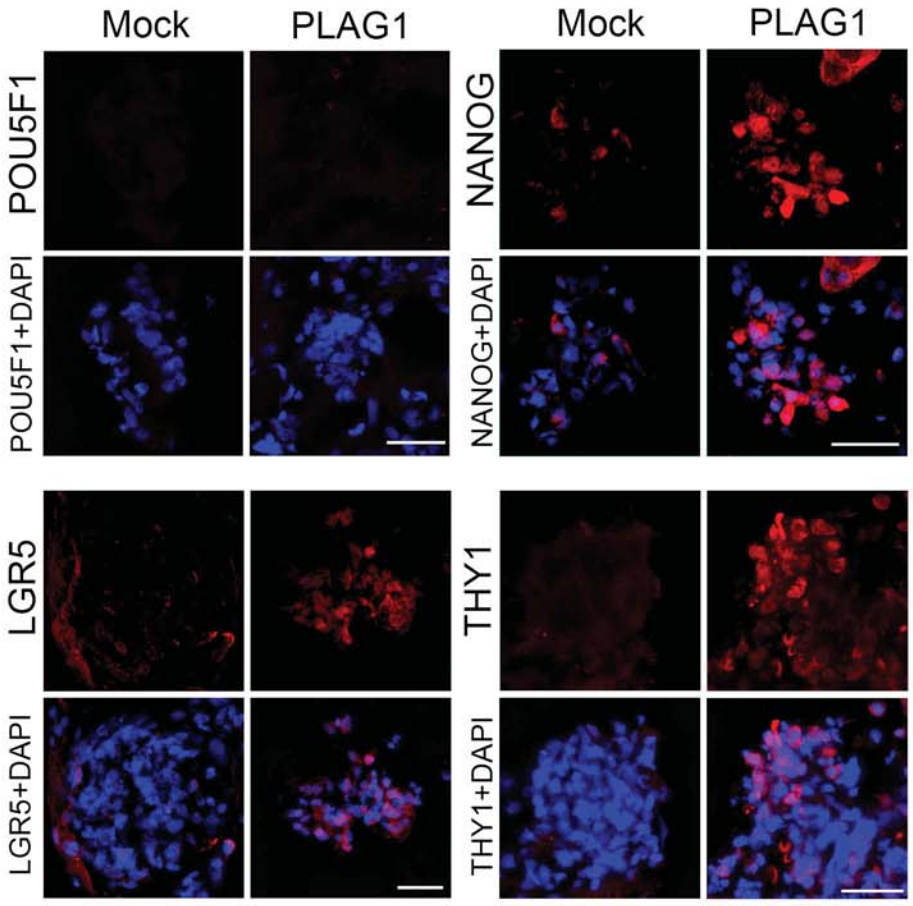
C


Mock
PLAG1
Mock
PLAG1
AQP5
CK14
AQP5+DAPI
CK14+DAPI
CK18
α-SMA
CK18+DAPI
α-SMA+DAPI

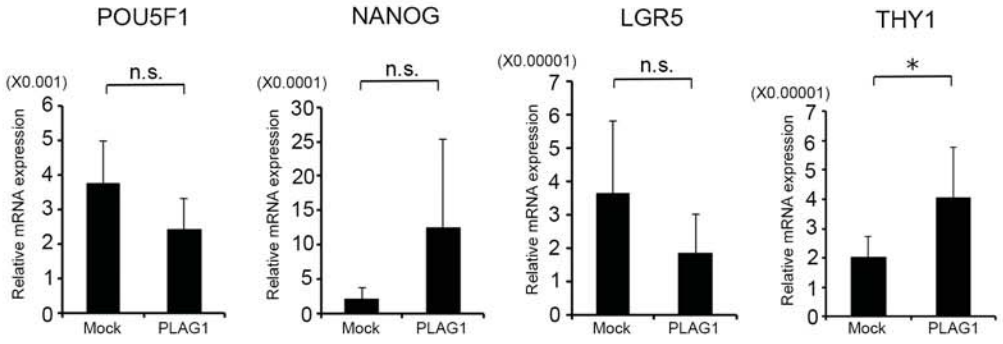
A


POU5F1
(X0.0001)
n.s.
Relative mRNA expression
Mock
PLAG1
NANOG
(X0.0001)
*
LGR5
(X0.0001)
***
THY1
(X0.00001)
**

B


Mock
PLAG1
POU5F1
POU5F1+DAPI
NANOG
NANOG+DAPI
LGR5
LGR5+DAPI
THY1
THY1+DAPI

A


POU5F1
NANOG
LGR5
THY1
(X0.001)
(X0.0001)
(X0.00001)
(X0.00001)
n.s.
n.s.
n.s.
*
Relative mRNA expression
Mock
PLAG1

B

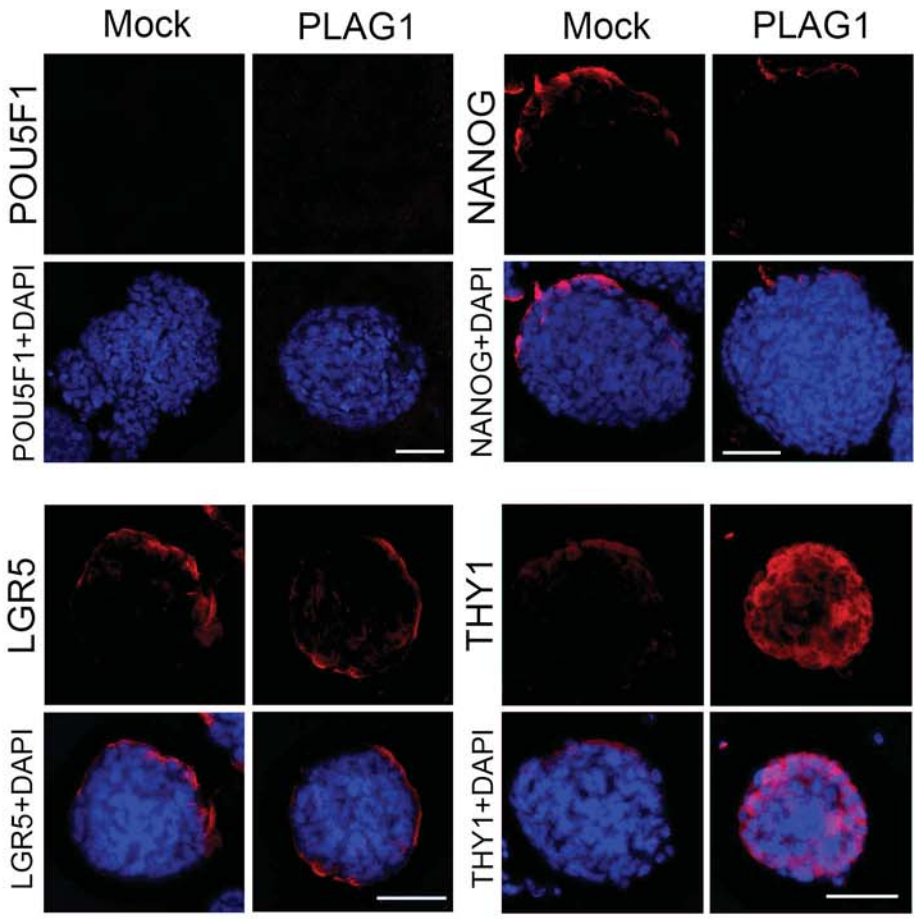

Mock
PLAG1
Mock
PLAG1
POU5F1
NANOG
POU5F1+DAPI
NANOG+DAPI
LGR5
THY1
LGR5+DAPI
THY1+DAPI

Table 1 Short list of the GO term enrichment analysis with annotation category of Kyoto Encyclopedia of Genes and Genomes pathways among up-regulated genes in acinar cells with PLAG1 overexpression

Pathway ID	Description	Gene name	P-value
hsa04015	Rap1 signaling pathway	FGF19, PRKCZ, ITGAL, FGF8, FLT1, FGFR3, PDGFB, PDGFA, ADORA2A, ADCY5, FGF17, GRIN1, PIK3CD, FGF11, FGF21, FGF12, RASSF5, LPAR5, ID1, RASGRP2, CALML6, RAPGEF3, NGFR, PLCB2, FGF3, FGF4	1.91E-07
hsa05200	Pathways in cancer	FGF19, WNT5A, FGF8, FGFR3, APC2, PDGFB, PDGFA, WNT3A, ADCY5, FGF17, GNG13, FGF11, FGF12, MMP1, SHH, GNG8, WNT1, RASGRP2, HHIP, AXIN2, PLCB2, FGF3, FGF4, CEBPA, RET, PIK3CD, BIRC7, FGF21, CBLC, RASSF5, WNT7B, LPAR5, WNT11, WNT7A	7.09E-06
hsa04014	Ras signaling pathway	FGF19, FGF8, FLT1, FGFR3, PDGFB, PDGFA, FGF17, GRIN1, PIK3CD, GNG13, FGF11, FGF21, FGF12, PAK6, GNG8, RASSF5, KSR2, RASGRP2, CALML6, NGFR, FGF3, FGF4	8.77E-05
hsa05031	Amphetamine addiction	ARC, GRIN2C, PPP1R1B, ADCY5, GRIN2D, GRIN1, TH, CALML6, CAMK2B, FOSB, CAMK2A	1.39E-04
hsa05217	Basal cell carcinoma	WNT5A, WNT1, WNT7B, APC2, WNT3A, WNT11, HHIP, AXIN2, WNT7A, SHH	1.41E-04
hsa05218	Melanoma	FGF19, FGF8, PDGFB, PDGFA, FGF17, PIK3CD, FGF11, FGF12, FGF21, FGF3, FGF4	2.58E-04
hsa04151	PI3K-Akt signaling pathway	FGF19, FGF8, FGFR3, PDGFB, PDGFA, STK11, FGF17, GNG13, FGF11, COL2A1, FGF12, GNG8, COMP, NOS3, PPP2R2C, FGF3, FGF4, IL2RB, FLT1, PIK3CD, NR4A1, FGF21, CD19, LPAR5, COL1A1, JAK3, NGFR	4.07E-04
hsa04010	MAPK signaling pathway	FGF19, FGF8, NTF4, FGFR3, PDGFB, PDGFA, FGF17, CACNG7, FGF11, CACNG4, NR4A1, FGF21, FGF12, DUSP2, RASGRP2, CACNA1G, MAPK8IP2, MAPK8IP1, DUSP9, CD14, FGF3, FGF4	4.21E-04
hsa04550	Signaling pathways regulating pluripotency of stem cells	WNT5A, FGFR3, APC2, WNT3A, PIK3CD, INHBB, WNT1, WNT7B, ID1, ID4, WNT11, JAK3, AXIN2, DUSP9, WNT7A	6.46E-04
hsa04514	Cell adhesion molecules	ITGAL, CADM3, CLDN9, NRXN2, CD8A, CLDN4, NFASC, LRRC4B, NTNG2, L1CAM, PDCD1LG2, PDCD1, CDH15, CD34, NLGN4X	7.45E-04

Table 2 Short list of the GO term enrichment analysis with annotation category of Kyoto Encyclopedia of Genes and Genomes pathways among up-regulated genes in ductal cells with PLAG1 overexpression

Pathway ID	Description	Gene name	P-value
hsa04151	PI3K-Akt signaling pathway	FGF19, FGF8, FLT1, FGF17, EFNA2, COL3A1, PIK3CD, GNG13, COL2A1, FGF21, FGF12, ITGB3, GNG8, CD19, CREB3L1, PDGFRB, NOS3, COL1A1, JAK3, PPP2R2C, FGF3, FGF4	2.35E-06
hsa04015	Rap1 signaling pathway	FGF19, ITGAL, FGF8, FLT1, ADORA2A, FGF17, EFNA2, ADCY5, GRIN1, PIK3CD, FGF12, ITGB3, FGF21, PDGFRB, CALML6, FGF3, FGF4	2.45E-06
hsa04916	Melanogenesis	WNT5A, WNT10A, WNT1, WNT5B, ADCY5, WNT3A, CREB3L1, CALML6, WNT11, POMC, WNT7A, CAMK2A	3.05E-06
hsa05031	Amphetamine addiction	ARC, GRIN2C, PPP1R1B, ADCY5, GRIN1, TH, CREB3L1, CALML6, CAMK2A	3.54E-05
hsa05200	Pathways in cancer	WNT5A, FGF19, WNT10A, RET, FGF8, WNT5B, FGF17, WNT3A, ADCY5, PIK3CD, BIRC7, GNG13, FGF21, FGF12, GNG8, WNT1, PDGFRB, WNT11, WNT7A, FGF3, FGF4	5.83E-05
hsa05218	Melanoma	FGF19, FGF8, FGF17, PIK3CD, PDGFRB, FGF12, FGF21, FGF3, FGF4	6.04E-05
hsa04014	Ras signaling pathway	FGF19, FGF8, FLT1, FGF17, EFNA2, GRIN1, PIK3CD, GNG13, FGF12, FGF21, GNG8, PDGFRB, CALML6, FGF3, FGF4	1.09E-04
hsa04261	Adrenergic signaling in cardiomyocytes	TNNT2, MYL3, ADCY5, ATP1A3, CACNG4, CREB3L1, SCN4B, CALML6, TNNI3, CAMK2A, PPP2R2C	3.15E-04
hsa05030	Cocaine addiction	GRM2, GRIN2C, PPP1R1B, ADCY5, GRIN1, TH, CREB3L1	3.30E-04
hsa04024	cAMP signaling pathway	HCN2, ADORA2A, ADCY5, PIK3CD, GRIN1, ATP1A3, TNNI3, PPP1R1B, GRIN2C, PDE4A, CREB3L1, CALML6, CAMK2A	4.20E-04