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NR2F1-associated Dormancy and Glutamine-dependency in H295R Adrenocortical Cancer Cells

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ABSTRACT

Adrenocortical cancer poses a significant clinical challenge due to its aggressiveness and limited treatment options. Herein, we investigated the role of NR2F1, as a potential dormancy factor, and its relationship with glutamine-dependency of adrenocortical cancer cells. Materials and Methods: NR2F1 expression was evaluated in adrenocortical cancer, in publicly available databases. H295R adrenocortical cancer cells and HAdCC normal adrenocortical cells transfected to overexpress NR2F1, were analyzed for cell viability, lysosome function, and protein expression of cell-cycle regulators, as well as for cell-cycle distribution. Kaplan-Meier analyses of publicly available databases showed a marginal positive association of NR2F1 expression with OS in adrenocortical cancer. Transient overexpression of NR2F1 in H295R cells resulted in suppressed proliferation and increased lysosome function. In normal human AdCC adrenocortical cells, lysosome activity was increased in glucose- and glutamine- deprived state, only when the cells were confluent. On the contrary, in H295R cells, lysosome activity was reduced in glucose- and glutamine- deprived states, only when the cells were confluent. Western blot analyses showed that the expression of NR2F1 was induced by confluency. Confluency also induced a marked increase in CDK1 and CDKN1A expression, which was significantly reduced by glutamine deprivation in H295R cells. Our findings provide new insights into the molecular mechanisms underlying the role of NR2F1 in cellular dormancy, as well as the expression patterns of CDK1 and CDKN1A in response to confluency and glutamine dependency, suggesting an interdependence of these pathways as potential therapeutic targets in adrenocortical cancer.

Keywords: Adrenocortical cancer, NR2F1, dormancy, glutamine deprivation.

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INTRODUCTION

Adrenocortical carcinoma (ACC) stands as a formidable clinical challenge characterized by its aggressive nature and limited therapeutic alternatives (1). ACC is a rare type of cancer occurring in 1-2 per million every year with a five-year survival rate of less than 60% (2). Nonetheless, the five-year survival rates for the different stages are 63-88% (stage 1) and 38-73% (stage 2) for the non-metastatic tumors, while they are 19-54% (stage 1) and 0-21% (stage 4) for the metastatic ones (3). Surgical resection may be successful in some cases but once lymph node metastasis is detected, the five-year survival rate (microscopically incomplete: 26%) is equivalent to stage 4. The prognosis is even worse when surgical resection is macroscopically incomplete (13%) (3).

Various clinical studies have been focused on metastatic ACC; however, with inadequate progression. The latest accomplishment marks the current first-line protocol, mitotane plus etoposide, doxorubicin, and cisplatin (EDP) in chemotherapy /more than ten years ago, in the randomized phase 3 trial (FIRM-ACT) (4). Since then, minor modifications have been suggested, such as the usage of etoposide and doxorubicin topoisomerase inhibitors in ACCs where TOP2A is highly expressed (5); and increasing etoposide dose due to the decreased efficacy of etoposide at standard dose in platinum-etoposide chemotherapy (6). Moreover, the use of insulin-like growth factor-1 receptor inhibitors in an international, double-blind, phase 3 study, showed no increase of efficacy for linsitinib (7). The multi-tyrosine kinase inhibitor, cabozantinib, developed partial response in three patients and five with stable disease with a median progression free survival of 16 weeks and a median overall survival of 58 weeks (8). A phase 2 trial for cabozantinib-S-malate has been initiated for pediatric solid tumors including ACC (9). Immune checkpoint inhibitors have yielded contradictory results in clinical trials, but a retrospective cohort study showed moderate response and acceptable safety profile as second line therapy compared to first line and modified protocols (10).

Recent developments in the elucidation of molecular mechanisms for ACC have found the pediatric cases to be associated with TP53 mutations and with a better prognosis as well (11). Adult primary ACCs are known for alterations in WNT–β-catenin pathway, cell cycle and p53 apoptosis pathway, chromatin remodeling and telomere maintenance pathway, cAMP–protein kinase A (PKA) pathway or DNA transcription and RNA translation pathways. In spite of the vast compiling research for ACCs, we only have clues of the driver genes responsible for the development of adrenocortical cancer, specific to the adrenal gland or to the stage of the cancer, such as IGF-2, DDR, WNT–β-catenin, PI3K, AKT–mTOR and TP53, which are ubiquitously expressed and not specific for the adrenal cortex (2).

In this study, we focused on NR2F1, a candidate dormancy factor (12), and its role in H295R adrenocortical carcinoma cells. Recent findings of NR2F1 show its importance in early dissemination of breast cancer cells (13, 14), positively correlated with poor prognosis in gastric cancer (15), and pancreatic cancer (16), but our search did not yield any articles detailing the function of NR2F1 in adrenocortical cancer. NR2F1 has been proved to induce dormancy by SOX9 and RARβ to a quiescent state (12). The problem of dormancy is whether it is deep enough to stay in senescent state by lowering lysosome function (17, 18, 19). Understanding the interplay between NR2F1, contact inhibition, and cell cycle dynamics is crucial for unraveling the complexities of dormancy mechanisms of H295R cells compared to normal adrenal cortex cells.

MATERIALS AND METHOD

Data collection

The transcripts per million (TPM) value of NR2F1 and NR5A1 genes in the normal human adrenal gland were downloaded from the GTEx portal (https://gtexportal.org/home/GTEx v8) (2013). The expression of the NR2F1 gene related to prognosis in adrenocortical carcinoma patients were analyzed by cBioPortal (https://www.cbioportal.org) using datasets from the TCGA, PanCarcinoma Atlas. Expression of NR2F1 gene in normal adrenal tissue, adrenal adenoma, and adrenocortical cancer was analyzed by bioGPS microarray database portal (http://biogps.org) using the following dataset, Accession: E-GEOD-10927.

Cell lines

H295R adrenocortical cancer cells (CRL 2128; ATCC, Manassas, VA, USA) were cultured using the following media: DMEM/Ham's F-12 (with L- glutamine, sodium pyruvate and HEPES; Nacalai Tesque Inc., Kyoto, Japan) for normal condition; and DMEM/Ham's F-12 (no Glucose) with L- glutamine and sodium pyruvate, (Nacalai Tesque Inc.) for glucose-deprived condition; and DMEM/Ham's F-12 with 15 mM HEPES and sodium bicarbonate, without L-glutamine and phenol red (Sigma-Aldrich Japan Co., Tokyo, Japan) for glutamine-deprived condition. The change of sodium pyruvate to sodium bicarbonate in glutamine deprived condition is to enhance glutamine deprivation in the absence of pyruvate (20). These three media were supplemented with Nu-Serum I (Corning Japan, Tokyo, Japan) and ITS+ Premix (Corning Japan) and Penicillin-Streptomycin Mixed Solution (Nacalai Tesque Inc.) as previously described (21).

Normal human adrenal cortical cells (HAdCC) were purchased (ScienCell Research Laboratories, Carlsbad, CA, USA; ScienCell fulfills the ethic requirements, including donor's

consent) and cultured using Human Mesenchymal Stem Cell Growth Medium Kit (ScienCell Research Laboratories) supplemented with penicillin-streptomycin mixed solution (Nacalai Tesque Inc.), following the manufacturer's instructions (ScienCell Research Laboratories). The cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C. Cells were used at a confluence of 70% or after being cultured three further days after 100% confluency changing the medium daily.

Lysosome labeling

LysoTracker Green DND-26 (Cell Signaling Technology Japan, Tokyo, Japan) was diluted to 1:20,000 directly into the media with Hoechst 33258 (Dojindo Laboratories, Kumamoto, Japan) diluted to 1:1,000 and analyzed immediately under a microscope (BZ-X710; Keyence).

Cell transfection

Transfection was conducted with Lipofectamine 3000 Transfection Reagent (Invitrogen Japan, Tokyo, Japan) following the manufacturer's instructions. In brief, six-well plates with 3□x105 cells/well were seeded the day before transfection. The following day, transfection was performed by mixing plasmid DNA in Opti-MEM (Gibco, Thermo Fisher Scientific, Tokyo, Japan) and Lipofectamine 3000 Transfection Reagent in Opti-MEM. The mixture was incubated at room temperature for 20 min. Plasmids were purchased at VectorBuilder (hNR2F1, VB900132-2927ynt, ORF_Stuffe, VB900123-9037ybc). The transfected cells were evaluated for LysoTracker under a microscope (BZ-X710; Keyence, Osaka, Japan) at the indicated timepoints. For stable transfectants, NR2F1-transfected H295R cells were selected by G418 (Nacalai Tesque Inc.).

Cell viability assay

Cells were fixed with 100% methanol for 15 min at room temperature. Stained with 0.5% crystal violet (Nacalai Tesque Inc.) in 20% methanol/water for 30 min. After washed with PBS, optical density at 595 nm was measured.

Immunoblot analyses

H295R or HAdCC cells were cultured by the indicated conditions washed twice with PBS and resuspended in 0.5 ml TRIzol reagent (Invitrogen). After passing through a 25G needle five times, chloroform was added, vortexed, and centrifuged. Protein was purified from the interphase and organic phase by precipitating with 6V of a solution for precipitating protein (50% ethanol, 24.5% acetone, 24.5% methanol, 1% distilled water). Twenty micrograms of protein were boiled in sample buffer and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane and incubated with primary

antibodies all at a concentration of 1:1000 anti-NR2F1 antibody (Proteintech, Tokyo, Japan), anti-CDK1 antibody (Proteintech), anti-CDKN1A antibody (Atlas Antibodies AB, Stockholm, Sweden), anti-CDKN1B antibody (Atlas Antibodies AB), anti-phospho-Akt (Ser473) antibody (Cell Signaling Technology), anti-phospho-p70 S6 kinase (pThr389) antibody (Sigma-Aldrich), and anti-β-actin antibody (MBL, Nagoya, Japan). Then, the primary antibodies were detected by the appropriate secondary antibody conjugated to peroxidase (rabbit: Vector Laboratories (Vector Laboratories, Burlingame, CA, USA), mouse: Cell Signaling) were used at a concentration of 1:5000 and visualized by Chemi-Lumi One L (Nacalai Tesque). The immunoblot experiments were replicated three times.

Flow cytometry

H295R cells were detached from the plates with trypsin and fixed with cold methanol; after washing with PBS, cells were stained with 1% propidium iodide in PBS and analyzed for nucleic acid content on a flow cytometer BD Accuri C6 Plus (Becton Dickinson iNippon Becton Dickinson Co., Ltd., Tokyo, Japan). Cell cycle analysis was performed using Flow Jo software (Becton DickinsonNippon Becton Dickinson Co., Ltd., Tokyo, Japan).

Statistical analysis

Statistical analyses were performed with Microsoft Excel. Continuous variables were compared between groups using Student t-test. For Kaplan Meier analyses, log-rank test was performed using the cBioPortal online platform (https://www.cbioportal.org). P-Value below 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Adrenocortical carcinomas with high NR2F1 expression were marginally associated with better prognosis

Before checking the expression of the dormancy factor, NR2F1 (previously called COUP-TF1) in adrenal tumors, we searched for its expression in the normal adrenal gland by the publicly available GTEx (Genotype-Tissue Expression (GTEx) portal (https://www.gtexportal.org/). GTEx portal is a comprehensive public resource to study tissue-specific gene expression with samples collected from 54 non-diseased tissue sites across nearly 1,000 individuals. The TPM value was 41.8 for NR2F1 in the normal adrenal gland. Though it is not possible to directly compare these values across tissues, these TPM values are a qualitative measure of relative expression, with TPM over 40 reported as highly expressed. Thus, NR2F1 was highly expressed in the normal adrenal gland. The expression of NR5A1 (Ad4BP/SF-1), the master regulator of the adrenal gland, is known to be overexpressed in adrenocortical cancer tissue and results in

hyper secretion of cortisol leading to a worse prognosis of adrenocortical cancer (22-24). The TPM value of NR5A1 in the normal adrenal gland was 216.7. Next, we searched whether NR2F1 had any relationship with the survival rate of adrenocortical carcinoma patients by Kaplan-Meier analysis using the cBioPortal (https://www.cbioportal.org) with datasets from the TCGA, PanCarcinoma Atlas. We found that a small group, designated as NR2F1 high, had better prognosis than those that were not designated as NR2F1 high (Figure 1A); however, at a significance level of 10% (p=0.0883), probably due to the small number of patients in the study. The same data set showed worse prognosis of a small group that overexpressed NR5A1 that also was not significant (p=0.916) (Figure 1B).

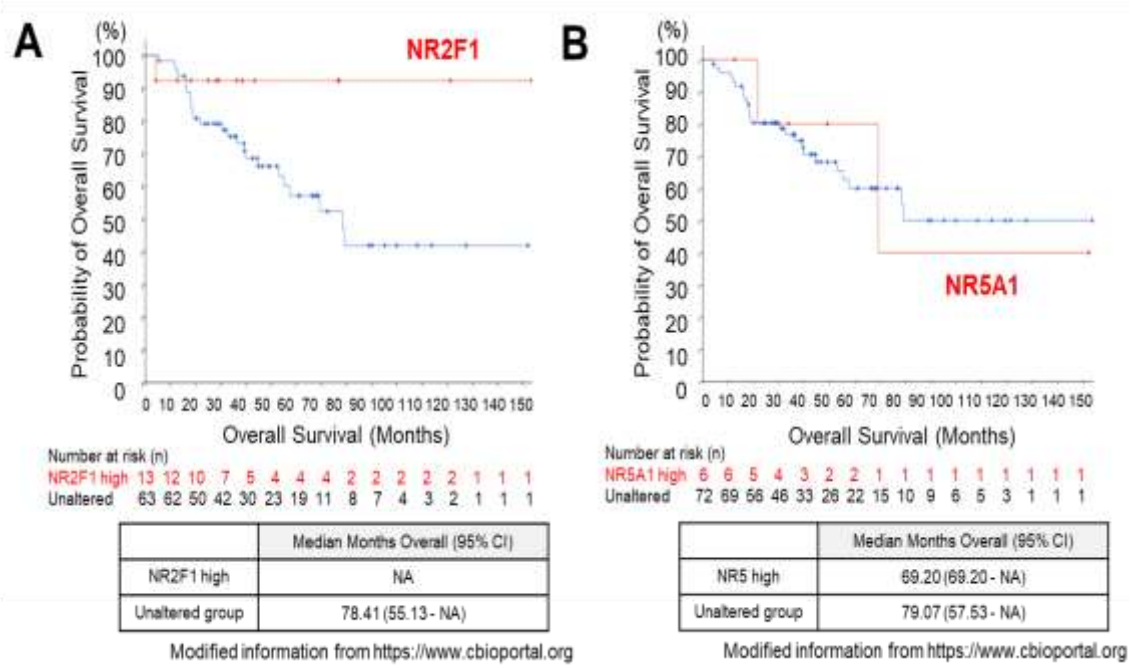

Figure 1: NR2F1 expression may be associated with good prognosis in adrenocortical carcinoma (ACC) patients. (A) Kaplan Meier curve of all ACC patients (blue line) and ACC patients with tumors with high NR2F1 expression (red line). (B) Kaplan Meier curve of all ACC patients (blue line) and patients with tumors with high NR5A1 expression (red line). The dots in the curves are the censored survival time points.

NR2F1 expression was attenuated in adrenocortical cancer and reduced viability in H295R cells

To check whether this highly expressed NR2F1 in normal adrenal gland changed in adrenocortical adenomas or carcinomas, we used the publicly available bioGPS microarray database portal (http://biogps.org). Analysis of a dataset of 33 adrenocortical carcinomas, 22 adrenocortical adenomas, and 10 normal adrenal cortex samples (25), revealed that the expression of NR2F1 was significantly attenuated to 93% in adenomas and 81% in carcinomas

compared to normal adrenal tissue (Figure 2A). However, the expression of NR5A1 was 107% in adenomas and 96% in carcinomas compared to normal adrenal tissue with no significant difference (Figure 2B). Based on the data presented, it is conceivable that reintroducing NR2F1 into adrenocortical cancer cells may mitigate their malignant proliferation. Thus, we tested transient expression of NR2F1 in H295R adrenocortical carcinoma cells and found that cell viability was significantly attenuated in NR2F1-transient-transfected H295R cells compared to mock-transfected H295R cells [day 2 after transfection/ day 0: control 1.41 vs. NR2F1 1.02 (p=0.04), day 3 after transfection/ day 0: control 1.87 vs. NR2F1 1.31 (p=0.04)] (Figure 2C).

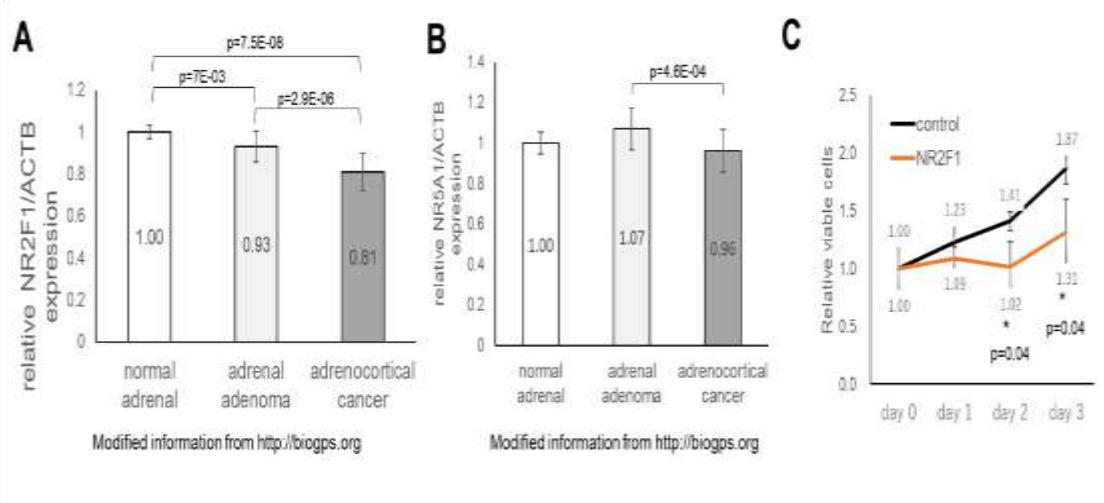

Figure 2: NR2F1 had low expression in adrenocortical carcinoma (ACC) and attenuated cell growth when overexpressed in H295R cells. (A) Expression of NR2F1/ACTB in normal adrenal, adrenal adenoma, and ACC tumors. (B) Expression of NR5A1/ACTB in normal adrenal, adrenal adenoma, and ACC tumors. (C) Relative viability of H295R cells transfected with NR2F1 or mock (control).

NR2F1 expression in H295R cells induced quiescence while maintaining regrowth potential through decreased CDKN1A expression

The relationship between the adrenal gland, autophagy, and carcinogenesis is a complex and multifaceted topic that involves intricate cellular mechanisms (26). While research in this area is ongoing, our investigation centered on elucidating the impact of NR2F1 on lysosome biogenesis in the context of contact inhibition and nutritional deprivation. When H295R cells transiently expressing NR2F1 were checked for their lysosomal mass by Lyso Tracker, a statistically significant increase was observed compared to the control cells (mock-transfected H295R cells) (1.8 vs. 1.0, p=0.04) (Figure 3A).

The cellular expression levels of cyclin-dependent kinases, specifically CDK1, along with its inhibitors CDKN1A and CDKN1B, as well as autophagy effectors AKT and P70-S6K, were

examined in these cells (Figure 3B). In H295R cells transiently expressing NR2F1, the expression of CDK1, identified as a potential therapeutic target for adrenocortical carcinoma (ACC) (27), exhibited a reduction (Figure 3B). The attenuated phosphorylation level of AKT (NR2F1/control ratio: 0.14±0.15, p=0.0008) and P70-S6K (NR2F1/control ratio: 0.38±0.11, p=0.0009) implies reduced activation of the mammalian target of rapamycin signaling resulting in reduced protein synthesis and cell growth and G1 cell cycle arrest (2) (Figure 3B). However, this quiescence state seems to be reversible, evidenced by the decreased expression of CDKN1A known to counteract irreversible senescence (28), though the nuclear localization was not examined (29) (Figure 3B). Although, differential expression of a single protein may not be sufficient evidence for predicting reversibility, it could indicate potential cellular changes or deviations from a quiescent state, suggesting the possibility of reversibility. NR2F1-stably-transfected H295R cells showed G0/G1 and G2/M cell cycle arrest by flow cytometry analyses (Figure 3C).

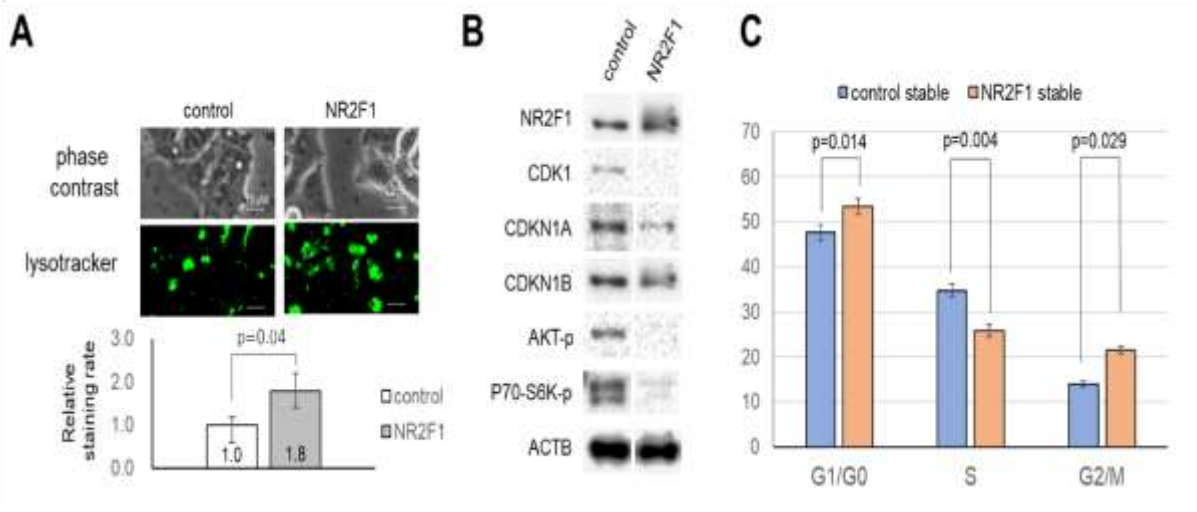

Figure 3: NR2F1 induced quiescence in NR2F1-transfected-H295R cells. (A) Cells that transiently expressed NR2F1 had increased lysosomal activity compared to mock-transfected cells (control). (B) Cell cycle-related protein and mTOR signal-related protein expression in NR2F1-expressing H295R cells and mock-transfected cells. (C) NR2F1 stable expression in H295R cells induced a G0/G1 arrest, and with a less but significant change of G2/M arrest, compared to control cells.

Lysosomal activity was higher in confluent H295R adrenocortical carcinoma cells than sub-confluent H295R cells or HAdCC normal adrenocortical cells and had differential response to glutamine deprivation.

The H295R cells act as pluripotent adrenocortical cells capable of being directed to produce each of the zone-specific steroids (30). Thus, this cell line has been used to study endocrine disrupters (30, 31) as well as ACC. In such studies, it is important to use sub-confluent H295R cells since confluency disrupts it steroidogenic profile. Here, we tested confluent H295R cells, in the sense that this may resemble the quiescent status induced by NR2F1. We also tested the impact of glucose and glutamine deprivation by checking lysosomal activity to test the impact of nutritional deprivation on confluency and NR2F1 expression. H295R cells were compared with HAdCC human normal adrenocortical cells (31). In sub-confluent conditions, lysosomal activity showed increase in both glucose- and glutamine-deprived conditions in HAdCC cells, compared to the control cells (0.87 vs. 0.01; p=0.0083 and 0.70 vs. 0.01; p=0.0025, respectively) (Figure 4A), but not in H295R cells (2.2 vs. 0.5; p=0.27 and 0.6 vs. 0.5; p=0.29, respectively) (Figure 4B). Lysosomal activity was increased in confluent HAdCC control cells compared to the sub-confluent HAdCC control cells (1.0 vs. 0.01, p=0.0025), while it was further increased in “no glucose” and “no glutamine” confluent conditions [comparison of confluent “no glucose” (7.9, p=1.1E-06) and “no glutamine” (9.5, p=7.3E-05) with confluent “control” (1.0)] (Figure 4A). In the H295R cells, the increase of lysosomal activity was even stronger in confluent control cells compared to the sub-confluent control cells (25.7 vs. 0.5, p=5.2E-05). However, it was attenuated in “no glucose” and “no glutamine” conditions [comparison of “no glucose” (19.1, p=0.020) and “no glutamine” (15.2, p=0.0030) with “control” (25.7)] (Figure 4B). In confluent HAdCC cells, glucose- and glutamine-deprivation increased lysosome activity compared to the control cells (Figure 4A). Conversely, in H295R cells, in confluent condition, lysosome activity was decreased in the glucose- and glutamine- deprivation groups compared to the control group (Figure 4B).

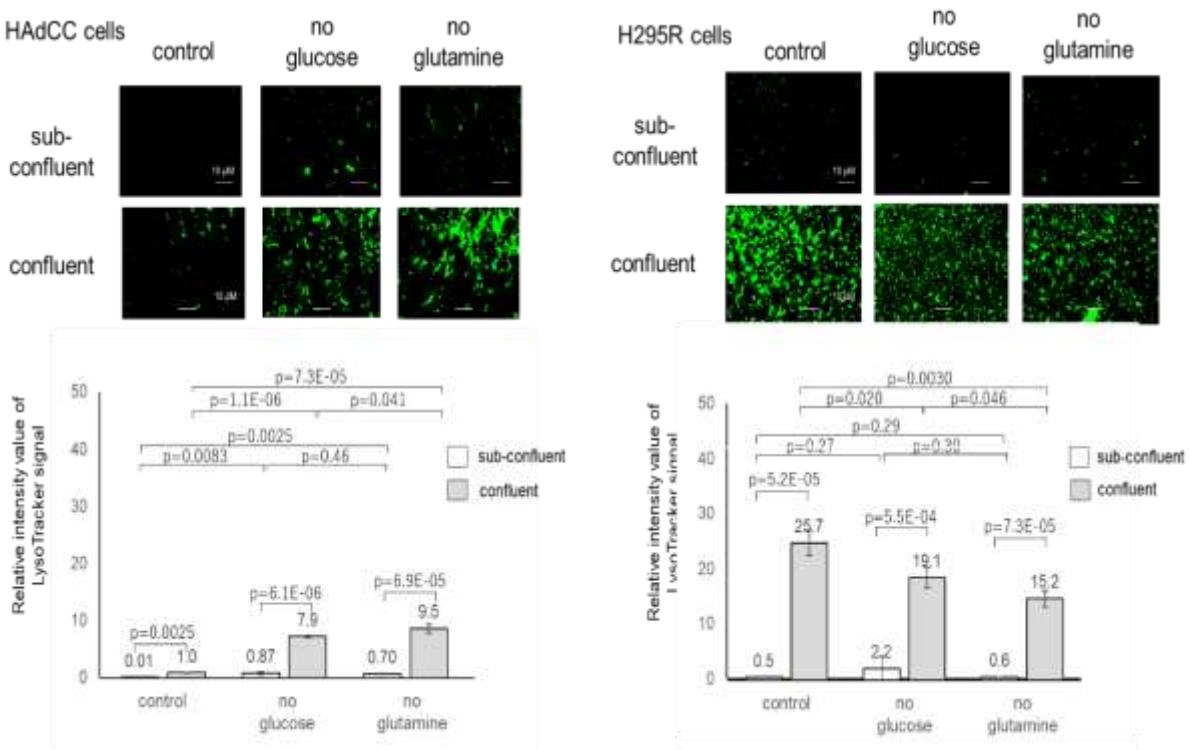

Figure 4: HAdCC normal adrenocortical cells and H295R cells showed differential lysosome activity in various conditions. Lysosome activity of HAdCC cells (A) or H295R cells (B) in normal conditions or glucose- or glutamine-deprived cells during sub-confluent and confluent conditions. The graphs present the quantified results from three independent experiments. The tables show the ratio of lysosome activity of glucose- or glutamine-deprived cells to that of control cells.

NR2F1, CDK1 and CDKN1A were induced by confluency in H295R cells, and this induction was attenuated by glutamine deprivation.

We compared the NR2F1 protein expression levels between sub-confluent and confluent conditions. NR2F1 showed an increase of 1.99-fold change (p=0.006) in confluent, compared to sub-confluent H295R cells (Figure 5A). Based on the differential lysosome activity of HAdCC and H295R cells in confluent conditions, we tested the expression of cell cycle proteins in H295R cells. CDK1 and CDKN1A were induced by confluency (Figure 5B and 5C: compare "sub-confluent" and "confluent" conditions of "control" for CDK1 and CDKN1A); however, this increase was attenuated by glutamine deprivation (Figure 5B and 5C: compare "control" and "no glutamine" in confluent condition for CDK1 and CDKN1A). Glucose deprivation did not affect the expression of these proteins as strong as glutamine deprivation (Figure 5B and 5C: compare "control" and "no glucose" with "control" and "no glutamine" in confluent condition for CDK1

and CDKN1A) in H295R cells. The expression of CDKN1B, phosphorylated AKT, and P70-S6K showed no significant change in these conditions.

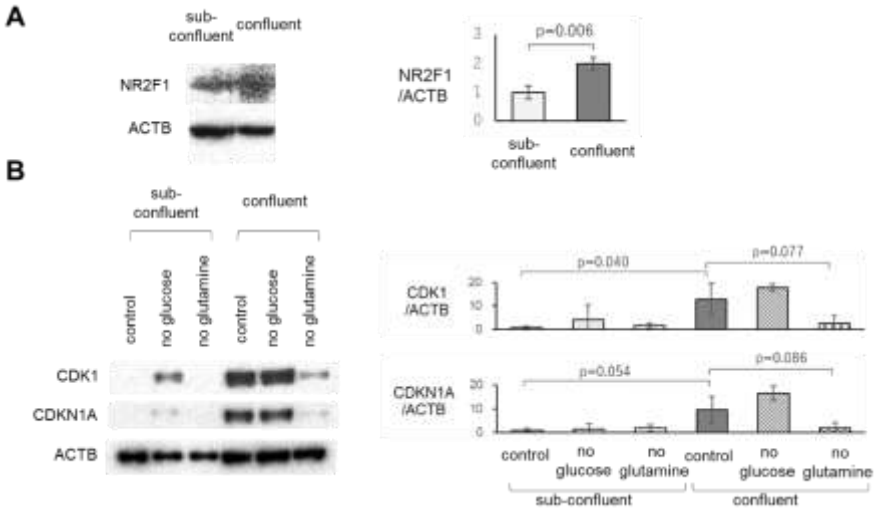

Figure 5: Differential expression of cell cycle-related and mTOR signal-related proteins of normal, glucose- and glutamine- deprived H295R cells, in sub-confluent and confluent conditions. (A) Quantification of NR2F1 protein in sub-confluent and confluent conditions. (B) Quantification of CDK1 protein in sub-confluent and confluent conditions with glucose and glutamine deprivation. Quantification of CDKN1A protein in sub-confluent and confluent conditions with glucose and glutamine deprivation. Values represent means ±standard deviation from 3 experiments and relative ratio of expression was compared to sub-confluent control.

DISCUSSION

ACC is well known for its very poor prognosis with very limited choices of therapy (2). Despite ongoing efforts, the intricate molecular mechanisms underlying regrowth at metastatic sites during ACC progression remain elusive, necessitating further exploration to uncover potential targets for intervention. Our study focused on examining the role of the dormancy factor, NR2F1 (12), in H295R adrenocortical cancer cells.

Initially, we analyzed publicly available databases and found that ACCs that highly expressed NR2F1 had a better prognosis, though not statistically significant, than those without high expression of NR2F1. Analysis of a dataset of a microarray study with normal adrenal, adrenal adenoma, and ACC samples using the BioGPS microarray database portal (http://biogps.org/), showed that NR2F1 gene expression is attenuated in adrenal adenoma compared to normal adrenal, while it is further decreased in ACC.

Transient over-expression of NR2F1 attenuated the growth of H295R adrenocortical cancer cells. NR2F1-stably-transfected H295R cells showed G0/G1 cell cycle arrest and G2/M cell cycle arrest by flow cytometry analyses. This is known to be induced by up regulated CDKN1B and CDKN2A via up regulation by SOX9 and retinoic acid (RA) receptor β (RARβ) (12). Our finding is in concert with the lack of down-regulation of CDKN1B (Figure 3B).

Immunoblot analyses were in line with a quiescent state supported by the reduced CDK1 expression (32), maintained CDKN1B expression (33), inhibited AKT phosphorylation (34) and P70-S6K phosphorylation (35) forming a pre-dormant state. On the other hand, in confluent H295R cells overexpressing NR2F1, there was a robust up regulation of CDK1 and CDKN1A protein expression, compared to sub-confluent H295R cells. Consequently, we posit that the heightened expression of NR2F1 may represent a feedback mechanism responding to the overexpression of CDK1 and CDKN1A, possibly as an attempt to down regulate these factors. To determine the validity of this feedback mechanism, further investigation is required. However, our study revealed that glutamine deprivation significantly attenuates the expression of CDK1 and CDKN1A without changing the expression of NR2F1, whereas glucose deprivation does not. CDK1 has recently been reported for its role in epithelial–mesenchymal transition, G2/M phase transition, and PANoptosis in ACCs (27). CDKN1A is known for its function in senescence (28), a state where quiescent cells cannot reactivate (16). Whether these H295R quiescent cells induced by NR2F1 or contact inhibition have a pro-tumorigenic (resistance to chemotherapy) or anti-tumorigenic (anti-proliferative) effect may be context-dependent (36). Though the interplay of NR2F1 and glutamine deprivation needs more investigation, we believe the two may serve independently or in combination as a novel target in ACCs.

The present study has several limitations. First, we could not find any direct association between NR2F1 and glutamine metabolism. The functional long noncoding RNA of NR2F1-AS1 is known to be involved in tumorigenesis and in glutamine metabolism in cancer (37). Future analyses of NR2F1-AS1 in glutamine deprivation of confluent H295R cells may uncover the precise mechanism of glutamine in advanced ACCs. Second, confluency is not a model of advanced ACC and glutamine deprivation may induce a chemo-resistant phenotype. Advanced screening of potent biomarkers in advanced ACCs found overexpression of wild type p53 as a potent biomarker (38) indicating that sensitivity to glutamine deprivation may not be a common feature of ACC. Third, the observed down regulation of CDKN1A needs to be checked of its nuclear-cytoplasmic localization, an important aspect of its function (29). Finally, the

discrepancy of CDKN1B protein level, phosphorylation level of AKT and P70-S6K in NR2F1-overexpressed cells and confluent cells needs further investigation.

In low-glutamine conditions, cells depend on pyruvate carboxylase for growth, and they end up using glucose-derived pyruvate rather than glutamine for anaplerosis (39). We added bicarbonate to the media instead of pyruvate to ensure glutamine-deprived conditions. The effect of glutamine deprivation is enhanced without pyruvate because of its proton-dependent uptake into the cell through the monocarboxylate transporter.

In summary, we have found that NR2F1 possesses its characteristics as a dormancy factor in adrenocortical cancer and that glutamine may be a key nutrient in controlling the expression of CDK1 and CDKN1A in adrenocortical cancer cells. Our results indicate a novel role for NR2F1 and shed light on potential therapeutic avenues for ACC. These findings not only expand our understanding of ACC pathogenesis but also pave the way for future exploration of targeted interventions in other cancer recurrences where NR2F1 is involved, to address this clinical challenge.

Conflicts of Interest

All the Authors have no conflicts of interest to disclose.

Authors' Contributions

Y.H. and K.O. conducted the experiments with the assistance of T.T. and K.O. . K.O. wrote the manuscript. M.E. supervised and arranged the experiments.

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