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Regular Article

Metabolic Reprogramming Drives Pituitary Tumor Growth through Epigenetic Regulation of TERT

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Pituitary adenomas are common, benign brain tumors. Some tumors show aggressive phenotypes including early recurrence, local invasion and distant metastasis, but the underlying mechanism to drive the progression of pituitary tumors has remained to be clarified. Aerobic glycolysis known as the Warburg effect is one of the emerging hallmarks of cancer, which has an impact on the tumor biology partly through epigenetic regulation of the tumor-promoting genes. Here, we demonstrate metabolic reprogramming in pituitary tumors contributes to tumor cell growth with epigenetic changes such as histone acetylation. Notably, a shift in histone acetylation increases the expression of telomerase reverse transcriptase (TERT) oncogene, which drives metabolism-dependent cell proliferation in pituitary tumors. These indicate that epigenetic changes could be the specific biomarker for predicting the behavior of pituitary tumors and exploitable as a novel target for the aggressive types of the pituitary tumors.

Key words: pituitary tumors, metabolic reprogramming, epigenetics, histone acetylation, TERT

I. Introduction

Metabolic reprogramming is an emerging, core hallmark of cancer, which meets an energetic demand of proliferating tumor cells [4, 6]. Recently, epigenetic changes including histone modifications through metabolic reprogramming were reported to be involved in neoplastic development, including pituitary tumorigenesis [8, 10, 20, 29, 30].

Most of the pituitary tumors are benign pituitary adenoma (PA) and accounts for 10–15% of intracranial tumors. Some PAs exhibit clinically aggressive behavior, and "aggressive pituitary tumors" or "high-risk PAs" (formerly designated as "atypical adenomas") are defined to refer to pituitary adenomas with rapid growth, resistance to conventional therapies and/or early/multiple recurrences although reliable pathological predictors or specific diagnostic criteria are absent [26, 27]. There is also a malignant pituitary carcinoma which represents 0.1–0.2% of all pituitary tumors and shows a 1-year mortality rate of 66% [26]. Tumorigenesis of pituitary tumors has remained to be well clarified, but recent studies have revealed the genetic basis of pituitary tumorigenesis in hereditary syndromes such as multiple endocrine neoplasia type 1 (MEN1), Carney complex and familial-isolated PA (FIPA), and sporadic cases with somatic mutations including *USP8*, *USP48*, *BRAF* and *GNAS* [5, 30]. Meanwhile, increasing evidences have shown the relationship between aggressiveness in pituitary tumors and epigenetic dysregulation such as a shift in histone modifications, DNA methylation changes in the oncogene promoters, and post-transcriptional regulation of the genes by microRNAs [29, 30]. Thus, genetic hits

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Table 1. *Antibodies used for immunohistochemical staining and Western blotting*

Antibodies	Source	Catalog number	Antibody dilution
H3K9ac	Cell Signaling	#9649	WB 1:1000, IHC 1:800, ICC 1:400
H3K18ac	Cell Signaling	#9675	WB 1:1000, IHC 1:800
H3K27ac	Cell Signaling	#4353	WB 1:1000, IHC 1:800
H3K4me2	Cell Signaling	#9725	WB 1:1000, IHC 1:1500
H3K9me2	Cell Signaling	#4658	WB 1:1000, IHC 1:200
H3K27me3	Cell Signaling	#9733	WB 1:1000, IHC 1:200
Total histone H3	Cell Signaling	#4499	WB 1:2000
GAPDH	Cell Signaling	#5174	WB 1:1000
Cleaved caspase-3	Cell Signaling	#9661	WB 1:1000
HRP-linked anti-rabbit IgG	Cell Signaling	#7074	WB 1:5000
HRP-linked anti-mouse IgG	Cell Signaling	#7076	WB 1:5000
Ki-67	Dako	M7240	IHC 1:500
ACTH	Dako	N1531	prediluted
GLUT-1	Chemicon International	AB1340	IHC 1:2000
TPIT	Abcam	ab243028	IHC 1:1000
PIT1	Santa Cruz	sc-393943	IHC 1:200
SF1	Abcam	ab168380	IHC 1:2000

WB, Western blotting; IHC, immunohistochemistry; ICC, immunocytochemistry.

could initiate the tumor formation in the pituitary gland, and epigenetic dysregulation may drive the progression of pituitary tumors via metabolic reprogramming.

In this study, we report metabolic reprogramming is the key phenomenon to promote pituitary tumor growth through the epigenetic regulation of telomerase reverse transcriptase (TERT), a tumor promoting gene. The findings indicate that a dynamic shift of histone acetylation could be the specific biomarker to predict the aggressiveness of the pituitary tumors as well as the druggable target to treat the aggressive types of pituitary tumors.

II. Materials and Methods

Cell culture and human samples

AtT-20 cell lines derived from ACTH-secreting mouse pituitary tumor cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC: Wiltshire, UK). Cells were cultured in DMEM supplemented with 10% FBS (Omega Scientific, Tarzana, CA) in a humidified 5% CO_2 incubator at 37°C. Surgical and autopsied cases of human pituitary adenoma (corticotroph adenoma), high-risk PA and pituitary carcinoma, a part of the collections from Tokyo Women's Medical University Hospital, were analyzed for glycolytic markers, Ki-67 and histone modifications. Additional 9 corticotroph adenomas, analyzed for histone H3K9 acetylation, were also from Tokyo Women's Medical University Hospital. Physicians obtained informed consent from the patients. All methods and experimental protocols related to human subjects were approved by each institutional review board of Ethics Committee, and the procedures related to human subjects were carried out in accordance with each institutional review board-approved protocol and Declaration of Helsinki, 2013.

Antibodies and reagents

Cell Signaling antibodies (Danvers, MA): H3K9ac (Cat# 9649), H3K18ac (Cat# 9675), H3K27ac (Cat# 4353), H3K4me2 (Cat# 9725), H3K9me2 (Cat# 4658), H3K27me3 (Cat# 9733), Histone H3 (Cat# 4499), GAPDH (Cat# 5174), cleaved caspase-3 (Cat# 9661), HRP-linked anti-rabbit IgG (Cat# 7074) and HRP-linked anti-mouse IgG (Cat# 7076). DAKO (Glostrup, Denmark) antibodies: Ki-67 (Cat# M7240) and ACTH (Cat# N1531). Chemicon International antibodies (Temecula, CA): GLUT-1 (Cat# AB1341). Abcam (Cambridge, UK) antibodies: TPIT (Cat# ab243028) and SF1 (Cat# ab168380). Santa Cruz (Dallas, TX) antibodies: PIT1 (Cat# sc-393943). Reagents used are sodium acetate (Sigma-Aldrich, St. Louis, MO; Cat# S5636), PP242 (Cayman Chemical, Ann Arbor, MI, Cat# 13643), BPTES (Selleck, Houston, TX, Cat# S7753), 2-Deoxy-D-Glucose (2-DG) (FUJIFILM Wako, Osaka, Japan, Cat# 040-06481) and L1H1-7OTD (Cosmo Bio, Tokyo, Japan, Cat# TAT-004). Information on antibodies used in the study was summarized in Table 1.

Quantitative immunohistochemical staining by the immunoperoxidase and immunofluorescence methods

Immunostaining was performed as described previously [8, 22]. Slides were counterstained with hematoxylin or DAPI (Invitrogen, Waltham, MA) to visualize nuclei. Negative control staining was also performed for each section without primary antibodies to determine the threshold for immunopositivity. Immunostained sections underwent the analyses in which the results were evaluated independently by two pathologists who were unaware of the findings of the molecular analyses. Immunofluorescence samples were analyzed with a fluorescent microscope (Olympus BX53 Digital Fluorescence Microscope, Shinjuku, Tokyo). Quantitative image analysis was performed with Soft

Table 2. *Results of histone modifications in pituitary tumors*

	Normal	Adenoma	High-risk adenoma	Carcinoma
H3K9me2	+	−	+/−	+
H3K27me3	+	−	++	++
H3K9ac	+	+	++	++
H3K27ac	+	+	++	++

Imaging System software (Olympus cellSens) or Image J-based scoring systems (ImageJ version 1.49, NIH) for nuclear and cytoplasmic proteins according to the manufacturer's instructions. Images from each immunostained section were captured from representative regions of the tumor with sufficiently high tumor cell content. Also, the percentage of histone modifications in human samples was semiquantitatively evaluated as +/− (a few weakly positive cells), + (positive cells < 50%), and ++ (positive cells ≥ 50%) (Table 2).

Western blotting

To extract whole-lysate protein, cultured cells were lysed and homogenized with a RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) from Boston BioProducts (Boston, MA). Histone protein was extracted by EpiXtract (R) Total Histone Extraction Kit (Abcam). Protein concentration of each sample was determined by the BCA Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Equal amounts of 15 μg whole lysate or 3 μg histone extract were separated by electrophoresis on 4% to 12% Miniprotean TGX Gel (Bio-Rad, Hercules, CA) in Tris/Glycine Buffer (Bio-Rad), and then transferred to a nitrocellulose membrane (BioRad). Primary antibodies used in this study were described above (Table 1). The immunoreactivity was detected with Super Signal West Pico Chemiluminescent Substrate or West Femto Trial Kit (Thermo Fisher Scientific), and quantitative densitometry analysis was performed with image analysis software (ImageJ version 1.49).

siRNA transfection

siRNAs used are SignalSilence® Control siRNA (Cell Signaling, Cat# 6568) and TERT siRNA (Santa Cruz Biotechnology, Cat# sc-36642). Transfection of siRNA into cell lines was carried out using Lipofectamine RNAiMAX (Thermo Fisher Scientific) in full serum, with medium change after 24 hr. siRNAs were used at 10 nmol/L, and cells were harvested 48 hr posttransfection.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted by the use of RNeasy Plus Micro Kit (QIAGEN, Hilden, Germany). Firststrand cDNA was synthesized with iScript™ RT Supermix for RT-qPCR (Bio-Rad). Real-time RT-PCR was performed with the SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Kyoto, Japan) on Thermal Cycler Dice Real Time System TP800 (Takara) following the manufacturer's instructions. β-actin was used as an endogenous control. Primer sequences were available upon request.

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP experiment was performed using SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer's instruction. H3K9ac ChIP was performed from 5×10^6 crosslinked AtT-20 cells treated with low or high glucose for 24 hr. Immunoprecipitated chromatin was washed and de-crosslinked, and purified DNA was quantified by TB-Green real-time quantitative PCR. Recoveries were calculated as percent of input according to the previously reported methods [8, 22].

Glucose-dependent assay on cell proliferation and Warburg effect

For determining the effect of glucose deprivation on cellular function, DMEM containing glucose (Thermo Fisher) or no-glucose DMEM (Thermo Fisher) were used. For the cell proliferation assay, 2.0×10^5 AtT-20 cells were seeded in six-well plates, 10% FBS and 1% penicillin and streptomycin medium with or without glucose (4.5 g/L) for 24 hr. The number of cells with 0.4% Trypan blue staining was quantified by cell counting with TC10 Automated Cell Counter (Bio-Rad). Data represent the mean ± SD of triplicates.

Statistical analysis

Statistical differences between the two groups were analyzed using Student's two-tailed unpaired t-test, and those among three or more groups using one-way ANOVA, followed by a Tukey test and Dunnett's test. Error bars represented standard deviation (SD) unless otherwise noted, and statistical significance was indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

III. Results

Overexpression of GLUT-1 is associated with aggressive types of human pituitary tumors

To understand the role of metabolic reprogramming in pituitary tumor growth, we first assessed glucose transporter-1 (GLUT-1) expression in human autopsy cases of pituitary tumors. GLUT-1 was reported to be expressed in various tumors, and overexpression of GLUT-1 indicates the intracellular influx of glucose which leads to the upregulation of cell metabolism [1, 36]. We compared our peculiar case of pituitary carcinoma developed from high-risk PAs, with autopsied cases of normal pituitary and PAs (Supplementary Fig. S1). A case of pituitary adenoma with normal adenohypophyseal tissue (49-year-old female) showed ACTH and TPIT immunopositivity, corresponding to corticotroph adenoma. A case of high-risk PA (79-year-

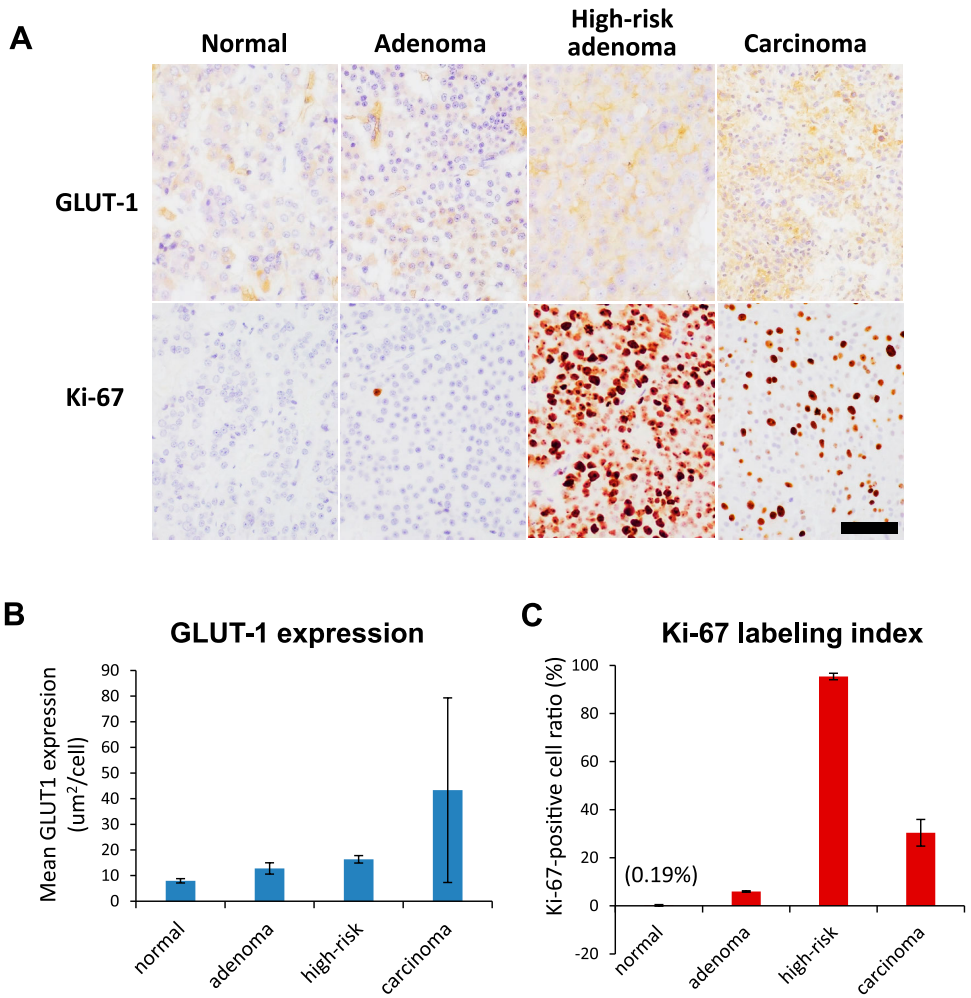

Fig. 1. GLUT-1 expression and cell proliferation in human pituitary tumors. (**A**) GLUT-1 and Ki-67 immunohistochemical staining in human pituitary tumors including pituitary adenoma, high-risk PAs and pituitary carcinoma. Bar = 40 μm. (**B, C**) Quantification of the immunohistochemical expression of GLUT-1 (**B**) and Ki-67 (**C**) showed its increase in high-risk PAs and pituitary carcinoma compared with normal pituitary and adenoma.

old male) was diagnosed by the following histopathological finding: a tumorous lesion invading the surrounding cavernous sinus showed diffuse proliferation of atypical neuroendocrine cells with round nuclei and basophilic cytoplasm, associated with focal ACTH and TPIT positivity, strong p53 positivity and high Ki-67 index. The diagnosis of pituitary carcinoma at the age of 81 was made by the massive dissemination of relatively uniform tumor cells with round nuclei through the neuraxis, associated with focal ACTH positivity and high Ki-67 index. The information on each case was summarized in Supplementary Table S1. Of note, high-risk PAs and pituitary carcinoma expressed high levels of GLUT-1 in comparison with its low expression in normal pituitary and adenoma tissue (Fig. 1A, B). The proliferative activity shown by Ki-67 labeling index was also increased in high-risk PAs and pituitary carcinoma compared with normal pituitary and adenoma tissue (Fig. 1A, C). The findings suggest that activated glucose metabolism could be associated with the clinically and histologically aggressive features of the human pituitary tumors.

High level of glucose promotes metabolic reprogramming and cell proliferation in pituitary tumor cells

High expression of GLUT-1 in human aggressive pituitary tumors suggests that increased uptake of glucose could drive cell proliferation in pituitary tumor cells. To identify the role of glucose in the phenotypes of pituitary tumor cells, we cultured murine pituitary adenoma cells (AtT-20) with or without glucose and assessed their cellular function. The presence of glucose promoted the Warburg effect (shown by a shift in media color of the cultured cells) as well as the expression of glycolytic enzymes, especially GLUT-1 in AtT-20 cells (Fig. 2A, B). Furthermore, proliferation of AtT-20 cells was dependent on glucose (Fig. 2C), and glucose significantly increased the expression of the cell cycle-related gene, cyclin D1 (*CCND1*) (Fig. 2D). The effect of glucose on another major cell

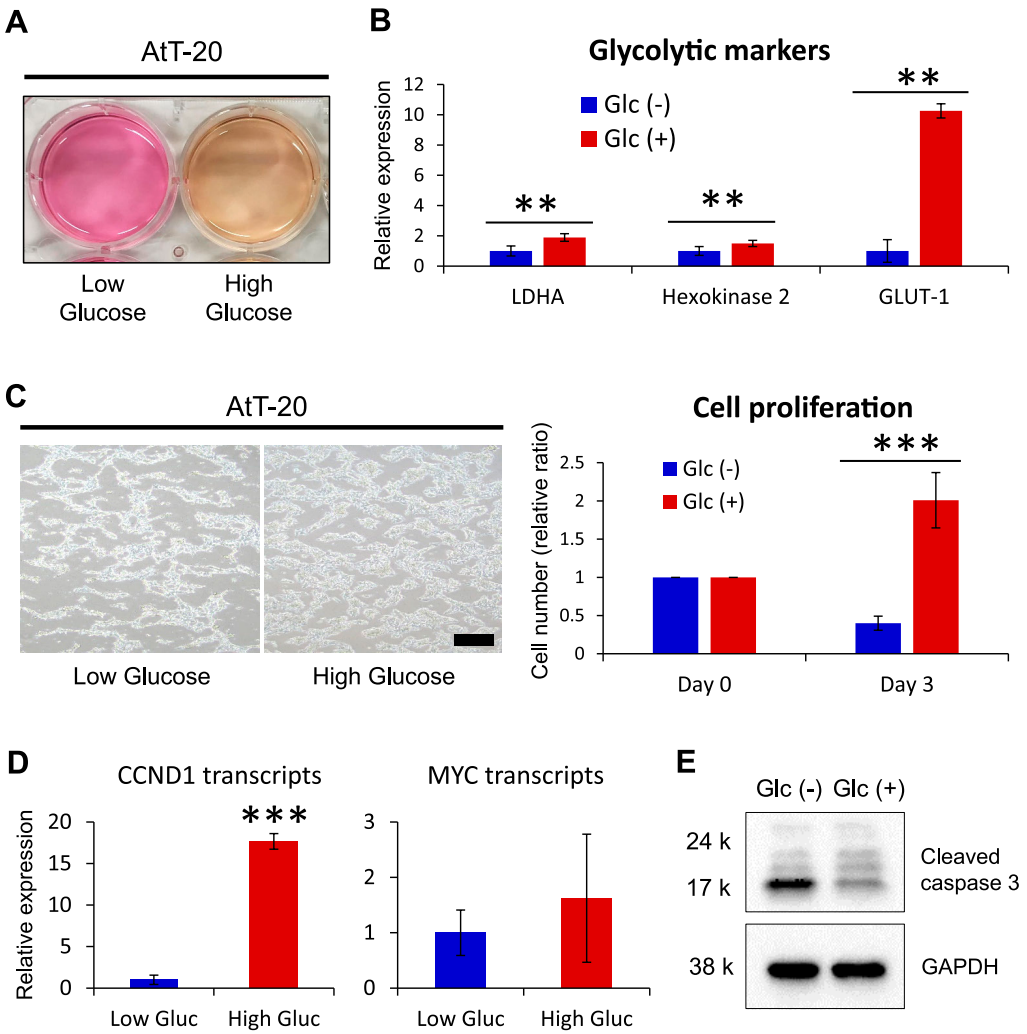

Fig. 2. Glucose drives cell proliferation in pituitary tumor cells. (**A**) The color of media of AtT-20 cells treated with or without glucose (4.5 g/dL) for 24 hr (n = 3). (**B**) RT-qPCR analyses on major glycolysis-related genes (lactate dehydrogenase A: LDHA, hexokinase 2 and GLUT-1) in AtT-20 cells, cultured in media with or without glucose for 24 hr. (**C**) Cell proliferation of AtT-20 pituitary tumor cells cultured in media with or without glucose for 24 hr (n = 3). Bar = 100 μm. (**D**) RT-qPCR analyses on cell cycle-related genes including cyclin D1 (CCND1) and c-Myc in AtT-20 cells, cultured in media with or without glucose for 24 hr. (**E**) Immunoblot detection of cleaved caspase 3 (apoptosis marker) in AtT-20 cells cultured in media with or without glucose showed an increase of cleaved caspase 3 in AtT-20 without glucose.

cycle-regulated gene *c-Myc* was mild (Fig. 2D). Additionally, glucose deprivation promoted apoptosis in AtT-20 cells (Fig. 2E). Of interest, another major nutrient such as acetate similarly increased cell proliferation and *CCND1* expression in AtT-20 cells (Supplementary Fig. S2). Taken together, major nutrients including glucose and acetate are necessary for driving cellular metabolism and proliferation in pituitary tumor cells.

Glucose-dependent metabolic reprogramming promotes histone acetylation in aggressive pituitary tumors

Metabolic reprogramming induces histone modification such as acetylation and methylation, and plays an important role in tumorigenesis [10, 20]. Importantly, glucose and acetate are the major source for modifying histone tails [10, 20]. Therefore, we hypothesized that activated metabolism with glucose and acetate would drive proliferation of pituitary tumor cells through the regulation of histone modifications. We assessed the histone modification status (H3K9ac, H3K18ac, H3K27ac, H3K4me2, H3K9me2, H3K27me3 and total H3) in AtT-20 with or without glucose, and found an increase of histone acetylation (especially H3K9 acetylation) in AtT-20 cells with glucose or acetate (Fig. 3A, B and Supplementary Fig. S2C). We previously reported that metabolic reprogramming promotes histone acetylation in glioblastoma cells through the activation of mammalian target of rapamycin (mTOR) and glutaminolysis pathways [22]. To test the possibility that glucose-dependent histone modifications are also regulated in pituitary tumor cells by mTOR and glutaminolysis pathways, we assessed several pharmacologic inhibitors related to cell metabolism including BPTES

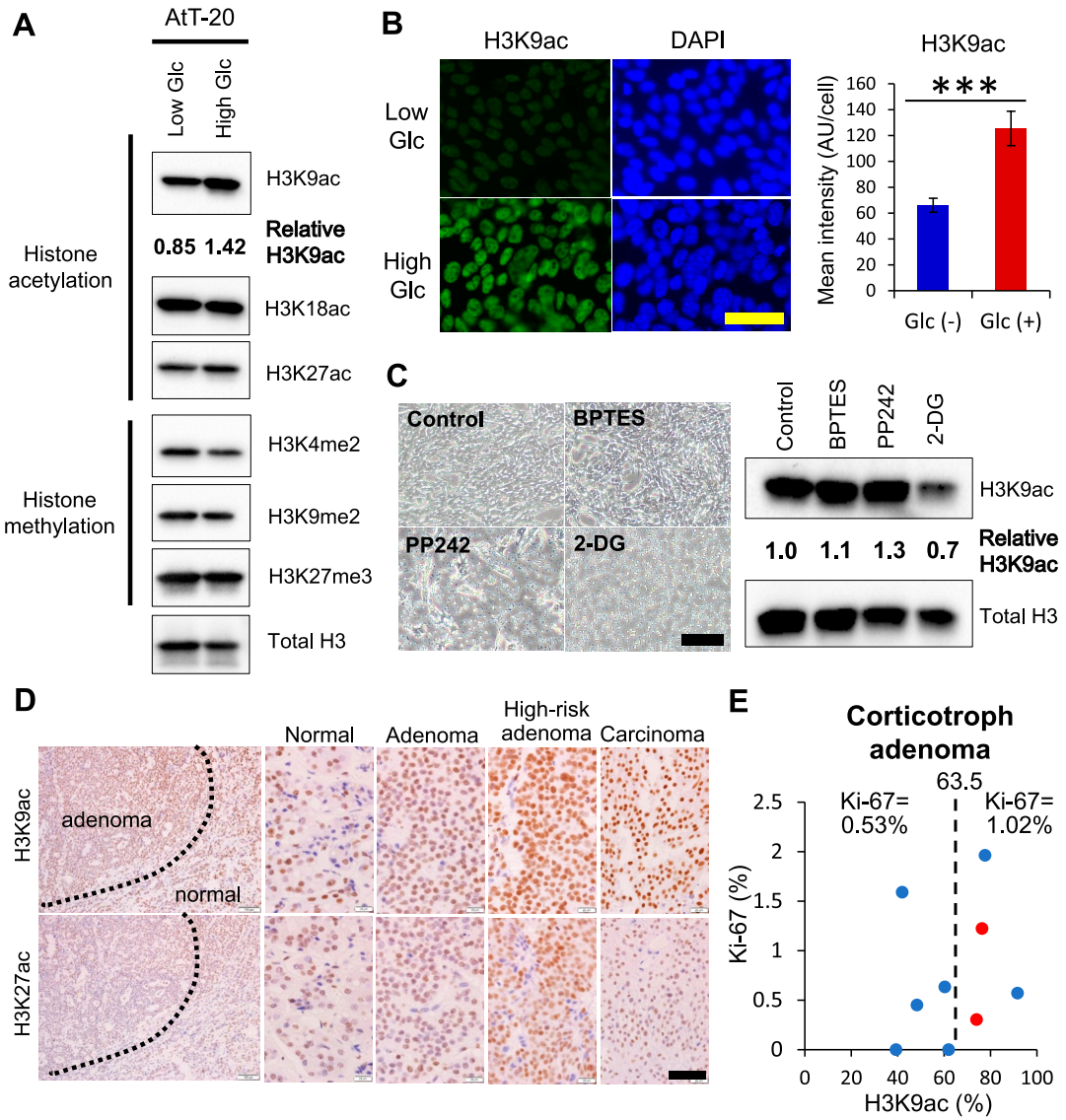

Fig. 3. Glucose-dependent metabolic reprogramming promotes H3K9 acetylation in pituitary tumors. (**A**) Immunoblot detection of histone H3 modifications including acetylation (H3K9ac, H3K18ac, H3K27ac) and methylation (H3K4me2, H3K9me2, H3K27me3) in AtT-20 pituitary tumor cells, cultured in media with or without glucose for 24 hr. H3K9ac showed a significant change in glucose-treated tumor cells, in comparison with other histone modifications. (**B**) Immunofluorescent staining of H3K9ac in AtT-20 cells treated with or without glucose for 24 hr. Green, H3K9ac staining; blue, DAPI staining. Bar = 40 μm. AU, arbitrary unit. (**C**) Immunoblot detection of H3K9ac treated with BPTES (glutaminolysis inhibitor), PP242 (mTOR inhibitor) and 2-DG (glycolysis inhibitor). Bar = 100 μm. (**D**) Immunohistochemical analyses of histone acetylation (H3K9ac and K27ac) in human pituitary tumors including normal pituitary, pituitary adenoma, high-risk PA and pituitary carcinoma. Bar = 40 μm. (**E**) Scatter plot of H3K9ac status in 7 non-recurrent (blue dots) and 2 recurrent cases (red dots) of corticotroph adenoma: the cut-off value (H3K9ac = 63.5%) was calculated by the average of H3K9ac in all 9 cases. Ki-67 indexes for H3K9ac-low and -high group were 0.53% and 1.02%, respectively.

(glutaminolysis inhibitor), PP242 (mTOR inhibitor), and 2-DG (glycolysis inhibitor). Interestingly, only 2-DG treatment significantly reduced H3K9 acetylation in AtT-20 cells with high glucose (Fig. 3C), suggesting that the regulatory mode of histone modifications may be different between pituitary and other brain tumors. We further observed that histone acetylation was increased in human aggressive pituitary tumors including high-risk PAs and pituitary carcinoma (Fig. 3D, Supplementary Fig. S3 and Table 2). Of note, we examined the level of H3K9ac in non-recurrent and recurrent cases of corticotroph adenoma (7 non-recurrent and 2 recurrent), and recurrent cases were included in the H3K9ac-high group (Fig. 3E). These indicate that glucose-dependent metabolic reprogramming modulates histone modifications, which could be associated with the clinically and histologically aggressive features of the human pituitary tumors.

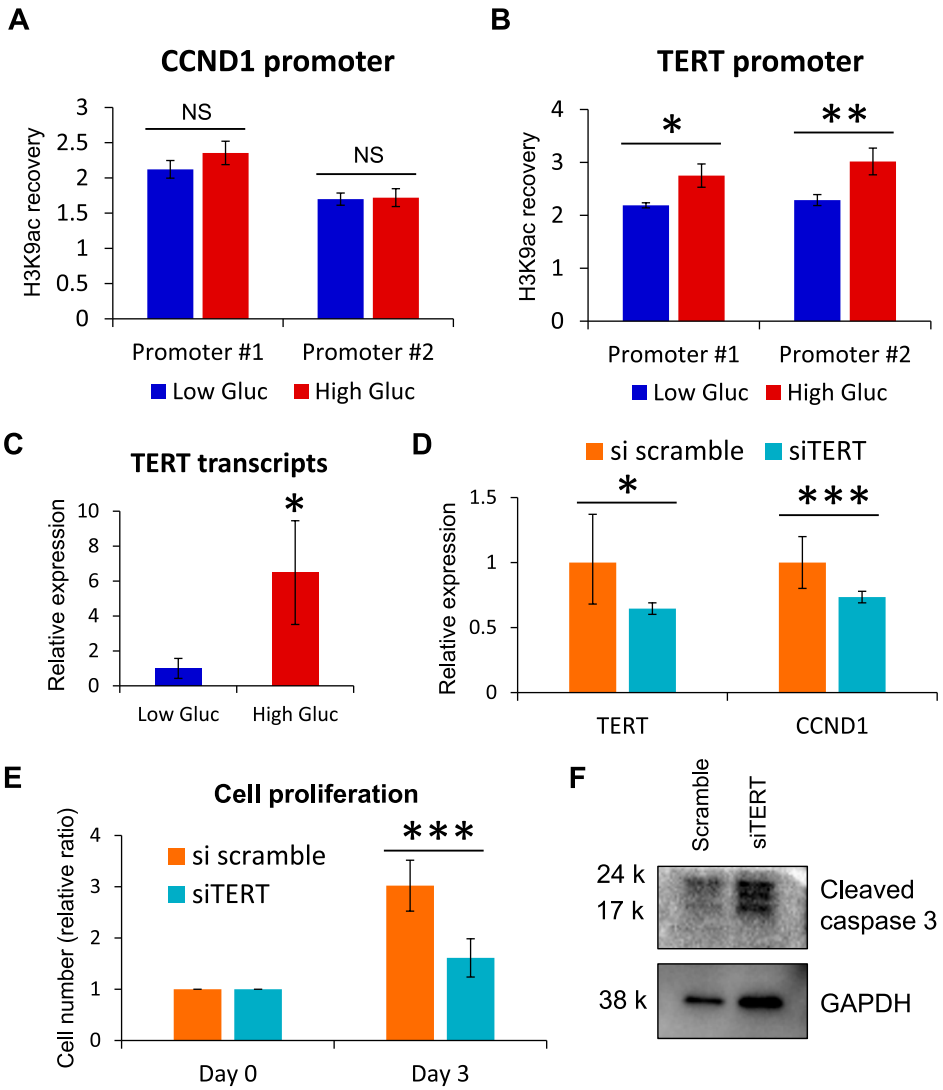

Fig. 4. Glucose-dependent metabolic reprogramming drives tumor cell proliferation via epigenetic regulation of TERT. (**A, B**) ChIP-qPCR analysis of AtT-20 cells cultured in media with or without glucose for 24 hr, assessing H3K9ac recovery (% input) on binding elements in CCND1 (**A**) and TERT (**B**) promoter regions. NS, not significant. (**C**) RT-qPCR analysis of TERT, cultured in media with or without glucose for 24 hr. (**D**) RT-qPCR of TERT and CCND1 in AtT-20 cells with siRNA treatment against scramble or TERT sequence. (**E**) AtT-20 cell growth was suppressed after siRNA-mediated knockdown of TERT for 3 days. (**F**) Immunoblot analyses showing an increase of cleaved caspase 3 (apoptotic marker) in AtT-20 cells with siRNA-mediated knockdown of TERT.

Epigenetic regulation of TERT drives metabolism-dependent cell proliferation in pituitary tumors

We next examined whether a shift in histone modifications is essential in metabolism-driven cell proliferation of pituitary tumors. Generally, histone modification occurs in promoter region of the genes, and increased histone acetylation is known to relax chromatin configuration and facilitate gene expression [10, 20]. Thus, we performed chromatin immunoprecipitation (ChIP) analysis to determine whether metabolic reprogramming induces a shift in histone acetylation in the promoter region of cell cycle-related genes including *CCND1*. Rather unexpectedly, however, there was no significant difference in H3K9ac peaks in the *CCND1* promoter region between AtT-20 with or without glucose (Fig. 4A). Recent reports demonstrated that the expression of telomerase reverse transcriptase (TERT) is epigenetically regulated in pituitary tumors [11, 24], and TERT could regulate the expression of *CCND1* in cancer cells [9, 16]. We thus hypothesized that metabolic reprogramming in pituitary tumors could upregulate *CCND1* and cell proliferation via epigenetic regulation of TERT. Consistent with the idea, upregulation of H3K9 acetylation was observed in *TERT* promoter as well as increase of *TERT* transcripts in AtT-20 cells with high glucose (Fig. 4C, D). Furthermore, genetic (siRNA) and pharmacologic (L1H1-7OTD) inhibition of TERT suppressed *CCND1* expression and cell proliferation, and promoted apoptosis in AtT-20 cells (Fig. 4D–F and Supple-

mentary Fig. S4). Together, glucose-dependent metabolism contributes to aggressiveness of pituitary tumors through epigenetic regulation of TERT.

IV. Discussion

Contributing factors to tumorigenesis and malignant transformation in pituitary tumors have remained unclear, but recent studies have reported molecular basis of PA formation in familial PA cases. In FIPA, approximately 20% show germline mutation in aryl hydrocarbon receptor interacting protein (AIP), an immunophilin-like protein found in the cytoplasm as part of a multiprotein complex, considered to link cyclin AMP (cAMP)-dependent protein kinase A (PKA) pathway to tumor cell growth [33, 34]. As for sporadic cases, the common somatic mutations in PAs include *GNAS*, *USP8*, *PIK3CA*, *HMGA2* and *IDH1* [30]. *GNAS* mutation disrupt the GTPase activity of the protein, leading to prolonged adenylyl cyclase activity and increase cAMP levels [30], and USP8 is associated with tumor cell proliferation with its de-ubiquitinase activity [12]. Genetic abnormality has so far revealed the mechanism of pituitary tumorigenesis. Epigenetic dysregulation could drive the progression in the pituitary tumors, and the present study supports the epigenetic contribution to the progression and aggressiveness of pituitary tumors. Of note, a dynamic epigenetic shift is driven by aerobic glycolysis known as Warburg effect or metabolic reprogramming [10, 20, 21]. Our results corroborate the idea that metabolic reprogramming and subsequent epigenetic changes could drive the progression of the pituitary tumors through promoting tumor cell proliferation. Genetic mutation of *TP53* gene was reported to be involved in the process of transformation to more aggressive growth of pituitary tumors, and p53 nuclear immunoreactivity was formerly defined as one of the histopathological markers for aggressive pituitary tumors [27, 32]. Interesting speculation would be that an interaction of metabolic and epigenetic reprogramming may become an alternative to genetic mutation in the progression of pituitary tumors, considering the interplay between epigenetic changes and the p53 protein [15].

Histone modifications are one of the major epigenetic regulatory mechanisms of the gene expression, involved in an array of physiological and pathological phenomena including cancer formation [10, 20]. Histone acetylation and methylation are two major histone modifications, and the former generally turns on the gene expression and the latter switches it on or off, depending on which lysine residues are methylated [2]. Recently, the roles of histone modification in pituitary tumors have been reported. Retinoblastoma protein-interacting zinc finger gene (RIZ1) acts as H3K9 methytransferase, and PA with high RIZ1 group had a long progression-free survival (PFS) compared with low RIZ1 group [25, 35]. Also, overexpression of histone H3K27-specific methyltransferase, enhancer of zeste homolog 2 (EZH2) in PAs has been reported to be associated with cell proliferation [28]. Our study revealed the importance of histone acetylation (H3K9ac) in aggressive pituitary tumors including high-risk PA and carcinoma via facilitating the expression of tumor-promoting genes. In contrast to histone acetylation, histone methylation was lost even in the early stages of PA in our cases, suggesting that histone methylation might be involved in the initiation of pituitary tumors whereas histone acetylation could contribute to its progression (Table 2 and Fig. 5). Further, an increase in histone acetylation and methylation in both high-risk adenomas and carcinomas might indicate that high-risk adenomas could represent the precancerous lesion or so-called "carcinoma *in situ*" status. The limitation of the present study is based on a small number of cases, especially for aggressive pituitary tumors including high-risk adenomas and pituitary carcinomas. It would be preferable to compare metastatic tumor and non-metastatic tumors in the same category (i.e. corticotroph adenomas) to determine whether histone acetylation is actually associated with the aggressive phenotypes of the pituitary tumors. Future studies with a sufficient number of cases will be necessary to unravel the role of epigenetics in the biology of pituitary tumors with histology-based analyses on histone modifications and other epigenetic mechanisms including DNA methylation and non-coding RNAs [17], as well as the development of specific *in vivo* models to recapitulate human pituitary tumorigenesis.

Up to 90% of human cancers are reported to show *TERT* overexpression and increased telomerase activity. The mechanism of *TERT* overexpression is mainly based on hotspot mutations in *TERT* promoter at C228T or

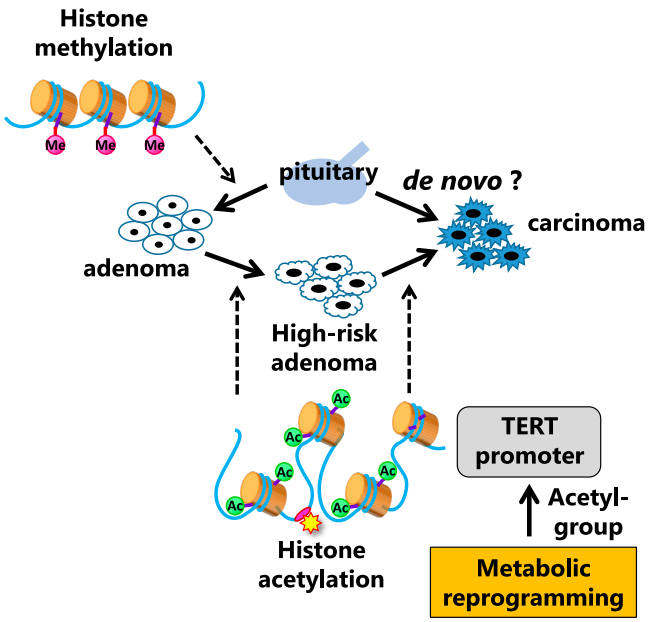

Fig. 5. Histone modifications in the biology of pituitary tumors. Metabolic reprogramming promotes histone acetylation, mainly H3K9, leading to upregulation of *TERT*, a key downstream effector in the progression of pituitary tumors.

C250T in various types of cancer including brain tumors, which are rare in pituitary tumors [3, 37]. Instead, some PAs present a high level of *TERT* promoter methylation, more likely to show disease progression and shorter PFS [11, 24]. The present study has also revealed the novel regulatory mode of TERT in pituitary tumors through histone acetylation, supporting the idea that its epigenetic regulation is more essential in the progression of pituitary tumors rather than its genetic regulation [23]. Notably, recent studies have indicated a non-canonical function of TERT such as involvement in stem cell proliferation and proliferating-related signaling pathways [9, 16, 18, 19], which could explain our findings on the relationship between TERT and *CCND1* expression in pituitary tumors. It awaits further investigation whether epigenetic regulation of TERT in pituitary tumors could also be associated with its canonical function to lengthen telomere.

In conclusion, we demonstrate a dynamic epigenetic status of histone acetylation regulated by metabolic reprogramming, which enables pituitary tumors to sustain cell survival. Promisingly, increased expression of histone acetylation and TERT, which was demonstrated herein to be associated with high-risk PAs and pituitary carcinomas, could be targeted as novel types of cancer therapeutics with protein acetylation modifiers and telomerase inhibitors [7, 14, 31]. This study thus provides a potential of H3K9ac as a useful histological biomarker to predict pituitary tumor aggressiveness and determine the post-operative management [13], which leads to novel therapeutic strategies aimed at disrupting metabolism-histone acetylation-TERT pathways for high-risk PAs and pituitary carcinomas [20].

V. Conflicts of Interest

The authors declared no competing interests.

VI. Acknowledgments

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