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Fukushima Medical University

学位論文

題　名：Upregulation of glucocorticoid receptor-mediated glucose transporter 4 in enzalutamide-resistant prostate cancer

（エンザルタミド耐性前立腺癌でのグルココルチコイド受容体により制御されるグルコーストランスポーター4 の亢進）

論 文 内 容 要 旨

氏名（しめい）	ほし　せいじ 星　誠二
学位論文題名	**Upregulation of glucocorticoid receptor-mediated glucose transporter 4 in enzalutamide-resistant prostate cancer**（エンザルタミド耐性前立腺癌でのグルココルチコイド受容体により制御されるグルコーストランスポーター4 の亢進）

（背景と目的）　前立腺癌は、アンドロゲン依存的に増殖し、アンドロゲンの分泌抑制およびアンドロゲン受容体（AR）阻害薬が治療に用いられる。第一世代の抗アンドロゲン薬に対する耐性を生じた前立腺癌は去勢抵抗性前立腺癌と呼ばれ根治不能であり、様々な薬剤が開発されている。エンザルタミドは、第二世代抗アンドロゲン薬であり、CRPC 患者の予後を延長させる薬剤として知られているが、治療過程で耐性を生じることが問題とされている。さらにエンザルタミドに耐性を生じた場合、他の AR シグナル阻害薬にも耐性を生じること（交叉耐性）から耐性機序に関する研究が進められている。近年では、エンザルタミドの耐性および交叉耐性に、グルココルチコイド受容体（GR）の発現亢進が関与していることが報告されているが、どのような過程で耐性に関与しているかは不明である。GR を阻害することで、耐性の克服を試みる研究はあるが、GR の複雑な制御や選択性の高い阻害薬が無いことから、有効な結果は得られていない。そのため、GR がどのような経路の制御を介して、エンザルタミド耐性および交叉耐性を獲得するかを解明することは、エンザルタミド耐性や交叉耐性を有する患者への新たな治療標的となる可能性がある。また一般的に癌の薬物耐性において、腫瘍の糖代謝特に糖取り込みを担うグルコーストランスポーター（GLUT）の働きは重要である。今回我々は、アンドロゲンに関連し前立腺癌に発現している GLUT である GLUT4 に着目した。前立腺癌のエンザルタミドの耐性過程において、GR と GLUT4 の発現変化や制御機構、薬剤耐性に対する関与を検討した。

（結果）　本研究では、まずエンザルタミド耐性株 LNEnzR 細胞を樹立し、エンザルタミド耐性および交叉耐性を生じていることを証明した。LNEnzR 細胞は GR および GLUT4 を高発現する細胞であった。次に、AR 陰性 GR 陽性の前立腺癌細胞株である PC3 細胞において、GR が AR と独立して GLUT4 を制御し得ることを証明した。最後に、LNEnzR 細胞において、GR および GLUT4 の阻害が細胞増殖を抑制し、エンザルタミド耐性および交叉耐性を回復し得ることを証明した。

（考察）　今回我々は初めて、前立腺癌のエンザルタミドの耐性および交叉耐性に、GR を介した GLUT4 の発現および機能亢進が関係していることを明らかにした。GLUT4 は選択的な阻害により薬剤耐性の改善や治療効果が期待され、特にエンザルタミドに対する耐性を有する患者で新たな治療標的となることが示唆された。また GLUT4 阻害は、致死的な合併症を来す可能性が低く、生体でも忍容性があることが予測されるため、臨床での応用が期待される。1054/1200

Title：Upregulation of glucocorticoid receptor-mediated glucose transporter 4 in enzalutamide-resistant prostate cancer（エンザルタミド耐性前立腺癌でのグルココルチコイド受容体により制御されるグルコーストランスポーター4 の亢進）

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概要： 前立腺癌は、本邦で男性におけるもっとも多い悪性腫瘍であり、一般的に予後良好な悪性腫瘍とされている。しかし、転移性癌では根治不能であり、本邦でも 2015 年の統計では年間 1 万 2 千人の患者が前立腺癌により死亡している。前立腺癌の患者数は年々増加しており、転移性前立腺癌の治療の発展が望まれている。現在、転移性前立腺癌の治療は、男性ホルモン抑制治療が第一選択とされている。男性ホルモン抑制治療は、中枢性のシグナル抑制により男性ホルモンであるアンドロゲンの分泌を抑制する LH-RH アナログとアンドロゲン受容体（AR）を遮断する抗アンドロゲン薬を併用することが一般的である。しかし、男性ホルモン抑制治療は多くの患者で有効であるものの、ほとんどの患者において 2-3 年程度で効果が不十分になり進行性の病態である去勢抵抗性前立腺癌（CRPC）に至る。CRPC 患者の治療に用いられる治療としては、タキサン系抗がん剤と第二世代抗アンドロゲン薬が用いられる。第二世代抗アンドロゲン薬としてはエンザルタミドおよびアビラテロンが広く用いられており、多くの臨

床試験で有効性が証明されている。しかし、これらの薬剤による治療の過程で、多くの患者が薬物耐性を獲得し、同時に他の AR シグナルを介した治療薬への耐性（交叉耐性）も有することがある。最近行われたクロスオーバー試験では、1 次治療としてエンザルタミドを投与した後に 2 次治療としてアビラテロンを投与した場合、アビラテロン投与後にエンザルタミド投与を行った場合と比較して、2 次治療の反応が不良であったと報告されている。そのため、エンザルタミドの治療後では、アビラテロンに比較し、AR シグナルを介した治療薬に対する耐性が生じやすいと考えられる。

エンザルタミドの耐性機序としては、AR 変異、AR スプライスバリアントの形成、AR 関連遺伝子の活性化などが報告されているが、近年グルココルチコイド受容体（GR）の発現亢進が、エンザルタミド耐性の原因として注目されている。GR が AR 遺伝子に結合して AR シグナルを活性化させたり、GR が AR と独立した経路を活性化させたりすることで、エンザルタミドの耐性を獲得するとされている。しかし実際に GR がどのような経路を活性化させて薬物耐性に寄与しているかは不明である。

一般的にがん細胞の増殖や薬剤耐性には脂質やアミノ酸の代謝亢進、特に糖代謝の亢進が密接に関与している。前立腺癌の薬物耐性に関しては、脂質やアミノ酸の代謝が注目されていたが、糖代謝に関する報告は少ない。がん細胞が薬

物耐性を獲得する過程で多くの糖を消費するが、消費した糖を補うため細胞表面に glucose transporter（GLUT）を発現させ細胞への糖輸送を亢進させることが知られている。GLUT は細胞表面に存在する 12 回膜貫通型の糖輸送タンパクで、14 のアイソフォームが報告されている。そのうち前立腺癌では GLUT1,3,4,12 の発現亢進が報告されている。これらの GLUT のうち、細胞増殖に関連しかつ AR シグナル依存的である GLUT として、GLUT1 と GLUT4 が報告されている。しかし、薬剤耐性獲得過程における GLUT1、GLUT4 の役割はわかっていない。

骨格筋や脂肪細胞において GR を阻害することにより GLUT4 の発現が抑制され、血中から筋肉や脂肪細胞への糖取り込みが低下することが報告されている。このことから、前立腺癌においても GLUT4 の発現が GR により制御されている可能性があり、エンザルタミドの耐性過程で GR の発現が亢進することにより GLUT の発現に変化が起きる可能性があると考えた。

本研究では、まず前立腺癌細胞株を用いてエンザルタミド耐性株を作成し、エンザルタミドの薬剤耐性獲得の過程での GR および GLUT4 の発現変化を検討した。次に、エンザルタミド耐性株における GR、GLUT4 の制御機構について検討した。最後に GLUT4 の阻害がエンザルタミドの耐性および AR シグナルを標的とする薬剤の交叉耐性へ関与するかについて検討し、GLUT4 の阻害が前立腺癌の治療やエンザルタミド耐性の回復に有効かを考察した。

Content：

1. Abbreviation：

Abi: abiraterone

ANOVA: analysis of variance

AR: androgen receptor

Bcl: bicalutamide

cDNA: complementary DNA

CRPC: castration-resistant prostate cancer

DHT: dihydrotestosterone

DMSO: dimethyl sulfoxide

Enz: enzalutamide

GLUT: glucose transporter

GR: glucocorticoid receptor

mRNA: messenger RNA

PET: positron emission tomography

PSA: prostate specific antigen

Rit: ritonavir

RT-PCR: reverse transcription-PCR

siRNA: small interfering RNA or short interfering RNA

2. Introduction：

Enzalutamide (Enz) and abiraterone acetate (abiraterone, Abi) are second-generation small-molecule inhibitors of androgen receptor (AR) signaling, and both are useful for the treatment of castration-resistant prostate cancer (CRPC). However, during treatment with these inhibitors, most patients acquire resistance to them and often show cross-resistance to other AR signaling inhibitors. A recent crossover trial showed that Enz retained clinical activity as a second-line drug following Abi treatment, whereas Abi retained low second-line activity following Enz treatment, even though Abi and Enz have similar first-line activity for metastatic CRPC.[1] Chronic Enz treatment may make CRPC cells acquire resistance to AR signaling inhibitors more easily than Abi.

Although several mechanisms of Enz resistance, including AR mutations, the induction of AR splice variants, and AR-related gene upregulation, have been reported,[2-5] the proposed mechanisms cannot fully explain Enz resistance. In recent reports, glucocorticoid receptor (GR) upregulation has been identified as a driver of Enz resistance.[6-8] Activation of AR signals by GR binding to the AR gene,[8, 9] or activation of an AR-independent pathway by GR, may contribute to Enz-resistance;[6, 8] however, the detailed mechanism of how GR-regulated pathways contribute to Enz resistance has not yet been clarified.

In general, anti-cancer drug resistance is closely related to increased metabolism of glucose[10] as well as lipids and amino acids.[11] Although lipid and glutamine metabolism are considered to play an important role in the process by which prostate cancer (PCa) cells acquire resistance to anti-cancer agents,[12-15] the role of glucose metabolism has remained unclear.[16-18] Cancer cells consume a lot of glucose in the process of acquiring drug resistance,[13, 17] and glucose transporters (GLUTs) are expressed on cell surfaces to supplement the consumed glucose.[19] GLUTs are 12-transmembrane glucose transport proteins that have been reported to have 14 isoforms. In PCa, upregulation of GLUT1, 3, 4, and 12 has been observed[16]. Of these GLUTs, both GLUT1 and GLUT4 are related to AR signaling-dependent cell proliferation.[16, 20] However, the detailed role of GLUTs in the process of acquiring drug resistance in PCa and CRPC has remained unknown.

In skeletal muscle and adipocytes, inhibition of GR suppresses GLUT4 expression and reduces glucose uptake from blood into muscle and adipocytes.[21, 22] Hence, we considered that GLUT4 may be regulated by GR in PCa as in skeletal muscle and adipocytes, and GLUT4 may be involved in acquiring resistance to Enz. In this study, first, we established an Enz-resistant PCa cell line to examine changes in GR and GLUT4 expression during the process of acquiring Enz resistance. Next, we examined the relationship between GR and GLUT4 and the underlying regulatory mechanism in the Enz-resistant cell line.

Finally, we evaluated the effect of GLUT4 inhibition on recovery from resistance to Enz and other AR signaling inhibitors, and discuss a possible new therapeutic strategy in the treatment of CRPC.

3. Materials and Methods：

Reagents and cell lines

Two human PCa cell lines, androgen-dependent LNCaP cells and androgen-independent PC-3 cells, were purchased from the American Type Culture Collection (ATCC) and were maintained in RPMI 1640 and Ham's F-12 Nutrient Mixture Medium, respectively, supplemented with 10% charcoal/dextran-treated FBS (cd-FBS) to perform the experiments. A previous report showed that LNCaP cells produce testosterone, even in cd-FBS medium[23]. In a preliminary study, prostate specific antigen (PSA) levels increased over time, even in cd-FBS medium (Figure 1). In addition, Enz and DHT+Enz administration significantly suppressed cell proliferation to the same levels in both FBS and cd-FBS media (Figure 2). These results suggest that LNCaP cells produce testosterone even in cd-FBS medium, and that Enz suppresses the proliferation of LNCaP cells by inhibiting AR-mediated signaling, regardless of the kind of FBS medium and presence or absence of DHT administration. The media and FBS were purchased from

Thermo Fisher Scientific. All cell lines were grown at 37°C in a humidified 5% CO_2 environment. The medium was changed every 2 days, and cultures were split once a week.

The AR signaling inhibitors Enz, bicalutamide (Bcl; Tokyo Chemical Industry), and Abi (Chemscene); GR inhibitor RU486 (Cayman Chemical); and GLUT4 inhibitors ritonavir (Rit; Chemscene) and dihydrotestosterone (DHT; Cayman Chemical) were used. These drugs (Enz: 10.0 μM, Bcl: 10.0 μM, Abi: 2.5 μM, RU486: 20 μM, Rit: 2 μM, DHT: 10 nM) were administered at doses corresponding to the plasma concentrations of the patients within each treatment.

Establishment of Enz-resistant cell line

To establish an Enz-resistant cell line, LNCaP was first maintained in RPMI medium with 10% cd-FBS. After culturing for 1 week, cells were transferred to the above medium with 10.0 μM Enz and cultured for at least 12 weeks.

Cell viability assay

For the determination of cellular proliferation and viability, water soluble tetrazolium (WST)-1 assays were carried out in 96-well plates using a Cell Proliferation Reagent (Roche Applied Science) according to the manufacturer's protocol. Briefly, 1-7 days after the incubation of cells with DHT, Bcl, Enz, Abi, RU486, Rit, and siRNA, WST-1 reagent was added to each well and incubated for 1 h at 37°C. Spectrophotometric absorbance of

the samples was measured using a microplate reader (Varioskan Flash, Thermo Scientific) and compared against that of nontreated cells.

cDNA construction and quantitative RT-PCR

cDNA construction was performed using a SuperPrep Cell Lysis & RT Kit for qPCR (Toyobo) according to the manufacturer's instructions at 24 h after treatments. PCR reagents for GLUT1 (Hs00892681_m1), GLUT4 (Hs00168966_m1), AR (Hs00171172_m1), and GR (Hs00230813_m1) were purchased from Applied Biosystems, and custom primers were used to amplify androgen receptor splice variant 7 (AR-V7), GLUT3 and GLUT12 (Table 1). Quantitative RT-PCR was carried out using the PowerTrack SYBR® Green Master Mix (Invitrogen) on a StepOne real-time PCR System (Applied Biosystems). The data were standardized against β-actin gene expression using β-actin control reagent (Applied Biosystems).

Western blotting analysis

At 24 or 72 h after drug administration, cells were lysed with RIPA buffer supplemented with protease inhibitors (Nacalai Tesque). Proteins from the LNCaP, LNEnzR, and PC3 cells were extracted and applied to SDS-PAGE. GLUT1 (ab15309), GLUT3 (ab48547), GLUT4 (ab33780), GLUT12 (ab202908), AR (ab9474), AR-V7 (ab19839) and GR (ab3579) were used as primary antibodies. Anti-β-actin antibody (Sigma) was used as an

internal control. Protein bands were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and imaged with the ChemiDoc XRS plus system (Bio-Rad). Individual bands were quantified with Image Lab 3.0 software (Bio-Rad) and normalized against the control value.

siRNA and transfection

We used Silencer™ Select Pre-Designed siRNA (Invitrogen), GR (s6186), GLUT4 (s12934, Table 2) and the negative control (Silencer™ Select Negative Control No. 1 siRNA). For siRNA transfection, Lipofectamine RNAi MAX (Invitrogen) was used according to the manufacturer's instructions. Briefly, 1 day prior to transfection, cells were seeded without antibiotics so as to be 60-80% confluent at the time of transfection. The siRNA-Lipofectamine complexes were prepared by mixing an adequate concentration of siRNA oligomer and Lipofectamine using Opti-MEM Medium (Gibco). The transfected cells were incubated at 37°C in a 5% CO_2 incubator for 24 h until treatment.

Flow cytometry

Cells were plated in six-well plates at 1.0×10^6 cells/well for 24 h. To quantify GLUT1, GLUT3, GLUT4 and GLUT12 expression, we used immunofluorescence staining coupled with flow cytometry. Cells were detached with 0.25% trypsin (Invitrogen) and

rinsed thoroughly in PBS with intermittent centrifugation. Cells were blocked for 10 min with PBS containing 10% normal goat serum and 0.3 M glycine. Permeabilization was skipped to evaluate subcellular GLUTs. Cells were stained for 30 min with anti-GLUT1 antibodies (ab15309), anti-GLUT3 antibodies (ab15311), anti-GLUT4 antibodies (ab48547), anti-GLUT12 antibodies (ab202908) and control antibody (ab91366) at a final concentration of 1 mg/ml in PBS. The cells were rinsed with On-chip T buffer (On-Chip Biotechnologies) and incubated with AlexaFluor 488-conjugated anti-rabbit antibodies (ab96879) and anti-mouse antibodies (ab91366) diluted 1:500 in PBS, respectively. Fluorescence was measured at 488 nm via a flow cytometer (On-Chip Biotechnologies).

Glucose uptake assay

The glucose uptake assay was done performed as described previously with minor modifications.[24] Briefly, cells were treated with 1 mM 2-deoxyglucose (2-DG) for 20 min. The reaction was stopped by harvesting the cells and washing them three times with ice-cold PBS. After removal of an aliquot for cell counting, the cell pellet was solubilized in 10 mM Tris-HCl (pH 7.4) by sonication (Bioruptor II, BM Bio), followed by determination of the amount of 2-DG using a 2DG Uptake Measurement Kit (Cosmo Bio) according to the manufacturer's instructions. Standardization of glucose concentration was accomplished by determining the protein concentration.

Statistical analysis

Determination of cell proliferation, half maximal inhibitory concentration (IC_{50}), mRNA expression level, western blotting analysis, and glucose uptake assays were repeated at least three times independently, and the results were expressed as the mean ± SE. Analyses were performed with SPSS Statistics 21 software (IBM Japan). Data were statistically evaluated using the unpaired two-tailed Student's *t* test for two groups and one-way ANOVA for three groups, and values were considered statistically significant when $P < 0.05$.

4. Results：

Establishment of a LNCaP-derived cell line with acquired resistance against enzalutamide

We selected LNCaP cells, a high AR and low GR expression PCa cell line, and chronically administrated 10 μM Enz to this cell line for at least 12 weeks. The surviving and proliferating resistant cells were pooled, maintained, and finally termed LNEnzR cells. LNEnzR cells were morphologically identical to LNCaP cells (Figure 3A).

A WST-1 assay was performed on LNCaP and LNEnzR cells exposed to various concentrations of Enz, Abi and Bcl. There was a clear reduction in cell viability of LNCaP

and LNEnZR cells after Enz (Figure 3B), Abi (Figure 3C) and Bcl (Figure 3D) administration in a dose-dependent manner. Specifically, the IC_{50} values for Enz, Abi and Bcl were 13.2±6.2, 2.5±1.0 and 15.5±5.2 μM, respectively, for LNCaP cells, and 143.5±21.3, 62.9±12.5 and 75.5±12.6 μM, respectively, for LNEnzR cells. The IC_{50} values obtained for Enz, Abi and Bcl in LNEnzR cells were significantly larger than those in LNCaP cells ($p<0.01$).

We examined the effect of Enz, Abi and Bcl administration on cell proliferation and glucose uptake in these cell lines. DHT administration increased cell proliferation in LNCaP cells ($p<0.01$), but not in LNEnzR cells. Enz administration significantly suppressed cell proliferation in LNCaP cells ($p<0.05$), but not in LNEnzR cells. These results indicate that LNEnzR cells have acquired resistance to Enz. We also examined whether LNEnzR cells exhibited cross-resistance to other AR-signaling inhibitors, i.e., Abi or bicalutamide (Bcl). As with Enz administration, cell proliferation was suppressed significantly after Abi or Bcl administration in LNCaP cells ($p<0.05$), but not in LNEnzR cells (Figure 4A). These results indicate that LNEnzR cells not only have resistance to Enz, but are also cross-resistant to other AR-signaling inhibitors, such as Abi and Bcl.

Glucose uptake in LNCaP cells was significantly increased by DHT administration ($p<0.01$) and decreased by Enz, Abi and Bcl administration ($p<0.05$). However, glucose

uptake in LNEnzR cells was not affected by DHT administration, but it was significantly increased after Enz, Abi and Bcl administration ($p<0.05$; Figure 4B). These findings indicate that Enz, Abi and Bcl treatment promotes glucose uptake by LNEnzR cells.

A question is how Abi, which suppressed the production of androgens by inhibiting CYP17A1, suppressed cell proliferation in LNCaP. A previous report showed that Abi inhibits AR signals by inhibiting intracellular testosterone metabolism in vitro[25]. In a preliminary study, we examined cell proliferation in LNCaP cells exposed to various concentrations of Abi. As shown in Figure 5, although Abi suppressed cell proliferation in a dose-dependent manner, Abi administration plus siRNA-induced AR knockdown did not exhibit the same dose-dependent reduction in cell proliferation as Abi alone. These results indicate that both Abi and Enz have an antiproliferative effect that is related to the inhibition of the AR signal and intracellular testosterone synthesis and production in LNCaP cells, even in a cd-FBS medium.

Alteration of AR and GR expression levels in LNEnzR cells

We compared AR and GR expression levels between LNCaP and LNEnzR cells to examine whether AR and GR expression levels changed in the process of acquiring resistance to Enz. Compared to LNCaP cells, LNEnzR cells showed statistically

significant decreases in AR expression ($p<0.05$) and increases in GR expression ($p<0.01$) at the mRNA and protein levels (Figure 6). Chronic exposure to Enz upregulated GR expression, as described in previous reports.[6, 8, 18] We also examined the expression level of AR-V7 in these cells and found that AR-V7 mRNA and protein expression was not observed in either cell lines (data not shown).

Next, we examined the changes in AR expression levels in these cell lines due to AR stimulation by DHT and AR signal inhibition by Enz (Figure 7A, B and E). AR mRNA expression levels were significantly reduced by DHT, Enz and DHT+Enz administration in LNCaP cells ($p<0.05$). AR protein levels were significantly increased by DHT administration and reduced by Enz and DHT+Enz administration in LNCaP cells ($p<0.05$). However, significant changes in AR mRNA and protein expression levels in response to the administration of these drugs were not observed in LNEnzR cells. These results indicate that LNEnzR cells lost the AR reactivity which was observed in LNCaP cells after DHT and Enz administration. We also examined the changes in GR expression levels in these cell lines due to DHT and Enz administration. In LNCaP cells, the GR expression level was not significantly changed by DHT, Enz and DHT+Enz administration, while in LNEnzR cells, it was significantly increased by Enz and DHT+Enz administration ($p<0.01$), but not by DHT administration. (Figure 7C, D, and

E). These results suggest that in LNEnzR cells, which have high GR expression levels, Enz and Abi treatment increases GR expression levels.

Alterations of GLUT1, GLUT3, GLUT4 and GLUT12 expression levels in LNEnzR cells

Since a previous study demonstrated that upregulation of GLUT1, 3, 4, and 12 has been observed in PCa,[16] we compared the expression levels between LNCaP and LNEnzR cells. No statistically significant difference in GLUT1, GLUT3 and GLUT12 mRNA and protein levels was observed between the two cell lines; however, significantly higher levels of GLUT4 expression were observed in LNEnzR cells compared to LNCaP cells ($p<0.01$; Figure 8A, B, and C). To evaluate the expression of activated GLUT1, GLUT3, GLUT4 and GLUT12 proteins on the cell surface, we performed flow cytometry using LNCaP and LNEnzR cells. Although there were no significant differences among GLUT1, GLUT3 and GLUT12 expression levels at the cell surface in either cell line, GLUT4 expression levels at the cell surface were significantly higher in LNEnzR cells than in LNCaP cells ($p<0.05$; Figure 8D, E, F and G). These results indicate that chronic Enz treatment not only induces increased expression, but it also activates GLUT4 expression.

To evaluate the effect of androgen stimulation and inhibition on GLUT1 and GLUT4

expression, both of which are related to AR signaling-dependent cell proliferation,[16, 20] we examined changes in GLUT1 and GLUT4 expression levels after DHT and Enz administration in LNCaP and LNEnzR cell lines (Figure 9). In androgen-dependent LNCaP cells, AR stimulation by DHT increased significantly GLUT1 and GLUT4 expression levels ($p<0.01$), but Enz and DHT+Enz administration suppressed these expression levels ($p<0.05$). Conversely, in LNEnzR cells, which lose the AR reactivity observed in LNCaP cells and have high GR expression, DHT administration did not change GLUT1 or GLUT4 expression levels, but Enz and DHT+Enz administration did increase GLUT4 expression levels ($p<0.01$). These findings showed that LNEnzR cells, which originally had high GLUT4 expression levels, Enz treatment promotes further increases in GLUT4 expression.

Effects of GR and GLUT4 inhibition on cell proliferation in PC3 cells with negative AR expression, but positive GR expression

As shown above, both GR and GLUT4 were upregulated in LNEnzR cells. To assess the correlation between GR and GLUT4 in GR-positive PCa cells, we examined the change in GR and GLUT4 expression levels by GR and GLUT4 inhibition in PC3 cells. Since it was possible that the expression of GLUT4 would be influenced by AR signals,

we first utilized PC3 cells, which are characterized by having negative AR but positive GR expression. siRNA-induced GR knockdown and administration of the GR inhibitor RU486 significantly decreased both GR and GLUT4 expression at the mRNA and protein levels, whereas siRNA-induced GLUT4 knockdown and administration of the GLUT4 inhibitor ritonavir (Rit), decreased only GLUT4 expression without changing GR expression (Figure 10A, B, and C). These results indicate that GR regulated GLUT4 expression, but GR expression was not influenced by GLUT4 inhibition.

We performed WST-1 assays to evaluate the effect of GR and GLUT4 inhibition on cell proliferation. siRNA-induced GR and GLUT4 knockdown and administration of a GLUT4 inhibitor significantly reduced cell proliferation at 3, 5, and 7 days after treatment compared to controls ($p<0.01$). GR inhibitor administration significantly reduced cell proliferation at 5 ($p<0.05$) and 7 days ($p<0.01$) after treatment compared to the control (Figure 10D). We also performed glucose uptake assays to evaluate the changes in glucose uptake by GR and GLUT4 inhibition. siRNA-induced GR and GLUT4 knockdown, as well as administration of GR and GLUT4 inhibitors, significantly reduced cellular glucose uptake compared to controls (Figure 10E; $p<0.05$). These results indicate that GR regulated GLUT4 expression independently of AR signals, and that GR and GLUT4 inhibition reduced cell proliferation and glucose uptake.

Effects of GR and GLUT4 inhibition on cell proliferation and glucose uptake in LNEnzR cells

To evaluate whether GR and GLUT4 inhibition influenced the proliferation and glucose uptake of LNCaP cells, which have low GR expression levels, and LNEnzR cells, which have high GR expression levels, we performed siRNA-induced GR and GLUT4 knockdown and GR and GLUT4 inhibitor administration on these cell lines, and measured cell proliferation by a WST-1 assay and glucose uptake by a glucose uptake assay. siRNA-induced GR knockdown and administration of the GR inhibitor RU486 did not reduce cell proliferation and glucose uptake in LNCaP cells, whereas siRNA-induced GLUT4 knockdown and administration of the GLUT4 inhibitor Rit significantly reduced cell proliferation and glucose uptake in this cell line (Figure 11). These results indicated that GR inhibition did not reduce cell proliferation and glucose uptake in LNCaP cells, because LNCaP cells have low GR expression, but GLUT4 inhibition reduced cell proliferation by inhibiting glucose uptake in this cell line. In contrast, siRNA-induced GR and GLUT4 knockdown, and administration of the GLUT4 inhibitor Rit, reduced cell proliferation and glucose uptake ($p<0.05$), but administration of the GR inhibitor RU486 significantly promoted cell proliferation and glucose uptake in LNEnzR cells, which have high levels of GR expression ($p<0.01$, Figure 11). As described above, RU486

administration significantly decreased the proliferation of PC3 cells. Completely opposite results were observed between LNEnzR cells, which are AR positive and have high levels of GR expression, and PC3 cells, which are AR negative and GR positive.

We evaluated whether the changes in cell proliferation by GR and GLUT4 inhibition resulted from changes in GR and GLUT4 expression. We examined the changes not only of GLUT4, but also of GLUT1 expression, because it is possible that GR and GLUT4 inhibition may induce changes in GLUT1 expression. In LNCaP cells, siRNA-induced GR knockdown significantly decreased GR expression ($p<0.05$), but use of the GR inhibitor RU486 did not change GR expression at the mRNA and protein levels. Neither siRNA-induced GR knockdown or the GR inhibitor influenced GLUT1 and GLUT4 expression levels in this cell line. siRNA-induced GLUT4 knockdown and administration of the GLUT4 inhibitor Rit significantly decreased GLUT4 expression without changing GR or GLUT1 expression levels in this cell line ($p<0.01$; Figure 12A, B and E). These results indicate that GR and GLUT4 do not regulate each other in AR signaling-dependent and GR signaling-independent LNCaP cells. On the other hand, in LNEnzR cells, siRNA-induced GR knockdown decreased both GR ($p<0.01$) and GLUT4 expression ($p<0.05$) at the mRNA and protein levels without changing the GLUT1 expression level. In addition, GLUT4 knockdown and the GLUT4 inhibitor Rit significantly decreased GLUT4

expression levels without changing GR and GLUT1 expression levels ($p<0.01$). These results indicate that GR regulated GLUT4 expression, and that, like PC3 cells, GR expression was not influenced by GLUT4 inhibition in LNEnzR cells. The GR inhibitor RU486 significantly increased GR and GLUT4 expression levels ($p<0.01$; Figure 12C, D and E). As described above, GR inhibitor administration significantly decreased both GR and GLUT4 expression at the mRNA and protein levels in PC3 cells. Thus, completely opposite results were observed between LNEnzR and PC3 cells.

Inhibition of GR and GLUT4 improves resistance to anti-androgens in LNEnzR cells

To evaluate whether the inhibition of GR and GLUT4 can increase drug resistance to AR signaling inhibitors in LNEnzR cells, we performed WST-1 assays to examine the effect of Enz or Abi treatment on cell proliferation when GR and GLUT4 are inhibited. Although the administration of Enz or Abi alone did not reduce cell proliferation, the administration of Enz or Abi combined with GR or GLUT4 inhibition, such as by siRNA-induced GR or GLUT4 knockdown or by a GLUT4 inhibitor, significantly reduced cell proliferation ($p<0.01$). These reductions were significantly larger than GR or GLUT4 inhibition alone ($p<0.05$; Figure 13). These results suggest that GR-mediated GLUT4

upregulation contributes to resistance to Enz in PCa cells, and that GR and GLUT4 inhibition can improve the resistance.

5. Discussion：

In this study, for the first time, we demonstrated that GR-mediated GLUT4 upregulation was involved in Enz resistance and cross-resistance in PCa treatment, and that GLUT4 inhibition improved resistance. Although it has been reported that GR is upregulated as a bypass for AR signal inhibition by Enz, no reports have showed that GLUT4 is regulated by GR and can contribute to acquiring resistance to Enz and other AR signaling inhibitors.

Previous reports have shown that the expression of GR changes during PCa treatment.[6,8,26] The expression levels of GR in untreated PCa cells is lower than in normal prostate cells, and in the process of acquiring resistance to second-generation anti-androgens (Enz and Abi), GR expression increases inversely with AR expression.[8] In addition, the upregulation of GR enhances the expression of KLK3 and FKBP5, which are androgen-responsive genes, and SGK1, which is a glucocorticoid-responsive gene, and might contribute to the acquisition of resistance to AR signaling inhibitors.[6] FKBP5 and SGK1 are involved in the expression and activation of GLUT1 and GLUT4 via the PIK3 pathway and AMPK pathway.[27-30] In this study, we demonstrated that GR and

GLUT4 were upregulated in LNEnzR cells, but not GLUT1. Since activation of the PIK3 and AMPK pathways due to upregulation of FKBP5 and SGK1 influences both GLUT1 and GLUT4 expression, [29-31] the upregulation of GLUT4 expression without alteration of GLUT1 expression may not be mediated only by these pathways when acquiring Enz resistance. Previous reports have shown that the functions of GLUT1 and GLUT4 in PCa cells depend on whether the cells are androgen dependent or independent,[20,32] and also that the expression of GLUT1 is lower and the expression of GLUT4 is higher in androgen-independent PCa cells than in androgen-dependent PCa cells.[20] Their findings suggest that GLUT4 may play a more significant role than GLUT1 in androgen-independent PCa cells, such as Enz-resistant cells. In this study, we also demonstrated that GLUT4, which was regulated by GR, was involved in Enz resistance and cross-resistance in LNEnzR cells. Therefore, GLUT4 may be regulated by GR via a different pathway from GLUT1 regulation in Enz resistance and cross-resistance in PCa cells; however, the detailed mechanism and pathway by which GR mediates GLUT4 to confer resistance remains unknown, and further study is needed.

In our study, although administration of Enz or Abi alone did not reduce cell proliferation, administration of Enz or Abi combined with GR or GLUT4 inhibition, significantly reduced cell proliferation. These reductions were significantly larger than

those for GR or GLUT4 inhibition alone. These findings showed that GR and GLUT4 inhibition can improve the resistance of LNEnZR cells to Enz or Abi. Here, we hypothesize how GR or GLUT4 inhibition facilitated the recovery of sensitivity to Enz or Abi in LNEnzR. Briefly, although AR expression levels were low in LNEnzR cells, expression still occurred and was not changed by GR or GLUT4 inhibition (Figure 14). Consequently, Enz may still have potential to inhibit cell proliferation via AR in LNEnzR cells. However, this potential may be masked by Enz administration, because Enz markedly increased cell proliferation in LNEnzR, which in turn showed higher levels of GR expression, increasing GLUT4 expression and activating glucose uptake. However, the administration of Enz combined with GR or GLUT4 inhibition improved resistance to Enz, possibly because GR and GLUT4 inhibition may unmask the potential of Enz to inhibit cell proliferation via AR in LNEnzR cells. Further studies are required to better clarify these mechanisms.

In our study, there was discrepancy in the effects on cell proliferation for LNEnzR cells between siRNA-induced GR knockdown and administration of the GR inhibitor RU486; siRNA-induced GR knockdown suppressed cell proliferation, but the GR inhibitor promoted it. Several reasons should be considered to explain this discrepancy. First, RU486 administration to LNEnzR cells, like Enz administration, induced GR

upregulation since it has anti-androgenic effects. Second, GR inhibitors can possibly induce iatrogenic tumor proliferation because of receptor-to-receptor interactions caused by structural similarities between AR and GR, [33-35] the suppression of tumor immunity [33], and activation of the p53 gene by therapeutic modification.[36,37] Third, RU486 does not have sufficient inhibitory activity.[38] Since siRNA-induced GR knockdown could suppress cell proliferation in LNEnzR cells, sufficient inhibition of GR with a highly-selective GR inhibitor, which is in development,[39] may be able to suppress cell proliferation in LNEnzR cells. Forth, the effects of RU486 on cell proliferation depend on the AR and GR expression levels, which were different among the three cell lines, PC3, LNCaP, and LNEnzR; RU486 administration reduced cell proliferation in PC3 cells, did not change cell proliferation in LNCaP cells, and promoted cell proliferation in LNEnzR cells. These differences may have been caused by the different expression levels of AR and GR in these cells.

Our study may have clinical significance for the treatment of patients with CRPC. First, GR expression in PCa cells may be increased in some CRPC patients with resistance to Enz and other AR signaling inhibitors, and GLUT4 inhibition may mitigate resistance to them in CRPC patients with increased GR expression. Second, although GR is a classic therapeutic target for CRPC, the therapeutic effect of the GR inhibitor RU486 was

limited,[40] as described above. Since GLUT4 inhibition did not affect GR expression, a GLUT4 inhibitor could provide good therapeutic effects without the iatrogenic cancer cell proliferation induced by GR inhibitors in some CRPC patients. Third, in our study, the expression of GLUT4 increased in the process of acquiring resistance to Enz and AR signaling inhibitors, and its inhibition mitigated the resistance. Therefore, the GLUT4 expression level, regardless of the GR expression level, may be a predictor for the therapeutic effect not only of Enz, but also of other AR signaling inhibitors. In general, glucose is the primary substrate for energy metabolism in tissues, and a continuous supply of glucose is required for cells to function. Thus, glucose utilization measured using fluorodeoxyglucose (FDG)-PET has become an established method for quantifying local functional activity in brain, heart, and most cancers. 2-Deoxy-D-glucose (2-DG) is a glucose analog that utilizes the GLUTs, including GLUT4, for entry into the cell. The kinetics of 2DG are similar to those of FDG. Since PCa cells have low expression levels of GLUT1, FDG-PET is not well suited for the detection of PCa. However, FDG-PET could potentially be used to evaluate the expression of GLUT4,[41] which could then be used to predict the therapeutic effect of AR signaling inhibitors in PCa in actual clinical settings.

GLUT4 is an insulin-sensitive glucose transporter that facilitates insulin-stimulated

glucose uptake in adipose tissue, skeletal muscle, and cardiac tissues.[42,43] Since GLUT4 expression is low in the central nervous system and most organs except the heart, GLUT4 inhibitors may lead to fewer fatal complications than GLUT1 inhibitors, since GLUT1 is expressed in many important organs. In fact, the GLUT4 inhibitor ritonavir is actually used for patients with acquired immunodeficiency syndrome due to human immunodeficiency virus infection, and it rarely induces fatal complications.[44,45] Additionally, a clinical trial using GLUT4-selective inhibitors for patients with melanoma has been reported.[46] Combination therapy with AR signaling inhibitors, including Enz and a GLUT4 inhibitor, may be a new therapeutic strategy for patients with CRPC.

There are several limitations to our study. First, we did not consider the effects of AR signals in the process of acquiring resistance to AR signaling inhibitors in our cell lines. In patients with CRPC, AR and GR interact with each other to control tumor survival and proliferation;[8] however, since AR expression is low in the cell line used in this study, it is unclear whether GR or GLUT inhibition is effective under the presence of AR expression and AR-GR interaction. Second, the assessment of GLUT4 expression in patients with PCa is difficult because the expression can be changed by the effects of insulin, insulin-like proliferation factors, and testosterone. Third, we did not show how glucose uptake was involved in Enz resistance and how GR regulates GLUT4. Fourth, this is an in vitro

study; detailed mechanisms will be clarified by in vivo studies or clinical samples compatible with Enz resistant conditions. Further study will be needed to establish a method for evaluating the expression of GR and GLUT4 *in vivo*.

In conclusion, GR-mediated GLUT4 upregulation by chronic Enz treatment may be involved in Enz resistance as well as cross-resistance to other AR signaling inhibitors. The inhibition of GLUT4 suppressed the proliferation of Enz-resistant PCa cells without changing GR expression, and recovered Enz resistance and cross-resistance. Our study may provide a new therapeutic strategy for Enz-resistant CRPC patients in the future.

6. References：

1 Khalaf DJ, Annala M, Taavitsainen S, et al. Optimal sequencing of enzalutamide and abiraterone acetate plus prednisone in metastatic castration-resistant prostate cancer: a multicentre, randomised, open-label, phase 2, crossover trial. *The Lancet Oncology*. 2019; 20: 1730-1739.

2 Pal SK, Patel J, He M, et al. Identification of mechanisms of resistance to treatment with abiraterone acetate or enzalutamide in patients with castration-resistant prostate cancer (CRPC). *Cancer*. 2018; 124: 1216-1224.

3 Tucci M, Zichi C, Buttigliero C, Vignani F, Scagliotti GV, Di Maio M. Enzalutamide-resistant castration-resistant prostate cancer: challenges and solutions. *OncoTargets and therapy*. 2018; 11: 7353-7368.

4 Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. *Nature medicine*. 2004; 10: 33-39.

5 Kregel S, Chen JL, Tom W, et al. Acquired resistance to the second-generation androgen receptor antagonist enzalutamide in castration-resistant prostate cancer. *Oncotarget*. 2016; 7: 26259-26274.

6 Arora VK, Schenkein E, Murali R, et al. Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. *Cell*. 2013; 155: 1309-1322.

7 Chen X, Chen F, Ren Y, et al. Glucocorticoid receptor upregulation increases radioresistance and

triggers androgen independence of prostate cancer. *The Prostate*. 2019; 79: 1386-1398.

8 Puhr M, Hoefer J, Eigentler A, et al. The Glucocorticoid Receptor Is a Key Player for Prostate Cancer Cell Survival and a Target for Improved Antiandrogen Therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2018; 24: 927-938.

9 Sahu B, Laakso M, Pihlajamaa P, et al. FoxA1 specifies unique androgen and glucocorticoid receptor binding events in prostate cancer cells. *Cancer research*. 2013; 73: 1570-1580.

10 Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell metabolism*. 2016; 23: 27-47.

11 Keenan MM, Chi JT. Alternative fuels for cancer cells. *Cancer journal (Sudbury, Mass)*. 2015; 21: 49-55.

12 Galbraith L, Leung HY, Ahmad I. Lipid pathway deregulation in advanced prostate cancer. *Pharmacological research*. 2018; 131: 177-184.

13 Giunchi F, Fiorentino M, Loda M. The Metabolic Landscape of Prostate Cancer. *European urology oncology*. 2019; 2: 28-36.

14 Stoykova GE, Schlaepfer IR. Lipid Metabolism and Endocrine Resistance in Prostate Cancer, and New Opportunities for Therapy. *International journal of molecular sciences*. 2019; 20.

15 Strmiska V, Michalek P, Eckschlager T, et al. Prostate cancer-specific hallmarks of amino acids metabolism: Towards a paradigm of precision medicine. *Biochimica et biophysica acta Reviews on cancer*. 2019; 1871: 248-258.

16 Gonzalez-Menendez P, Hevia D, Mayo JC, Sainz RM. The dark side of glucose transporters in prostate cancer: Are they a new feature to characterize carcinomas? *International journal of cancer*. 2018; 142: 2414-2424.

17 Wade CA, Kyprianou N. Profiling Prostate Cancer Therapeutic Resistance. *International journal of molecular sciences*. 2018; 19.

18 Prekovic S, van den Broeck T, Linder S, et al. Molecular underpinnings of enzalutamide resistance. *Endocrine-related cancer*. 2018; 25: R545–r557.

19 Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *Journal of cellular physiology*. 2005; 202: 654-662.

20 Gonzalez-Menendez P, Hevia D, Rodriguez-Garcia A, Mayo JC, Sainz RM. Regulation of GLUT transporters by flavonoids in androgen-sensitive and -insensitive prostate cancer cells. *Endocrinology*. 2014; 155: 3238-3250.

21 Weinstein SP, Paquin T, Pritsker A, Haber RS. Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin- and non-insulin-related stimuli. *Diabetes*. 1995; 44: 441-445.

22 Kim HI, Moon SH, Lee WC, et al. Inhibition of cell growth by cellular differentiation into adipocyte-like cells in dexamethasone sensitive cancer cell lines. *Animal cells and systems*. 2018;

22: 178-188.

23 Sedelaar M, Isaacs JT. Tissue Culture Media Supplemented with 10% Fetal Calf Serum Contains a Castrate level of Testosterone. *The Prostate.* 2009; 69: 1724-1729

24 Wang X, Wang L, Zhu L, et al. PAQR3 modulates insulin signaling by shunting phosphoinositide 3-kinase p110α to the Golgi apparatus. *Diabetes*. 2013; 62: 444-456.

25 Li R, Evaul K, Sharma KK, et al. Abiraterone inhibits 3β-hydroxysteroid dehydrogenase: a rationale for increasing drug exposure in castration-resistant prostate cancer. *Clinical cancer research*. 2012; 18: 3571-3579

26 Kassi E, Moutsatsou P. Glucocorticoid receptor signaling and prostate cancer. *Cancer letters*. 2011; 302: 1-10.

27 Jeyaraj S, Boehmer C, Lang F, Palmada M. Role of SGK1 kinase in regulating glucose transport via glucose transporter GLUT4. *Biochemical and biophysical research communications*. 2007; 356: 629-635.

28 Palmada M, Boehmer C, Akel A, et al. SGK1 kinase upregulates GLUT1 activity and plasma membrane expression. *Diabetes*. 2006; 55: 421-427.

29 Lang F, Görlach A, Vallon V. Targeting SGK1 in diabetes. *Expert opinion on therapeutic targets*. 2009; 13: 1303-1311.

30 Balsevich G, Häusl AS, Meyer CW, et al. Stress-responsive FKBP51 regulates AKT2-AS160 signaling and metabolic function. *Nature communications*. 2017; 8: 1725.

31 Sidibeh CO, Pereira MJ, Abalo XM, et al. FKBP5 expression in human adipose tissue: potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes. *Endocrine*. 2018; 62: 116-128.

32 González Menéndez P, Hevia D, Alonso R, Gonzalez-Pola I, Mayo J, Sainz R. GLUT1/GLUT4 balance is a marker of androgen-insensitivity in prostate cancer. *European Journal of Cancer*. 2016; 61: S41.

33 Ayroldi E, Cannarile L, Delfino DV, Riccardi C. A dual role for glucocorticoid-induced leucine zipper in glucocorticoid function: tumor growth promotion or suppression? *Cell death & disease*. 2018; 9: 463.

34 Montgomery B, Cheng HH, Drechsler J, Mostaghel EA. Glucocorticoids and prostate cancer treatment: friend or foe? *Asian journal of andrology*. 2014; 16: 354-358.

35 Pufall MA. Glucocorticoids and Cancer. *Advances in experimental medicine and biology*. 2015; 872: 315-333.

36 Sengupta S, Vonesch JL, Waltzinger C, Zheng H, Wasylyk B. Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. *The EMBO journal*. 2000; 19: 6051-6064.

37 Li H, Qian W, Weng X, et al. Glucocorticoid receptor and sequential P53 activation by

dexamethasone mediates apoptosis and cell cycle arrest of osteoblastic MC3T3-E1 cells. *PloS one*. 2012; 7: e37030.

38 Clark RD. Glucocorticoid receptor antagonists. *Current topics in medicinal chemistry*. 2008; 8: 813-838.

39 Kach J, Long TM, Selman P, et al. Selective Glucocorticoid Receptor Modulators (SGRMs) Delay Castrate-Resistant Prostate Cancer Growth. *Molecular cancer therapeutics*. 2017; 16: 1680-1692.

40 Taplin ME, Manola J, Oh WK, et al. A phase II study of mifepristone (RU-486) in castration-resistant prostate cancer, with a correlative assessment of androgen-related hormones. *BJU international*. 2008; 101: 1084-1089.

41 Van den Abbeele AD, Gatsonis C, de Vries DJ, et al. ACRIN 6665/RTOG 0132 phase II trial of neoadjuvant imatinib mesylate for operable malignant gastrointestinal stromal tumor: monitoring with 18F-FDG PET and correlation with genotype and GLUT4 expression. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. 2012; 53: 567-574.

42 Fukumoto H, Kayano T, Buse JB, et al. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *The Journal of biological chemistry*. 1989; 264: 7776-7779.

43 Charron MJ, Brosius FC, 3rd, Alper SL, Lodish HF. A glucose transport protein expressed predominately in insulin-responsive tissues. *Proceedings of the National Academy of Sciences of the United States of America*. 1989; 86: 2535-2539.

44 Croxtall JD, Perry CM. Lopinavir/Ritonavir: a review of its use in the management of HIV-1 infection. *Drugs*. 2010; 70: 1885-1915.

45 Murata H, Hruz PW, Mueckler M. The mechanism of insulin resistance caused by HIV protease inhibitor therapy. *The Journal of biological chemistry*. 2000; 275: 20251-20254.

46 Wei C, Bajpai R, Sharma H, et al. Development of GLUT4-selective antagonists for multiple myeloma therapy. *European journal of medicinal chemistry*. 2017; 139: 573-586.

7. Figure legends：

Figure 1：

Change in PSA levels in the culture solution of LNCaP cells under cd-FBS medium at 1, 3, 5 and 7 days after culturing the cells on a culture dish.

Figure 2:

Effects of DHT and/or Enz administration on cell proliferation (A), and GLUT4 expression at the mRNA (B) and protein levels (C, D) in LNCaP cells under FBS and cd-FBS medium.

Figure 3:

Evaluation of cell morphology and resistance to Enz and other AR signaling inhibitors in LNCaP-derived Enz-resistant LNEnzR cells. Morphological comparison between LNCaP cells and LNEnzR cells in a 40× field of view under an inverted microscope (A). Proliferation of these cells under various concentrations of Enz (B), Abi (C) and Bcl (D) (WST-1 assay). Enz: enzalutamide, Abi: abiraterone, Bcl: bicalutamide, WST: water soluble tetrazolium.

Figure 4:

Effects of DHT, Enz, Abi and Bcl on cell proliferation and glucose uptake in LNCaP and LNEnzR cells. DHT: Dihydrotestosterone, Enz: enzalutamide, Abi: abiraterone, Bcl: bicalutamide, WST: water soluble tetrazolium.

Figure 5

Effects of various concentrations of Abi administration and Abi administration plus siRNA-induced AR knockdown on cell proliferation in LNCaP cells.

Figure 6:

Expression analysis of AR and GR in LNCaP and LNEnzR cells. The mRNA and protein expression levels of AR and GR were analyzed by quantitative RT-PCR (A) and western blotting (B and C), respectively. AR: androgen receptor; GR: glucocorticoid receptor.

Figure 7:

Effects of DHT and/or Enz administration on AR and GR expressions in LNCaP and LNEnzR cells. The mRNA (A and C) and protein levels (B, D, and E) for AR (A, B, and E) and GR (C, D, and E) were analyzed by quantitative RT-PCR and western blotting, respectively. DHT: dihydrotestosterone, Enz: enzalutamide, AR: androgen receptor; GR: glucocorticoid receptor.

Figure 8:

Expression analysis of GLUT1, GLUT3, GLUT4 and GLUT12 in LNCaP cells and

LNEnzR cells. Analysis of mRNA (A) and protein levels (B and C) for GLUT1, GLUT3, GLUT4 and GLUT12 by quantitative RT-PCR and western blotting in both cells. The expression of GLUT1 (D), GLUT3 (E), GLUT4 (F) and GLUT12 (G) on the surface of both cells was analyzed by flow cytometry.

Figure 9:

Effects of DHT and/or Enz administration on GLUT1 and GLUT4 expression in LNCaP and LNEnzR cells. mRNA (A and C) and protein levels (B, D, and E) of GLUT1 (A, B, and E) and GLUT4 (C, D, and E) were analyzed by quantitative RT-PCR (qRT-PCR) and western blotting in both cells. DHT: dihydrotestosterone, Enz: enzalutamide.

Figure 10:

Effects of GR and GLUT4 inhibition on GR and GLUT4 expression, cell proliferation, and glucose uptake in the AR-negative and GR-positive PCa cell line, PC3 cells. GR and GLUT4 mRNA (A) and protein expression (B and C) levels under siRNA control (si control), siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown, and administration of the GR inhibitor, RU486 or the GLUT4 inhibitor, Rit in PC3 cells. WST-1 assay (D) and glucose uptake assay (E) were performed in PC3 cells after administration of these drugs. Rit: ritonavir; WST, water soluble tetrazolium.

Figure 11:

Effects of GR and GLUT4 inhibition on cell proliferation and glucose uptake in LNCaP and LNEnzR cells. Cell proliferation (A) and glucose uptake (B) under siRNA control (si control), siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown and administration of the GR inhibitor, RU486 or the GLUT4 inhibitor, Rit. GR: glucocorticoid receptor, Rit, ritonavir.

Figure 12:

Effects of GR and GLUT4 inhibition on GR and GLUT expression in LNCaP (A and B) and LNEnzR (C and D) cells. mRNA (A and C) and protein (B, D, and E) expression levels for GR, GLUT1, and GLUT4 under siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown and administration of the GR inhibitor, RU486 or GLUT4 inhibitor, Rit were analyzed by quantitative RT-PCR and western blotting, respectively. GR: glucocorticoid receptor, Rit, ritonavir.

Figure 13:

Effects of GR and GLUT4 inhibition on drug resistance to Enz and Abi in LNEnzR cells.

WST-1 assay was performed to evaluate the effect of Enz or Abi treatment with or without siRNA-induced GR (siGR) or GLUT4 (siGLUT4) knockdown or the GLUT4 inhibitor, Rit on cell proliferation. GR: glucocorticoid receptor, WST: water soluble tetrazolium, Enz: enzalutamide; Abi: abiraterone, Rit: ritonavir.

Figure 14

Change in AR expression at the mRNA (A) and protein levels (B) by siRNA-induced GR and GLUT4 knockdown in LNEnzR cells.

8. Figures and Tables

Figure 1

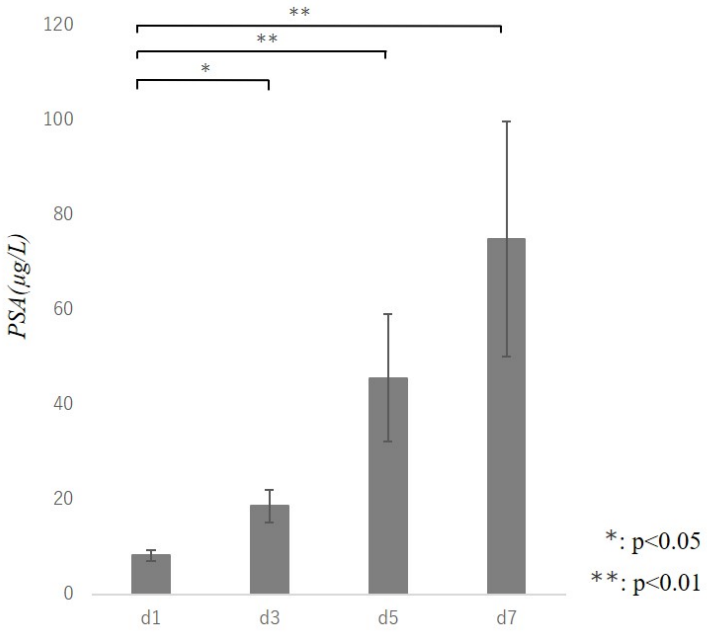
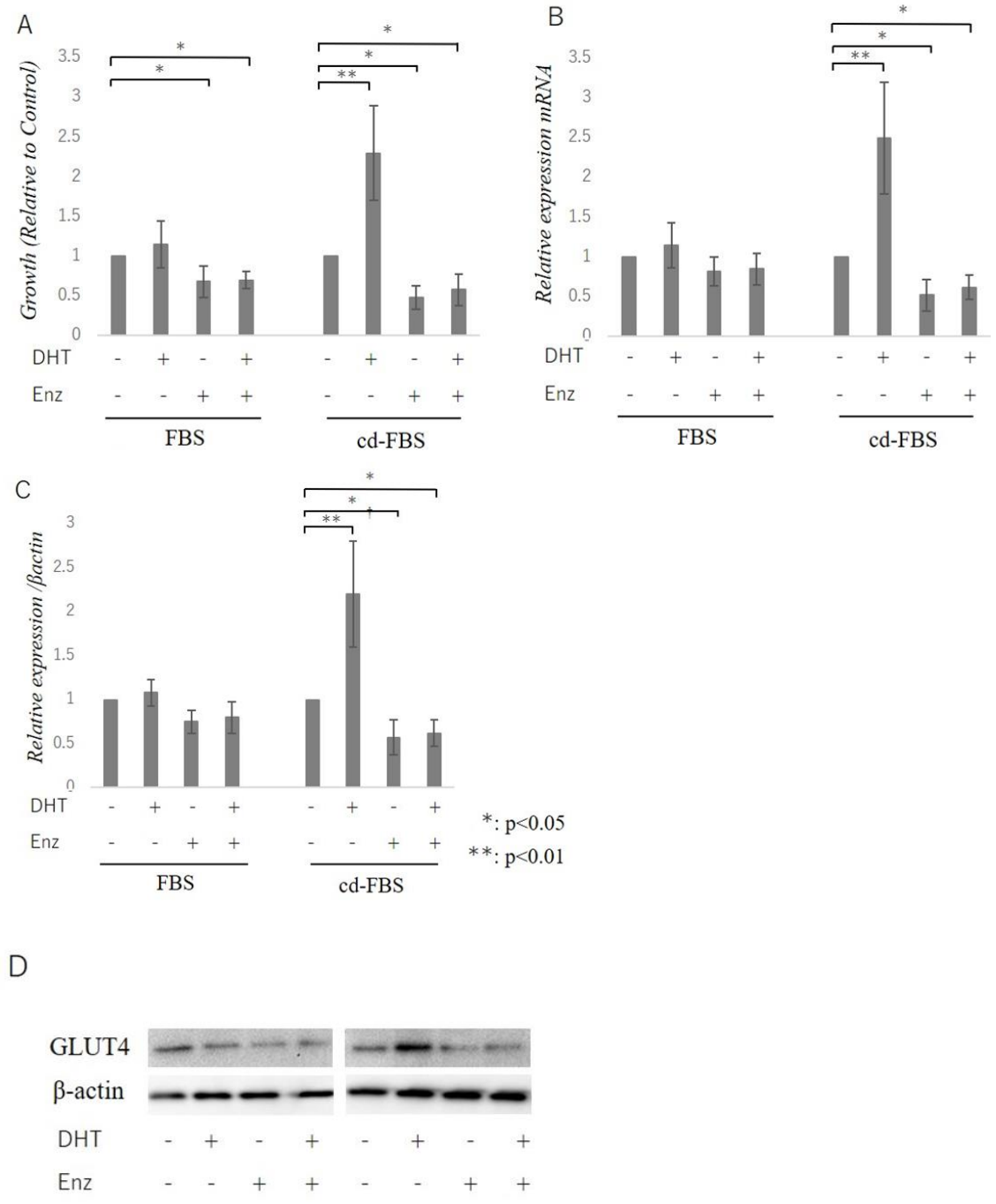


Figure 2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Growth (Relative to Control)
3.5
3
2.5
2
1.5
1
0.5
0
*
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DHT - + - + - + - +
Enz - - + + - - + +
FBS
cd-FBS
B
Relative expression mRNA
*
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C
Relative expression /βactin
*: p<0.05
**: p<0.01
D
GLUT4
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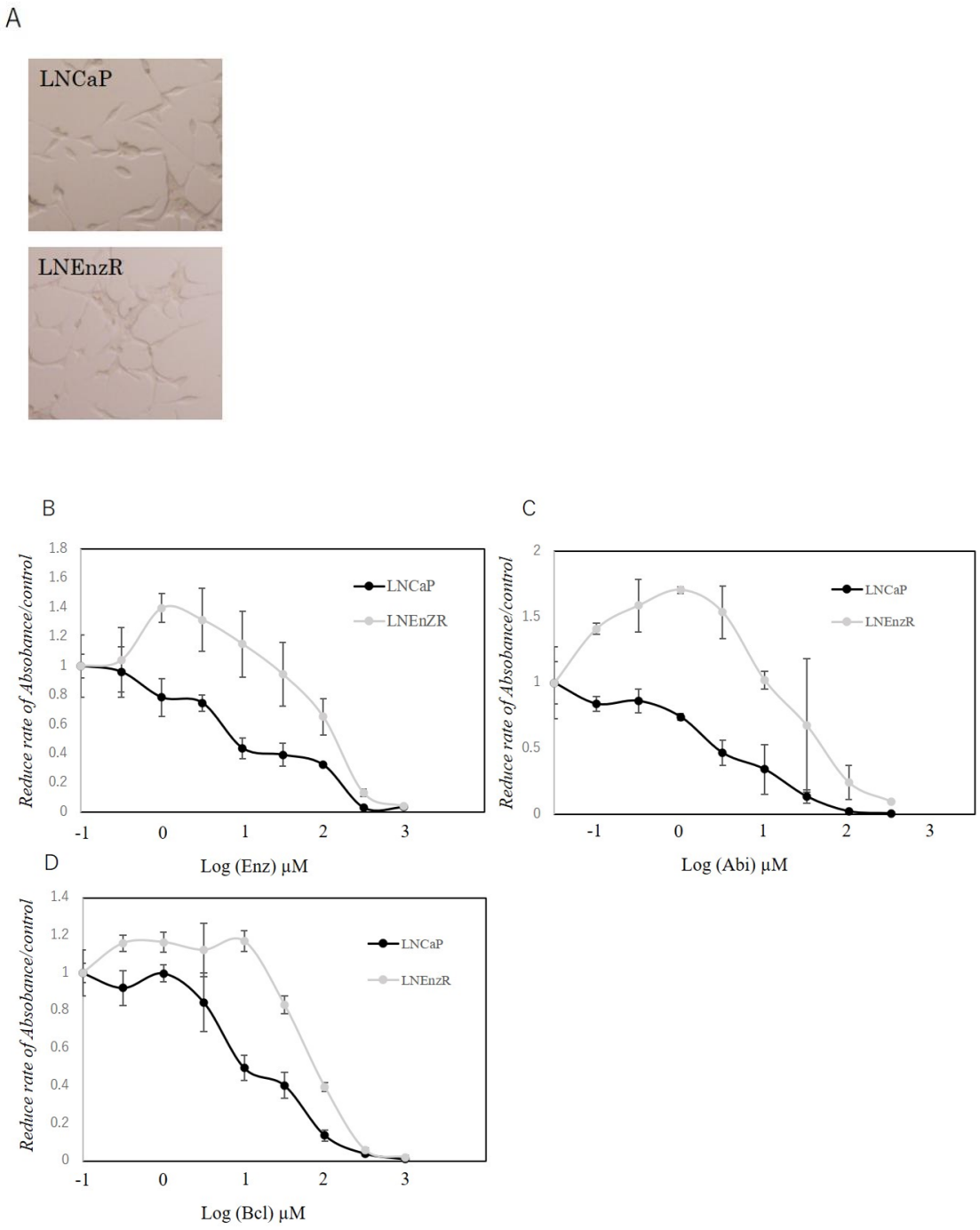
DHT - + - + - + - +
Enz - - + + - - + +

Figure 3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LNCaP
LNEnzR
B
Reduce rate of Absobance/control
LNCaP
LNEnZR
Log (Enz) µM
C
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D
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LNCaP
LNEnzR
Log (Bcl) µM

Figure 4

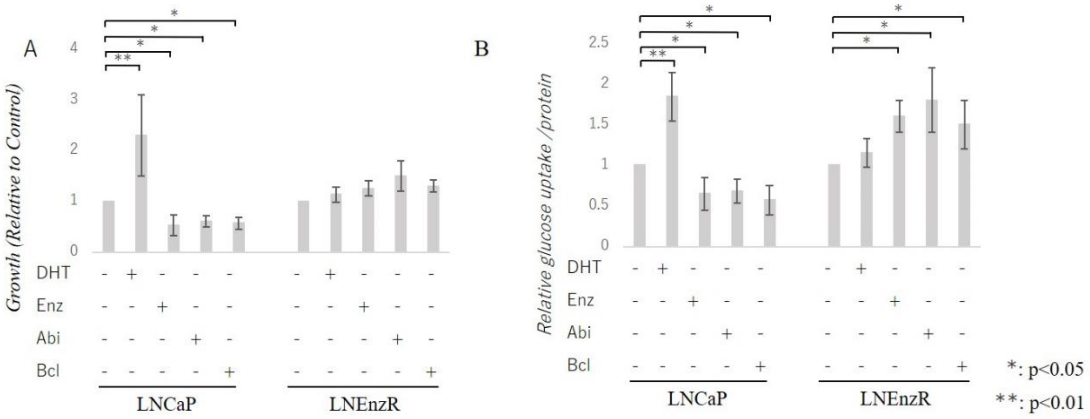


Figure 5

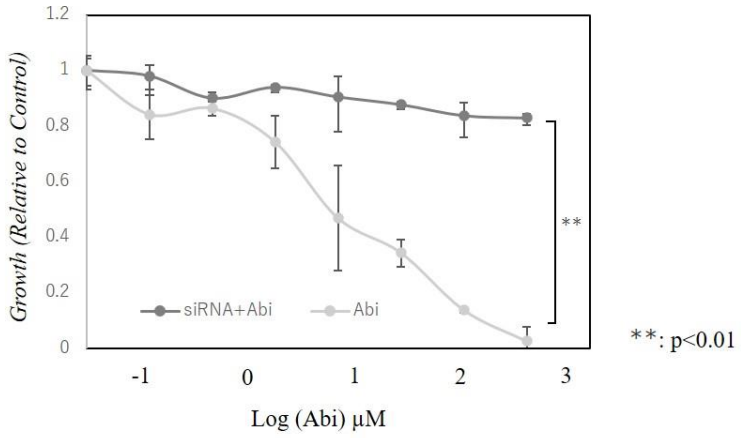


Figure 6

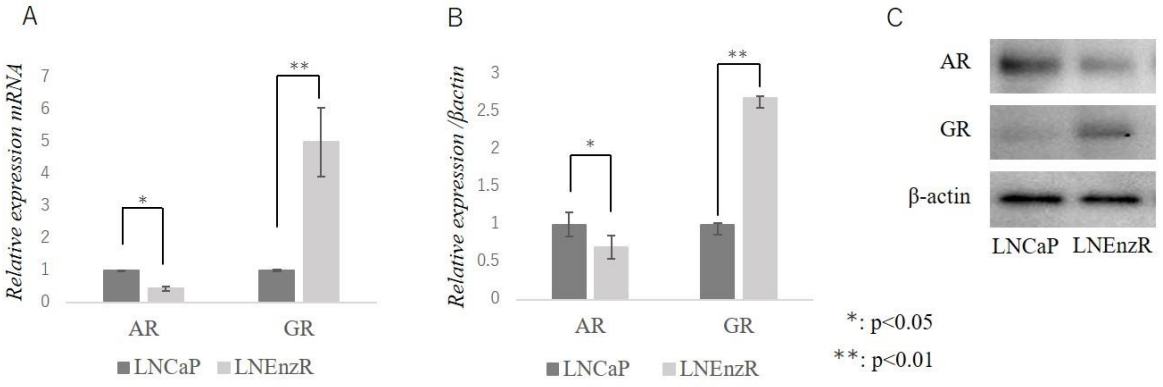
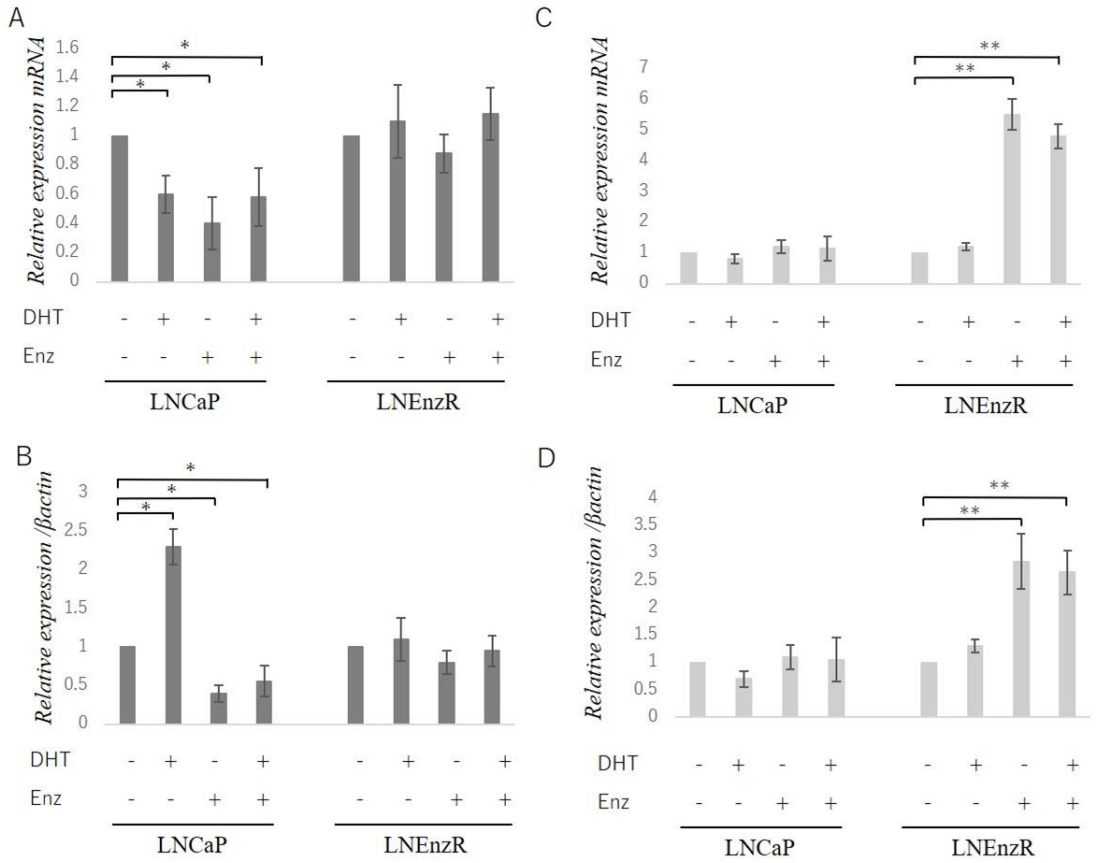

A
Relative expression mRNA
7
6
5
4
3
2
1
0
*
**
AR
GR
LNCaP
LNEnzR
B
Relative expression /βactin
3
2.5
2
1.5
1
0.5
0
*
**
AR
GR
LNCaP
LNEnzR
C
AR
GR
β-actin
LNCaP
LNEnzR
*: p<0.05
**: p<0.01

Figure 7



*: $p<0.05$

**: $p<0.01$

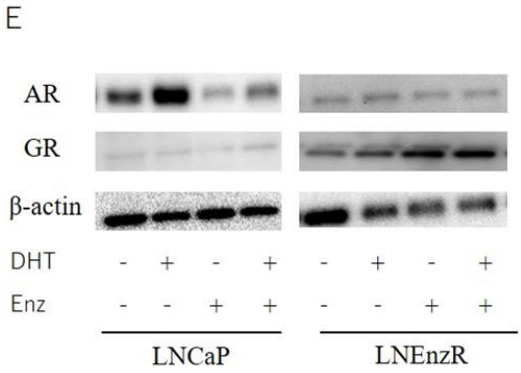
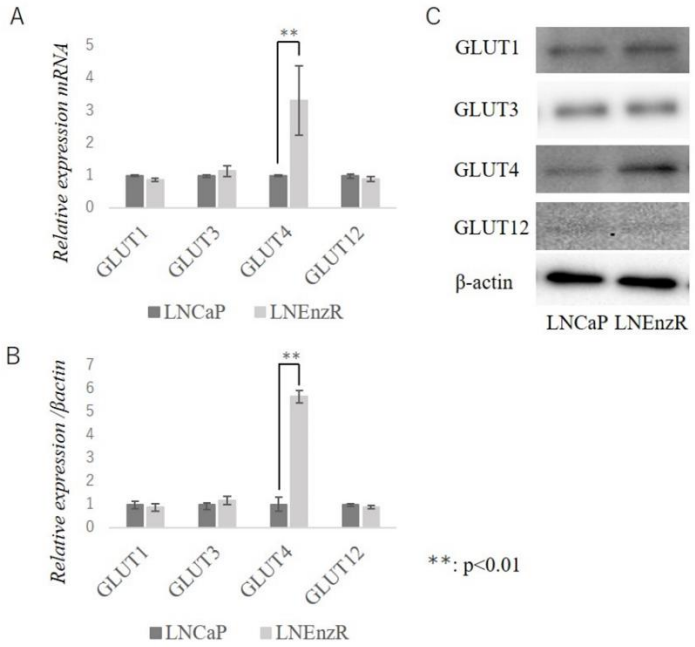


Figure 8


A
Relative expression mRNA
**
GLUT1
GLUT3
GLUT4
GLUT12
LNCaP
LNEnzR
B
Relative expression /βactin
**: p<0.01
C
GLUT1
GLUT3
GLUT4
GLUT12
β-actin
LNCaP LNEnzR

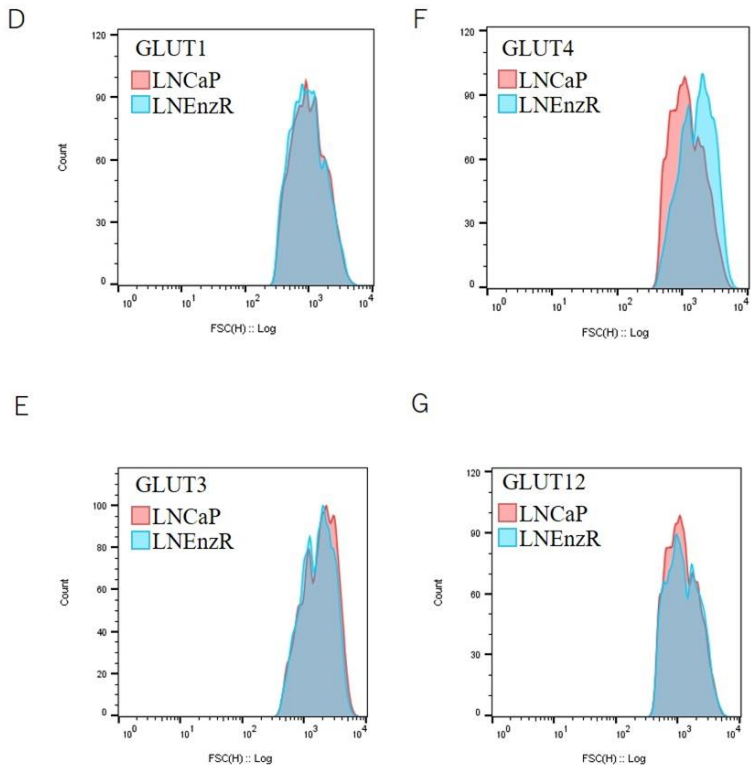
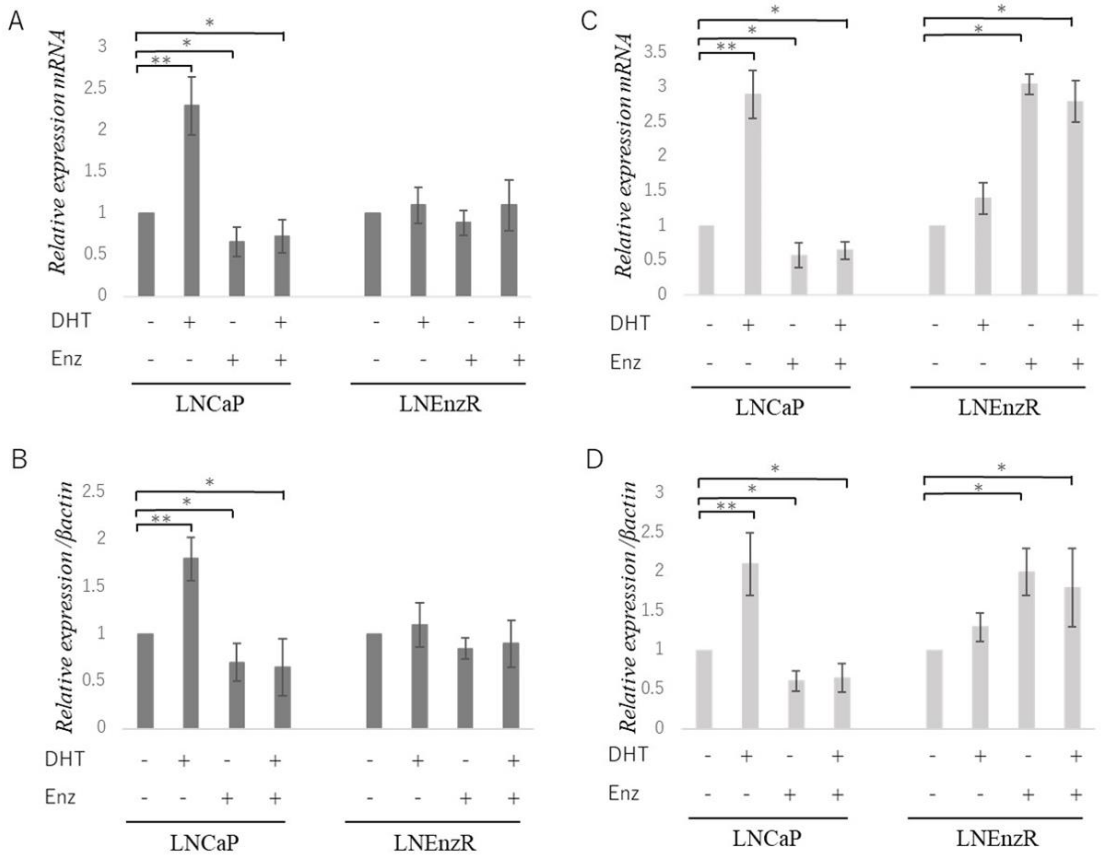

D
GLUT1
LNCaP
LNEnzR
Count
FSC(H) :: Log
F
GLUT4
LNCaP
LNEnzR
FSC(H) :: Log
E
GLUT3
LNCaP
LNEnzR
Count
FSC(H) :: Log
G
GLUT12
LNCaP
LNEnzR
Count
FSC(H) :: Log

Figure 9



*: p<0.05

**: p<0.01

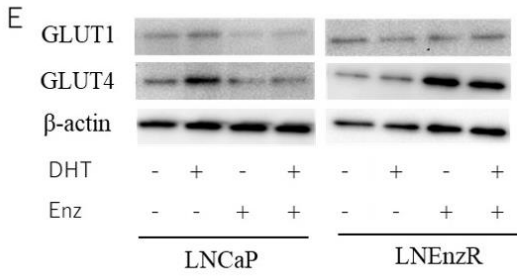


Figure 10

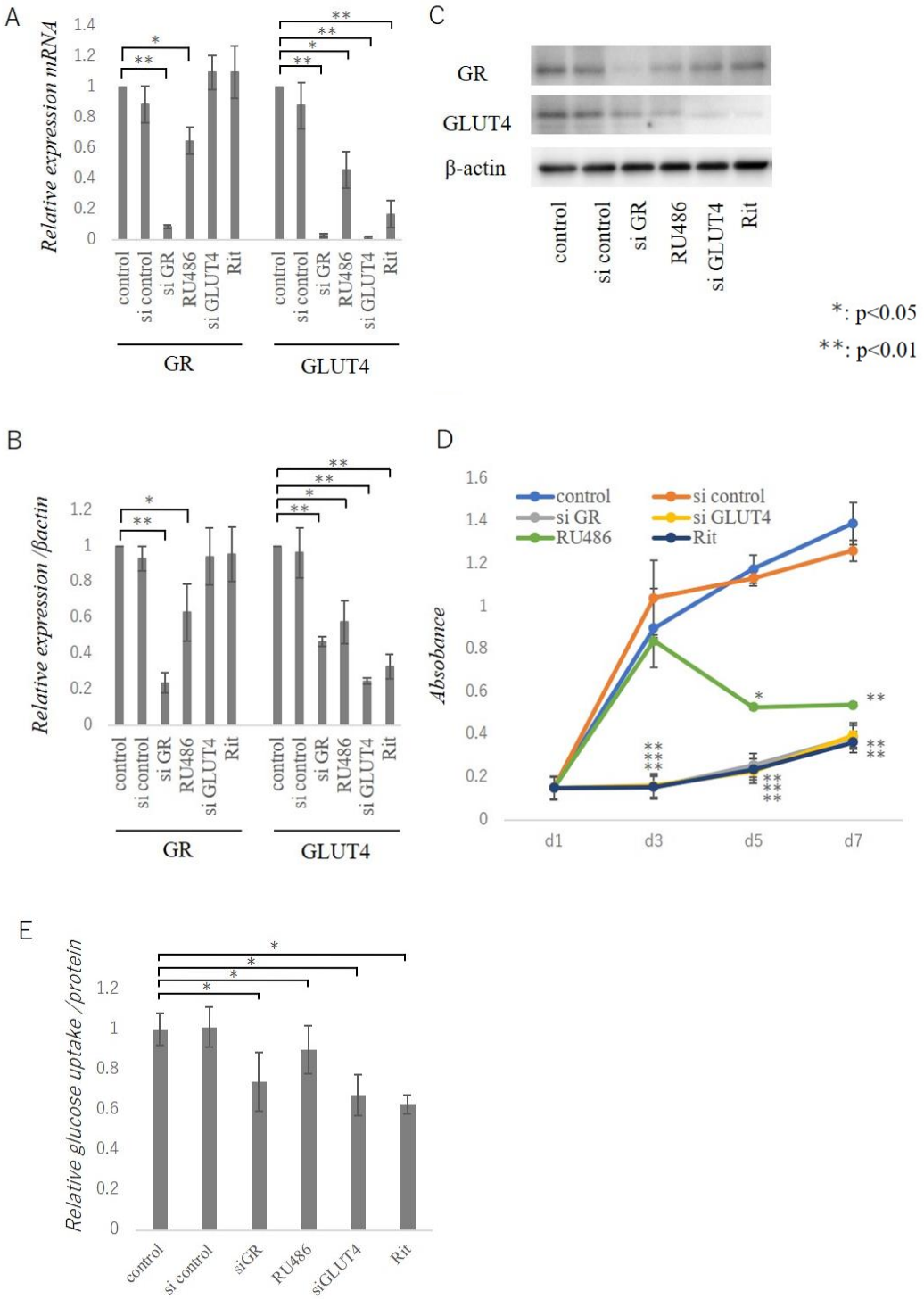

A
Relative expression mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
si control
si GR
RU486
si GLUT4
Rit
GR
GLUT4
C
GR
GLUT4
β-actin
control
si control
si GR
RU486
si GLUT4
Rit
*: p<0.05
**: p<0.01
B
Relative expression /βactin
D
Absobance
control
si control
si GR
si GLUT4
RU486
Rit
d1
d3
d5
d7
E
Relative glucose uptake /protein
control
si control
siGR
RU486
siGLUT4
Rit

Figure 11

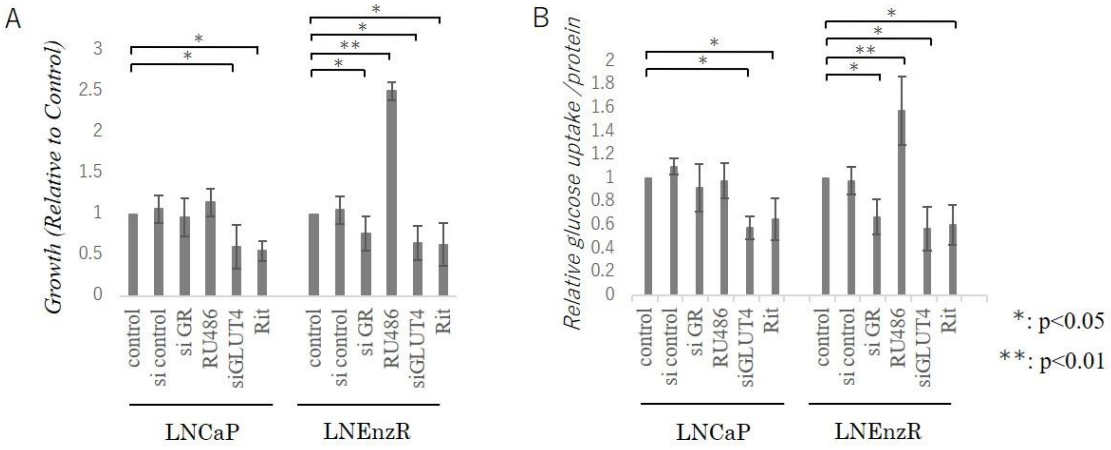

A
Growth (Relative to Control)
3
2.5
2
1.5
1
0.5
0
control
si control
si GR
RU486
siGLUT4
Rit
LNCaP
LNEnzR
B
Relative glucose uptake /protein
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*: p<0.05
**: p<0.01

Figure 12

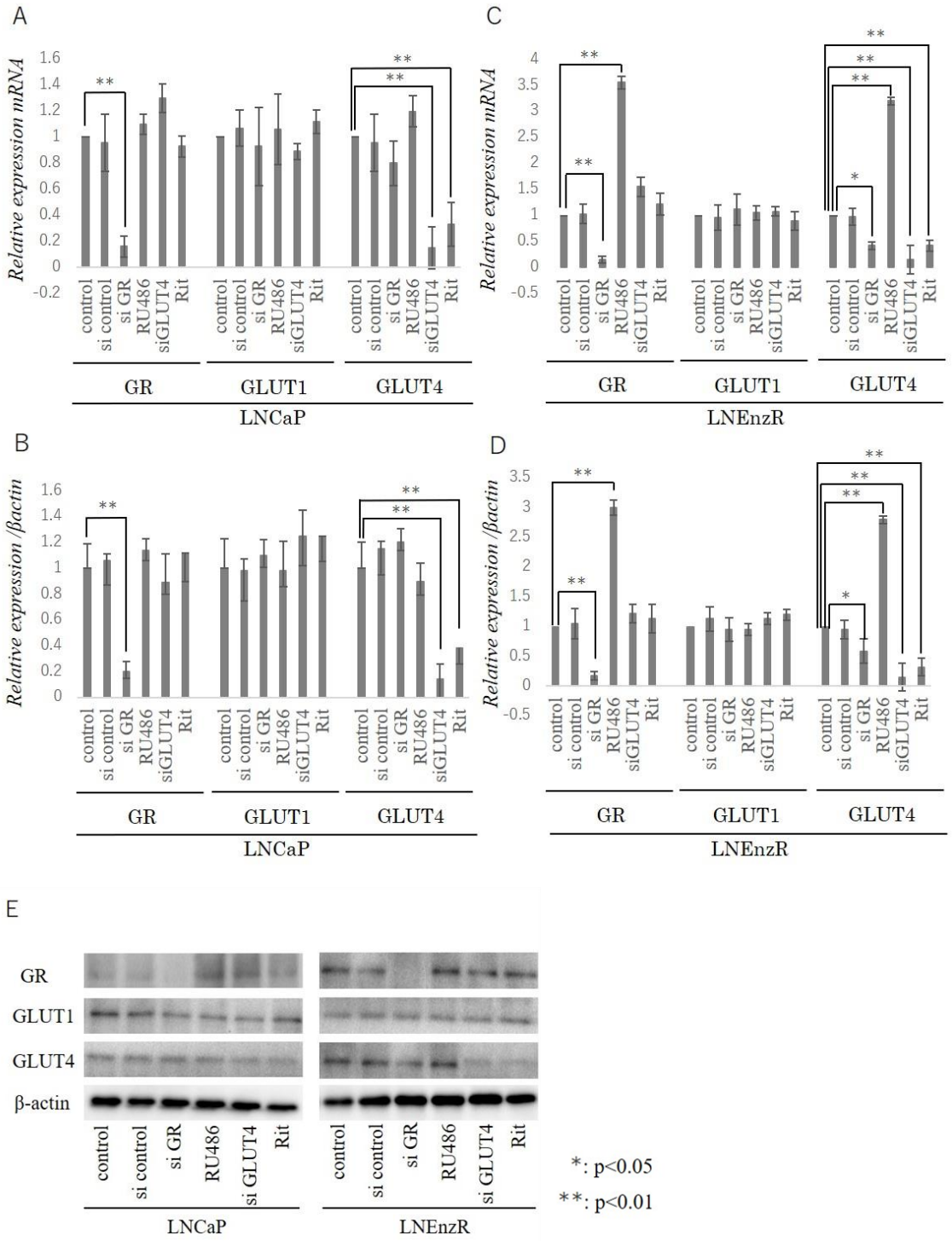

A
Relative expression mRNA
control
si control
si GR
RU486
siGLUT4
Rit
GR
GLUT1
GLUT4
LNCaP
C
LNEnzR
B
Relative expression /βactin
D
E
GR
GLUT1
GLUT4
β-actin
*: p<0.05
**: p<0.01

Figure 13

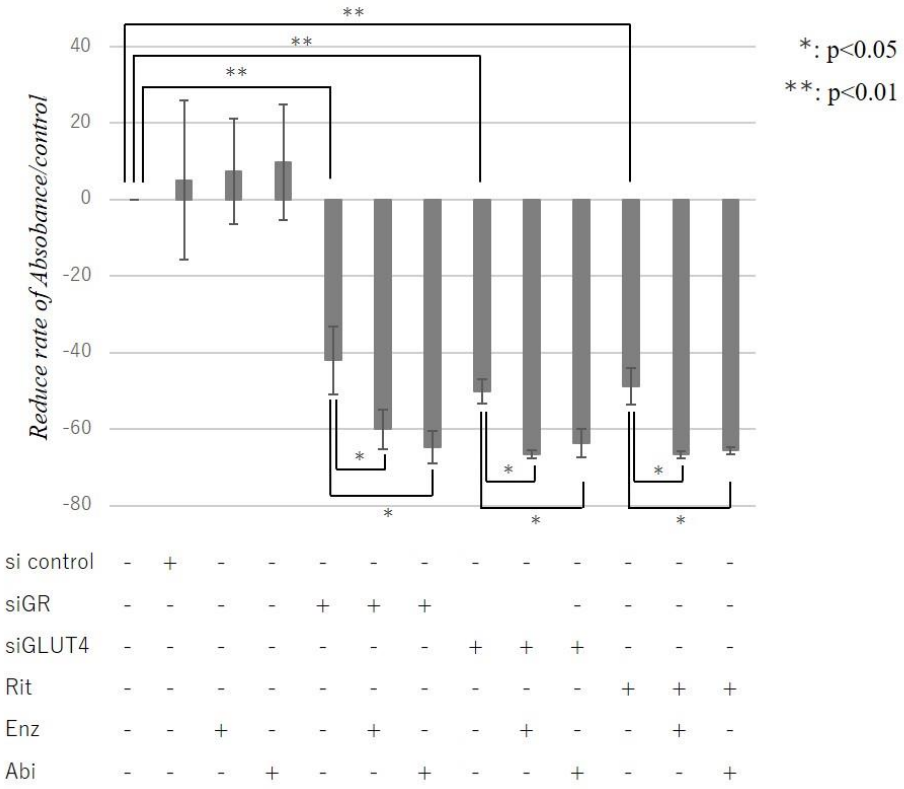


Figure 14

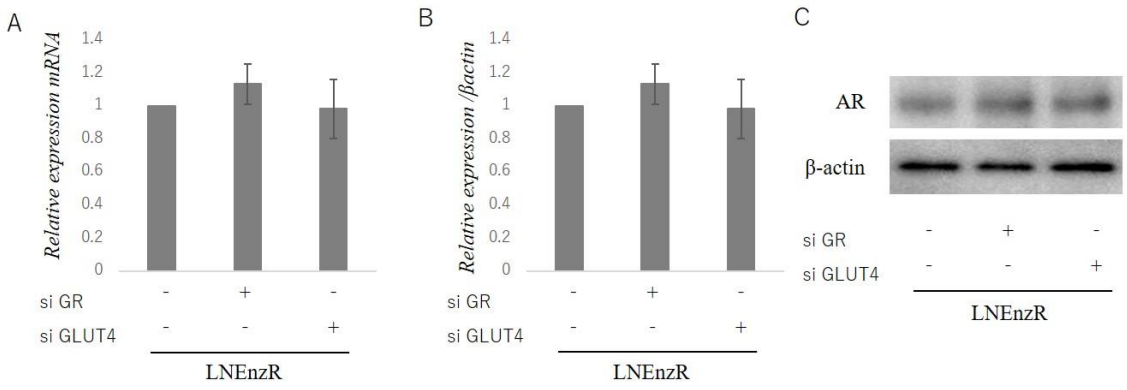


Table 1: Custom primers designed siRNA

Custom primers

	Forward (5′ to 3′)	Reverse (5′ to 3′)
AR-v7	TTTGAATGAGGCAAGTCAGCCTTCT	CCATCTTGTCGTCTTCGGAAATGTTATGA
GLUT3	ATGGGGACACAGAAGGTCACC	AGCCACCAGTGACAGCCAAC
GLUT12	CTCGAGTTAGATCTCTGAAGAAAGCTGC	CTCGAGATGGTACCTGTTGAAAACGCAG

Table 2: Custom designed siRNA

Designed siRNA

	Sense (5′ to 3′)	Antisense (5′ to 3′)
AR	GGGAGGUUACACCAAAGGGtt	CCCUUUGGUGUAACCUCCCtt
GR	GCAGGAUCAGAAGCCUAUUtt	AAUAGGCUUCUGAUCCUGCtg
GLUT4	CCUUCUUAAGAGUACCUGAtt	UCAGGUACUCUUAAGAAGGtg

9. Acknowledgment：

We gratefully acknowledge the work of past and present members of our laboratory.