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著者	Arakawa Hiroshi, Nakanishi Takeo, Yanagihara Chihiro, Nishimoto Tomohiro, Wakayama Tomohiko, Mizokami Atsushi, Kawai Keiichi, Tamai Ikumi
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Enhanced expression of organic anion transporting polypeptides (OATPs) in androgen receptor-positive prostate cancer cells: Possible role of OATP1A2 in adaptive cell growth under androgen-depleted conditions

Hiroshi Arakawa[1], Takeo Nakanishi[1], Chihiro Yanagihara[1], Tomohiro Nishimoto[1], Tomohiko Wakayama[2], Atsushi Mizokami[3], Keiichi Kawai[4], and Ikumi Tamai[1]

[1]Department of Membrane Transport and Biopharmaceutics, Faculty of Pharmaceutical Science, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, [2]Department of Histology and Embryonology, and [3]Integrative Cancer Therapy and Urology, Faculty of Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, [4]Department of Quantum Medical Technology, Faculty of Health Sciences, Graduate School of Medical Sciences, Kanazawa University, 5-11-80 Kodatsuno, Kanazawa 920-0942, Japan

Running Title

Role of OATP1A2 in cell growth of CRPC

Correspondence: Ikumi Tamai, Ph.D.,

Department of Membrane Transport and Biopharmaceutics, Faculty of Pharmaceutical Sciences, Kakuma-machi, Kanazawa, Ishikawa, 920-1192, Japan,

Tel: +81-76-234-4479, Fax: +81-76-264-6284,

E-mail: tamai@p.kanazawa-w.ac.jp

Abbreviations

AR; androgen receptor; STX64; 6-oxo-6,7,8,9,10,11-hexahydrocyclohepta[c]chromen-3-yl sulfamate, CRPC; castration-resistant prostate cancer, STS; steroid sulfatase, OATP; organic anion transporting polypeptide, DHEA; dehydroepiandrosterone, DHEAS; dehydroepiandrosterone sulfate, DHT; dihydrotestosterone, ADT; androgen deprivation therapy, SRB; sulforhodamine B, PSA; prostate specific antigen, HPRT1; hypoxanthine guaninephosphoribosyl-transferase HSD; hydroxysteroid dehydrogenase, AP-1; activator protein-1, NTCP; Na^+-dependent taurocholate cotransporting polypeptide, E3S; estrone 3-sulfate, BSP; bromosulfophthalein, TCA; taurocholate, PRO; probenecid, SAL; salicylate, PAH; *p*-aminohippurate, TEA; tetra-ethylammonium, MPP^+; 1-methyl-4-phenylpyridinium

Keywords: prostate cancer, OATP1A2, DHEAS, androgen receptor, steroid sulfatase

Abstract

The biological mechanisms underlying castration resistance of prostate cancer are not fully understood. In the present study, we examined the role of organic anion transporting polypeptides (OATPs) as importers of dehydroepiandrosterone sulfate (DHEAS) into cells to support growth under androgen-depleted conditions. Cell growth and mRNA expression of OATP genes were studied in human prostate cancer LNCaP and 22Rv1 cells under androgen-depleted conditions. The stimulatory effect of DHEAS on cell growth was investigated in LNCaP cells in which OATP1A2 had been silenced. Growth of both cell lines was stimulated by DHEAS and the effect was attenuated by STX64, an inhibitor of steroid sulfatase which can covert DHEAS to DHEA. OATP1A2 mRNA expression was increased most prominently among various genes tested in LNCaP cells grown in androgen-depleted medium. Similar results were obtained with 22Rv1 cells. Furthermore, the characteristics of [^{3}H]DHEAS uptake by LNCaP cells were consistent with those of OATP-mediated transport. Knockdown of OATP1A2 in LNCaP cells resulted in loss of the DHEAS sensitivity of cell growth. Our results suggest that enhanced OATP1A2 expression is associated with adaptive cell growth of prostate cancer cells under androgen-depleted conditions. Thus, OATP1A2 may be a pharmacological target for prostate cancer treatment.

1. Introduction

Gonadal androgens play a critical role in protein synthesis and cell survival in prostate tumors [1]. Therefore, androgen deprivation therapy (ADT) to remove gonadal testosterone or to antagonize androgen receptors is currently a mainstream treatment for prostate cancer. Once the disease progresses to castration-resistant prostate cancer (CRPC), it no longer responds to androgen deprivation therapy. CRPC tumor progression is considered to involve enhanced androgen receptor (AR) function, mainly due to *AR* gene amplification/overexpression [2], stabilization of AR protein and increased sensitivity of AR to androgens [3], and constitutive, ligand-independent activation [4].

The organic anion transporter polypeptide (OATP/*SLCO*) family members generally mediate Na^+-independent transport of amphipathic organic anion compounds, including bile salts, steroid conjugates, thyroid hormones, and oligopeptides [5, 6]. The physiological roles of these transporters are not yet fully understood, but there is compelling evidence that certain members of the OATP family promote cell proliferation and survival of human malignant tissues. We have previously shown that transport of estrone 3-sulfate via OATPs sustains the growth of hormone-dependent human breast cancer cells [7, 8], because estrone-3-sulfate can be hydrolyzed by steroid sulfatase (STS) to estrone and eventually estradiol. We recently showed that OATP1B3 contributes to estrone 3-sulfate uptake in estrogen receptor-positive human breast cancer MCF-7 cells [9]. In addition to estrone 3-sulfate, these OATPs translocate dehydroepiandrosterone sulfate (DHEAS) [10, 11]. DHEAS is a thousand fold more abundant than testosterone in human serum [12], and is essentially unaffected by ADT. DHEAS is

hydrolyzed to DHEA by STS [13], and DHEA can be converted to androstenedione in prostate cancer [14], resulting in activation of AR function [15]. More recently, clinical observations have suggested that several OATPs are upregulated in castration-resistant metastatic prostate tumor tissues derived from human patients [16]; however, it remains unclear whether OATPs play a role in prostate cancer cell survival under androgen-depleted conditions. We hypothesized that increased expression of OATPs does contribute to cell survival of androgen-dependent prostate cancer cells under androgen deprivation, by providing the cells with an increased supply of DHEAS as a precursor of active androgen.

In the present study, we investigated the expression of functional OATPs in, and the growth of, androgen-dependent prostate cancer cells. Among the OATP family members tested, OATP1A2 was found to be remarkably upregulated in AR-positive human prostate cancer cells cultured under androgen-depleted conditions. We propose that enhanced expression of OATP1A2 in these cells plays a role in progression of prostate cancer by increasing the availability of DHEAS as a precursor of active androgen, under conditions of androgen depletion.

2. Materials and methods

2.1. Materials

DHEAS, dihydrotestosterone (DHT), and [^{3}H]DHEAS sodium salt (3.50 TBq/mmol) were purchased from Tokyo Chemical Industry (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), and PerkinElmer Life Science (Boston, MA), respectively. Human prostate

cancer LNCaP and 22Rv1 cell lines were purchased from American Type Culture Collection (Manassas, VA).

2.2. Cell culture and growth assays

LNCaP and 22Rv1 cells were cultured in RPMI1640 (Wako Pure Chemical Industries) with penicillin (100 units/mL, Nakalai Tesque, Kyoto, Japan), streptomycin (100 μg/mL, Wako Pure Chemical Industries) and 10% fetal bovine serum (FBS, Invitrogen). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37^{o}C. For the cell growth assay, cells were cultured in RPMI1640 with penicillin (100 units/mL), streptomycin (100 μg/mL) supplemented with FBS or charcoal-stripped FBS (CSS, Invitrogen) in the presence or absence of androgen and/or STX64 (also known as 667 coumate or 6-oxo-6,7,8,9,10,11-hexahydrocyclohepta-[c]chromen-3-yl sulfamate, Sigma Aldrich, St. Louis, MO), which is a tricylic coumarin-based sulfamate that inhibits steroid sulfatase. The growth-stimulatory effect of androgens, including DHEAS, was evaluated by measuring cell growth after cells were plated at a density of 1.5 to 2.4 × 10^4 cells/cm^2 on a 96-well tissue culture plate. In general, once cells became attached to the plate, a test androgen or DMSO (at 0.1% of the final concentration) was added to the well, and cell growth was monitored for up to 9 days by means of sulforhodamine B (SRB, Sigma Aldrich) assay as previously described [17].

2.3. Gene expression profiling assay

LNCaP cells were cultured in RPMI1640 with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% CSS for 6 days. Then, total RNA was prepared using Isogen reagent (Nippon

Gene, Tokyo, Japan). Microarray experiments were performed and data were analyzed according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). In brief, 300 ng of total RNA was subjected to linear amplification and Cy3 labeling using the Low RNA Input Linear Amplification Kit (Agilent Technologies), and then hybridized to a 44-K Whole Human Genome Microarray (Agilent Technologies). Data were extracted using Feature Extraction software (version 9.5.3) and analyzed using GeneSpring GX software (version 11.0).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from cells using Isogen reagent (Nippon Gene). mRNA expression of OATP1A2, OATP1B1, OATP1B3, OATP2B1, OAT2, OAT3, and OAT4 was measured by quantitative RT-PCR (qRT-PCR), using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies). The fold change in mRNA expression of transporter genes was normalized to hypoxanthine guaninephosphoribosyl-transferase 1 (HPRT1) using the 2-ΔΔ threshold cycles (Ct) method [18]. Primer sequences specific to transporter genes tested are listed in Table 1.

2.5. [^{3}H]DHEAS cellular uptake assay

Intracellular accumulation of DHEAS was evaluated by means of uptake assays as described previously [17]. Briefly, cells were plated at a density of 1.0 x10^5 cells/cm^2 on a 24-well tissue-culture plate in culture medium supplemented with 10% FBS or CSS in the absence or presence of DHT (1 nM) two days before assay containing 0.1% DMSO of the final concentration. The assay was initiated by adding transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4,1.2 mM, $MgSO_4$, and 25 mM HEPES,

adjusted to pH 7.4) containing 0.5 μCi/mL [^{3}H]DHEAS (10 nM) with or without inhibitors at 37^{o}C. Sodium dependence of uptake was evaluated by replacing Na^+ with Li^+, K^+ or N-methylglucamine (NMG^+). At the end of the assay, cells were washed with ice-cold transport buffer and solubilized in 1% (v/v) Triton X-100 (Wako Pure Chemical Industries). The radioactivity in the resultant cell lysate was measured using a liquid scintillation counter (Aloka, Tokyo, Japan). Intracellular [^{3}H]DHEAS accumulation was normalized by protein content determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and is shown as the ratio of accumulation to the initial concentration of DHEAS, i.e., cell-to-medium (C/M) ratio (μL/mg).

2.6. Western blot

To determine protein expression of OATP1A2, cells were collected and lysed in RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris at pH 8.0) by sonication. A 50 μg aliquot of total cell lysate was subjected to SDS polyacrylamide gel electrophoresis, and then electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blots were probed with anti-human OATP1A2 rabbit polyclonal antibody (Assay Biotechnology Company, San Francisco, CA) at 1 to 500 dilution or with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) at 1 to 2000 dilution, followed by appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ).

2.7. Construction of OATP1A2-Transfected LNCaP Cells

LNCaP cells were transfected with pcDNA3.1/OATP1A2 or pcDNA3.1 alone (Invitrogen), and then selected with 800 μg/mL of G418 (Wako Pure Chemicals). Enhanced OATP1A2 expression was verified by uptake study of [^{3}H]DHEAS (0.5 μCi/mL) for up to 5 min and Western blotting using anti-human OATP1A2 rabbit IgG.

2.8. OATP1A2 knockdown in LNCaP cells

To develop prostate cancer cell lines with knockdown of OATP1A2, double-stranded DNA containing a hairpin sequence for the targeted site (5'–tattctttggctgccatacct-3') and (5'-aagtgtcatctacctggtgaa-3') of OATP1A2 mRNA was inserted into the microRNA-adapted vector pcDNA 6.2-GW/EmGFP-miR (Invitrogen). Knockdown of OATP1A2 was performed using the BLOCK-iT Pol II miR RNAi expression vector kit according to the manufacturer's instructions (Invitrogen). The pcDNA6.2-GW/EmGFP-miR-negative control plasmid contains an oligonucleotide not related to OATP1A2, which serves as a negative control. LNCaP cells were transfected with these plasmid DNAs using LipofectAMINE 2000 reagent (Invitrogen). Two days later, selection of transfected cells was started with blasticidin (2 μg/mL, Invitrogen). After about 3 weeks, blasticidin-resistant and EmGFP-positive cells were used for the proliferation studies and subjected to RT-PCR analysis.

3. Results

3.1. DHEAS-induced cell growth and PSA expression in androgen receptor-positive prostate cancer cells

In order to determine whether DHEAS can stimulate cell growth of androgen receptor-positive human prostate cancer LNCaP cells, cell growth was measured in androgen-depleted medium. The cell growth was stimulated by DHEAS in a concentration-dependent manner. At a physiologically relevant serum concentration of DHEAS (5 μM), the growth increased about 174% compared to that in the absence of DHEAS, corresponding to about half of the effect of DHT, an active androgen, at 1 nM (Figure 1A). DHEAS also exhibited a stimulatory effect on cell growth of 22Rv1 cells (16% ± 1.82 % on Day 9) compared to that in the absence of DHEAS (data not shown). The stimulatory effect observed in LNCaP cells was significantly attenuated in the presence of STX64, a specific inhibitor of STS (Figure 1B), suggesting that activation of cell growth by DHEAS requires its conversion to DHEA mediated by STS. Expression of STS showed no change in LNCaP cells grown in normal and CSS-containing medium (Figure 1C). In parallel, DHEAS induced mRNA expression of prostate specific antigen (PSA) in LNCaP cells, reflecting the effect of DHEAS on the cell growth (Figure 1D).

3.2. Alteration in organic anion transporter levels in androgen receptor-positive prostate cancer cells

DHEAS is an organic anion at physiological pH, and its uptake requires carrier-mediated transport across plasma membranes. We therefore performed a microarray analysis to compare the gene expression profiles of transporters that might be involved in DHEAS uptake between LNCaP cells grown in normal and androgen-depleted culture medium. Table 2 summarizes the genes whose expression altered by more than 1.5 times under the androgen-depleted condition; there were significant increases in several OATP genes, including OATP5A1, 1A2, and 1B1.

This was confirmed by qRT-PCR (Figure 2A). OATP1A2 mRNA expression was increased most markedly among the genes tested, and was 3.6- and 6.4-fold greater in LNCaP cells cultured in CSS-containing medium on Days 3 and 7, respectively, than on Day 1; however, no such increase was observed in LNCaP cells cultured for 7 days in normal medium (data not shown). Similarly, mRNA expression levels of OATP2A1 and 1B1 on Day 7 were increased 3.4- and 7.4-fold, respectively (Figure 2A). Table 3 summarizes the fold difference in mRNA expression of OATP genes tested on Days 3 and 7. Western blotting analysis indicated a time-dependent increase of OATP1A2 protein expression (Figure 2B). More interestingly, the stimulation of OATP1A2 mRNA expression under the androgen-depleted condition was almost completely abolished by the addition of DHT (Figure 2C), suggesting that *OATP1A2* gene transcription is negatively regulated by androgens. Expression of DHEAS transporters was also studied in 22Rv1 cells grown in both normal and androgen-depleted medium, and qualitative PCR results are shown in Figure 2D. Significant mRNA expression of OATP2A1, 2B1, 3A1, 4A1 and SLC10A4 was detected in 22Rv1 cells as well. Similarly, transcription of OATP1A2 and SLC10A4 appeared to be upregulated when the medium was deprived of androgen. Further qRT-PCR analysis showed that OATP1A2 mRNA expression was increased 3.7-fold in 22Rv1 cells, confirming DHT-mediated negative regulation of the transcription (Figure 2E).

3.3. Enhanced cellular uptake of DHEAS under androgen deprivation

To study the contribution of OATPs to cellular uptake of DHEAS in LNCaP cells, intimal uptake of [^{3}H]DHEAS was measured in the absence or presence of several inhibitors of organic anion transport systems. The uptake was significantly reduced in the presence of unlabeled

DHEAS, estrone 3-sulfate and BSP, which are inhibitors of OATPs, but inhibitors of OAT (salicylate and *p*-aminohippouric acid) and organic cation transporter inhibitors (tetra-ethylammonium and 1-methyl-4-phenylpyridinium) had no effect (Figure 3A). The uptake was tolerant of Na^+ replacement with Li^+, K^+ and NMG^+ (Figure 3B). Accordingly, [^{3}H]DHEAS uptake by LNCaP cells has the characteristics of OATP-mediated transport. Furthermore, [^{3}H]DHEAS uptake was significantly enhanced in LNCaP cells cultured with CSS-containing medium for 2 days, but addition of DHT (1 nM) to the medium significantly decreased the uptake, implying that cellular uptake of DHEAS is regulated by OATPs whose expression is negatively regulated by androgen, such as OATP1A2 (Figure 3C).

3.4. Contribution of OATP1A2 to DHEAS-induced cell growth in LNCaP cells

To further examine the role of OATP1A2 in DHEAS-induced growth of prostate cancer cells, growth of *OATP1A2*-transfected LNCaP cells was examined in CSS-containing medium in the absence or presence of 5 μM DHEAS. In the presence of DHEAS, growth of OATP1A2-transfected cells was stimulated by 41 %, whereas that of cells transfected with an empty-vector plasmid increased by only 11 %. Furthermore, the stimulatory effect of DHEAS was evaluated in LNCaP cell lines in which OATP1A2 had been silenced, designated as KD16 and KD34, compared with control cell lines (C3 and C9). As shown in Figure 4A, mRNA expression of OATP1A2 in KD16 and KD34 cells was almost completely knocked down. The effect of DHEAS on growth of the cell lines tested was normalized with respect to that in the absence of DHEAS (1.00) on Day 3 through Day 7 (Figure 4B). In the two control cells lines, DHEAS significantly stimulated cell growth with the highest stimulation on Day 5 or 6, even

though there were differences in degree of stimulation, whereas there was no statistically significant effect on growth of KD16 and KD34 cells during the experiments (p-value of 0.207 for KD16 and 0.056 for KD34 by one-way ANOVA). The finding that growth of OATP1A2-knockdown cells was insensitive to DHEAS suggests that OATP1A2 plays a predominant role in DHEAS-induced cell growth under androgen-depleted conditions.

4. Discussion

Our present findings indicate that OATP-mediated DHEAS transport plays a major role in survival and proliferation of androgen-receptor-positive prostate cancer cells under conditions of androgen depletion. In other words, enhanced import of DHEAS mediated by increased expression of OATPs, most likely predominantly OATP1A2, can be utilized to provide an alternative source of androgen under conditions of androgen depletion, allowing prostate cancer cells scope to acquire castration resistance, because the high serum concentration of DHEAS is little altered during the course of ADT.

To date, little is known about the role of OATPs in prostate cancer cells, although enhanced expression of OATPs in prostate tumors has been reported [16, 19]. Among them, OATP1B3 may be an important player for driving cell proliferation of prostate cancer cells, because it facilitates testosterone uptake into cells [19]. A more recent study with patient-derived prostate tumor specimens indicated that mRNA expression of six *SLCO* genes, including *SLCO1B3* and *2B1*, was enhanced several-fold in CRPC metastases, compared to untreated prostate cancer, implying their association with prostate cancer-specific motility [16]. In the present study, we

observed remarkable upregulation of OATP1A2 mRNA expression, as well as significant increases of OATP2A1 and 1B1 on Day 7 in CSS-containing culture medium, whereas expression of OATP2B1 and OATP1B3 was increased only moderately and slightly, respectively (Figure 2). We also found a stimulatory effect of DHEAS on growth of LNCaP and 22Rv1 cells (Figure 1); therefore, we focused here on DHEAS transport by OATPs, even though several other substrates are also commonly transported by the OATPs whose expression is enhanced.

Several classes of SLC transporters have been shown to mediate DHEAS uptake, including OATP1A2, 1B1, 1B3, and 2B1 [10, 11], OAT2, 3, and 4 [20-22], and Na^+-dependent taurocholate cotransporting polypeptide (NTCP) [23]. Hence, we conducted gene expression profiling of human prostate cancer LNCaP cells cultured under androgen-depleted conditions (Table 2A and Table 3). Microarray analysis suggests that OATP molecules are preferentially upregulated in LNCaP cells cultured in the absence of androgens, and this idea was supported by evidence that the functional characteristics of DHEAS transport in LNCaP cells resemble those of OATP-mediated transport (Figure 3A and 3B). Among several OATPs whose expression is enhanced by androgen deprivation, OATP1A2 showed the greatest increase in expression, and interestingly, its expression is negatively regulated by androgen signaling (Figure 2C). The increase of OATP1A2 mRNA was confirmed by Western blotting, showing two distinct bands around 75 and 100 kDa. This result agrees with two bands previously shown for OATP1A2 expressed in breast cancer cells in Western blotting, suggesting that OATP1A2 is glycosylated [24]. We found that expression of OATP1A2 was increased in LNCaP and 22Rv1

cells cultured under conditions of hormone deprivation, and the increase was consistent with the increase of initial uptake of DHEAS in LNCaP cells (Figure 3C). Ligand-bound nuclear hormone receptors activate transcription in the classical mode of receptor action, by binding to the regulatory sequence of the target gene promoter in dimer form. However, they also silence gene expression in various ways, such as through recruiting co-repressor, changing chromatin structure and accessibility, or inhibiting signaling pathways via cross-talk. For instance, androgen receptor has been shown to act as a ligand-dependent *trans*-repressor of activator protein-1 (AP-1) activity, resulting in attenuation of AP-1-activated gene expression [25]. The proximal promoter region of OATP1A2 has been characterized, and contains active *cis*-elements for several hepatocyte nuclear factors, a TAATAT box, and a putative AP-1 binding site [26]; it was therefore speculated that androgen-dependent negative regulation may be due to inhibition of AP1 by ligand-bound androgen receptor in LNCaP cells. Further study is needed to clarify the precise mechanism of repression of OATP1A2 expression by active androgens.

Because DHEAS is an inactive precursor of DHEA, it has to be activated by desulphation. It was reported that DHEAS is hydrolyzed by STS in LNCaP cells [27] and the expression of STS is increased in prostate cancer cells, compared to non-malignant prostate tissues, as determined by immunohistochemical staining [28]. In the present study, expression of STS mRNA was detected in LNCaP cells, and this expression was not affected under androgen-depleted conditions (Figure 1C). Furthermore, cell growth of LNCaP cells was significantly reduced in the presence of STX64 (Figure 1B), while PSA mRNA expression in LNCaP cells was

increased in the presence of DHEAS (Figure 1D). These results suggest that DHEAS undergoes metabolism at least to DHEA, mediated by STS. DHEA thus generated may have a weak androgenic potential to activate AR directly, but is more likely converted to T or DHT via 3b-hydroxysteroid dehydrogenase (3bHSD), since cell proliferation of LNCaP cells in the presence of DHEA was reported to be suppressed by inhibition of 3bHSD [15]. Although DHEA is present in human serum (~ 5 nM), the DHEAS concentration in serum (3 ~ 5 μM) is almost a thousand fold greater [12]; therefore, DHEAS may be taken up by prostate cancer cells via OATPs as a source of DHEA to compensate for the shortage of androgen in serum. This notion is supported by the experimental finding that knockdown of OATP1A2 in LNCaP cells resulted in loss of the cell growth response to DHEAS (Figure 4). Thus, concomitant use of a blocker of OATP1A2 may be pharmacologically important to more efficiently eradicate prostate cancer cells during the course of ADT. It is also noteworthy that, among non-synonymous polymorphisms which have been reported in *SLCO1A2* gene, decreased transport of estrone sulfate was reported in c.767A>C (rs11568563, E172D) and c.655A>T (rs45502302, N135I) [29, 30]. Therefore, the efficacy of ADT might be affected in prostate cancer patients with these genotypes, of which c.767A>C is relatively frequent in Europeans and Americans (5.3%).

5. Conclusion

In conclusion, our results indicate that OATP1A2 plays a role in the growth of prostate cancer cells under androgen-depleted conditions by supplying DHEAS as an alternative source of androgen, so that the cells can survive to acquire castration resistance. This is a novel mechanism underpinning the progression of advanced prostate cancer to the castration-resistant

state. Hence, OATPs (especially OATP1A2) may be a pharmacological target for more effective treatment of prostate cancer. In other words, pharmacological inhibition of OATP1A2-mediated influx transport of DHEAS in conjunction with ADT might be effective to improve the prognosis of prostate cancer patients.

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Conflict of interest

There are no conflicts of interest.

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Table 1

Gene specific primers for RT-PCR

Symbol Gene	Protein		Primer sequence (5’ to 3’)	Length (bp)	Amplicon (bp)
SLCO1A2	OTAP1A2	Fw	AAGACCAACGCAGGATCCAT	20	101
		Rev	GAGTTTCACCCATTCCACGTACA	23	
SLCO2A1	OATP2A1	Fw	CTGTGGAGACAATGGAATCGAG	22	147
		Rev	CACGATCCTGTCTTTGCTGAAG	22	
SLCO1B1	OATP1B1	Fw	GAATGCCCAAGAGATGATGCTT	22	154
		Rev	AACCCAGTGCAAGTGATTTCAAT	23	
SLCO2B1	OATP2B1	Fw	CGCACTCACTGATTCCTACACATT	24	278
		Rev	AAGCCATACCATCACTCATTACAC	24	
SLCO5A1	OATP5A1	Fw	TCATTGGAAYGGGCTCCAC	19	213
		Rev	TGAAACGAGGGTCATTCTGG	20	
SLCO1B3	OATP1B3	Fw	GTCCAGTCATTGGCTTTGCA	20	111
		Rev	CAACCCAACGAGAGTCCTTAGG	22	
SLCO3A1	OATP3A1	Fw	TACGACAATGTGGTCTACCGATAC	24	309
		Rev	CAGAGTTCAGCCCTCCTTTAGTCA	24	
SLCO4A1	OATP4A1	Fw	GCACCAGTTGAAGGACAGC	19	88

		Rev	GAGCCAGATGGAGAGAGG	18	
STS		Fw	GGAGAAATTCATGGCGGAAG	20	134
		Rev	GTGGGCTCATCAATCTTCTGG	21	
KLK3	PSA	Fw	AGAGGAGTTCTTGACCCCAAAG	22	182
		Rev	GCACACCATTACAGACAAGTGG	22	
HPRT1	HPRT	Fw	TGACACTGGCAAAACAATGCA	21	194
		Rev	GGTCCTTTTCACCAGCAAGCT	21	

Table 2

Alterations of transporter gene expression in LNCaP cells under androgen-depleted conditions, as determined by microarray assay

Gene	Protein	Major substrate	Fold change
SLC10A4	P4	Bile acid	4.85
SLCO5A1	OATP5A1	Organic anion	4.39
SLCO1A2	OATP1A2	Organic anion	3.87
SLCO1B1	OATP1B1	Organic anion	2.47
SLCO2B1	OATP2B1	Organic anion	1.74
SLC22A6	OAT1	Organic anion	0.654
SLC10A1	NTCP	Taurocholate	0.606
SLC22A13	OAT10	Organic anion	0.486
SLC04A1	OATP4A1	Organic Anion	0.411

Table 3

Quantitative changes in mRNA expression of OATP transporters in LNCaP cells under androgen-depleted condition, as determined by qRT-PCR

Protein name	Fold change relative to Day 1	
	Day 3	Day 7
OATP1A2	3.6 ± 0.20*	6.4 ± 1.2*
OATP2A1	1.7 ± 0.31*	3.4 ± 0.23*
OATP5A1	0.96 ± 0.30*	0.93 ± 0.36
OATP3A1	0.49 ± 0.15*	2.3 ± 1.2
OATP1B1	1.9 ± 0.59*	7.4 ± 2.9*
OATP1B3	0.72 ± 0.20	0.80 ± 0.37
OATP2B1	1.7 ± 0.25*	2.2 ± 0.38*
OATP4A1	1.0 ± 0.21	0.98 ± 0.88

All data are presented as mean ± S.E. (n=5 or 6). * indicates a significant difference from day 1 ($p < 0.05$) by Student's t-test.

Figure Legends

Figure 1: DHEAS-induced cell proliferation and PSA expression in LNCaP cells. (A) Growth of LNCaP cells was monitored by means of SRB assay for up to 7 days. LNCaP cells cultured in RPMI1640 with 10% CSS for 7 days were seeded at 8,000 cells each in the presence or absence of androgen. Each bar represents the mean ± S.E. (n=6), and * indicates a significant difference from the control ($p < 0.05$) by Student's t-test. (B) Effect of STS inhibitor STX64 (10 μM) on stimulation of LNCaP cell growth by DHEAS. LNCaP cells cultured in RPMI1640 with 5% CSS for up to 9 days were seeded at 8,000 cells. Their growth was monitored in the absence (open, control) and presence (closed) of DHEAS (5 μM) with (square) or without (circle) STX64 (10 μM). Experiments were done in quadruplicate and repeated at least twice. Each point represents the mean ± S.E. (n = 4), and * indicates a significant difference from the control ($p < 0.05$) by Student's t-test. (C) Expression change of STS or (D) PSA in LNCaP cells was measured by qRT-PCR. LNCaP cells were cultured in RPMI1640 supplemented with 10% FBS or 10% CSS in the presence or absence of androgen. Each bar represents the mean ± S.E. (n=3), and * indicates a significant difference from the control ($p < 0.05$) by Student's t-test.

Figure 2: Changes of expression of organic anion transporters in androgen receptor-positive prostate cancer cells. (A) mRNA expression of OATP transporter genes in LNCaP cells was measured by qRT-PCR. LNCaP cells were cultured in RPMI1640 with 10% CSS for up to 6 days. Each bar represents the mean ± S.E. (n=5 or 6), and * indicates a significant difference from the control ($p < 0.05$) by Student's t-test. (B) OATP1A2 protein expression was measured

by Western blotting. LNCaP cells were cultured in RPMI1640 supplemented with 10 % CSS in the presence or absence of DHT (1 nM) for up to 6 days. A typical Western blot showing increased OATP1A2 expression is presented. Each bar (sum of the two bands shown) represents the mean value of densitometric analysis of three individual blots ± S.E. (n=3), and * indicates a significant difference from the control ($p < 0.05$) by Student's t-test. (C) Effect of DHT (1 nM) on mRNA expression of OATP1A2 in LNCaP and (E) 22Rv1 cells cultured in RPMI1640 supplemented with 10% FBS or 10% CSS in the presence or absence of DHT (1 nM) for 2 days. Each bar represents the mean ± S.E. (n=3). * and † indicate a significant difference from the expression of OATP1A2 in LNCaP cultured in RPMI1640 supplemented with 10% FBS or 10% CSS ($p < 0.05$) by Student's t-test, respectively. (D) mRNA expression of organic anion transporters and HPRT in 22Rv1 cells was determined by RT-PCR. 22Rv1 cells were cultured in RPMI1640 supplemented with 10% FBS or 10% CSS for 2 days. PCR products were visualized on 2% agarose gel.

Figure 3: Characterization of [^{3}H]DHEAS uptake by LNCaP cells. (A) Cellular uptake of [^{3}H]DHEAS (10 nM) was measured in the presence or absence of the indicated compounds at pH 7.4 for 5 min in LNCaP cells cultured in RPMI1640 with 10% CSS for 2 days. Each bar represents the mean ± S.E. (n=3 or 4), and * indicates a significant difference from the control ($p < 0.05$) by Student's t-test. E3S; Estrone 3-sulfate, BSP; bromosulfophthalein, TCA; taurocholate, PRO; probenecid, SAL; salicylate, PAH; *p*-aminohippurate, TEA; tetra-ethylammonium, MPP^+; 1-methyl-4-phenylpyridinium. (B) Cellular uptake of [^{3}H]DHEAS (10 nM) was measured in the presence or absence of extracellular Na^+. Extracellular Na^+ was

replaced with K^+, Li^+, or N-methylglucamine (NMG^+) at pH 7.4 for 5 min in LNCaP cells cultured in RPMI1640 with 10% CSS for 2 days. Each value represents the mean ± S.E. (n=3 or 4). (C) Cellular uptake of [^{3}H]DHEAS (10 nM) in LNCaP cells was measured. LNCaP cells were cultured in RPMI1640 with 10% FBS (open, circle) or 10% CSS (closed) in the absence (circle) or the presence (square) of DHT (1 nM) for 2 days. Triangle symbols represent uptake of [^{3}H]DHEAS (10 nM) in the presence of DHEAS (1 mM). Each bar represents the mean ± S.E. (n=3 or 4), and * indicates a significant difference from the uptake rate of [^{3}H]DHEAS in LNCaP cells cultured with 10% CSS in the absence of DHEAS (1 mM) ($p < 0.05$) by Student's t-test.

Figure 4: Impact of OATP1A2-mediated DHEAS transport on proliferation of LNCaP cells. (A) Quantification of OATP1A2 mRNA expression in OATP1A2-knockdown (KD16 and KD34, closed bars) cells, compared with that in control (C3 and C9, open bars). Results are shown as the mean fold change (log2) ± S.E. Quantification of mRNA expression by qRT-PCR was repeated three times in duplicate. (B) Stimulatory effect of DHEAS on growth of OATP1A2-knockdown (open) and control cells (closed) in SRB assay. Cells were plated at 8,000 cells in each well. The ratio of cell growth to that without DHEAS (5 μM) was calculated for C3 (circle), C9 (square), KD16 (circle), and KD34 (square) each day. Each value is the mean of 7 or 8 individual results with S.E. (n=4).

Figure 1

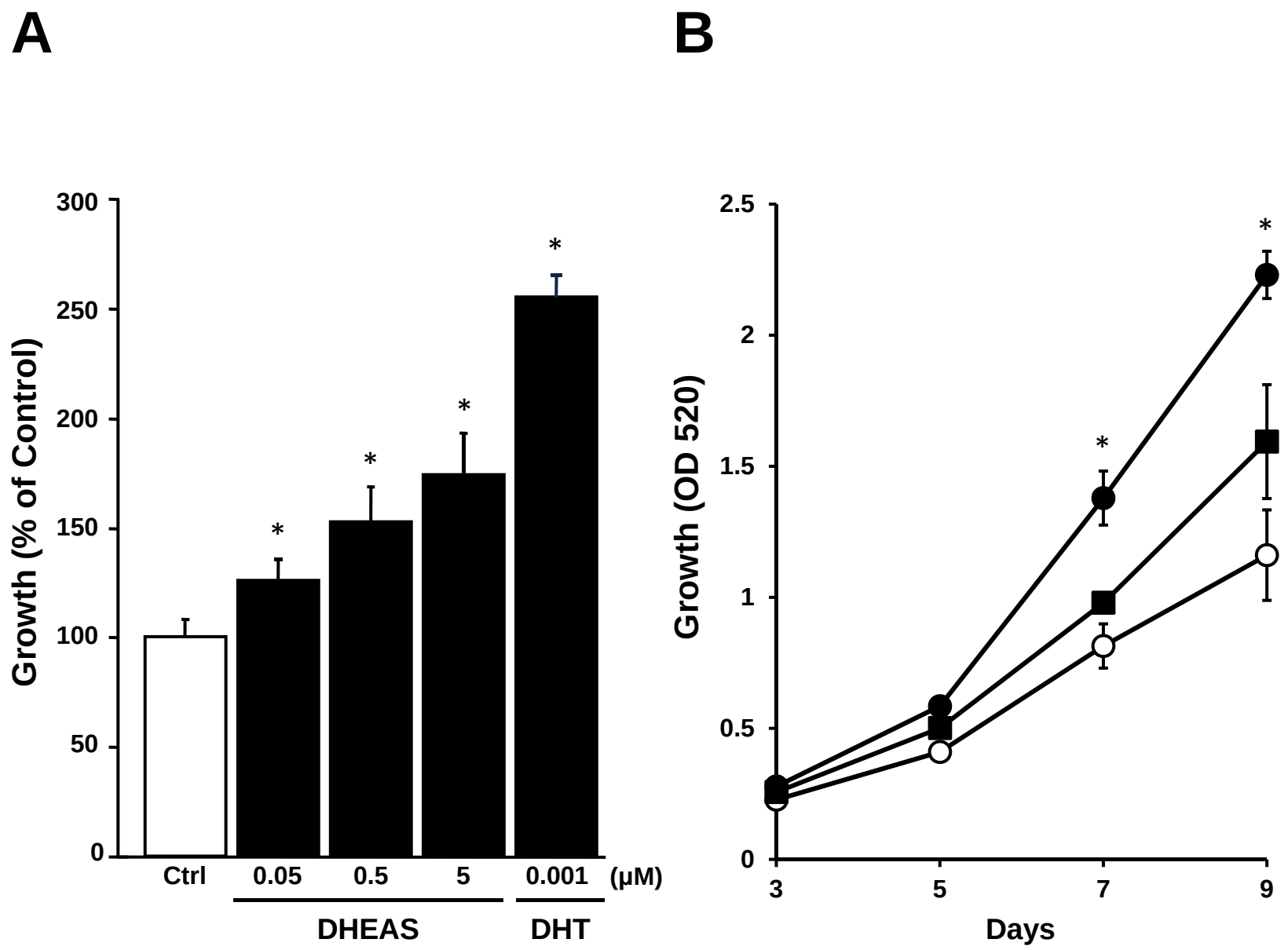

A
B
Growth (% of Control)
300
250
200
150
100
50
0
Ctrl
0.05
0.5
5
0.001
(μM)
DHEAS
DHT
Growth (OD 520)
2.5
2
1.5
1
0.5
0
3
5
7
9
Days

Figure 1

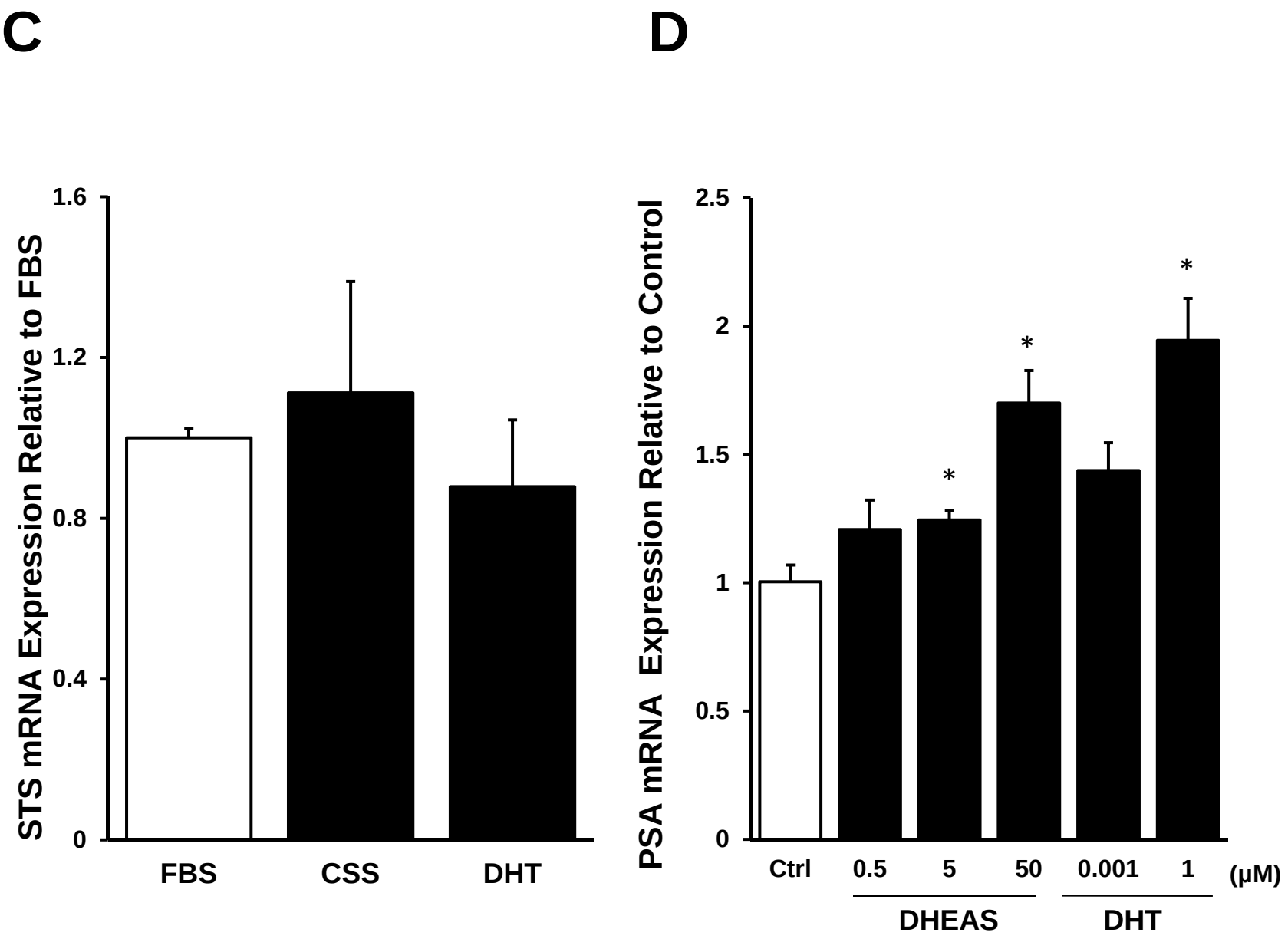

C
D
STS mRNA Expression Relative to FBS
1.6
1.2
0.8
0.4
0
FBS
CSS
DHT
PSA mRNA Expression Relative to Control
2.5
2
1.5
1
0.5
0
*
*
*
Ctrl
0.5
5
50
0.001
1
(μM)
DHEAS
DHT

Figure 2

A

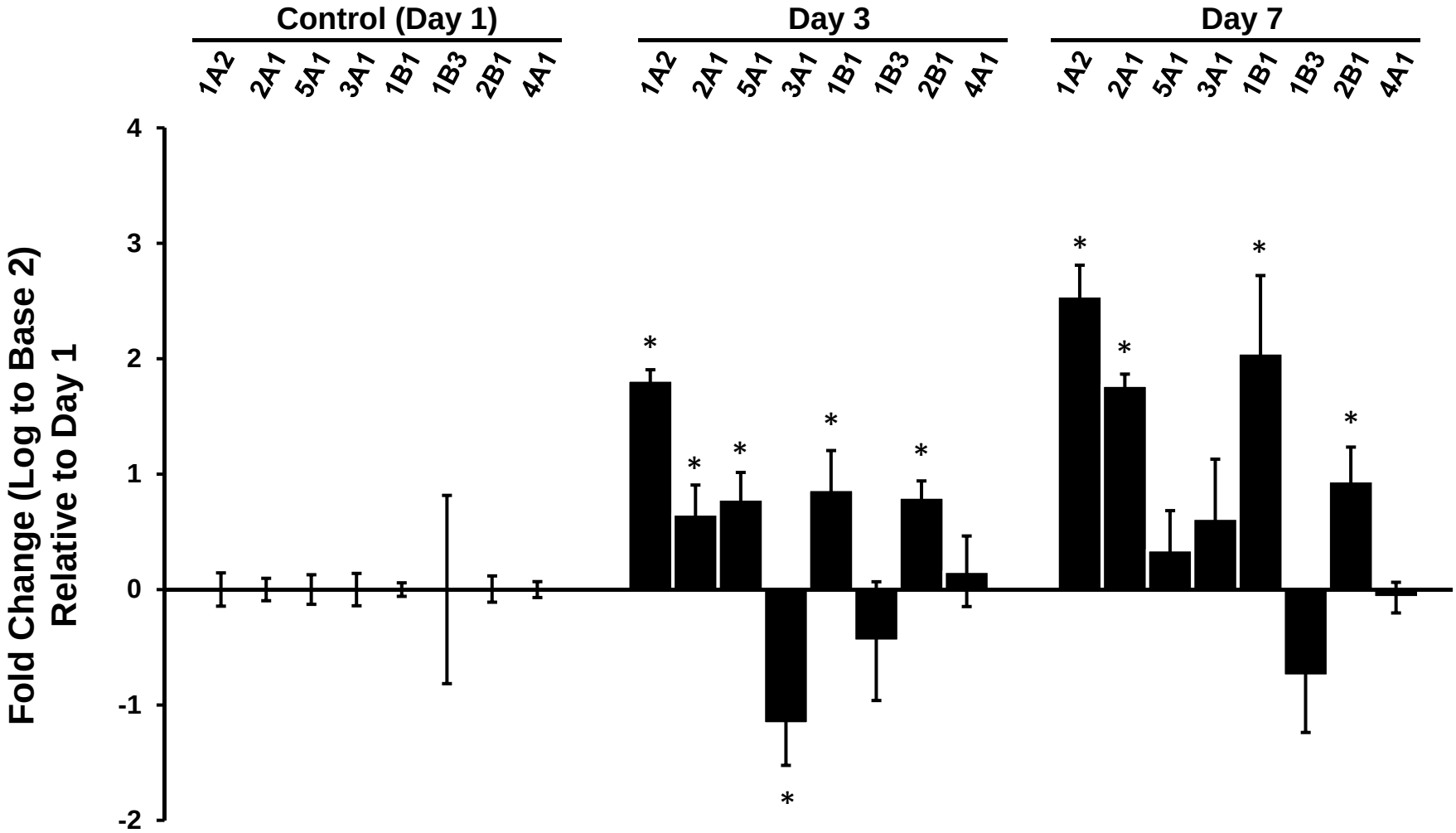
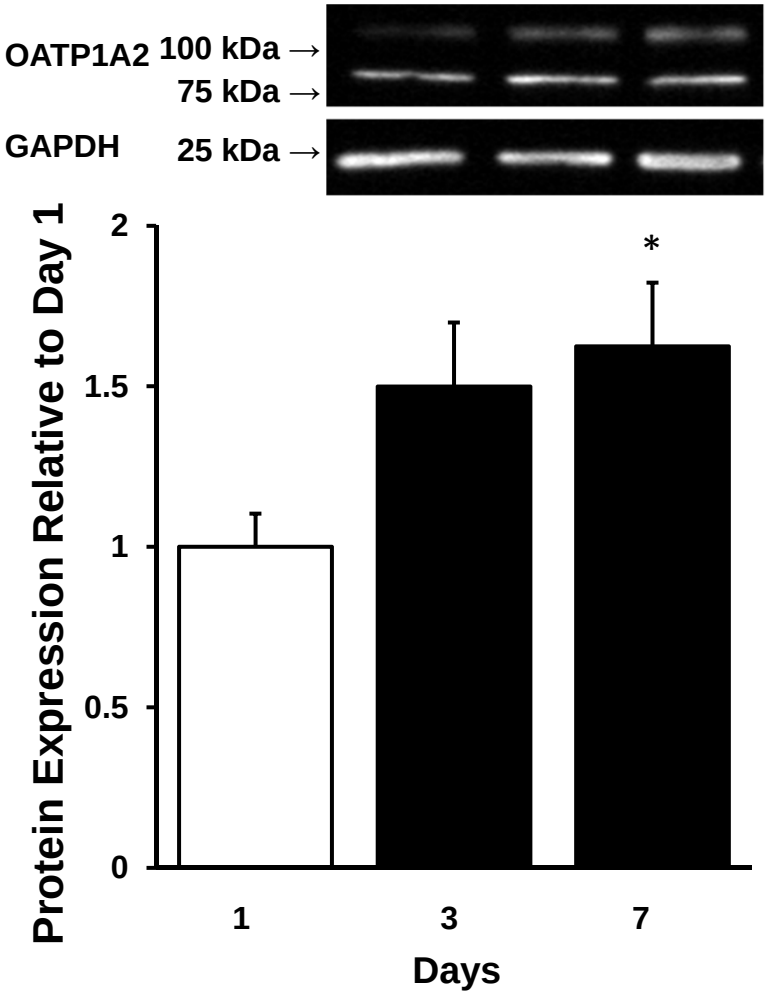

Control (Day 1)
Day 3
Day 7
1A2
2A1
5A1
3A1
1B1
1B3
2B1
4A1
Fold Change (Log to Base 2)
Relative to Day 1
4
3
2
1
0
-1
-2

Figure 2

B


OATP1A2
100 kDa →
75 kDa →
GAPDH
25 kDa →
Protein Expression Relative to Day 1
2
1.5
1
0.5
0
*
1
3
7
Days

C

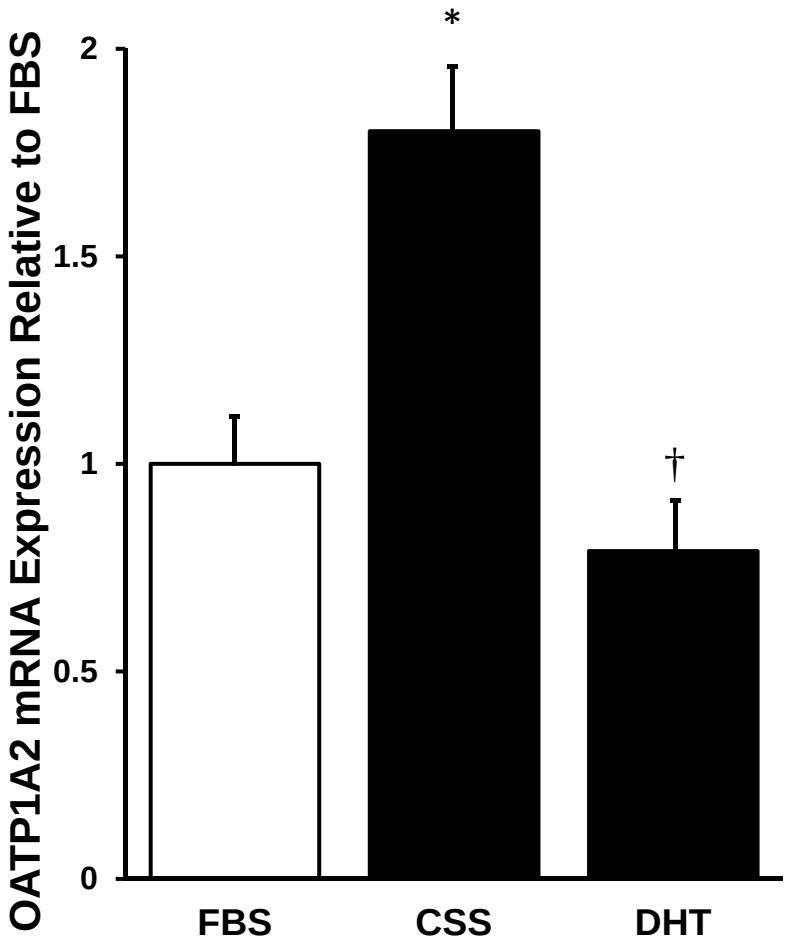

OATP1A2 mRNA Expression Relative to FBS
2
1.5
1
0.5
0
*
†
FBS
CSS
DHT

Figure 2

D

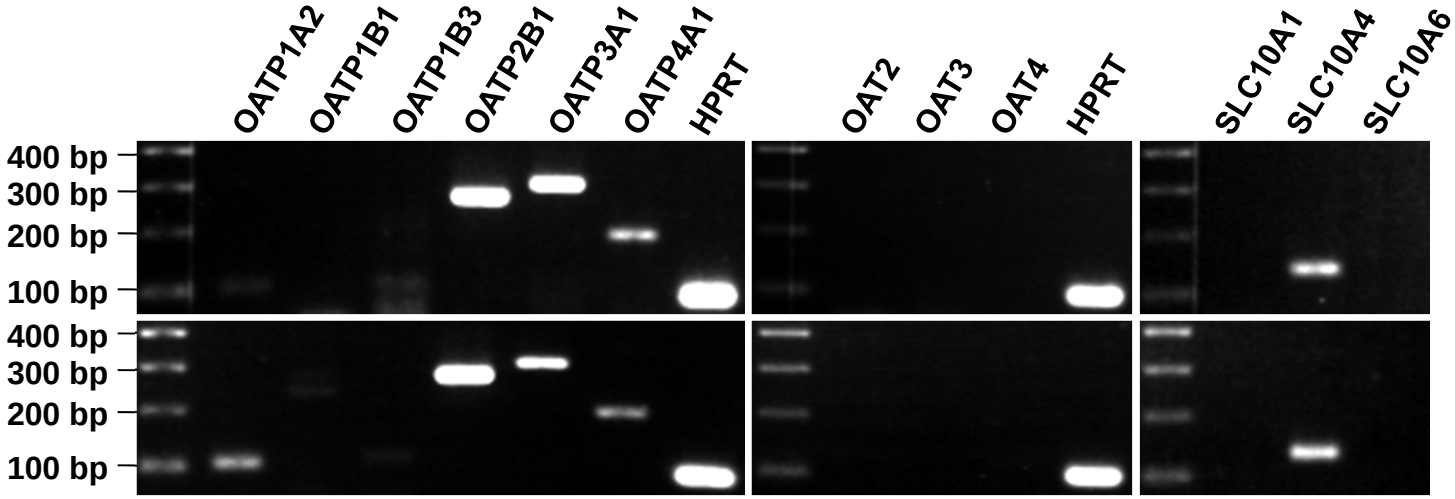

OATP1A2
OATP1B1
OATP1B3
OATP2B1
OATP3A1
OATP4A1
HPRT
OAT2
OAT3
OAT4
HPRT
SLC10A1
SLC10A4
SLC10A6
400 bp
300 bp
200 bp
100 bp
400 bp
300 bp
200 bp
100 bp

Figure 2

E

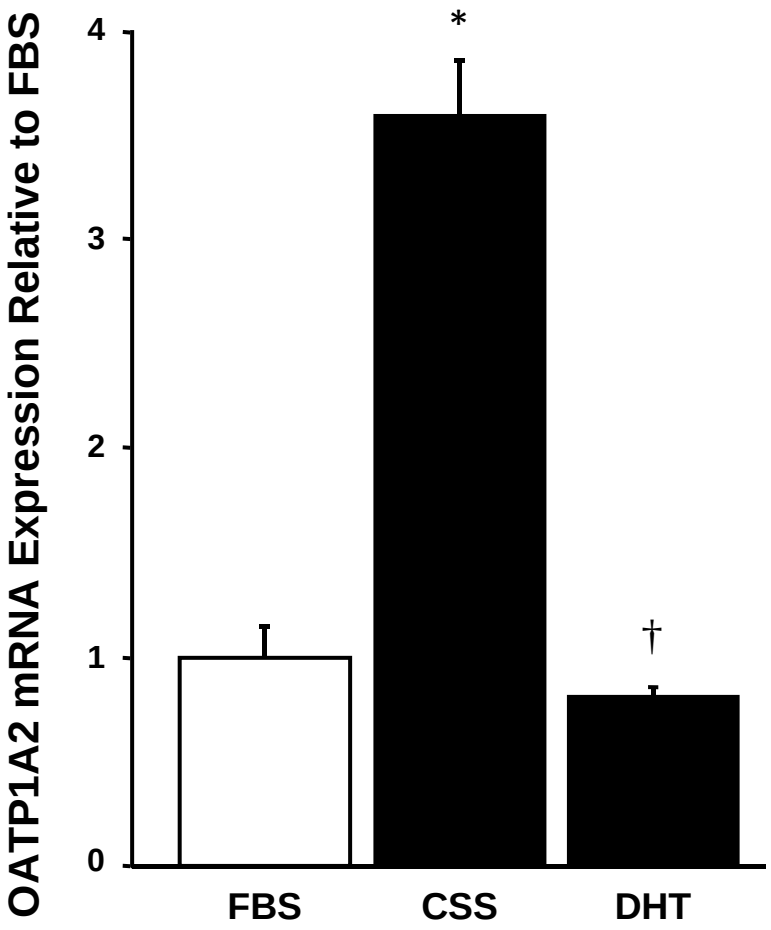


Figure 3

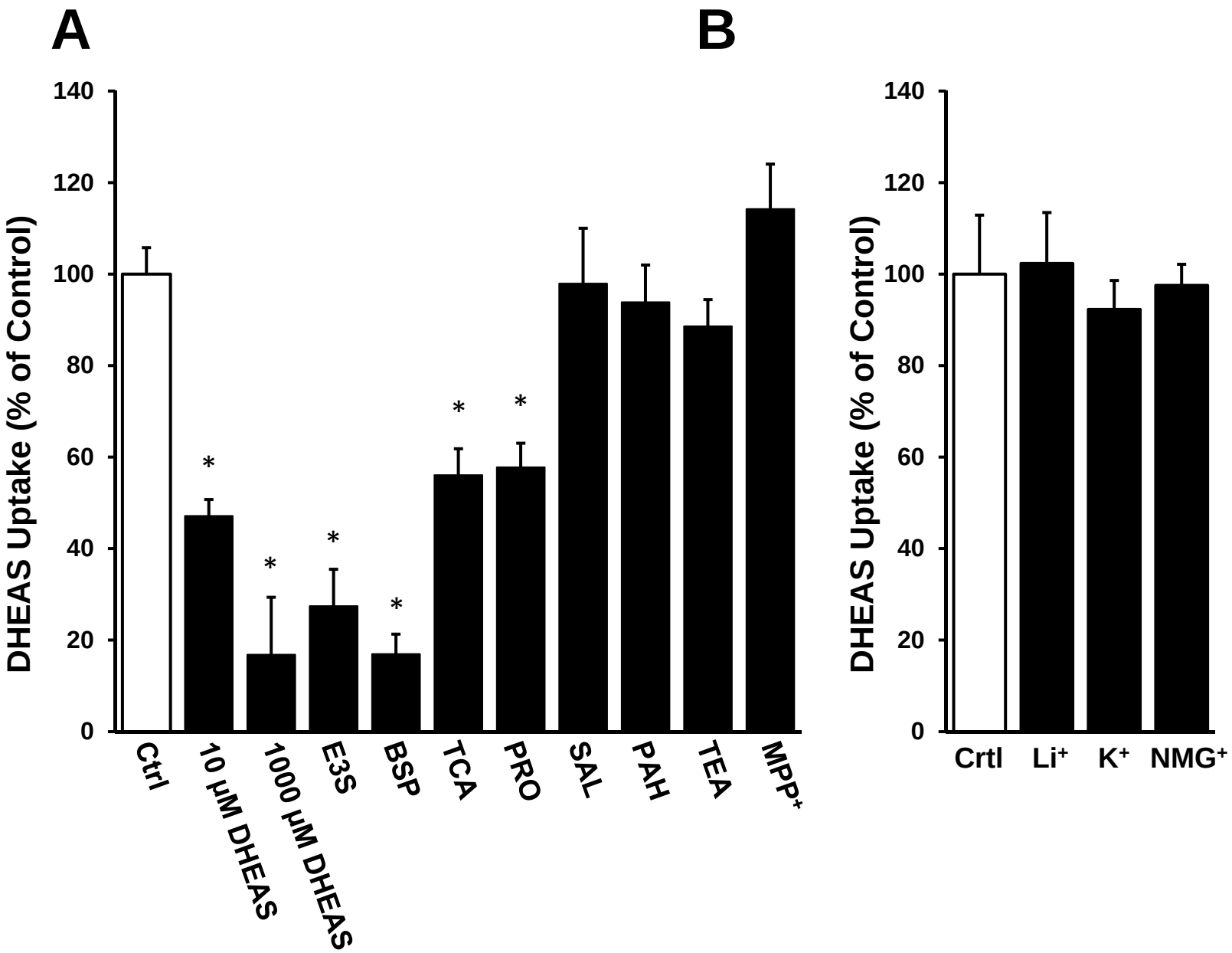

A
B
DHEAS Uptake (% of Control)
140
120
100
80
60
40
20
0
*
Ctrl
10 μM DHEAS
1000 μM DHEAS
E3S
BSP
TCA
PRO
SAL
PAH
TEA
MPP+
Crtl
Li+
K+
NMG+

Figure 3

C

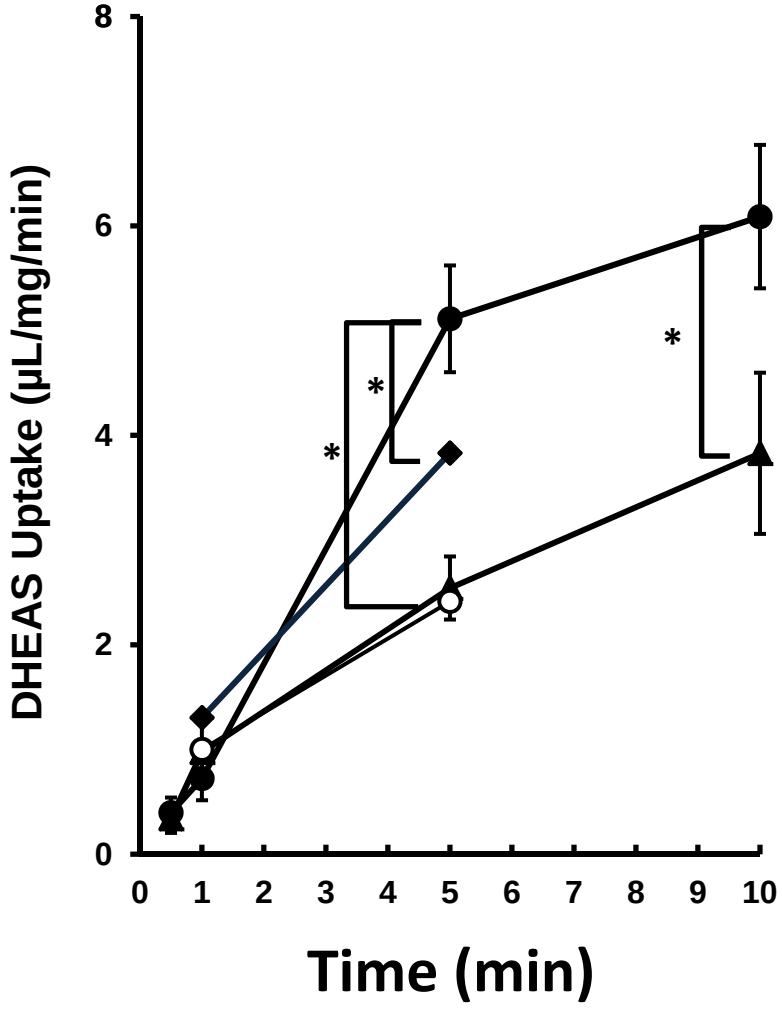
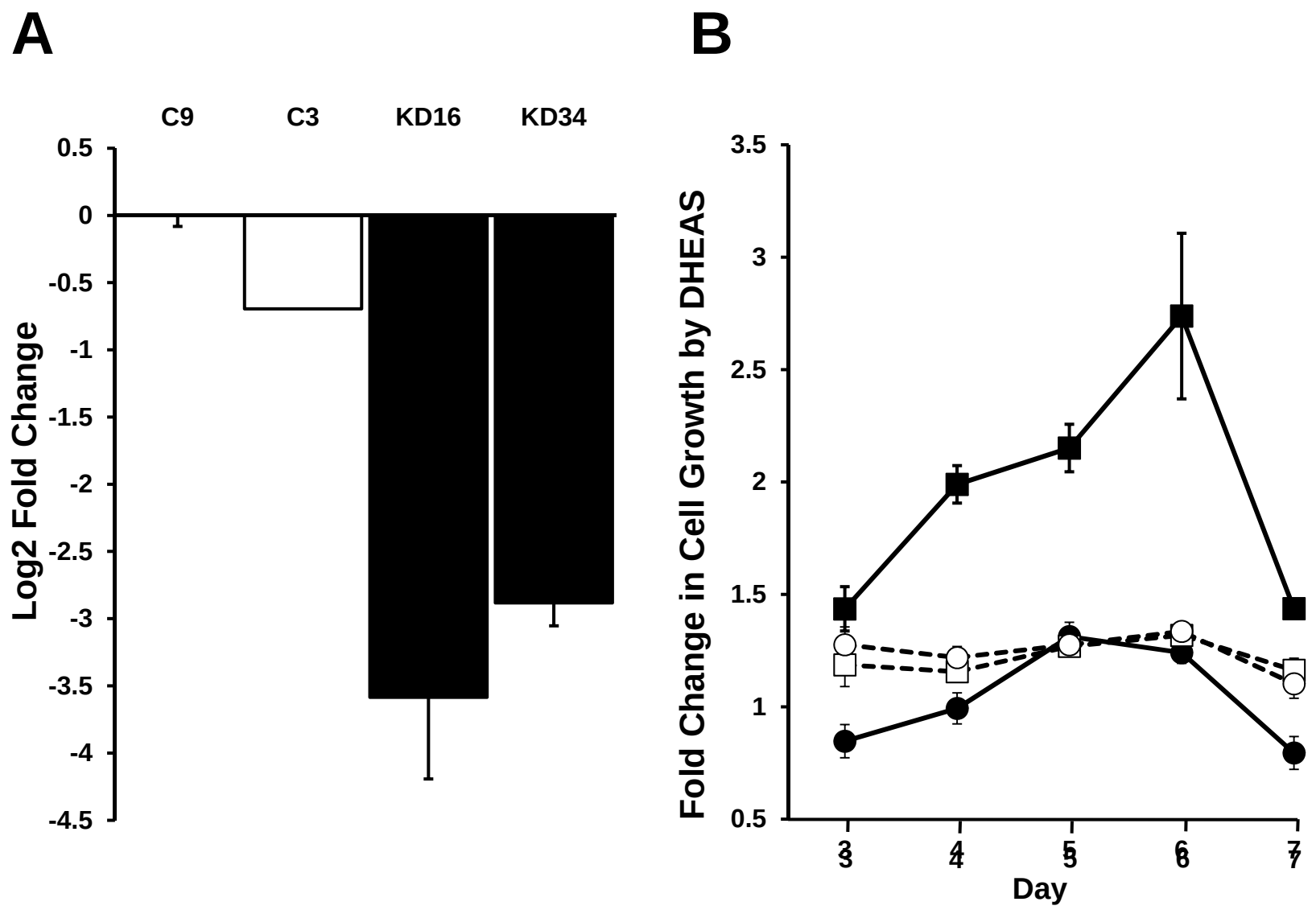


Figure 4


A
B
C9
C3
KD16
KD34
Log2 Fold Change
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-4.5
Fold Change in Cell Growth by DHEAS
3.5
3
2.5
2
1.5
1
0.5
3
4
5
6
7
Day