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Original Article

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

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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 PLAG1 roles in the acinar and ductal cells of normal human salivary glands to clarify the early events that occur during the histogenesis of salivary gland tumors.
Methods: Normal salivary gland cells with acinar and ductal phenotypes were transfected with PLAG1 plasmid DNA. Subsequently, PLAG1 overexpressed and mock cells were examined by cell proliferation, transwell migration, and salisphere formation assays. Differentiation and salivary and pluripotent stem cell marker expression levels were evaluated by quantitative reverse transcription-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.

1. Introduction

Pleomorphic adenoma gene 1 (PLAG1) is a crucial oncogene involved in the development of pleomorphic adenomas in salivary glands [1]. Tumorigenesis is caused by the swapping of promoters between PLAG1 and beta-catenin through chromosomal reciprocal translocations [2]. These events cause PLAG1 ectopic overexpression, which is driven by the constitutively active beta-catenin promoter. The definite oncogenic capacity of PLAG1 has been demonstrated in vivo [3]. Human insulin-like growth factor (IGF) 2 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

Abbreviations: α-SMA, alpha-smooth muscle actin; AQP5, aquaporin 5; CAGE, cap analysis of gene expression; CK14, cytokeratin 14; CK18, cytokeratin 18; Ct, cycle threshold; DAPI, 4′-6-diamidino-2-phenylindole; EGF, epithelial growth factor; FGF, fibroblast growth factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; HE, hematoxylin and eosin; HSPC, hematopoietic stem/progenitor cells; IGF2, human insulin-like growth factor 2; KEGG, the Kyoto Encyclopedia of Genes and Genomes; LGR5, leucine-rich repeat-containing G-protein-coupled protein 5; PLAG1, pleomorphic adenoma gene 1; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SD, standard deviation; Wnt, Wingless/integrated.

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overexpress PLAG1. PLAG1 influences tumorigenesis, at least in part, via the mitogenic action of IGF2, presumably by the activation of IGF1 receptor (IGF1R) and the Ras/Raf/Mitogen-activated protein kinase (MAPK) signaling pathway in NIH3T3 cells [4,5].

The histogenesis of salivary gland tumors is not well understood because they are generally well-developed during surgical resection. Among the few hypothetical concepts, the most novel concept 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 [6]. 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 [7]. These results overlap with recent evidence suggesting that differentiated acinar cells are self-duplicating and maintained without significant input from stem/progenitor cells in the intercalated duct in adult salivary glands [8,9]. Furthermore, it has been shown that there are more than one progenitor cell population in the adult salivary gland [10]. 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 [11,12].

In this study, we report the roles of PLAG1 in acinar and ductal cells of normal human salivary glands 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′-ends of the full-length cDNAs by PCR amplification using the following primers: 5′-GAATTCCGCCACTGTCATTCCTGGT-3′ (for the addition of the EcoRI site to the 5′-end) and 5′-AGATCTCTACTGAAAAGCTTGATGGAA-3′ (for the addition of the BglII site to the 3′-end). The PCR product was subcloned by insertion into a TOPO TA vector (Thermo Fisher 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 [13]. 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. The cells were grown to 70–80% confluency and transfected with the human PLAG1 construct or an empty vector (negative control) using the FUGENE HD transfection reagent (Promega Corporation, WI, USA). At 24 h after transfection, photographs of the cell morphology were captured using the Keyence BZ-X710 microscope (Keyence, Tokyo, Japan; Fig. 1A). After confirming PLAG1 overexpression in the cells by immunoblotting (Fig. 1B), the transfected cells were used for subsequent analysis.

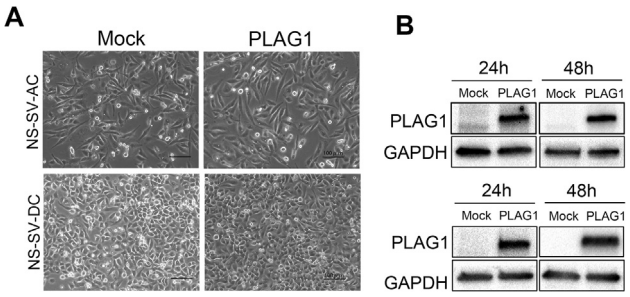

Fig. 1. PLAG1 overexpression does not affect the morphology of acinar and ductal cells. Acinar (NS-SV-AC) and ductal (NS-SV-DC) cells were transfected with PLAG1 and empty vector using the lipofection method (A) Cell morphology of acinar (upper) and ductal (lower) cells 24 h after transfection. Scale bar = 100 μm (B) Immunoblotting of acinar and ductal cells 24 and 48 h after PLAG1 transfection. GAPDH was used as the control.

2.3. Western blot analysis

The cells were washed with cold phosphate-buffered saline (Takara, Shiga, Japan) and lysed in radioimmunoprecipitation assay buffer (RIPA buffer; Nacarai Tesque). After centrifugation, the supernatant was collected and protein concentrations were determined by microplate assay (DC Protein Assay Kit, Bio-Rad Laboratories, CA, USA). Five micrograms of protein were loaded and resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membrane was blocked in 2.5% ECL Blocking Agent (GE Healthcare, CT, USA) for 30 min and then probed with mouse monoclonal anti-human PLAG1 primary antibody (Abnova, Taipei, Taiwan) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody (Santa Cruz Biotechnology, CA, USA) at 25 °C for 1 h. After washing in Tris-buffered saline (Takara, Kyoto, Japan) containing 0.1% tween 20 (Sigma–Aldrich), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Buckinghamshire, UK) at 25 °C for 1 h. The antibody used for GAPDH detection was diluted with Can Get Signal solution (TOYOBO CO., Osaka, Japan). The blots were visualized with ECL or ECL Select Western Blotting Detection Reagent (GE Healthcare) followed by scanning with the ChemiDoc XRS Plus System and analysis with Image Lab Software (Bio-Rad Laboratories).

2.4. Cell proliferation assay

Human PLAG1 or empty vector-transfected cells were plated into a 96-well plate (1×10^4 cells per well), and after 5 days of culture, assessed by addition of 10 μl Cell Count Reagent SF (Nacalai Tesque), 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, 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 cell culture inserts (Corning, NY, USA). The inserts were coated with BD Matrigel Basement Membrane Matrix (Corning) 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 chamber 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. 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) and counted.

2.6. Salisphere culture

The transfected cells were suspended in DMEM/F12 (Gibco, NY, USA) containing antibiotic and N2 supplement (Gibco) along with 20 ng/ml human recombinant fibroblast growth factor 2 (FGF2) and EGF, 10 μg/ml insulin, and 1 μM dexamethasone (Sigma–Aldrich) and mixed with Corning Matrigel Basement Membrane Matrix in a 2:1 ratio. The cells were plated in a 24-well plate (density, 1×10^4 cells per well) and cultured for 12–14 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).

2.7. Immunohistochemistry

The salispheres were embedded in ipGell (Genostaff, Tokyo, Japan) and fixed with 4% paraformaldehyde (Nacalai Tesque). After sucrose replacement, the fixed salispheres were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and sectioned (thickness, 6 μm). The sections were stained with hematoxylin and eosin (HE), or incubated with primary aquaporin 5 (AQP5; Invitrogen, Waltham, USA), cytokeratin 18 (CK18; Protein Tech, IL, USA), cytokeratin 14 (CK14; AbD Serotec Ltd., Oxford, UK), alpha-smooth muscle actin (α-SMA; GeneTex International Corporation, Hsinchu, Taiwan), POU5F1 (Abcam, Cambridge, UK), NANOG (Abcam), leucine-rich repeat-containing G-protein-coupled protein 5 (LGR5; Invitrogen), and THY1 (Abcam) antibodies. For the immunofluorescent staining, the sections were incubated with Alexa Flour anti-mouse IgG 568 (Abcam) or anti-Rabbit IgG 594 (Invitrogen) and stained with 4′-6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich). The stained images were observed using the Keyence microscope BZ-X710.

2.8. Quantitative reverse transcription-polymerase chain reaction (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). qRT-PCR was carried out in triplicate using a 7500 Real-Time PCR system (Applied Biosystems). Nine μl diluted cDNA, 1 μl PCR primer set, and 10 μl 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′-TGGTCACCACA-CAGTCTGCT -3′ and reverse: 5′-CCAAGGCATCACCAAGATTA -3′; CK14, forward: 5′-TGAGCCGCATTCTGAACGAG-3′ and reverse: 5′-GATGACTGCGATCCAGAGGA-3′; α-SMA, forward: 5′-CGATAGAA-CACGGCATCATC-3′ and reverse: 5′-CATCAGGCAGTTCGTAGCTC -3′; POU5F1, forward: 5′-AAGCGATCAAGCAGCGACTA-3′ and reverse: 5′-GAAGTGAGGGCTCCCATAGC-3′; NANOG, forward: 5′-CAGAAAAA-CAACTGGCCGAA -3′ and reverse: 5′-GGCCTGATTGTTCCAGGATT-3′; LGR5, forward: 5′-AGGATCTGGTGAGCCTGAGAA-3′ and reverse: 5′-CATAAGTGATGCTGGAGCTGGTAA-3′; THY1, forward: 5′-GACAGCCT-GAGAGGGTCTTG-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). 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)

CAGE library preparation, sequencing, mapping, and gene expression analysis were performed (DNAFORM). In brief, RNA quality was assessed with a Bioanalyzer (Agilent, CA, USA) to ensure that the RIN (RNA integrity number) was above 7.0 and the A260/280 and 260/230 ratios were 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 [14] using the Paraclu algorithm [15] with default parameters. The upregulated genes in PLAG1 transfected acinar and ductal cells than in the mock cells were identified with a threshold log2 fold change of >2.0, whereas the downregulated genes in those 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 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 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 promotes 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 promoted the transwell migration of both acinar and ductal cells (Figs. 2B and C).

3.2. PLAG1 enhances luminal cell-like profiles only in acinar cells

We utilized a salisphere culture that has been used to grow adult salivary gland epithelial cells into 3D clusters for in vitro differentiation studies [16]. In salispheres composed of acinar cells, PLAG1 induced the mRNA expression of AQP5 and CK18, but not of CK14 and α-SMA (Fig. 3B). These results were compatible with those of

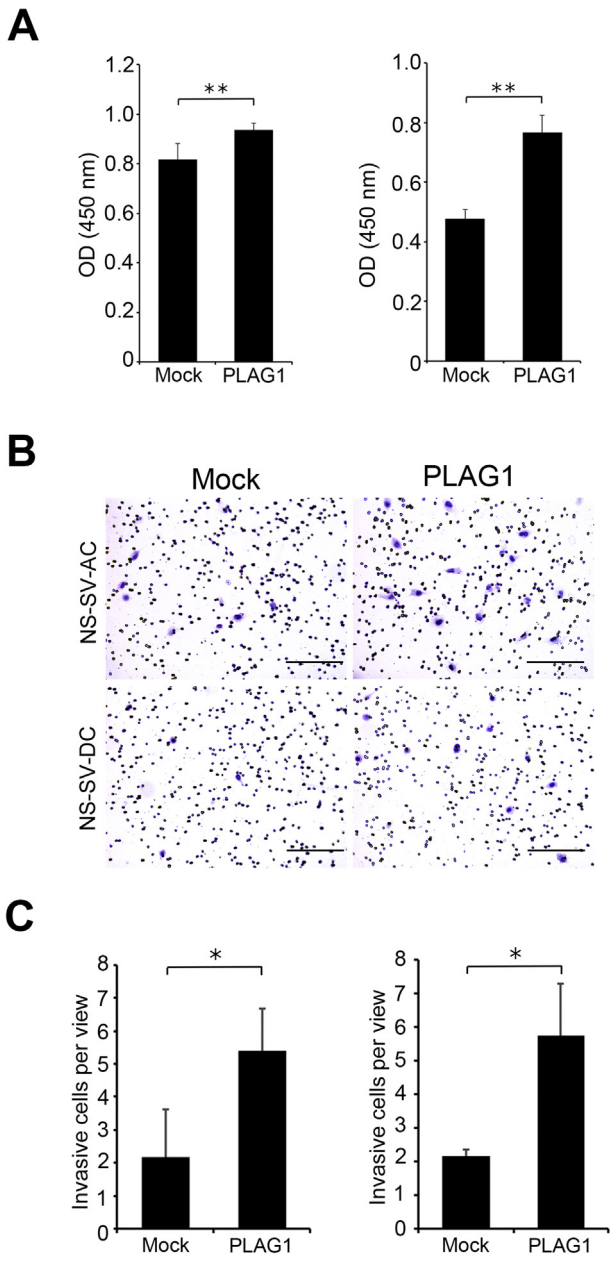

Fig. 2. PLAG1 promotes proliferation and migration of acinar and ductal cells (A) Cell proliferation assay of acinar and ductal cells transfected with PLAG1. The absorbance of acinar (left) and ductal (right) cells at 450 nm were measured five days after seeding the cells transfected with PLAG1 or an 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. Scale bar = 200 μm. All values are shown as mean ± standard deviation (SD) of septuplicate (A) or quadruplicate (C) measurements. *P < 0.05; **P < 0.01.

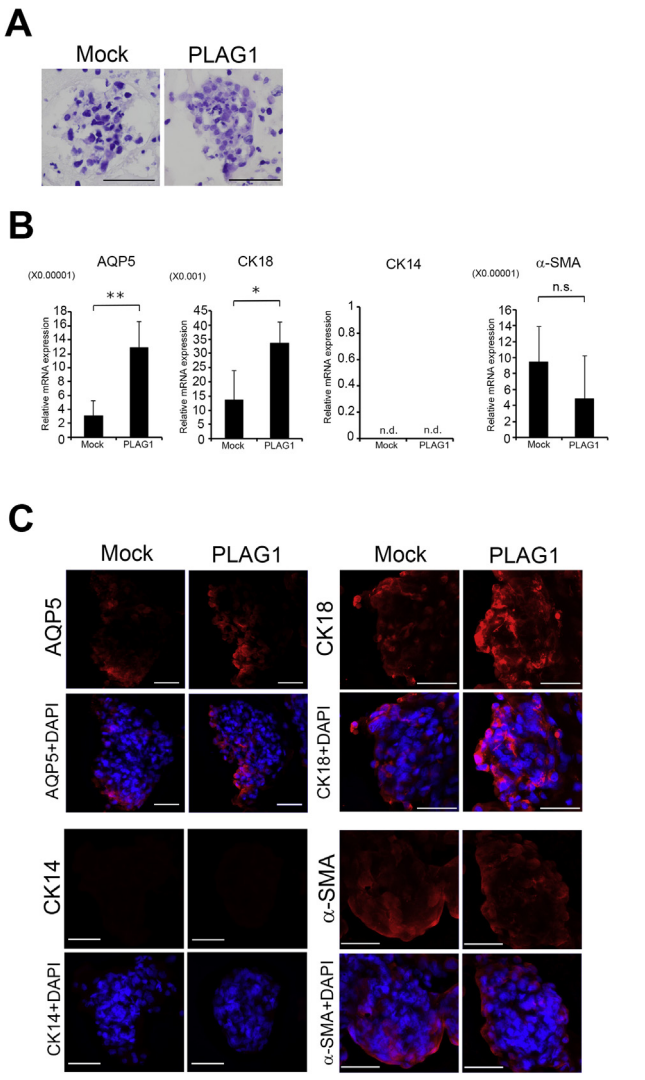

Fig. 3. PLAG1 enhances luminal cell-like profiles in acinar cells (A) Hematoxylin and Eosin (HE) staining of salispheres formed from PLAG1-transfected acinar cells (B) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis on salispheres formed from PLAG1-transfected acinar cells. All values are shown as 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 α-SMA (red) in salispheres formed from PLAG1-transfected acinar cells. The nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI; blue). The salispheres for all experiments were cultured for 12 or 13 days. Scale bar = 50 μm.

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 of 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.

3.3. *PLAG1 enhances the stemness profile in acinar cells*

Several stem/progenitor cell markers have been reported in human salivary gland cells [17–19]. Interestingly, the mRNA and protein expression levels of NANOG, LGR5, and THY1 were markedly upregulated in the acinar cells (Fig. 5), whereas in the ductal cells, only THY1 was significantly upregulated (Fig. 6).

3.4. *Genes participating in cancer- and stemness-related pathways were among the differentially upregulated genes in acinar cells with PLAG1 overexpression*

CAGE analysis revealed 849 upregulated and 120 downregulated genes in the acinar cells and 445 upregulated and 80 downregulated genes in the ductal cells that overexpressed PLAG1. The results of the GO term analysis indicated that the genes

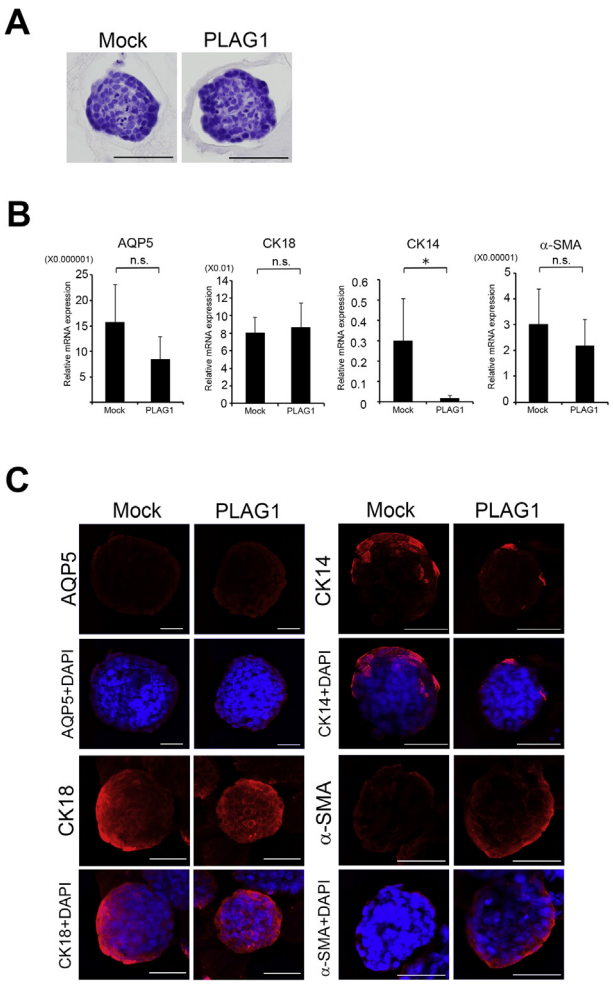

Fig. 4. PLAG1 slightly reduces the basal cell-like character in ductal cells (A) HE staining of salisphere formed from PLAG1-transfected ductal cells (B) qRT-PCR analysis of salispheres formed by PLAG1-transfected ductal cells. All values shown are mean ± standard deviation (SD; n = 4–6). *P < 0.05;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

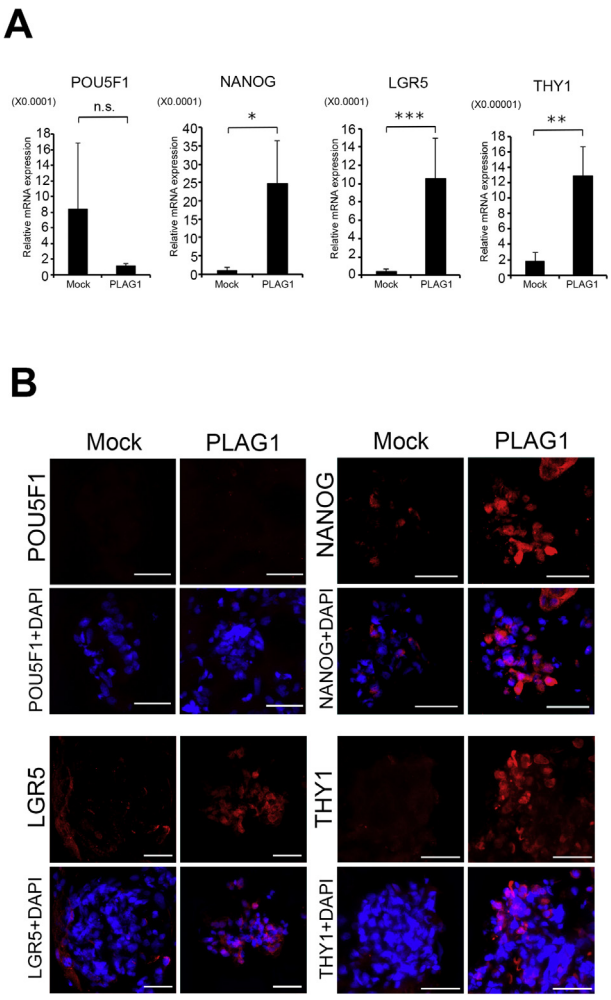

Fig. 5. PLAG1 enhanced the stemness profiles in acinar cells (A) qRT-PCR analysis of stem cell markers. All values shown are mean ± standard deviation (SD; n = 3–6). *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant (B) Immunofluorescence staining of POU5F1, NANOG, LGR5, and THY1 (red) in salisphere formed by PLAG1-transfected acinar cells. The nuclei were stained with DAPI (blue). The salispheres for all experiments were cultured for 12 or 13 days. Scale bar = 50 μm.

upregulated in PLAG1-overexpressed acinar cells predominantly participated in cancer- and stemness-related pathways (Table 1). Likewise, the genes upregulated in PLAG1-overexpressed ductal cells appeared to predominantly participate in the cancer-related pathways (Table 2).

4. Discussion

Little is known about the involvement of PLAG1 in stemness. PLAG1 is known to play a potential role in stemness in the hematopoietic [20] and neuronal systems [21]; 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 PLAG1 overexpression 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 stemness induction in normal human salivary gland cells remains unclear. Wingless/integrated (Wnt) is a key molecule in most types of stem cells in adult mammals [22]. Earlier studies have demonstrated that the Wnt signaling pathway is activated through β-catenin upregulation in salivary gland tumors induced by PLAG1 overexpression in transgenic mice [23]. Therefore, it is suggested that the Wnt/β-catenin signaling pathway plays 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 upregulated 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 PLAG1-overexpressed acinar cells. Recently, it has been reported that the Rap1 activation status in hematopoietic stem/progenitor cells (HSPCs) is important for maintaining HSPCs at the bone marrow niche for long-term hematopoiesis in adults [24]. 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 neuronal gene expression regulator and causes neuronal differentiation in neural progenitor

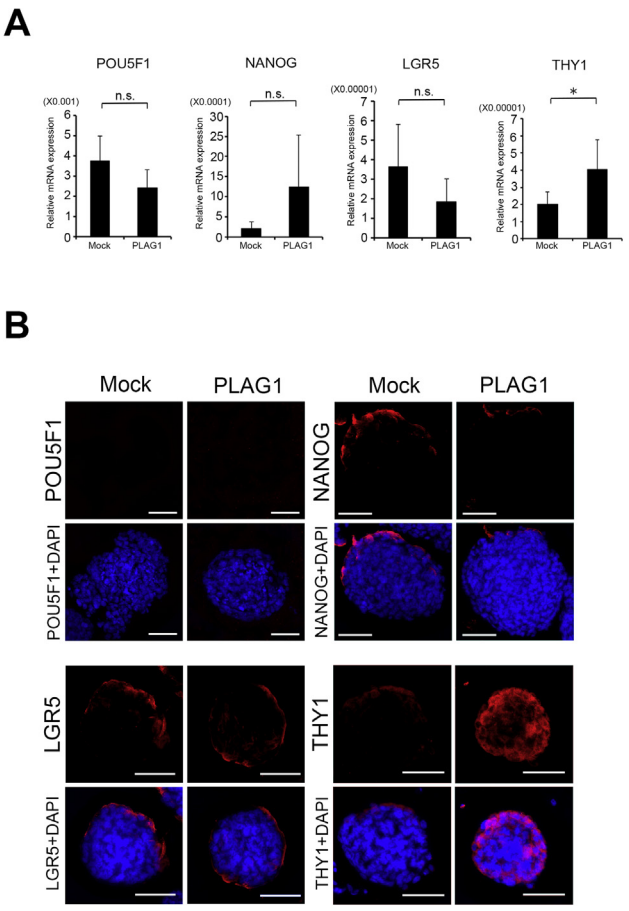

Fig. 6. PLAG1 increases THY1 expression in ductal cells (A) qRT-PCR analysis of stem cell markers. All values shown are mean ± standard deviation (SD; n = 4–6). *P < 0.05; n.s., not significant (B) Immunofluorescence staining of POU5F1, NANOG, LGR5, and THY1 (red) in salispheres formed by PLAG1-transfected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

cells in the developing mouse neocortex [25]. 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 [26]. Regulation of zygotic genome activation is thought to be closely involved with pluripotency control and lineage differentiation [26].

The constitutive renewal and aberrant expansion of the stem cell pool caused by uncontrolled Wnt signaling are required for the development of cancerous tissues [27]. Thus, PLAG1 may aid 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 due to uncontrolled Wnt signaling.

Our results showed that PLAG1 overexpression 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 [28]. The roles of PLAG1 in IGF2 regulation in Hep3B and JEG-3 cells are different [28]. 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 [28]. Epigenetic control at the H19–IGF2 locus for the maintenance of adult stem cells has been demonstrated in hematopoietic cells [29]. Furthermore, DNA methylation is required for stem cell differentiation control in the digestive system [30]. 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 [5]. 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 [5]. PLAG1 has been reported to inhibit cell migration through miR-218-1 by directly targeting RET and PLAG1 [31]. The GO term enrichment analysis in

Table 1
Short list of the gene ontology term enrichment analysis with annotation category of Kyoto Encyclopedia of Genes and Genomes pathways among upregulated 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 gene ontology term enrichment analysis with annotation category of Kyoto Encyclopedia of Genes and Genomes pathways among upregulated 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

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) [32]. In addition, almost all ACCs overexpressed c-Kit [33,34], which is thought to be a therapeutic molecular target in ACCs [35–37]. The PI3K/AKT, MAPK, and Ras/Raf pathways are known to be the downstream effectors of c-Kit [36]. The Ras/RAF/MEK/MAPK and PIK3/AKT/mTOR pathways are highly conserved pathways necessary for growth, proliferation, differentiation [38], and tumorigenesis [39,40]. Moreover, the PI3K and MAPK pathways mediate salivary gland branching [41]. It has been 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. 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.

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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Conflicts of interest

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

CRediT authorship contribution statement

Yuriko Goto: Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation, Writing - original draft. **Miho Ibi:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation, Writing - original draft. **Hirotaka Sato:** Methodology, Visualization. **Junichi Tanaka:** Methodology, Resources. **Rika Yasuhara:** Methodology, Resources. **Keiko Aota:** Resources. **Masayuki Azuma:** Resources. **Toshiyuki Fukada:** Methodology, Resources, Formal analysis, Funding acquisition. **Kenji Mishima:** Methodology, Resources, Formal analysis. **Tarou Irié:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

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References

[1] Van Dyck F, Declercq J, Braem CV, Van de Ven WJ. PLAG1, the prototype of the PLAG gene family: versatility in tumour development (review). Int J Oncol 2007;30:765–74.
[2] Kas K, Voz ML, Roijer E, Astrom AK, Meyen E, Stenman G, et al. Promoter swapping between the genes for a novel zinc finger protein and beta-catenin in pleiomorphic adenomas with t(3;8)(p21;q12) translocations. Nat Genet 1997;15:170–4.
[3] Declercq J, Van Dyck F, Braem CV, Van Valckenborgh IC, Voz M, Wassef M, et al. Salivary gland tumors in transgenic mice with targeted PLAG1 proto-oncogene overexpression. Can Res 2005;65:4544–53.
[4] Juma AR, Damdimopoulou PE, Grommen SV, Van de Ven WJ, De Groef B. Emerging role of PLAG1 as a regulator of growth and reproduction. J Endocrinol 2016;228:R45–56.
[5] Hensen K, Van Valckenborgh IC, Kas K, Van de Ven WJ, Voz ML. The tumorigenic diversity of the three PLAG family members is associated with different DNA binding capacities. Can Res 2002;62:1510–7.
[6] Dwivedi N, Agarwal A, Raj V, Chandra S. Histogenesis of salivary gland neoplasms. Indian J Canc 2013;50:361–6.
[7] Denny PC, Chai Y, Klauser DK, Denny PA. Three-dimensional localization of DNA synthesis in secretory elements of adult female mouse submandibular gland. Adv Dent Res 1990;4:34–44.
[8] Aure MH, Konieczny SF, Ovitt CE. Salivary gland homeostasis is maintained through acinar cell self-duplication. Dev Cell 2015;33:231–7.

[9] Aure MH, Arany S, Ovitt CE. Salivary glands: stem cells, self-duplication, or both? J Dent Res 2015;94:1502–7.
[10] Arany S, Catalan MA, Roztocil E, Ovitt CE. Ascl3 knockout and cell ablation models reveal complexity of salivary gland maintenance and regeneration. Dev Biol 2011;353:186–93.
[11] Adams A, Warner K, Nor JE. Salivary gland cancer stem cells. Oral Oncol 2013;49:845–53.
[12] Emmerson E, Knox SM. Salivary gland stem cells: a review of development, regeneration and cancer. Genesis 2018;56:e23211 (New York, NY : 2000).
[13] Azuma M, Tamatani T, Kasai Y, Sato M. Immortalization of normal human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous phenotype by transfection with SV40 ori-mutant deoxyribonucleic acid. Lab Invest 1993;69:24–42.
[14] Haberle V, Forrest AR, Hayashizaki Y, Carninci P, Lenhard B. CAGEr: precise TSS data retrieval and high-resolution promoterome mining for integrative analyses. Nucleic Acids Res 2015;43:e51.
[15] Frith MC, Valen E, Krogh A, Hayashizaki Y, Carninci P, Sandelin A. A code for transcription initiation in mammalian genomes. Genome Res 2008;18:1–12.
[16] Pringle S, Maimets M, van der Zwaag M, Stokman MA, van Gosliga D, Zwart E, et al. Human salivary gland stem cells functionally restore radiation damaged salivary glands. Stem Cell 2016;34:640–52 (Dayton, Ohio).
[17] Pringle S, Van Os R, Coppes RP. Concise review: adult salivary gland stem cells and a potential therapy for xerostomia. Stem Cell 2013;31:613–9 (Dayton, Ohio).
[18] Yi T, Lee S, Choi N, Shin HS, Kim J, Lim JY. Single cell clones purified from human parotid glands display features of multipotent epitheliomesenchymal stem cells. Sci Rep 2016;6:36303.
[19] Shin HS, Lee S, Hong HJ, Lim YC, Koh WG, Lim JY. Stem cell properties of human clonal salivary gland stem cells are enhanced by three-dimensional priming culture in nanofibrous microwells. Stem Cell Res Ther 2018;9:74.
[20] Belew MS, Bhatia S, Keyvani Chahi A, Rentas S, Draper JS, Hope KJ. PLAG1 and USF2 co-regulate expression of Musashi-2 in human hematopoietic stem and progenitor cells. Stem Cell Rep 2018;10:1384–97.
[21] Alam S, Zinyk D, Ma L, Schuurmans C. Members of the Plag gene family are expressed in complementary and overlapping regions in the developing murine nervous system. Dev Dynam 2005;234:772–82.
[22] Nusse R, Clevers H. Wnt/beta-catenin signaling, disease, and emerging therapeutic modalities. Cell 2017;169:985–99.
[23] Zhao X, Ren W, Yang W, Wang Y, Kong H, Wang L, et al. Wnt pathway is involved in pleomorphic adenomas induced by overexpression of PLAG1 in transgenic mice. Int J Canc 2006;118:643–8.
[24] Imai T, Tanaka H, Hamazaki Y, Minato N. Rap1 signal modulators control the maintenance of hematopoietic progenitors in bone marrow and adult long-term hematopoiesis. Canc Sci 2019;110:1317–30.
[25] Sakai H, Fujii Y, Kuwayama N, Kawaji K, Gotoh Y, Kishi Y. Plag1 regulates neuronal gene expression and neuronal differentiation of neocortical neural progenitor cells. Gene Cell : devoted to molecular & cellular mechanisms 2019;24:650–66.
[26] Madissoon E, Damdimopoulos A, Katayama S, Krjutskov K, Einarsdottir E, Mamia K, et al. Pleomorphic adenoma gene 1 is needed for timely zygotic genome activation and early embryo development. Sci Rep 2019;9:8411.
[27] Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature 2005;434:843–50.
[28] Akhtar M, Holmgren C, Gondor A, Vesterlund M, Kanduri C, Larsson C, et al. Cell type and context-specific function of PLAG1 for IGF2 P3 promoter activity. Int J Oncol 2012;41:1959–66.
[29] Venkatraman A, He XC, Thorvaldsen JL, Sugimura R, Perry JM, Tao F, et al. Maternal imprinting at the H19-Igf2 locus maintains adult haematopoietic stem cell quiescence. Nature 2013;500:345–9.
[30] Sheaffer KL, Kim R, Aoki R, Elliott EN, Schug J, Burger L, et al. DNA methylation is required for the control of stem cell differentiation in the small intestine. Gene Dev 2014;28:652–64.
[31] Tang W, Tang J, He J, Zhou Z, Qin Y, Qin J, et al. SLIT2/ROBO1-miR-218-1-RET/PLAG1: a new disease pathway involved in Hirschsprung's disease. J Cell Mol Med 2015;19:1197–207.
[32] He Q, Zhou X, Li S, Jin Y, Chen Z, Chen D, et al. MicroRNA-181a suppresses salivary adenoid cystic carcinoma metastasis by targeting MAPK-Snai2 pathway. Biochim Biophys Acta 2013;1830:5258–66.
[33] Mino M, Pilch BZ, Faquin WC. Expression of KIT (CD117) in neoplasms of the head and neck: an ancillary marker for adenoid cystic carcinoma. Mod Pathol 2003;16:1224–31.
[34] Holst VA, Marshall CE, Moskaluk CA, Frierson Jr HF. KIT protein expression and analysis of c-kit gene mutation in adenoid cystic carcinoma. Mod Pathol 1999;12:956–60.
[35] Hotte SJ, Winquist EW, Lamont E, MacKenzie M, Vokes E, Chen EX, et al. Imatinib mesylate in patients with adenoid cystic cancers of the salivary glands expressing c-kit: a Princess Margaret Hospital phase II consortium study. J Clin Oncol 2005;23:585–90.
[36] Tetsu O, Phuchareon J, Chou A, Cox DP, Eisele DW, Jordan RC. Mutations in the c-Kit gene disrupt mitogen-activated protein kinase signaling during tumor development in adenoid cystic carcinoma of the salivary glands. Neoplasia 2010;12:708–17 (New York, NY).
[37] Bruce IA, Slevin NJ, Homer JJ, McGown AT, Ward TH. Synergistic effects of imatinib (STI 571) in combination with chemotherapeutic drugs in head and neck cancer. Anti Canc Drugs 2005;16:719–26.
[38] Shalmon B, Drendel M, Wolf M, Hirshberg A, Cohen Y. Mutational analysis of PI3K/AKT and RAS/RAF pathway activation in malignant salivary gland tumours with a new mutation of PIK3CA. Int J Oral Maxillofac Surg 2016;45:721–5.
[39] Aravalli RN, Cressman EN, Steer CJ. Cellular and molecular mechanisms of hepatocellular carcinoma: an update. Arch Toxicol 2013;87:227–47.
[40] Chen MH, Lin KJ, Yang WL, Kao YW, Chen TW, Chao SC, et al. Gene expression-based chemical genomics identifies heat-shock protein 90 inhibitors as potential therapeutic drugs in cholangiocarcinoma. Cancer 2013;119:293–303.
[41] Patel VN, Rebustini IT, Hoffman MP. Salivary gland branching morphogenesis. Differentiation 2006;74:349–64.