THE ROLE OF OATPs IN MAMMALIAN STEROID HORMONE TRANSPORT

By

Avery Morse

A capstone project submitted for graduation with University Honors

May 8, 2024

University Honors

University of California, Riverside

APPROVED

Dr. Naoki Yamanaka
Department of Entomology

Dr. Sachiko Haga-Yamanaka
Department of Molecular, Cell and Systems Biology

Dr. Richard Cardullo, Howard H Hays Jr. Chair
University Honors

ABSTRACT

It is taught in textbooks that steroid hormones are lipid based molecules that passively diffuse through the cell membrane into the cell. However, recent studies have shown that the fruit fly *Drosophila melanogaster* has a membrane transporter for ecdysone, a steroid hormone in insects, called Ecdysone Importer (EcI). This implies that other steroid hormones, including mammalian steroid hormones, might also require transportation across the cell membranes, challenging our current understanding of this pathway. EcI is a member of a highly conserved superfamily of genes called Organic Anion Transporting Polypeptides (OATPs), which are also conserved in mammals. In these experiments, we investigated the potential role of OATPs that are highly expressed in HEK293T cells in cortisol cellular import. We tested for the transport of cortisol into cells by measuring cortisol-induced GR activation through a luciferase assay. Using qPCR, we analyzed the differences in expression levels of glucocorticoid-inducible genes in wild type HEK293T cells, OATP-overexpressing HEK293T cells, and double-knockout HEK293T cells where the two most highly expressed OATP genes are knocked out. Lastly, we utilized A549 lung cancer cells whose proliferation is known to be inhibited by glucocorticoids to further analyze the role OATPs play in glucocorticoids cellular import. In wildtype A549 cells and OATP-overexpressing A549 cells, growth was monitored with the addition of different doses of either a methanol control, cortisol, or dexamethasone. This research will help to elucidate the mechanisms behind steroid hormone transport, opening up pathways to be used in more precise and effective medicine and research.

ACKNOWLEDGMENTS

I would like to thank Dr. Sachiko Haga-Yamanaka for her incredible mentorship these last three years. I am incredibly grateful to have received the opportunity to complete research in her lab, which has changed the course of my life and helped me gain confidence in myself and my skills. I would also like to thank Dr. Mitchell Masterson for his guidance and endless support throughout this project, and for helping define the researcher I am today. Thank you as well to Dr. Naoki Yamanaka for putting faith in me with this project. I look forward to working with you further in graduate school this coming fall. I am grateful to the community and support I have found at UCR and in these labs. I would also like to acknowledge the Undergraduate Mini-Grant program, whose funding jump started my experiences with independent research projects two years ago. Finally, I am endlessly thankful for my family and friends who have put up with me during my stressful late nights, and who have offered unwavering patience and support.

TABLE OF CONTENTS

INTRODUCTION

In most introduction to biology courses, students are taught about the different types of transport across cellular membranes. One of the textbook examples given for passive diffusion across a membrane is that of steroid hormone signaling pathways. Steroid hormones are known to have intracellular receptors, and are believed to reach those receptors through passive diffusion through the membrane. We are taught that due to their small lipophilic structure they are able to diffuse through the phospholipid membrane with no aid. Yet, recent research has been done in the fruit fly *Drosophila melanogaster* that contradicts this model. In studies done by Okamoto et al., it was shown that there is a membrane transporter named Ecdysone Importer (EcI) in *Drosophila* that is required for the transport of ecdysone, an important steroid hormone in *Drosophila* (Okamoto et al., 2018). This research shifts the idea that steroids can traverse membranes through passive diffusion alone to a possible model of facilitated diffusion. This research begs the question of if this is true for other steroid hormone pathways in other organisms.

Organic Anion Transporting Polypeptides

EcI is a member of the highly conserved SLCO solute carrier gene family, which is also present in mammals (Obaidat et al., 2012). In mammals the gene products of these SLCO genes are a class of membrane bound transporters called organic anion transporting polypeptides, more commonly known as OATPs (Obaidat et al., 2012). In humans, this family contains eleven members, belonging to six major families (Hagenbuch & Stieger, 2013). All OATPs are membrane bound, with twelve transmembrane domains (Hagenbuch & Stieger, 2013). Members of the OATP superfamily are known to transport statins, antibiotics, bile acids, xenobiotics,

hormones, steroid hormone conjugates and a multitude of drugs (Hagenbuch & Stieger, 2013; Obaidat et al., 2012; Wright et al., 2011).

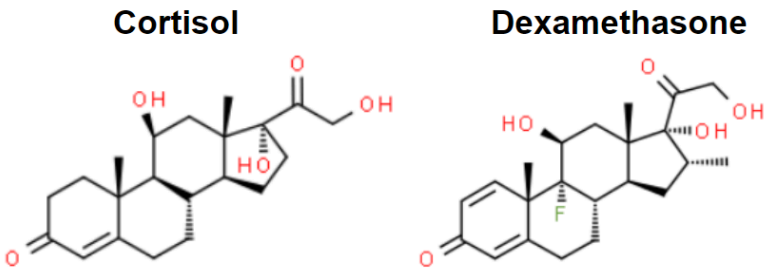
There is research correlating high OATP expression with greater steroid hormone precursor uptake. Dehydroepiandrosterone sulfate (DHEAS) is a male sex hormone, and a precursor for the synthesis of many other steroid hormones (Hagenbuch & Stieger, 2013). Prostate cancer xenografts in mice showed a significant increase in DHEAS uptake when expressing OATP1B1 and OATP2B1s compared to those not expressing those OATPs (Green et al., 2017). Similarly, estrogen sulfate (E1S) is a precursor to the production of endogenous estrogen, and is a substrate of OATP2B1 (Pizzagalli et al., 2003). These previous studies imply that members of the OATP family may play a role in the transport of some steroid hormone precursors, and that they may be clinically relevant to steroid dependent cancers such as breast and prostate cancer.

There is evidence of increased levels of OATPs in treatment resistant metastases and developed forms of steroid dependent cancers, such as prostate and breast cancer (Pizzagalli et al., 2003; Sarakbi et al., 2006; Wright et al., 2011). In these studies, high OATP2B1, OATP3A1 and OATP4A1 expression is associated with developed breast cancer (Pizzagalli et al., 2003; Sarakbi et al., 2006), while high OATP4A1 and OATP1B1 expression is associated with prostate cancer (Wright et al., 2011). Having high levels of OATPs in these developed cancers establishes a possible linkage between OATP expression and disease evolution (Green et al., 2017). If members of this OATP family are important to steroid hormone transport, then they may be potential targets for treating these steroid dependent cancers.

Glucocorticoids

There are many different kinds of steroid hormones in mammals, among the most important of which are glucocorticoids. Glucocorticoids are involved in many different processes in mammals, including anti-inflammatory pathways, immune response, growth, metabolism, development, and responses to psychological and physical stress (Khadka et al., 2023; Timmermans et al., 2019). Like many steroid hormones, they are synthesized from cholesterol, and are normally produced in the adrenal gland (Timmermans et al., 2019). Important glucocorticoids include cortisol, the mammalian stress hormone, as well as many synthetic glucocorticoids such as prednisolone and dexamethasone that are used for pharmaceutical purposes. Although they bind the same receptor, they are treated differently within the cell and have different properties. For example, dexamethasone is about 30 times more potent than cortisol in terms of eliciting cellular responses (Papich, 2016).

Figure 1: Comparison of cortisol and dexamethasone chemical structures, as retrieved from ChemSpider online



database (http://www.chemspider.com/).

All glucocorticoids bind to an internal glucocorticoid receptor (GR), encoded by the *nr3c1* gene. GR is localized in the cytoplasm when inactive. When activated by glucocorticoid binding, nuclear localization and DNA binding domains are activated (Timmermans et al., 2019; Weikum et al., 2017). Once in the nucleus, active GR-glucocorticoid complexes bind glucocorticoid response elements (GRE) and interact with cofactors to form transcription

regulatory complexes (Weikum et al., 2017). Here they can associate with cell-type specific transcription factors within the nucleus, allowing for glucocorticoids to elicit different responses under different conditions or in different cell types (Weikum et al., 2017). There are multiple GRE consensus sequences for direct GR-glucocorticoid complex binding, but other mechanisms for GR regulation include indirect activation through association with NF-κB and AP-1 transcription factors (Weikum et al., 2017).

As ubiquitous hormones involved in many different pathways, glucocorticoids have a large range of direct and indirect target genes. Tryptophan 2,3-dioxygenase (TDO2) is a gene related to the metabolism of tryptophan, and is of interest to research focused on behavior and impulsivity. It includes multiple GREs in the promoter region, implying that it is directly regulated by glucocorticoids (Soichot et al., 2013). Phosphoenolpyruvate carboxykinase 1 (PCK1) is an important enzyme to the regulation of gluconeogenesis, and also is directly regulated by the GR receptor dimer in response to glucocorticoids (Yu et al., 2021). Period 1 (PER1) is a clock gene essential to regulation of circadian oscillations, and shows a strong, quick response when treated by glucocorticoids such as dexamethasone (Balsalobre et al., 2000). This implies a direct regulation by GR as well.

Glucocorticoids in Cancer

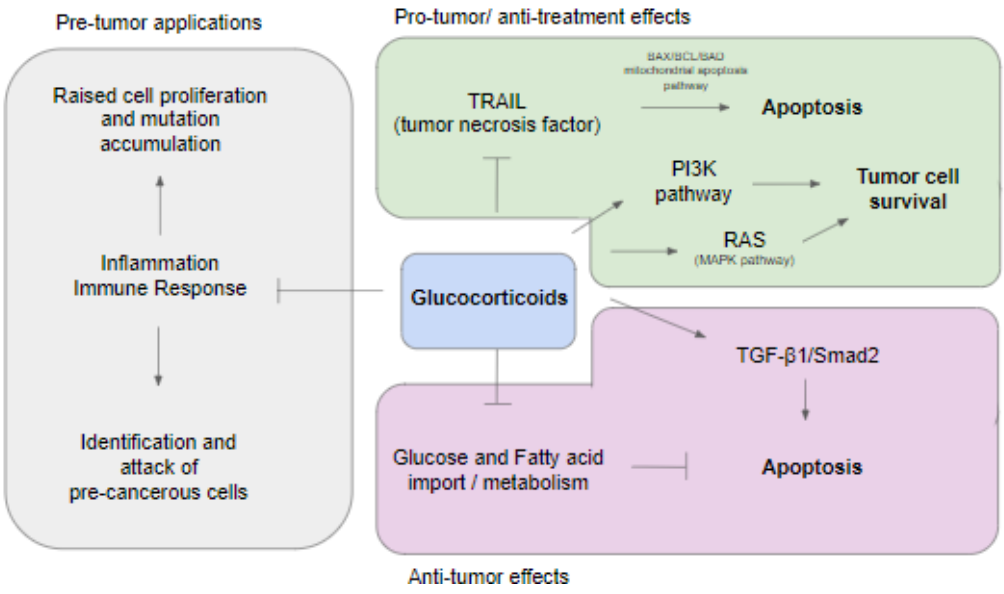


Figure 2: Summary of the different effects glucocorticoids can have on tumor treatment, development and progression as described below.

Glucocorticoids are usually used as a treatment for inflammation, a process that plays a large role in cancer development. Inflammation is inherently linked with increased cell proliferation, which both increases the chances of cancer causing mutations to develop through simple DNA replication errors and corresponds with the proliferative phenotype of cancer cells. The major inflammation pathway focuses on the activation and nuclear localization of the nuclear factor-kappa B (NF-κB) transcription factor. NF-κB is usually sequestered in the cytoplasm and inhibited by IκB, before it is activated by IκB kinase (IKK) (Taniguchi & Karin, 2018). Once activated, NF-κB can translocate to the nucleus and act as a transcription factor for genes associated with cell proliferation, apoptosis inhibition, and epithelial to mesenchymal transition (EMT) which increases cell motility for metastasis (Taniguchi & Karin, 2018). Mutations within the NF-κB pathway can often be associated with cancer, as they allow cancer cells to proliferate with less regulation, avoid immune detection, and to invade other tissues (Taniguchi & Karin, 2018). However, inflammation occurs in response to infection because it

also brings with it the body's immune response. NF-κB is important to the induction of many immunoregulatory genes, and any inhibition of this pathway will also interrupt the resulting immune response (Auphan et al., 1995).

Glucocorticoids are intertwined with both ends of the inflammation pathway, both aiding and worsening cancer outcomes depending on the cancer type (Khadka et al., 2023). There are multiple proposed mechanisms of this. One possible mechanism is that glucocorticoids increase expression of IκBα, part of the NF-κB inhibition mechanism (Auphan et al., 1995). Active GR-glucocorticoid complexes can bind and directly repress inflammatory transcription factor complexes composed of NF-κB or AP-1, meaning glucocorticoid signaling can inhibit inflammatory responses through the NF-κB pathway (Dougherty et al., 2016). Because of this, glucocorticoids are often used to treat various inflammatory diseases (Khadka et al., 2023). Through the same mechanisms, glucocorticoids can be used as a preventative treatment for many cancers. Cancers such as lung and gastric cancer commonly have chronic inflammation precursors that are often used as predictors for cancer outcomes (Khadka et al., 2023). In these cases glucocorticoids can help to decrease inflammation, and prevent accumulation of oncogenic mutations in patients that could lead to cancer in these organs.

Glucocorticoids can also be important to prevent further development of already developed cancer, as seen in breast cancer (Khadka et al., 2023). Synthetic glucocorticoids such as dexamethasone and prednisolone are especially important to the treatment of leukemias and other non-solid tumors. In acute lymphoblastic leukemia, a cancer commonly affecting children, resistance to glucocorticoids can even be a precursor for relapse and more aggressive forms of the cancer (Chougule et al., 2019). Most often, glucocorticoids are given alongside

chemotherapy in order to reduce side effects, though there are negative effects that come with glucocorticoid treatment that makes them not suitable for all cancer treatments.

Downregulating the inflammatory pathway can have negative side effects. Since inflammation as a response to disease or infection is primarily for increasing immune response, glucocorticoid mediated inhibition of this pathway can prevent recognition of cancer cells by the body's immune system. Through this mechanism, skin cancer cells may accumulate mutations that raise internal levels of glucocorticoids when they become metastatic and start to invade other tissues (Khadka et al., 2023). Glucocorticoids can also lead to increased metastasis in some cancers. There are reports of glucocorticoid treatments increasing breast cancer metastasis, despite its effects to prevent some aspects of its development (Liu et al., 2017). In aggressive forms of pancreatic cancer, excess glucocorticoid signaling can lead to metastasis as active GR positively regulates expression of transforming growth factor-β (TGF-β), which induces many mechanisms associated with metastasis (Liu et al., 2017).

Glucocorticoids are also involved in the RAS cell proliferation pathway ((Khadka et al., 2023; Liu et al., 2017). Inactive GR is able to bind RAS, preventing it from participating in the proliferation pathway. When glucocorticoids activate GR, it causes GR to detach from RAS, which indirectly activates that cell proliferation pathway (Khadka et al., 2023). Pancreatic cancer cell lines which often have highly active or mutated RAS pathways also rarely have functional GR, possibly because of their participation in this pathway (Gower et al., 1994). Although this glucocorticoid induced proliferation model has been shown in A549 lung cancer cells, in practice dexamethasone also activates the apoptotic TGF-β1/Smad2 pathway and *in vitro* can be used to induce apoptosis and slow proliferation of A549 cells (Feng et al., 2018; Khadka et al., 2023).

Considering the positive and negative roles of glucocorticoid signaling in various cancers, it follows that having a complete understanding of these pathways is essential to having the most safe and effective treatments. Any changes in this understanding may inform when glucocorticoids can be appropriately used as treatment, and the roles they play in cancer progression. Following the evidence that there is a steroid hormone transporter of ecdysone in *Drosophila*, this research aims to clarify the route of entry of glucocorticoids into human cell lines in order to strengthen our understanding of this pathway (Okamoto et al., 2018). This research focused on OATPs as a possible transporter due to their homology to EcI, as well as their recorded overexpression in glucocorticoid promoted cancers (Okamoto et al., 2018; Pizzagalli et al., 2003; Sarakbi et al., 2006; Wright et al., 2011). If there is a transporter for glucocorticoids in human cells, it may be a druggable target to limit side effects of cancer treatments, or to help minimize progression of glucocorticoid promoted cancers.

METHODS

Cell Culture

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, 10-013-CV), supplemented with 10% fetal bovine serum (FBS, Gibco, 10437028) and 1% penicillin/streptomycin (Gibco, 15140122). A549 cells were purchased from ATCC. A549 cells were cultured in Kaighn's modified Ham's F-12 Medium (F-12K, Gibco, 21127030) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37°C and 5% CO_2, and passaged twice a week upon reaching confluency.

Table 1: Summary of cell types and abbreviations used in the paper.

Cell Line	Abbreviation	Cell Line	Abbreviation
HEK293T - Wild Type	H-WT	HEK293T- OATP4A1 Knockout	H-4A1KO
HEK293T - OATP3A1/4A1 Knockout	H-dKO	A549 - Wild Type	A-WT
HEK293T - OATP3A1 Overexpression	H-3A1OE	A549 - OATP3A1 Overexpression	A-3A1OE

qPCR of SLCO genes in HEK293T cells

HEK293T cells were grown to confluency in 10 cm dishes. They were then washed with PBS before being collected into TRIzol (ThermoFisher, 15596018) in order to lyse cells. Chloroform was added to the TRIzol cell mixture and separated RNA from the gDNA. RNA was collected from the aqueous phase, and RNA was extracted using the QIAGEN RNeasy RNA Isolation kit. All samples were reverse transcribed using Oligo(dT) Primers (Invitrogen, 18418012), dNTP (ThermoFisher, 18427013) and RNaseOUT (ThermoFisher, 18080051) to prime the reaction for reverse transcription, and the SuperScript™ III Reverse Transcriptase kit from Invitrogen. The resulting cDNA was then set up for a qPCR reaction in 96 well plates using iQ SYBR® Green Supermix from BIO-RAD. qPCR was carried out using the CFX connect

real-time PCR detection system (Bio-Rad). Resulting Cq values were normalized against the *GAPDH* housekeeper's Cq to obtain the ΔCq value. All primers used listed below (Table 2).

Table 2: Lists the primer sequences for all qPCR primers used in the qPCR of SLCO genes in HEK293T cells.

Gene ID	Forward Primer Sequence	Reverse Primer Sequence
SLCO1A2	5' TGT CCT CAA ACA GTT TCT TGT GT 3'	5' GGA CGT ACA CCC ACA TTA ATG A 3'
SLCO1B1	5' TGT TGG TTT TAT TGA CGG AAG CT 3'	5' CCC ATA ATG AAA CAA CCG ATT CC 3'
SLCO1B3	5' TGG AGC AAC AGT ACG GTC AG 3'	5' TGC TTT CGC AGA TTA GAG GGA 3'
SLCO1B7	5' TGC TTT CCT CTG TGC AGT TG 3'	5' GCA TAA TCA TTG AAT GGA AGC CG 3'
SLCO1C1	5' GGA GTT GGA ACA CTG CTC ATT 3'	5' CTT GAC TCT AGG AGA CAC GGA 3'
SLCO2A1	5' CAG CGA CAC CTC TAC TAG CC 3'	5' GTA CAG GAG TTG GCA GAG CT 3'
SLCO2B1	5' CAG ATT GCG GGC ATC ACA C 3'	5' CAG ACA GGG TTA AAG CCG TC 3'
SLCO3A1	5' TCT CCT ACA TCG ACG ACC AC 3'	5' TGG TAC AGA AAG AGC CCA GG 3'
SLCO4A1	5' GGC ATC CTC GTC TTC TCA CT 3'	5' TTA GGT TCA GGT CTT CG 3'
SLCO4C1	5' TGA GAC TGG GAG CAC TT 3'	5' ACT GGT GCT ATT CCT TGT TGT 3'
SLCO5A1	5' TGT CTG GCT GGC TGT GTT AA 3'	5' CAG ACA CAG CAT AGC CGT TC 3'
SLCO6A1	5' TGA CAA ACT GCG TTC TCT GG 3'	5' AGA TTG ATG GTC CAG GAA TAG TC 3'
GAPDH	5' CCC ACT CCT CCA CCT TTG AC 3'	5' CCA CCA CCC TGT TGC TGT A 3'

Transfection for Dual-Glo Luciferase Assay

To optimize the seeding densities for transfection, we did a test GFP transfection at variable densities for each cell line so we could visualize the transfection efficacy. For WT and 4A1 KO we wanted cells to be at 80% confluency the day of transfection, while we wanted 3A1/4A1 dKO cells to be at 60% to increase transfection efficiency. Each cell type was transfected with Firefly Luciferase and Renilla Luciferase plasmids from the Promega Nano-Glo® Dual-Luciferase® Reporter Assay System, as well as *nr3c1*, the gene encoding for GR. Transfections were done using the QIAGEN Attractene transfection reagent and associated protocol.

Dual-Glo Luciferase Assay

One day after transfection, cells were seeded into 24 well plates in DMEM with 10% stripped FBS and 1% penicillin and streptomycin. On the day after seeding into 24 well plates cells were treated with a methanol control, 1 nM, 3 nM, or 10nM cortisol. They then were incubated in 37°C and 5% CO_2 for three hours before being lysed and treated for firefly luminescence activation. After shaking at 37°C for 30 min, cells were seeded in technical triplicate into 96 well plates. Then, the firefly luminescence was recorded using a VICTOR X3 luminometer (Perkin Elmer) according to the manufacturer instructions. Next the firefly luminescence is quenched for a 10 minute period, and renilla luciferase is activated according to the Promega Nano-Glo® Dual-Luciferase® Reporter Assay System protocol.

Firefly luciferase is only produced in response to the glucocorticoid receptor/glucocorticoid complex. Renilla luciferase is constitutively expressed in the cell independent of cortisol uptake, and acts as a control to account for transfection efficiency. First, the relative luminescence is calculated by normalizing the firefly response to our test renilla. Relative luminescence is defined as firefly luminescence divided by renilla luminescence for a given variable. Then the relative luminescence is normalized by dividing each value by the 0 nM control response of their respective cell type and assay run in order to give the fold change.

Cortisol Treatment and qPCR of HEK293T cells

Wildtype HEK293T cells and OATP3A1 overexpression HEK293T cells were grown to confluence and split into Poly-D-Lysine treated 24 well plates. OATP3A1 and OATP4A1 double knockout cells were also grown to confluence before splitting, but seeded at approximately 1.6 times density to account for slower growth. The cells were then treated with 1 µg Hydrocortisone, and incubated at 37°C and 5% CO_2 for either 0, 30, or 60 minutes. After treating

for their respective times, cells were taken up into the QIAGEN RNeasy RNA Isolation kit lysis buffer, and gDNA was eliminated using the kit's gDNA Eliminator columns. RNA isolation was carried out according to the QIAGEN RNeasy RNA Isolation kit protocol. As with the qPCR for SLCO genes, the resulting cDNA was set up for a qPCR reaction in 96 well plates using iQ SYBR® Green Supermix from BIO-RAD. Resulting Cq values were normalized against the *RPL30* ribosomal housekeeper Cq for the specific sample to get the ΔCq value. They were then normalized against the 0 minute controls for their respective cell types to obtain the ΔΔCq value. Primers used listed below (Table 3).

Table 3: Lists the primer sequences for all qPCR primers used in the qPCR of glucocorticoid responsive genes in the cortisol response experiments.

Gene ID	Forward Primer Sequence	Reverse Primer Sequence
TDO2[1]	5' TCC TCA GGC TAT CAC TAC CTG C 3'	5' ATC TTC GGT ATC CAG TGT CGG 3'
PCK1[2]	5' CCA GGA TCG AAA GCA AGA CG 3'	5' GTA CAT GGT GCG ACC TTT CAT 3'
PCK2[3]	5' GGC TGA GAA TAC TGC CAC ACT 3'	5' ACC GTC TTG CTC TCT ACT CGT 3'
PER1[4]	5' CAG TGC TCC TGT TCC TGC ATC 3'	5' CCC GCC AAC TGC AGA ATC T 3'
PER3[5]	5' AGC TAC CTG CAC CCT GAA GA 3'	5' CGA ACT TTA TGC CGA CCA AT 3'
NR3C1[2]	5' GGT GTG CTC TGA TGA AGC TTC 3'	5' CCT TCC AGC ACA TAG GTA ATT GT 3'
RPL30[6]	5' ACA GCA TGC GGA AAA TAC TAC 3'	5' AAA GGA AAA TTT TGC AGG TTT 3'

Primers from [1](Zhao et al., 2021),[2] Haga-Yamanaka lab [3](Zhuang et al., 2023), [4](Bellet et al., 2021), [5](Li et al., 2021), [6](de Jonge et al., 2007).

A549 Cell Proliferation Assay

A549 cells were grown to confluence and then split at a $3x10^4$ cell density into 6 well plates. The following day, the cells were treated with either cortisol or dexamethasone. For the cortisol treatment, cells were treated with either methanol control, 10 nM, 30 nM, 100 nM, 300 nM or 1000 nM chemically synthesized cortisol (hydrocortisone). For the dexamethasone treatment, cells were treated with methanol control, 0.3 nM, 1 nM, 3 nM, 10 nM, or 30 nM dexamethasone. Both media and treatment was refreshed on days 4 and 7 after seeding. Cells

were counted on days 3, 5, 7 and 9 after seeding. To count, cells were washed with DPBS, treated with Trypsin, then incubated at 37°C and 5% CO_2 for 10 minutes. Cells were then taken up into F12-K media to dilute the trypsin 1:4 while counting. They were then mixed 1:2 with Trypan Blue and counted using the Bio-Rad TC20 cell counter. Counts were also done with a hemocytometer when they were too low to be consistently read by the cell counter.

RESULTS

Given the evidence that EcI acts as a steroid hormone transporter in *Drosophila* (Okamoto et al., 2018), we chose to look at transporters from the same family of OATPs in mammals in order to search for one of similar function. In order to identify potential OATPs of interest when working with HEK293T cells, we used qPCR to calculate relative copy numbers of all twelve human OATPs in the HEK293T cell line (Figure 3A). Resulting Cq values were normalized to the GAPDH housekeeping gene in order to determine the relative copy numbers. In results aligning with preliminary data from the Haga-Yamanaka lab, the highest expression levels were of OATP4A1, followed by OATP3A1 and OATP5A1. This data informed the choice to explore OATP3A1 and OATP4A1 as main targets of interest under the assumption that higher expression could correlate with a more clear effect when testing for the necessity of these transporters to glucocorticoid transport.

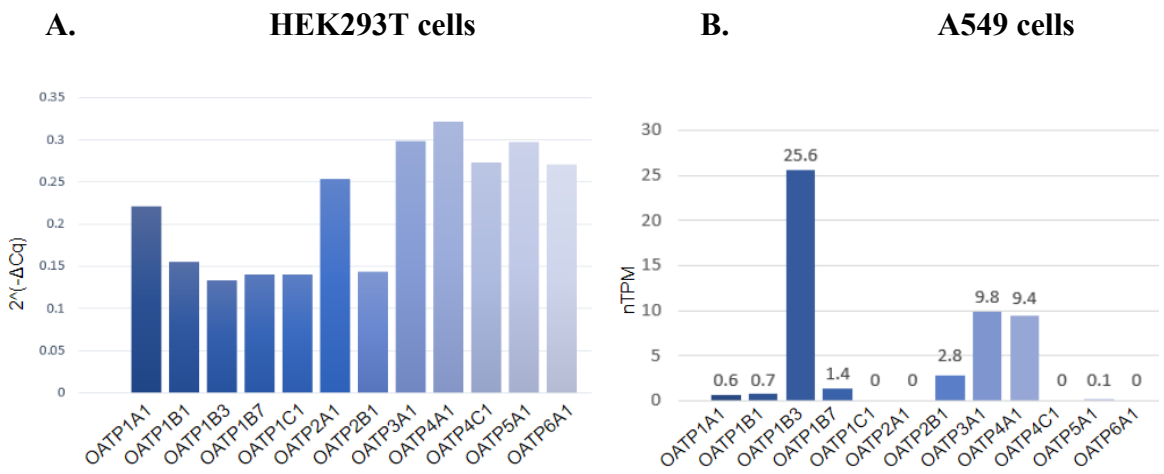


Figure 3: OATP gene expression in HEK293T and A549 cell lines
A) qPCR of OATP encoding genes (SLCOs) in HEK293T cells. Data reflects relative copy number determined from the average response of three biological replicates (n=3). B) OATP gene expression in A549 cells. Data from the Protein Atlas online database: Lung Cancer/SLCOs/Cell Line available from v23.proteinatlas.org (Karlsson et al., 2021; *The Human Protein Atlas V23.0*, 2023). Expression levels are conveyed in transcripts per million (nTPM).

The next step was to examine how changes in expression of these target genes affect the import of glucocorticoids. First we used a Dual-Glo luciferase assay to analyze the response of a glucocorticoid target luciferase gene with and without OATP4A1 and OATP4A1+OATP3A1. H-WT, H-dKO and H-4A1KO cells were transfected with a firefly luciferase gene containing a GRE so that its transcription is glucocorticoid responsive. They were then exposed to varying concentrations of cortisol for three hours before measuring firefly luminescence, then quenching and reading the renilla background luminescence in order to normalize the data. Statistical analysis was performed using GraphPad Prism to complete a two-way ANOVA test using Tukey multiple comparison testing.

All three cell types showed dose dependent response to cortisol, while the renilla luminescence remained constant for each cell line across time points (Figure 4A-B). We see a significant decrease in the response to 3 nM cortisol treatment in H-4A1KO cells compared to H-WT cells (Figure 4D). Interestingly, we also see an increase in response to 10 nM cortisol treatment in H-dKO cells when compared to H-WT cells (Figure 4D). There is also a significantly higher relative luminescence value of H-4A1KO cells when compared to H-WT cells at the 10 nM concentration. This data works in conversation with preliminary data completed by the Haga-Yamanaka lab, where luciferase assays completed at smaller incubation time points showed a significant decrease in cortisol response in both OATP knockout cell lines compared to H-WT cells.

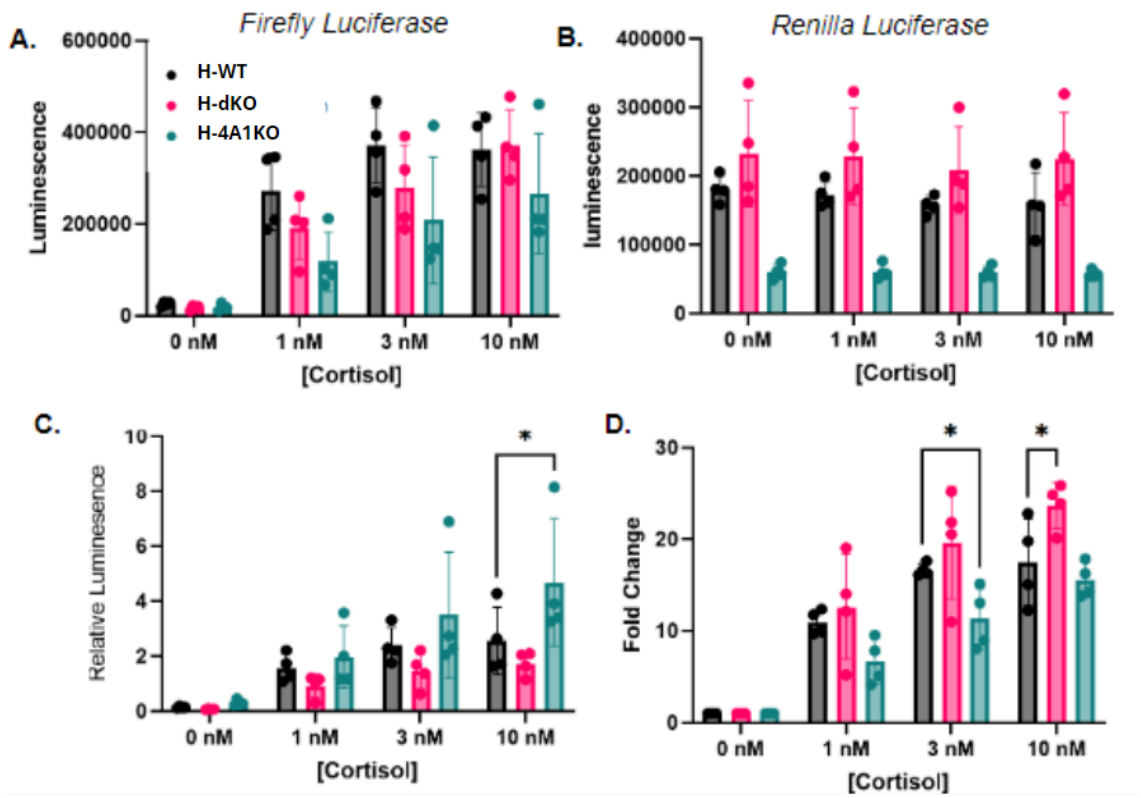


Figure 4: Dual-Glo Luciferase Luminescence in Response to Cortisol
* denotes $p < 0.05$ **A.** Average raw luminescence intensity derived from firefly luciferase activity (n=4) **B.** Average raw luminescence intensity derived from constitutively expressed renilla luciferase activity (n=4) **C.** Relative luminescence obtained by dividing firefly by renilla luciferase-derived luminescence intensity. **D.** The fold change obtained by normalizing relative luminescence data to the average 0 nM response in their respective runs.

The role of OATP3A1 in glucocorticoid transport is tested using qPCR to measure how glucocorticoid induced gene response differs with changes in OATP3A1 expression. H-WT, H-3A1OE and H-dKO cells were treated with 1uM cortisol for either 0, 30, or 60 minutes. They were then processed and used to complete a qPCR, where the measurement of known glucocorticoid responsive genes were measured. The resulting qPCR Cq levels were normalized to the RPL30 ribosomal housekeeper gene in order to account for differences in cell density and viability. They were then normalized to the 0 min controls for their individual cell types to determine the ΔΔCq values. There are not yet sufficient replicates to draw conclusions on the differences between the cell type variables.

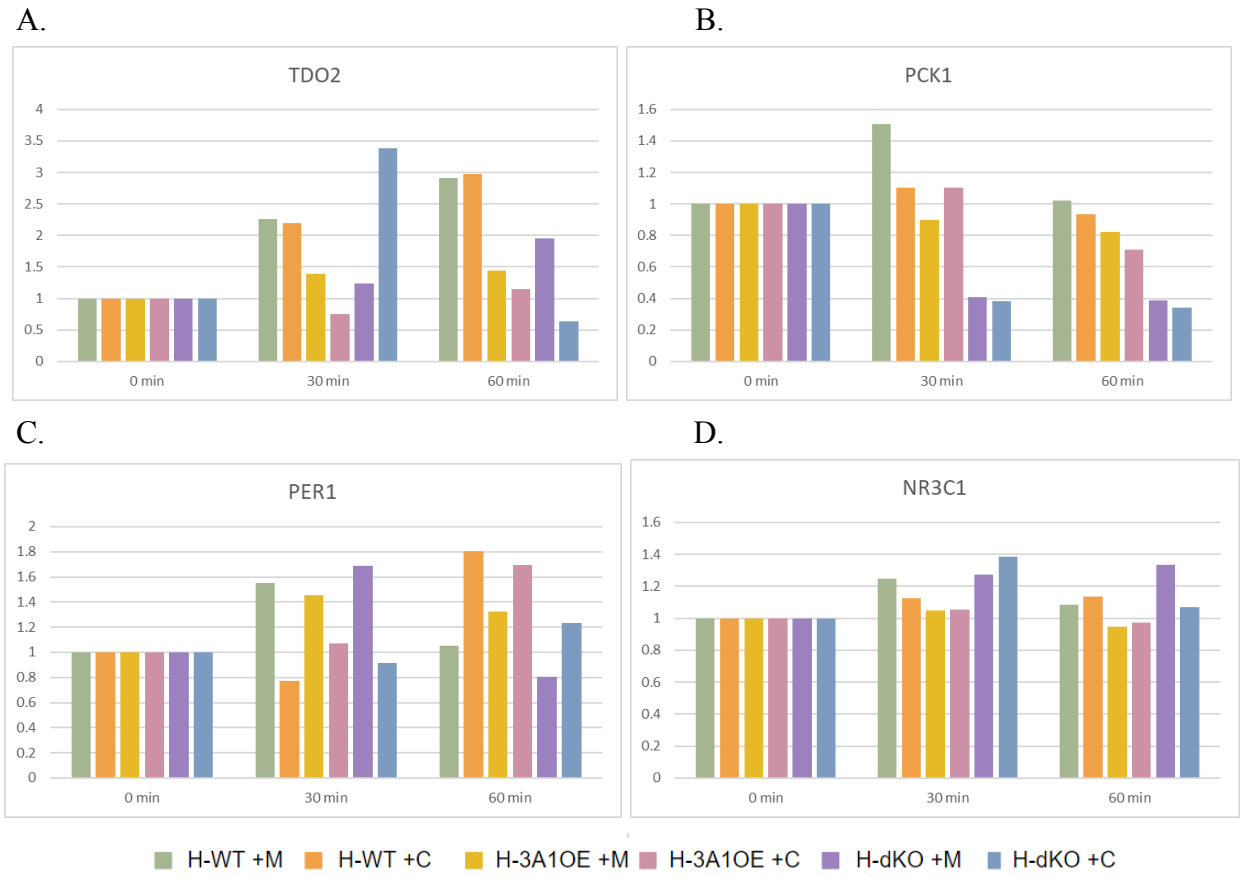


Figure 5: qPCR of GR Target Genes in Response to Cortisol Treatment in HEK293T cells
+M denotes methanol solvent control treatment, +C denotes 1µM cortisol treatment. Values represent the average between the two replicates. H-dKO+M, H-WT+M, and H-WT+C are (n=1), all other samples are (n=2).

A549 cells are a lung cancer cell line, and were used as a model due to their recorded response to glucocorticoids. Their growth is known to be inhibited by glucocorticoid signaling (Feng et al., 2018). As with the HEK239T cell experiments, OATP expression levels were analyzed in order to test if it would be an appropriate cell line for these experiments (Figure 3B). Using the Protein Atlas online database, published RNA-seq data was compiled to give insight on OATP gene expression in A549 cells (Karlsson et al., 2021; *The Human Protein Atlas V23.0*, 2023). OATP1B3 had the highest expression at 25.6 nTPM, followed by OATP3A1 at 9.8 nTPM and OATP4A1 at 9.4 nTPM (Figure 3B). Other OATPs, such as OATP1C1, OATP2A1, OATP4C1, and OATP6A1 had no recorded gene expression in A549 cells.

We hypothesized that A549-3A1OE cells would have a higher response to glucocorticoid signaling than A549-WT cells, and would therefore exhibit a stronger growth inhibition. Both cell lines were seeded at a density of $3x10^4$ cells on day 0, and were treated with either cortisol or dexamethasone at varying concentrations on day 1. Treatment was refreshed on days 4 and 7. Their growth was measured through counting viable cells with Trypan Blue stain on days 3, 5, and 7. There is a visible decrease in cell growth at concentrations of cortisol at or over 100 nM (Figure 6A and 6C), as well as at concentrations of dexamethasone at or over 3 nM (Figure 6B and 6D). There are not enough replicates to determine significance in any category.

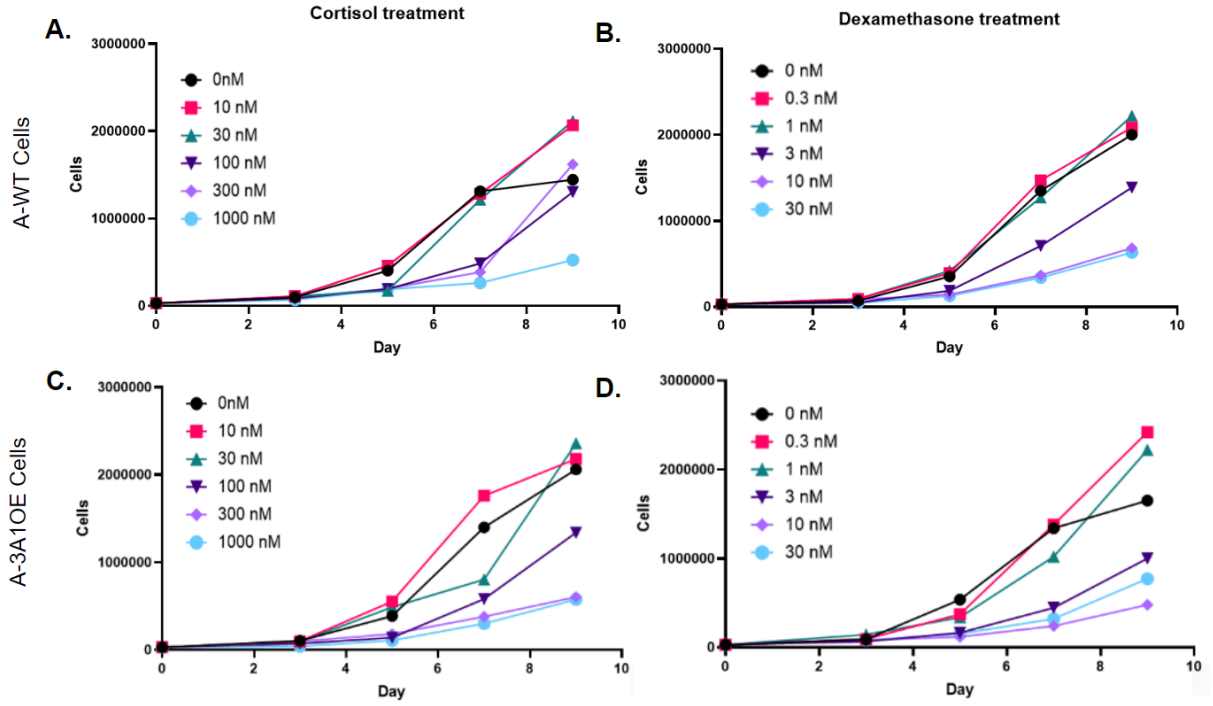


Figure 6: Proliferation Assay of Glucocorticoid Treated A549 Cells
A-B) Dots represent the average of experimental replicates (n=2) A) Response of A-WT cells to cortisol. B) Response of A-WT cells to dexamethasone. C) Response of A-3A1OE cells to cortisol (n=1) D) Response of A-3A1OE cells to dexamethasone (n=1).

DISCUSSION

In order to determine OATPs of interest in HEK293T cells, we completed a qPCR to determine the relative expression levels of OATPs in our specific cultured cells. Here we determined the highest two OATPs to be OATP4A1 and OATP3A1 (Figure 3A). This informed the choice to focus on those transporters in the HEK293T experiments. When choosing to explore the role of a possible steroid hormone transporter in glucocorticoid related cancers, OATP expression was an important factor in determining an appropriate cell line. If the cell line had no OATP expression, then it would not be appropriate for knockout experiments or the proliferation assays. Using the Protein Atlas database, data was compiled for a large range of cell lines (data not shown)(Karlsson et al., 2021; *The Human Protein Atlas V23.0*, 2023). A549 stood out as a possible model because it had high expression of genes encoding OATP3A1 and OATP4A1 (Figure 3B), and because it was known to be glucocorticoid responsive (Feng et al., 2018). In future experiments, it may be interesting to explore the possible role of OATP1B3 due to its very high expression in A549 cells despite its low expression in HEK293T cells (Figure 3).

The luciferase assay was completed in order to determine if OATP4A1 and OATP3A1 expression levels affect glucocorticoid induced gene response. There is a significant increase in the H-WT cell response when treated with cortisol at all concentrations when compared to the methanol control. This indicates that the system is functioning, and responsive to glucocorticoids. When looking at the relative luminescence, there was a significant difference between the 10 nM 4A1 KO response and the WT response, though on closer inspection of the data the highest data points correspond to the highest data points in the raw firefly luminescence reads, identifying it as an outlier (Figure 4B). These experiments build cumulatively on preliminary data done in the Haga-Yamanaka lab (data not shown). In conversation with the data

at low exposure time points, we see an interesting increase in cortisol induced luminescence in the H-dKO cell line (Figure 4D). Although this may be partially explained by the presence of an outlier, it may also indicate the presence of feedback loops over the extended three hour time period. A negative feedback loop in the H-WT cells after the increased exposure time would decrease the H-WT response to cortisol and the resulting luciferase expression, while a positive feedback loop in H-dKO cells could increase the receptivity to low amounts of cortisol and increase luciferase expression. Both are possible explanations for the different results at 3 hr exposure compared to smaller exposure times.

In the cortisol response qPCR experiments, we tested if OATP3A1 expression levels affected the expression levels of glucocorticoid induced genes. This experimental system is designed to have increased expression of genes with known GRE and glucocorticoid regulation such as TDO2, PCK1, and PER1. To prove that this experimental system is working as intended, H-WT cells should show increased expression of these genes when treated with cortisol compared to their methanol control. Although there were not enough replicates to come to significant conclusions, we do see that the methanol controls, which were expected to be lower in response than the cortisol treated counterparts, are around the same expression level or higher (Figure 5). The fact that the controls are not producing the expected results implies that the experimental system needs revision before further experiments.

In order to troubleshoot this issue, we have used new cortisol stocks and greatly increased the cortisol concentrations from 10 nM to 100 nM and finally 1μM. Given that there is no response at this level, despite being 1000x higher than what was able to exhibit a response in the luciferase assay experiments (Figure 4D), we are seeing signs that the system is not working as intended. There is evidence that HEK293 cells exhibit low endogenous GR expression, which

may be hindering the results (Bladh et al., 2005; Dougherty et al., 2016; Paakinaho et al., 2014). To fix this, NR3C1 can be transfected into cells, as done in the luciferase assay experiments.

With the proliferation assay in A549 cells, we establish a working system of glucocorticoid response with which we can test the importance and necessity of OATP3A1 to glucocorticoid import. After experimental optimization, concentration ranges were established for both cortisol and dexamethasone treatments that established a broad range of growth inhibition. In both cell types the lower concentrations (10-30 nM cortisol and 0.3-1 dexamethasone) did not generally show signs of growth inhibition when compared to the 0 nM methanol control, while greater concentrations did show slowed growth compared to the 0 nM methanol control (Figure 6). Although more tests need to be done to determine significance for any factors, at this point the data trends towards confirmation of the statements that A549 cells have glucocorticoid inhibited growth (Feng et al., 2018). With only one replicate with A-3A1OE cells, there is not sufficient data to determine a difference in inhibition response at this time. This data does show that this experimental model is appropriate to observe how different factors, such as transporter expression, affect glucocorticoid induced response.

In future experiments with A549 cells, we will complete more replicates overall in order to determine the effects of overexpressing OATP3A1 in A549 cells. If there is a significantly larger growth inhibition response in A-3A1OE cells, experiments could be done to rescue the phenotype by blocking the transporter. Perhaps more interestingly, this assay could be completed with OATP3A1 knockout cells in order to test if there is less growth inhibition and if they are closer to the methanol control response. This could help to show if OATP3A1 is necessary or not for long term glucocorticoid import.

This research addresses the role of highly expressed OATPs to glucocorticoid import in both a normal human cell line (HEK293T) and a cancerous human cell line (A549). It explores both the necessity of these OATPs to glucocorticoid transport, but also the effects of overexpression. It examined the necessity of both OATP4A1 and OATP3A1/OATP4A1 together using luciferase assays, and further explore the necessity of OATP3A1 and OATP3A1/OATP4A1 together using qPCR to determine cortisol induced gene expression response. It utilizes an A549 cell model to explore how glucocorticoid induced growth inhibition response can be altered by different factors.

Steroid hormones are essential to many of the functions in living organisms and their development. They also play a large role in the progression and treatment of various cancers, and thus having a complete understanding of their pathways is very important to general life science and medicinal fields. These experiments aim to elucidate the pathway of steroid hormones, specifically glucocorticoids in human cells. If glucocorticoid cellular import is transporter mediated, it not only changes our understanding of these essential pathways, but provides medicinal targets to increase safety and effectivity of glucocorticoid treatments in cancers, and could be used to decrease the negative effects that prevent their use in many solid tumors.

REFERENCES

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., & Karin, M. (1995). Immunosuppression by Glucocorticoids: Inhibition of NF-κB Activity Through Induction of IκB Synthesis. *Science*, *270*(5234), 286–290.

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schütz, G., & Schibler, U. (2000). Resetting of Circadian Time in Peripheral Tissues by Glucocorticoid Signaling. *Science*, *289*(5488), 2344–2347. https://doi.org/10.1126/science.289.5488.2344

Bellet, M. M., Stincardini, C., Costantini, C., Gargaro, M., Pieroni, S., Castelli, M., Piobbico, D., Sassone-Corsi, P., Della-Fazia, M. A., Romani, L., & Servillo, G. (2021). The Circadian Protein PER1 Modulates the Cellular Response to Anticancer Treatments. *International Journal of Molecular Sciences*, *22*(6), 2974. https://doi.org/10.3390/ijms22062974

Bladh, L.-G., Lidén, J., Pazirandeh, A., Rafter, I., Dahlman-Wright, K., Nilsson, S., & Okret, S. (2005). Identification of Target Genes Involved in the Antiproliferative Effect of Glucocorticoids Reveals a Role for Nuclear Factor-κB Repression. *Molecular Endocrinology*, *19*(3), 632–643. https://doi.org/10.1210/me.2004-0294

Chougule, R. A., Shah, K., Moharram, S. A., Vallon-Christersson, J., & Kazi, J. U. (2019). Glucocorticoid-resistant B cell acute lymphoblastic leukemia displays receptor tyrosine kinase activation. *Npj Genomic Medicine*, *4*(1), 1–7. https://doi.org/10.1038/s41525-019-0082-y

de Jonge, H. J. M., Fehrmann, R. S. N., de Bont, E. S. J. M., Hofstra, R. M. W., Gerbens, F., Kamps, W. A., de Vries, E. G. E., van der Zee, A. G. J., te Meerman, G. J., & ter Elst, A. (2007). Evidence Based Selection of Housekeeping Genes. *PLoS ONE*, *2*(9), e898. https://doi.org/10.1371/journal.pone.0000898

Dougherty, E. J., Elinoff, J. M., Ferreyra, G. A., Hou, A., Cai, R., Sun, J., Blaine, K. P., Wang, S., & Danner, R. L. (2016). Mineralocorticoid Receptor (MR) trans-Activation of Inflammatory AP-1 Signaling: DEPENDENCE ON DNA SEQUENCE, MR CONFORMATION, AND AP-1 FAMILY MEMBER EXPRESSION. *The Journal of Biological Chemistry*, *291*(45),

23628–23644. https://doi.org/10.1074/jbc.M116.732248

Feng, X.-L., Fei, H.-Z., & Hu, L. (2018). Dexamethasone induced apoptosis of A549 cells via the TGF-β1/Smad2 pathway. *Oncology Letters*, *15*(3), 2801–2806. https://doi.org/10.3892/ol.2017.7696

Gower, W. R., Risch, R. M., Godellas, C. V., & Fabri, P. J. (1994). HPAC, a new human glucocorticoid-sensitive pancreatic ductal adenocarcinoma cell line. *In Vitro Cellular & Developmental Biology - Animal*, *30*(3), 151–161. https://doi.org/10.1007/BF02631438

Green, S. M., Kaipainen, A., Bullock, K., Zhang, A., Lucas, J. M., Matson, C., Banks, W. A., & Mostaghel, E. A. (2017). Role of OATP transporters in steroid uptake by prostate cancer cells in vivo. *Prostate Cancer and Prostatic Diseases*, *20*(1), 20–27. https://doi.org/10.1038/pcan.2016.42

Hagenbuch, B., & Stieger, B. (2013). The *SLCO* (former *SLC21*) superfamily of transporters. *Molecular Aspects of Medicine*, *34*(2), 396–412. https://doi.org/10.1016/j.mam.2012.10.009

Karlsson, M., Zhang, C., Méar, L., Zhong, W., Digre, A., Katona, B., Sjöstedt, E., Butler, L., Odeberg, J., Dusart, P., Edfors, F., Oksvold, P., von Feilitzen, K., Zwahlen, M., Arif, M., Altay, O., Li, X., Ozcan, M., Mardinoglu, A., … Lindskog, C. (2021). A single–cell type transcriptomics map of human tissues. *Science Advances*, *7*(31), eabh2169. https://doi.org/10.1126/sciadv.abh2169

Khadka, S., Druffner, S. R., Duncan, B. C., & Busada, J. T. (2023). Glucocorticoid regulation of cancer development and progression. *Frontiers in Endocrinology*, *14*. https://doi.org/10.3389/fendo.2023.1161768

Li, Q., Xia, D., Wang, Z., Liu, B., Zhang, J., Peng, P., Tang, Q., Dong, J., Guo, J., Kuang, D., Chen, W., Mao, J., Li, Q., & Chen, X. (2021). Circadian Rhythm Gene PER3 Negatively Regulates Stemness of Prostate Cancer Stem Cells via WNT/β-Catenin Signaling in Tumor Microenvironment. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.656981

Liu, L., Aleksandrowicz, E., Schönsiegel, F., Gröner, D., Bauer, N., Nwaeburu, C. C., Zhao, Z., Gladkich, J., Hoppe-Tichy, T., Yefenof, E., Hackert, T., Strobel, O., & Herr, I. (2017). Dexamethasone

mediates pancreatic cancer progression by glucocorticoid receptor, TGFβ and JNK/AP-1. *Cell Death & Disease*, *8*(10), e3064–e3064. https://doi.org/10.1038/cddis.2017.455

Obaidat, A., Roth, M., & Hagenbuch, B. (2012). The Expression and Function of Organic Anion Transporting Polypeptides in Normal Tissues and in Cancer. *Annual Review of Pharmacology and Toxicology*, *52*(Volume 52, 2012), 135–151. https://doi.org/10.1146/annurev-pharmtox-010510-100556

Okamoto, N., Viswanatha, R., Bittar, R., Li, Z., Haga-Yamanaka, S., Perrimon, N., & Yamanaka, N. (2018). A Membrane Transporter Is Required for Steroid Hormone Uptake in Drosophila. *Developmental Cell*, *47*(3), 294-305.e7. https://doi.org/10.1016/j.devcel.2018.09.012

Paakinaho, V., Kaikkonen, S., Makkonen, H., Benes, V., & Palvimo, J. J. (2014). SUMOylation regulates the chromatin occupancy and anti-proliferative gene programs of glucocorticoid receptor. *Nucleic Acids Research*, *42*(3), 1575–1592. https://doi.org/10.1093/nar/gkt1033

Papich, M. G. (2016). Dexamethasone. In M. G. Papich (Ed.), *Saunders Handbook of Veterinary Drugs (Fourth Edition)* (pp. 217–219). W.B. Saunders. https://doi.org/10.1016/B978-0-323-24485-5.00195-9

Pizzagalli, F., Varga, Z., Huber, R. D., Folkers, G., Meier, P. J., & St-Pierre, M. V. (2003). Identification of Steroid Sulfate Transport Processes in the Human Mammary Gland. *The Journal of Clinical Endocrinology & Metabolism*, *88*(8), 3902–3912. https://doi.org/10.1210/jc.2003-030174

Sarakbi, W. A., Mokbel, R., Salhab, M., Jiang, W. G., Reed, M. J., & Mokbel, K. (2006). The Role of STS and OATP-B mRNA Expression in Predicting the Clinical Outcome in Human Breast Cancer. *Anticancer Research*, *26*(6C), 4985–4990.

Soichot, M., Vaast, A., Vignau, J., Guillemin, G. J., Lhermitte, M., Broly, F., & Allorge, D. (2013). Characterization of Functional Polymorphisms and Glucocorticoid-Responsive Elements in the Promoter of TDO2, a Candidate Gene for Ethanol-Induced Behavioural Disorders. *Alcohol and Alcoholism*, *48*(4), 415–425. https://doi.org/10.1093/alcalc/agt028

Taniguchi, K., & Karin, M. (2018). NF-κB, inflammation, immunity and cancer: Coming of age. *Nature*

Reviews Immunology, *18*(5), 309–324. https://doi.org/10.1038/nri.2017.142

The Human Protein Atlas v23.0. (2023, June 19). https://www.proteinatlas.org/

Timmermans, S., Souffriau, J., & Libert, C. (2019). A General Introduction to Glucocorticoid Biology. *Frontiers in Immunology*, *10*, 1545. https://doi.org/10.3389/fimmu.2019.01545

Weikum, E. R., Knuesel, M. T., Ortlund, E. A., & Yamamoto, K. R. (2017). Glucocorticoid receptor control of transcription: Precision and plasticity via allostery. *Nature Reviews. Molecular Cell Biology*, *18*(3), 159–174. https://doi.org/10.1038/nrm.2016.152

Wright, J. L., Kwon, E. M., Ostrander, E. A., Montgomery, R. B., Lin, D. W., Vessella, R., Stanford, J. L., & Mostaghel, E. A. (2011). Expression of SLCO Transport Genes in Castration-Resistant Prostate Cancer and Impact of Genetic Variation in SLCO1B3 and SLCO2B1 on Prostate Cancer Outcomes. *Cancer Epidemiology, Biomarkers & Prevention*, *20*(4), 619–627. https://doi.org/10.1158/1055-9965.EPI-10-1023

Yu, S., Meng, S., Xiang, M., & Ma, H. (2021). Phosphoenolpyruvate carboxykinase in cell metabolism: Roles and mechanisms beyond gluconeogenesis. *Molecular Metabolism*, *53*, 101257. https://doi.org/10.1016/j.molmet.2021.101257

Zhao, Y., Tao, F., Jiang, J., Chen, L., Du, J., Cheng, X., He, Q., Zhong, S., Chen, W., Wu, X., Ou, R., Xu, Y., & Tang, K.-F. (2021). Tryptophan 2, 3-dioxygenase promotes proliferation, migration and invasion of ovarian cancer cells. *Molecular Medicine Reports*, *23*(6), 445. https://doi.org/10.3892/mmr.2021.12084

Zhuang, W., Shi, X., Gao, S., & Qin, X. (2023). Restoring gluconeogenesis by TEF inhibited proliferation and promoted apoptosis and immune surveillance in kidney renal clear cell carcinoma. *Cancer & Metabolism*, *11*(1), 11. https://doi.org/10.1186/s40170-023-00312-4