EXPRESSION AND ROLE OF 17BETA-HYDROXYSTEROID DEHYDROGENASE TYPE 1, 5 AND 7 IN EPITHELIAL OVARIAN CANCER

Mémoire

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Sous la direction de :

Sheng-Xiang Lin, directeur de recherche

RÉSUMÉ

Expression et rôle des 17ß-hydroxystéroïdes déshydrogénases types 1, 5 et 7dans le cancer de l'ovaire épithélial

Le cancer de l'ovaire est l'une des cinq causes les plus fréquentes de décès par cancer chez les femmes dans le monde développé. Environ 90% des cancers de l'ovaire proviennent de l'épithélium que l'on nomme cancer de l'ovaire épithélial (EOC). Le EOC est un cancer hormono-dépendant et les stéroïdes sexuels jouent un rôle crucial en favoriant la prolifération et de la survie des cellules. Les 17β-hydroxystéroïdes déshydrogénases (17β-HSDs) jouent un rôle important pour le contrôle de la concentration intracellulaire de tous les stéroïdes sexuels actifs. Le mécanisme qui reculent le fonctionnent et l'expression des 17β-HSDs dans le EOC sont très peu compris. L'inhibition de certains 17β-HSDs pourrait être un traitement de l'EOC et ette approche thérapeutique doit être étudiée. Les résultats de notre étude ont démontré que les 17β-HSD types 1, 5 et 7 sont tous exprimés dans les cellules OOC-3, mais que la type 1 est la plus abondante. L'expression des 17β-HSD types 1 et 7 dans les tumeurs ovariennes épithéliales que dans les ovaires normaux (type 1, 2.2 fois; type 7, 1.9 fois). Mais l'expression de la 17β-HSD 5 est significativement plus faible dans les tumeurs, suite au développement de l'EOC (-5.217 fois). De plus, la prolifération cellulaire a diminué à la suite du knockdown la 17β-HSD type 1 ou type 7 par des siRNAs spécifiques dans les cellules OVCAR-3, mais, le knockdown de la type 5 a un effet contraire. Nous suggérons que la 17β-HSD 5 peut être impliquée dans une signalisation d'hormones stéroïdiennes pour le développement du cancer de l'ovaire épithélial. Les 17β-HSD 1 et 7 pourraient être des biomarqueurs importants pour l'EOC diagnostiqué tôt et ils peuvent également être de nouvelles cibles pour le traitement de l'EOC.

SUMMARY

Expression and role of 17β- hydroxysteroid dehydrogenase type 1, 5 and 7 in epithelial ovarian cancer

Ovarian cancer is one of the top five commonest causes of female cancer death in the developed world. About 90% of ovarian cancer have epithelial origins. Epithelial ovarian cancer (EOC) is a hormone-dependent cancer, in which the sex steroids play a crucial role in maintaining the cell proliferation and survival. The 17β-hydroxysteroid dehydrogenases (17β-HSDs) are important in the control of intracellular concentration of all active sex steroids. The function and expression of 17β-HSDs in EOC is not fully understood. Whether or not 17β-HSDs could be a therapeutic approach for the EOC treatment needs to be studied. Our results showed that 17β-HSD types 1, 5 and 7 are all expressed in EOC cells OVCAR-3 and type 1 is the highest one. The expression of 17β-HSD types 1 and 7 is higher in epithelial ovarian tumor tissues than in normal ovaries (type1, 2.2-fold; type7, 1.9-fold), but the expression of 17β-HSD type 5 is significantly lower in the tumor, following the EOC development (-5.2-fold). We found that cell proliferation was decreased after 17β-HSD type 1 or 7 knockdown by specific siRNAs in OVCAR-3 cells. While knocking down type 5 has the opposite effect. We suggest that 17β-HSD type 5 may be involved in steroid hormone signaling in EOC development. Moreover, 17β-HSD types 1 and 7 could be important biomarkers for early diagnosed EOC and novel targets for EOC treatment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

17β-HSDs	17β-hydroxysteroid dehydrogenases
2-D gel	two-dimensional gel
4-dione	androstenedione
3β-diol	5 α -androstane-3 β ,17 β -diol
5-diol	androst-5-ene-3α,17β-diol
5-diol-FA	androst-5-ene-3α,17β-diol fatty acid
5-diol-S	androst-5-ene-3α,17β-diol sulfate
ACTH	adrenocorticotropic hormone
A-dione	5α-androstane-3,17-dione
ADT	androsterone
AIB1	AR-associated protein amplified in breast 1
AR	androgen receptor
ARA70	AR-associated protein 70
AKR	aldoketo-reductase
BC	breast cancer
CCT	column cytotrophoblast
COF	cofactor binding site
CRH	corticotropin releasing hormone
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
EOC	epithelial ovarian cancer
E1	estrone
E1-S	estrone sulfate
E2	estradiol
E2-S	17β-estradiol 3-sulfate
E3	estriol
EMT	epithelial–mesenchymal transition
epi-ADT	epiandrosterone
ER	estrogen receptor
ER+	ER-positive
EREs	estrogen response elements
EVCT	extravillous cytotrophoblast
FIGO stage	international federation of gynecology and obstetrics staging
GnRH	gonadotropin releasing hormone
GRP78	78 kDa glucose-regulated protein

hr	Hours
HSD	hydroxysteroid dehydrogenase
HSPA5/GRP78	heat shock 70 kDa protein 5 / 78 kDa glucose-regulated protein
IC_{50}	half maximal inhibitory concentration
IPA	ingenuity pathway analysis
LH	luteinizing hormone
min	minute
mg	milligram
MS	mass spectrometry
nM	nanomolar
NSCLCs	non-small cell lung carcinomas
OC	ovarian cancer
PGE2	prostaglandin E2
PGK1	protein phosphoglycerate kinase 1
PVDF	polyvinylidenedifluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RoDH-1	Ro dehydrogenase 1
PR	progesterone receptor
RT-PCR	one-step reverse transcription polymerase chain reaction
RT-qPCR	reverse transcription quantitative real-time PCR
sec	second
SDR	short chain dehydrogenase/reductase
SDS-Page	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
ST	syncytiotrophoblast
STX1040	selected compound III
Sult2B1	sulfotransferase 2B1
TCGA	The Cancer Genome Atlas
Testo / T	testosterone
UGT2B28	uridine glucuronosyl transferase 2B28
UGT1A1	uridine glucuronosyl transferase 1A1
VCT	villous cytotrophoblast
µl	microliter
µM	micromolar

LIST OF PUBLICATIONS

1. **Ruixuan Wang** & Sheng-Xiang Lin. Critical reductive 17β- hydroxysteroid dehydrogenases in epithelial ovarian cancer cells. **(article under submission)**

2. Wanhong He, Misra Gauri, Tang Li, **RuixuanWang**, Sheng-Xiang Lin. A Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1). **Gene 588 (2016) 54–61.**

3. Dan Xu, Juliette A. Aka, **Ruixuan Wang**, Sheng-Xiang Lin. 17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation. **J. Steroid Biochem. Mol. Biol. (In Press, Accepted Manuscript)**

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FOREWARD

This mémoire is submitted to the Faculté des études supérieures et postdoctorales of Université Laval for the requirement of the master's degree in science. Except for the summary of the mémoire and the summary of each article which are in French, the mémoire is written in English in the form of one scientific manuscript.

Chapter I

The general introduction on ovarian cancer, endocrine function in ovaries and the relationship between steroid hormones and ovarian cancer were introduced. We summarized the treatment for ovarian cancer, especially hormonal therapy including hormone replacement therapy and ovarian cancer endocrine treatment now used are summarized. The human 17β-hydroxysteroid dehydrogenases (17β-HSDs) types 1, 5 and 7 are also introduced. The working hypothesis, research objectives and methodologies are also described in this chapter.

Chapter II

The article "Critical reductive 17β-hydroxysteroid dehydrogenases in epithelial ovarian cancer cells" is in preparation. This article is presenting the expression of reductive 17β-HSD types 1, 5 and 7 in epithelial ovarian cancer (EOC) cells and tissues comparion with the normal ovary tissue. Biological function of 17β-HSD types 1, 5 and 7 in EOC cells OVCAR-3 was also studied. All experimental work in this publication was my individual contribution.

Chapter III

The Discussion and Conclusion & Perspectives contains the expression of 17β-HSD types 1, 5 and 7 in epithelial ovarian cancer. We suggest that the study of reductive 17β-HSDs in epithelial ovarian cancer could benefit endocrine therapy and early diagnosis of EOC.

Appendices

This appendix includes two articles which I participated during my master study. The first article entitled “Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1)” from Wanhong He, Misra Gauri, TangLi, RuixuanWang and Sheng-Xiang Lin is a gene wiki review published in Gene (2016). In this article, I was working on the written materials collection. The second article entitled “17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation” from Dan Xu, Juliette A. Aka, Ruixuan Wang and Sheng-Xiang Lin was accepted by the Journal of Steroid Biochemistry and Molecular Biology and it is in press. In this article, I was working on western blots and cell proliferation experiments.

The references of Chapter I and Chapter III are listed at the end. References of each article are listed after the text of each manuscript. Appendices articles are written in their published forms.

CHAPTER I

Introduction

1.1. General introduction ovarian cancer

Ovarian cancer (OC) has the highest mortality rate among gynaecological malignancies[1]. Ovarian, lung, breast and colorectal cancer are the most common causes of cancer death among women in the United States and Europe[2]. OC occurs only in reductive tissues of the ovary[3]. About 90% ovarian cancer originates from epithelial tissue while 5% originates from stromal tissue, and less than 5% from germ cells[4]. The major subtypes of epithelial ovarian cancer (EOC) are either of low-grade or high-grade serous (70%), others are the endometrioid, the clear cell, the mucinous and the undifferentiated or unclassified[5]. Representative examples of EOC subtypes are shown in **Figure 1.1**.

Most women develop EOC during their post-menopausal years. According to National Cancer Institute data, OC is most frequently diagnosed among women aged 55-64 and only 12% before 44 years[6]. In general, around 70% of cases are diagnosed in advanced stages leading to very poor survival rates[7][5]. Only 30% of women with OC can expect to survive five years[5]. Most patients are asymptomatic until the cancer has been widely metastasized within the abdomen[2].

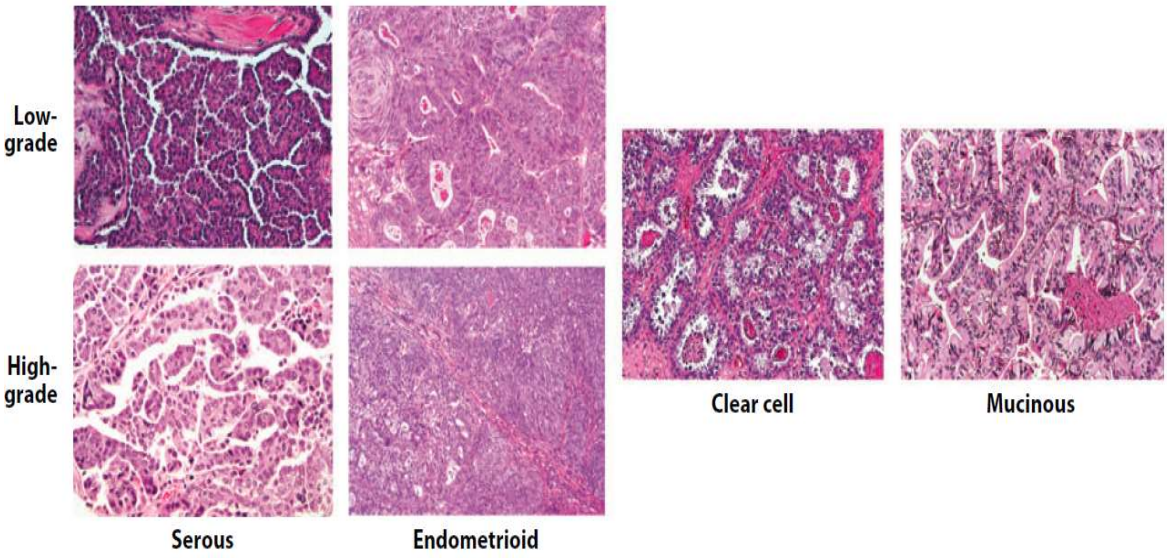

Figure 1.1 Representative examples of different histological types of epithelial ovarian carcinoma (Cho & Shih 2009)

1.2. Endocrine function in ovaries

1. The internal and external sex organs: the uterus, fallopian tubes and the ovaries make up the female reproductive system[8][9](**Figure 1.2**). The pair of ovaries locates in the side walls of the pelvis, one on each side of the uterus. They function in human reproduction. Ovaries are the main source of female hormones in reproductive life.

In reproductive years, ovaries secrete estrogen and progesterone following ovulation. Ovaries release a follicle at each menstrual cycle. The ovaries also secrete androgen. In women, 30%-60% of androstenedione (4-dione), 25-35% of testosterone (Testo) and around 20% of dehydroepiandrosterone (DHEA) are produced by the ovaries and released directly into the blood stream[10][11].

At the end of reproductive life, secretion of ovarian hormones estrogen and progesterone is reduced[12]. As the menopausal transition progresses, menstrual cycles become irregular, and ultimately will cease, as does ovulation[13]. The secretion of estradiol and progesterone by the ovary will stop when the ovary becomes completely depleted of estrogen-producing follicular eggs[14].

DHEA becomes the unique source of hormonal steroids in post-menopausal women[15][16]. In post-menopause, approximately 20% of circulating DHEA is released from the ovaries, and another 80% originate from the adrenal[17](**Figure 1.3**). Hence, the postmenopausal ovary continues to be a source of steroid hormones in women.

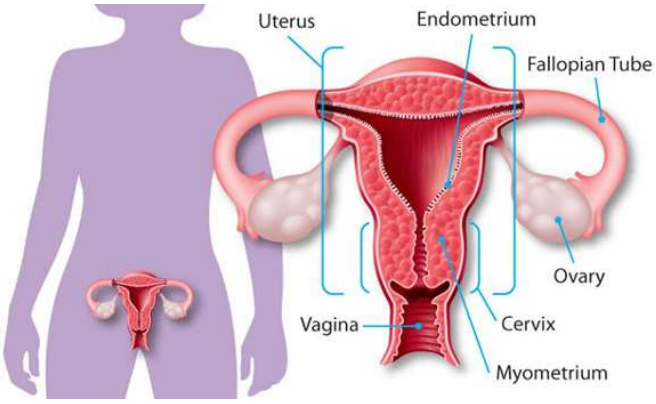

Figure 1.2 Female reproductive system

The pair of ovaries locates in the side walls of the pelvis, one on each side of the uterus. They function in human reproduction. Ovaries are the main source of female hormones in reproductive life. (https://www.womenshealth.gov/publications/our-publications/fact-sheet/images/ovarian-cysts-lg.jpg)

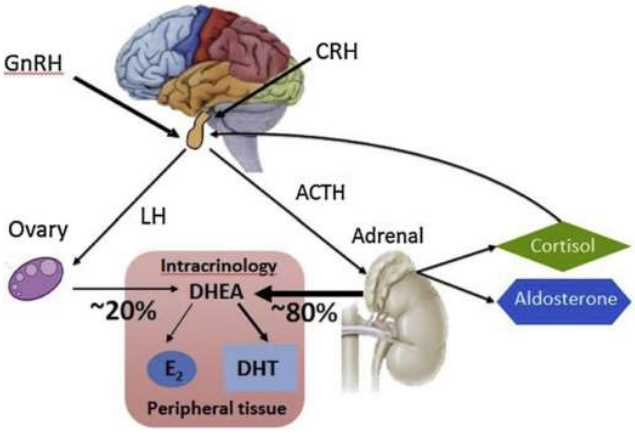

Figure 1.3 Source of sex steroids in post-menopause

After menopause, all estrogens and all androgens are made locally from DHEA in peripheral target tissues by the mechanisms of intracrinology. Approximately 20% of circulating DHEA is released from the ovary, the rest (about 80%) is from the adrenal. GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; CRH, corticotropin releasing hormone; ACTH, adrenocorticotropic hormone. (Labrie & Labrie 2013)

1.3. Relationship between steroid hormones and ovarian cancer

Ovarian cancer is a hormone-dependent cancer much like breast cancer. Based on epidemiological evidence, steroid hormones such as estrogens, progestins, and androgens play critical roles in ovarian cancer. They maintain the proliferation and survival of ovarian tumorigenic cells.

Sex steroid hormone nuclear receptors have been demonstrated to be highly expressed in ovarian tumors. Estrogen receptor (ER) is expressed in 61-79% of OC, and ERα is expressed in expressed in 60-80% of EOC[4]. ERβ is highly expressed throughout the normal ovary, but its expression is progressively lost during OC development and progression[18][19]. The progesterone receptor (PR) is expressed in about 25-50% of ovarian tumors[18]. The androgen receptor (AR) is expressed in up to 90% of OC[20].

1.3.1. Estrogens promote the invasion of ovarian cancer cells

Estrone (E1), estradiol (E2), and estriol (E3) are the major naturally occurring forms of estrogens in women. Results in EOC cell experiments have shown that estrogens stimulate the growth of ovarian cancer cells expressing ER[21]. As mentioned, ERα has been found to be overexpressed in ovarian carcinogenesis, but the expression of ERβ is quite low[4]. Epidemiological studies have indicated that estrogen replacement therapy may increase ovarian cancer incidence and mortality in postmenopausal women[21]. It has also been suggested that estrogens might promote the invasion of ovarian cancer cells via activation of the PI3K/AKT pathway in an estrogen receptor-independent manner[22].

Estrogens stimulate OC cell proliferation and epithelial–mesenchymal transition (EMT) via ERα, leading to induction of cell migration propensity and functional alterations

through up-and down-regulation of EMT-related genes as well as related transcription factors[2](**Figure 1.4**). Overall, data support the estrogens promote the OC progress and metastasis.

1.3.2. Progesterone inhibits the growth of ovarian cancer cells

The ovary is the main site for producing progesterone before menopause. It was found that women with progesterone deficiency have a high OC rate[23]. Epidemiological evidence proves that progesterone in ovarian surface epithelium cells reduces ovarian cancer development and progression[24]. Moreover, unlike estrogen, progesterone is responsible for interrepting the EMT process. In ovarian cancer cells, it reveals anti-proliferative and antimetastasis effects through the regulation of tumor suppressor genes and intracellular signaling pathways[2] (**Figure 1.4**).

1.3.3. Androgens increase ovarian cancer cellular proliferation and decreases cell death

Androgens are effectively stimulating EOC cells, increasing cellular proliferation and decreasing cell death, thus, potentially influencing ovarian tumor transformation. Androgen receptor (AR) is highly expressed in ovarian cancer tumors. Comparing with normal ovarian surface epithelium, the AR-associated protein amplified in breast 1 (AIB1) is found in 25% of EOCs and AR-associated protein 70 (ARA70) has been found to be overexpressed in the majority of EOC[25][26]. The AR-associated proteins could enhance the potential of androgen receptor signaling by co-regulating AR's transactivation potential[27].

In vitro studies also found that 4-dione and Testo increased the viability of EOC cells OVCAR-3. They could increase the activity, expression, and phosphorylation of

telomerase in the cells[28]. The activity of telomerase helps cancer cells to live longer than any other somatic cell type, avoiding their death[29]. Androgens have a similar effect in androgen-sensitive prostate cancer[30].

In EOC detailed mechanistic studies are lacking, and models to study hormone responses in vitro and in vivo are very limited.

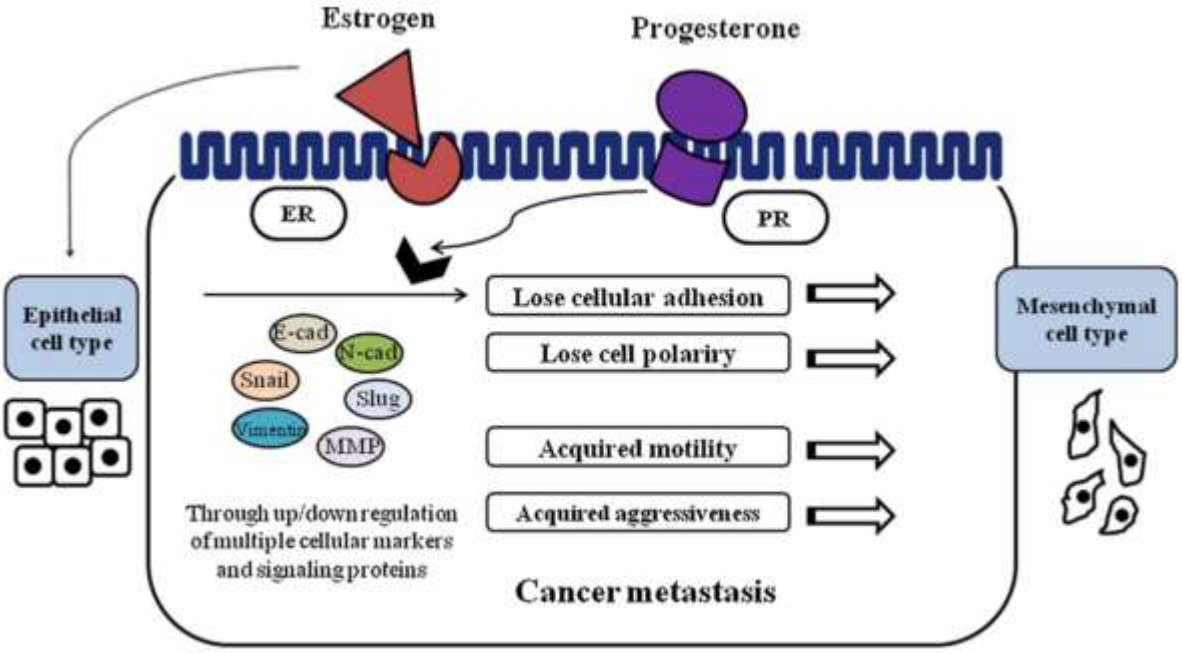

Figure 1.4 Effects of estrogen and progesterone on the EMT process in ovarian cancer cells. (Jeon et al. 2016)

1.4. Treatment of ovarian cancer

The major treatments for OC are surgery, cytotoxic chemotherapy, endocrine treatment, hormone replacement therapy or other treatment strategies [7][31].

Surgery will be performed to remove the cancer tissue as much as possible and help to provide a histopathological diagnosis to establish staging according to the International

Federation of gynecology and obstetrics (FIGO stage)[32].The staging could prevent the adjuvant chemotherapy in the early stage when disease is limited to the ovaries. But in about 30% of patients, the surgery might identify occult metastatic stages such as abdominal disease or affected lymph nodes[33].

Based on a recent clinical trial, adjuvant chemotherapy only improves overall survival ratio by 8% in early stage EOC[34]. Adjuvant chemotherapy has been suggested no benefit in patients who underwent complete debulking and staging[35]. In optimally debulked stage III OC patients, intraperitoneal chemotherapy (intravenous paclitaxel plus intraperitoneal cisplatin and paclitaxel) improves progression-free survival and overall survival[36]. But most women who present with advanced EOC will develop recurrence within 18 months and evolve to chemotherapy resistance[7].

BRCA gene mutations, BRCAness, and ploy (ADP-ribose) polymerase inhibitors are targets for new treatment strategies in OC. BRCA1 and BRCA2 gene mutations have been found in about 15% of OC cases, which is mostly serous[7].

1.4.1. Hormonal therapy

Evidence shows that EOC is a hormone-responsive cancer, much like endometrial, breast and prostate cancers[37]. Sex hormone receptors are highly expressed in EOC. The therapeutic strategy of targeting hormone receptors is successful in hormone-responsive cancers[4]. Hormonal therapy is well known as a relatively nontoxic anticancer therapy and it is easily administered and well-tolerated.

1.4.1.1 Hormone replacement therapy

Because patients younger than 50 years that will be exposed to estrogens before their menopause, hormone replacement therapy is a safe and appropriate way for these patients[38][39]. Studies showed that ovarian cancer cell proliferation decreased, and apoptosis increased after progesterone treatment. In vivo experiments with a simian model have shown that, epithelial ovarian cancer cell apoptosis is increased four-fold to six-fold in progesterone-treated group compared with the control and estrogen treated groups[40].

1.4.1.2 Endocrine treatment

The selective ER modulator Tamoxifen competitively inhibits ER, blocking its downstream signaling to generate anti-estrogenic effects. Tamoxifen has been tested in ovarian cancer phase II clinical trials with patients having heavily pretreated, recurrent disease[4]. As mentioned the majority of women are diagnosed with OC in their post-menopausal years. Aromatase inhibitors (AI) such as letrozole and anastrozole, block the production of estrogens and have been investigated for the treatment of recurrent or persistent OC[41]. In clinical trials on recreant cancers, antiandrogenic compounds are used in OC management. The treatment include gonadotropin-releasing hormone (goserelin, triptorelin, and leuprolide) or AR antagonists (bicalutamide and flutamide) [42]. The novel CYP17 inhibitor abiraterone, which blocks the generation of adrenal steroids downstream of CYP17, was also evaluated in clinical trials[43](**Figure 1.5**). Understanding the mechanism of action of hormones in EOC will likely benefit the hormonal therapy in ovarian cancer.

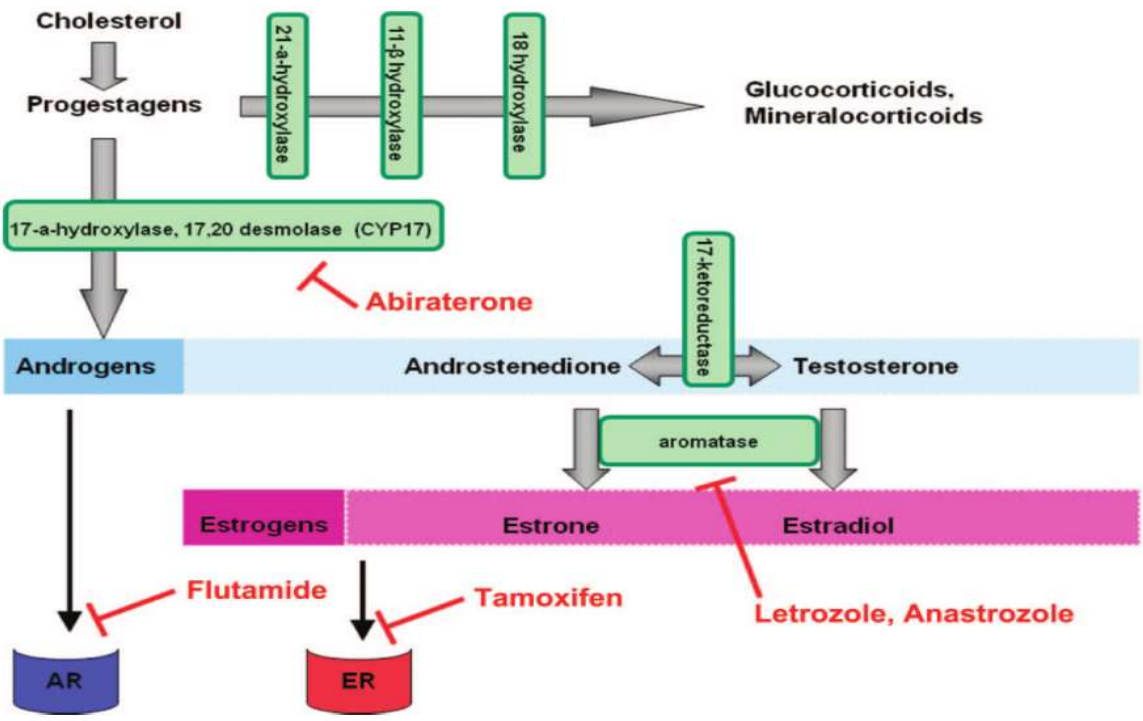

Figure 1.5 Steroid synthesis pathway and aromatization (Papadatos-Pastos et al. 2011**)**

1.5. Human 17β-hydroxysteroid dehydrogenases (17β-HSDs)

Human 17beta-HSDs are playing a key role in sex steroid biology and are therefore a unique site of action for the control of the intracellular concentration of all active sex steroids[44](**Figure 1.6**). They catalyze the last and key step of formation of all the active androgens and estrogens[45], while also catalizing the first step of their degradation. To data, 15 types of 17β-HSDs have been described, all of them except type 5 are belong to the short chain dehydrogenase/reductase (SDR) superfamily[46]. 17β-HSD5 is a member of the aldoketo-reductase (AKR) family[47]. 17β-hydroxysteroid dehydrogenases have been demonstrated to be expressed in the post-menopausal ovary[48].

The reductive 17β-HSD family comprises key enzymes involved in the formation of E2, the last step of estrogen activation[49]. They play important roles in various endocrine-related cancers. 17β-HSD types 1, 5 and 7 are the most important reductive members in estrogen synthesis; they have been widely studied in hormone-dependent breast cancer[50][51][52].

1.5.1. 17β-HSD type 1

17β-HSD type 1 plays a critical role in the synthesis of E2, the most potent estrogen, from E1 and also in the synthesis of E2 from of Testo 4-dione[53]. Enzyme kinetics and X-ray crystal lographic studies performed with type 1 also showed that this enzyme inactivates the most active androgen dihydrotestosterone (DHT)[46]. 17β-HSD type 1 plays a crucial role in the development of estrogen-dependent cancer including up-regulation of breast cancer cell growth. In the ovarian tumor, the increasing E2/E1 ratio and high levels of 17β-HSD1 mRNA point to a potential role in OC development[54].

1.5.2. 17β-HSD type 5

17β-HSD type 5 is also known as AKR1C3 and synthesize 5-diol from DHEA. It also catalyze the 4-dione reduction to Testo, which can be further converted to E2. AKR1C3 also participates in the production of prostaglandin E2 (PGE2)[55]. PGE2 is the major factor stimulating aromatase expression. Aromatase is expressed from the CYP19 gene; it is responsible for the synthesis of E1 from the preferred substrate 4-dione and of E2 from Testo[56]. In breast cancer studies, 17β-HSD5 has down-regulating effect in breast cancer development. The results showed that 17β-HSD5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1 in breast cancer[51].

1.5.3. 17β-HSD type 7

The 17β-HSD type 7 involved in cholesterogenesis as well as steroidogenesis. Recently, 17β-HSD7 was found to possess dual enzymatic activity. Similarly to 17β-HSD1, 17β-HSD7 has critical roles in the regulation of E2[52]. It also inactivates the most active androgen DHT into the weak estrogen 5α-androstane-3β,17β-diol (3β-diol) during steroid biosynthesis[13].

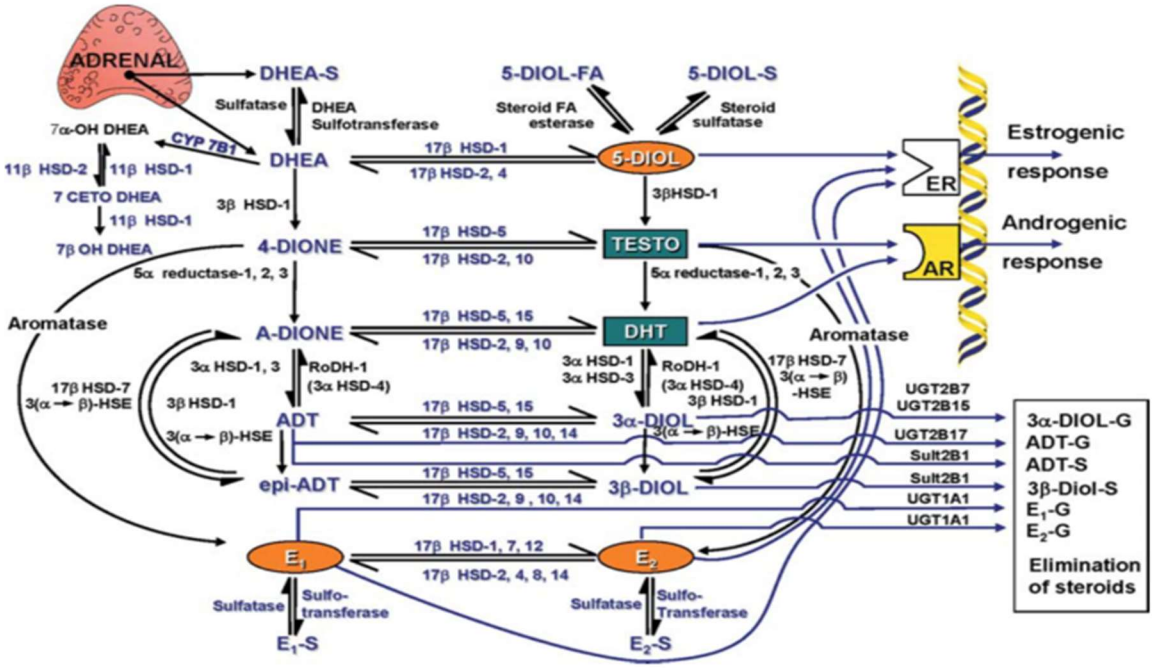

Figure 1.6. Human steroidogenic and steroid-inactivating enzymes in peripheral intracrine tissues.

A-dione, 5α-androstane-3,17-dione; ADT, androsterone; epi-ADT, epiandrosterone; 4-dione, androstenedione; E1, estrone; E1-S, estrone sulfate; E2, 17β-estradiol; E2-S, estradiol sulfate; 5-diol, androst-5-ene-3α, 17β-diol; 5-diol-FA, 5-diol fatty acid; 5-diol-S, 5-diol sulfate; HSD, hydroxysteroid dehydrogenase; Testo, testosterone; RoDH-1, Ro dehydrogenase 1; ER, estrogen receptor; AR, androgen receptor; UGT2B28, uridine glucuronosyl transferase 2B28; Sult2B1, sulfotransferase 2B1; UGT1A1, uridine glucuronosyl transferase 1A1. (**Labrie & Labrie 2013**)

1.6. Rationale and objectives of the research

1.6.1. Working hypothesis

Based on the above introduction, it is known that sex steroids play a critical role in EOC. Specifically, estrogen promotes the development of EOC. Reductive 17β-HSD enzymes are involved in the formation of E2, the most potent estrogen. The expression of the most important reductive enzymes 17β-HSD types 1, 5 and 7 in EOC needs to be confirmed. It is also relevant to investigate the possible impact of knocking down 17β-HSD types 1, 5 and 7, individually. Understanding the reductive 17β-HSD enzymes may help identify major targets for EOC treatment.

1.6.2. Objectives

Objective 1: To perform extensive characterization of the expression of 17β-HSD types 1, 5 and 7 in epithelial ovarian cancer cells and tissues.

Objective 2: To evaluate of the biological function of 17β-HSD types 1, 5 and 7 in epithelial ovarian cancer cells.

1.6.3. Methodological and approaches

Objective 1

Cell culture

The epithelial ovarian cancer cells OVCAR-3 originate from the malignant ascites of a Caucasian woman with progressive ovarian adenocarcinoma. The estrogen, androgen, and progesterone receptors are all positive in this cell line; it is useful to investigate sex steroid hormone-related enzymes in EOC. According to the information of OVCAR-3

from the American Type Culture Collection, it is also a suitable transfection host and an appropriate model system to study drug resistance in EOC.

Quantitative real-time PCR

Total RNA of cells was extracted from OVCAR-3 cells. The mRNA levels of 17β-HSD types 1, 5 and 7 in OVCAR-3 cells were obtained by quantitative RT-PCR.

Comparative expressions of 17β-HSDs

For comparing the mRNA expression of 17β-HSD types 1, 5 and 7 in epithelial ovarian tumor vs. normal ovary tissue, we interrogated the Oncomine database. Different fold change analysis about 17β-HSDs expression in normal tissue vs. serous ovarian cancers, early stage, and advanced stage tumors.

Western Blot

The expression resultsof 17β-HSD types 1, 5 and 7 in OVCAR-3 cells were confirmed on protein levels by western blot.

Objective 2

siRNA synthesis and transfections

Sense and antisense sequences of target protein siRNAs were transfected into OVCAR-3 cells by Lipofectamine 2000. The siRNA final concertation used was 100nM.

RT- PCR

The transfection effects of 17β-HSD1 or 7 were studied by one-step reverse transcription (RT)-PCR.

Western blot and Quantitative real-time PCR

Western blot was used to validate change of the target enzymes in OVCAR-3 cells after transfection with specific 17β-HSD5 siRNAs.

The mRNA levels of the control condition and siRNA condition after knockdown 17β-HSD type 1 or type 7 by siRNA transfection were obtained by quantitative real-time PCR.

Cell proliferation assay

Cell proliferation changes after siRNA transfections were measured by CyQuant cell proliferation kit. The kit determines cell number by staining nucleic acids (DNA and RNA).

CHAPTER II

Critical reductive 17β- hydroxysteroid dehydrogenases in epithelial ovarian cancer cells

RÉSUMÉ

Rôle critique des 17β-hydroxystéroïdes déshydrogénases réductrices dans les cellules du cancer de l'ovaire épithélial

Le cancer de l'ovaire épithélial (EOC) est un cancer hormono-dépendant, et les stéroïdes sexuels jouent un rôle crucial pour le maintien de la prolifération et de la survie des cellules. Les 17β-hydroxystéroïdes déshydrogénases (17β-HSDs) réductrices de types 1, 5 et 7 sont des enzymes clés dans la formation d'estradiol, et sont impliquées dans la dernière étape de l'activation des œstrogènes. Nos résultats ont démontré que ces trois types sont exprimés dans les cellules EOC OVCAR-3 et que type 1 est le plus élevé. L'expression des 17β-HSDs type 1 et type 7 dans les tissus de tumeurs ovariennes épithéliales est plus élevée que dans les ovaires normaux. Cependant, l'expression de la 17β-HSD5 est significativement plus faible dans la tumeur. La prolifération cellulaire a diminué dans les cellules OVCAR-3 où il y a un knockdown de la 17β-HSD type 1 ou de la type 7. Le knockdown du type 5 a eu un effet opposé. La 17β-HSD type 5 pouvait être impliquée dans la signalisation des hormones stéroïdiennes impliquées dans le développement de l'EOC. L'étude des 17β-HSDs types 1, 5 et 7 peut donc nous donner un nouvel espoir de traitement pour les patientes atteintes de l'EOC.

SUMMARY

Critical reductive 17β-hydroxysteroid dehydrogenases in epithelial ovarian cancer cells

Epithelial ovarian cancer (EOC) is a hormone-dependent cancer, in which sex steroids play a crucial role in maintaining the cell proliferation and survival. The reductive 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1, 5 and 7 are involved in the formation of estradiol and, consequently in the last step of estrogen activation. Our results showed that all three types are expressed in EOC cells OVCAR-3. Notably, the expression of type 1 is the highest. The expressions of 17β-HSD type 1 and type 7 in epithelial ovarian tumor tissues are higher than in normal ovaries. However, the expression of 17β-HSD5 is significantly lower in the tumor than normal ovaries, suggesting its role in EOC development. Cell proliferation was shown to be decreased in 17β-HSD type 1 or type 7 knockdown EOC cells. But knocking down type 5 had the opposite effect. The 17β-HSD5 may be involved in steroid hormone signaling in EOC development. The study of 17β-HSD types 1, 5 and 7 may give us new hope for the treatment of EOC.

Critical reductive 17β-hydroxysteroid dehydrogenases in epithelial ovarian cancer cells

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Abstract

Epithelial ovarian cancer (EOC) is considered as hormone-dependent cancer, the sex steroid hormones such as estrogens help to maintain the cell proliferation and survival. The reductive 17β-HSD types 1, 5 and 7 are involved in the formation of estradiol (E2). Our results showed that 17β-HSD types 1, 5 and 7 are all expressed in EOC cells OVCAR-3 and type 1 is the highest one. The expression of 17β-HSD type 1 and type 7 in epithelial ovarian tumor tissues is significantly higher compared to normal ovaries. But the expression of type 5 is lower in the tumor which further decrease following the EOC development. The cell proliferation decreased in 17β-HSD type 1 or type 7 knockdown EOC cells. In converse, knocking down type 5 has opposite effect. 17β-HSD5 may be a steroid hormone signaling in epithelial ovarian cancer development. 17β-HSD1 and 17β-HSD7 may be targets for EOC treatment; type 1 could be an important biomarker for early diagnosed EOC.

1. Introduction

Ovarian cancer has the highest mortality rate among gynaecological malignancies[1]. Ovarian, lung, breast and colorectal cancer are the most common causes of cancer death among women in the United States and Europe[2]. About 90% ovarian cancers originated from epithelial cells; the major subtypes of epithelial ovarian cancer (EOC) are the low-grade and high-grade serous (about 70%)[3][4]. Most women develop EOC during their post-menopause years. In general, 70% of this cancer is diagnosed in advanced stages leading to very poor survival rates[5][3]. Most patients are asymptomatic until it has been widely metastasized within the abdomen[2]. Epithelial ovarian cancer is considered as a hormone- dependent cancer as breast cancer. Based on epidemiological evidence, steroid hormones (primarily estrogens) effectively stimulate EOC cells, increases cell proliferation and decreases cell death, thus, potentially influencing ovarian tumor transformation[6][7]. The sex steroid hormone nuclear receptors have been proved widely expressed in ovarian tumors[8]. The reductive 17β-HSD family comprised key enzymes involved in the formation of estradiol (E2) and, consequently in the last step in estrogen activation. These enzymes had been proved expressed in the post-menopausal ovary, and they play important roles in various endocrine-related cancers[9][10]. Reductive types 1, 5 and 7 are the most important enzymes in the 17β-HSD family; they have been widely studied in hormone-related breast cancer[11][12][13].

In EOC, the increasing E2/ estrone (E1) ratio and high levels of 17-HSD1 mRNA point out type 1's pivotal role in ovarian tumor[14]. 17β-HSD7 from E1 to the regulation of E2[13]. 17β-HSD type 5 (AKR1C3) is a member of the aldoketo-reductase (AKR) family; it is the only one not belonging to the short chain dehydrogenase/reductase (SDR) superfamily. For the later suerfamily, 15 types of 17β-HSDs have been reported[15][16]. AKR1C3 synthesizes androst-5-ene-3β, 17β-diol (5-diol) from dehydroepiandrosterone (DHEA) and catalyzes androstenedione (4-dione) reduction to

testosterone (Testo), which is further converted to E2 by aromatase. AKR1C3 also participate in the production of prostaglandin E2 (PGE2)[17]. In another hormonal-dependent cancer breast cancer studies, 17β-HSD5 has down-regulating effect in breast cancer development. The results showed that 17β-HSD5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1[12].

Here we extensively verified the expression of 17β-HSD types 1, 5 and 7 in EOC tissues and cells, and evaluated the biological function of 17β-HSD types 1, 5 and 7 in EOC cells.

2. Material and methods

2.1 Cell culture

The epithelial ovarian cancer (EOC) cells OVCAR-3 were a gift from Dr. Donald Poirier. OVCAR-3 cells are from the malignant ascites of a Caucasian woman with progressive ovarian adenocarcinoma. The estrogen, androgen, and progesterone receptors are all positive in this cell line; it is useful for investigating sex steroid hormones related enzymes in EOC. OVCAR-3 is also an appropriate model system to study drug resistance in EOC. The cells were cultured in no phenol red RPMI-1640 medium (Gibco, Life Technologies, Paisley, Scotland), supplemented with 20% fetal bovine serum (FBS) (Sigma, Oakville, ON, Canada). Cells grew in a 5% CO_2 atmosphere at 37℃. When plating cells, we were using the RPMI-1640 medium containing 20% dextran-coated charcoal (Sigma, St. Louis, MI, USA)-stripped FBS as hormone free culture medium.

2.2 Western Blot

The cultured OVCAR-3 cells were washed with 2 ml cold phosphate buffered saline (PBS 1X) and total proteins from cells were extracted by RIPA buffer (Invitrogen, Burlington, ON, Canada) with 1% protease inhibitor cocktail (EMD Chemicals, Gibbstown, NJ, 100:1 v/v). The method for quantifying proteins was the Bradford method. Twenty μg total proteins were separated on a 12% SDS-PAGE Gel, then electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Amersham Hybond-PTM, GE Healthcare, QC, Canada) overnight at 60V. The PVDF membranes were blocked with 5% skimmed milk in pH 7.5 TBS-Tween buffer for 1 hour at room temperature. After that, the PVDF membranes were hybridized to the primary antibodies against the target proteins for overnight at 4℃. The primary antibodies were anti-17β-HSD 1 (SAB1403946) (Sigma, St. Louis, MI, USA)1:500, anti-AKR1C3 (17β-HSD 5) (ab84327) (Abcam, Cambridge, MA, USA) 1:1000, anti-17β-HSD 7 (ab112006) (Abcam, Cambridge, MA, USA) 1:500 and anti-β-actin (ab3289) (Abcam, Cambridge, MA, USA) 1: 5000. The anti-β-actin antibody was used for loading control. The goat-anti-rabbit IgG-HRP (sc-2004) 1:5000 and goat-anti-mouse IgG-HRP (sc-2005) (Santa Cruz Biotechnology, CA, USA) 1:2000 were used as secondary antibodies. After washing with TBS-Tween, blots were visualized with Western Lighting Plus ECL (PerkinElmer, MA, USA) enhanced chemiluminescence substrate for western blotting, followed by exposure to X-ray films. The target bands were quantified for density using the Image program (Molecular Dynamics, Sunnyvale, CA). Each sample was performed in triplicate and repeated in three independent experiments.

2.3 siRNA synthesis and transfections

Sense and antisense sequences of target protein siRNAs (**Table 2.1**) were synthesized and purified by HPLC by Gene Pharma (Shanghai, China). The 100 nM mixer duplex siRNAs were transfected into OVCAR-3 cells by Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada), according to manufacturer instruction. Control cells were

transfected with control siRNA provided by Gene Pharma (Shanghai, China) as a negative control (**Table 2.1**).

2.4 Quantitative real-time PCR

Total RNA from OVCAR-3 cells was extracted using RNeasy Plus mini kit (Qiagen, Hilden, DE) and synthesized to the first-strand cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, ON, Canada). Total cDNA for each sample (30 ng) was subjected to a quantitative real-time polymerase chain reaction (qRT-PCR) using the Fast Start Essential DNA Green Master (Roche Diagnosis, Mannheim, DE). Reactions were performed with a final concentration of 0.5 µM of each primer (**Figure 2.2**) in a final volume of 20 µl. The qRT-PCR programs according to the instrument protocol from the Fast Start Essential DNA Green Master manufacturer was carried out in the LightCycler® 96 Real-Time PCR System (Roche Diagnosis, Mannheim, DE). Several qRT-PCR reactions were tested by Plateforme de Séquençage et de Génotypage des Génomes (CHU de Québec, QC, Canada) subjected to DNA sequencing to confirm the specificity of the reactions. The LightCycler Software supplied by the manufacturer was used to calculate data and create the standard curves. The mRNA level per 1 mg total RNA was calculated from the level of specific cDNA template obtained by qRT-PCR and the molecular weight of each double-stranded specific cDNA sequence. The mRNA levels were expressed as mRNA copies/mg total RNA and SDs were <10% of triplicates. All the primers were designed using online software Primer3web version 4. 0. 0 **(**http://primer3.ut.ee/**)** and synthesized by Integrated DNA Technologies (IA, USA).

2.5 RT- PCR

OVCAR-3 cells were plated in 6-well plates at a density of 2 x 10^5 and transfected with 100 nM mixed target siRNAs for 96 h. Total RNA was extracted from cells using RNeasy Plus mini kit (Qiagen, Hilden, DE):1 µg of total RNA for each sample was

subjected to a one-step reverse transcription polymerase chain reaction (RT-PCR) using the Titanium One-Step PT-PCR kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The housekeeping gene human 18S was used as an internal control. The primers of 17β-HSD type 1, 17β-HSD type 7 and 18S were used as listed in **Table 2.2**. Samples were incubated at 50℃ for 60 min, then 94℃ for 5 min followed by 30 cycles at denaturation temperate 94℃ at 30 sec, annealing temperate 65℃ at 30 sec, elongation temperature 68℃ for 1 min, followed by 2 min final elongation. The program was carried out in the Eppendorf Mastercycler Gradient (Eppendorf, Mississauga, ON, Canada). The RT-PCR products were separated on a 1.2% agarose gel with 0.05% RedSafe Nucleic Acid Staining Solution. Results bands were photographed under UV light.

2.6 Cell proliferation assay

Cell proliferation changes were measured by CyQuant cell proliferation kit (Molecular Probes, Invitrogen, ON, Canada). The kit determines cell number by staining nucleic acids (DNA and RNA). OVCAR-3 cells were plated at a density of 3 x 10^3 cells per well in 96-well plates. After 24 h cultured with hormone-free medium, the cells were transfected with 100 nM siRNA, and the medium was changed with medium containing substrate E1 or different concentrations of DHEA after 5 h. The culture medium was changed every 48 h and removed after 72 h or 96 h. The cells were washed twice by 1xPBS and frozen more than 24 h in 96-well plates at -80℃. Each well was added 200 µl of CyQuant GR dye/cell-lysis buffer after the plates were thawed at room temperature for more than 15 min. The fluorescence data were obtained using the fluorescence microplate reader at 489 nm excitation and 520 nm emission. Quadruplicate wells were used for each condition and repeated in three independent experiments.

2.7 Integrative analysis of clinical datasets

For further validating the expression of 17β-HSD types 1, 5 and 7 in EOC, we interrogated the Oncomine database (https://www.oncomine.org). The Oncomine™ Platform is a web-based data-mining platform proving web applications and translational bioinformatic services, a useful cancer microarray database. Yoshihara Ovarian Dataset has been chosen, which included gene expression data for 43 serous ovarian cancers (8 stage I and 35 stage III/IV) as well as 10 normal peritoneum tissues as the reference[18]. The serous ovarian cancer is the biggest subtype of EOC. The datasets were used to analyzed the expression of 17β-HSD types 1,5 and 7 associated with tumor tissue vs. normal tissue. Data analysis was performed using SPSS software. Fold Change is adopted as a method of differences evaluation. The one-way ANOVA analysis was used to determine the significance of differences observed between normal tissue and tumor tissue. *, Correlation was significant <0.05 vs. Normal (2-tailed); **, Correlation is significant<0.001 vs. Normal (2-tailed).

2.8 Statistical analysis

Each result was calculated using Microsoft Excel 2010. We used unpaired, two-sided Student test for all two groups comparing. The statistical difference was considered significant with P values < 0.05 and P values <0.001. All data were presented as means± S.D. Analysis of data from the Oncomine clinical database was using IBM SPSS Statistics 20 software.

3. Results

3.1 17β-HSD types 1, 5 and 7 message RNA levels in EOC cells

The mRNA levels of 17β-HSD types 1, 5 and 7 in EOC cell OVCAR-3 were evaluated by qRT-PCR. Data showed as the mRNA copies number per mg of total RNA from OVCAR-3 (**Table 2.3**). 17β-HSD1 mRNA was detected at high level (1.79E+07 copies

/mg) in OVCAR-3, the type 5 at a lower level (1.72E+05 copies /mg) was around 100 times lower than type 1. The mRNA level of Type 7 is around half of type 1 (7.56E+06 copies /mg).

3.2 Analysis of 17 β -HSD types 1, 5 and 7 expressions in EOC cells

The expression of 17β-HSD types 1, 5 and 7 in epithelial ovarian cancer cell OVCAR-3 cells on protein levels confirmed by western blot. Total protein was extracted from OVCAR-3 cells, each specific band was recognized by a monoclonal or polyclonal anti-17β-HSD types 1, 5 or 7 antibodies. Based on the results, all three proteins had strong and clear bands (**Figure 2.1**). 17β-HSD types 1, 5 and 7 are all expressed in EOC cells OVCAR-3 on the protein levels.

3.3 Elevated expression of 17β-HSDs in EOC tissue compared with normal ovary tissue

The comparison between the expression of 17β-HSD1, 5 and 7 mRNA in EOC tissue or normal ovary tissue using clinical samples data of Yoshihara Ovarian Dataset in the Oncomine database (**Figure 2.2**).

17β-HSD types 1, 5 and 7 are widely expressed in normal ovary peritoneum tissues and ovarian serous adenocarcinoma, the biggest subtype of epithelial ovarian cancer. 17β-HSD1 is expressed at a higher level in tumor compared with normal (fold change 2.081, P-value 0.005); fold change in early stage tumor (stage I) is 2.188, P-value 0.032 and in advanced stage (stage III or IV) is 2.081, p-value 0.007 (**Figure 2.2 A**). There is no significant change in expression of type 1 during the cancer progress.

The expression of 17β-HSD7 is also increased in tumor tissue compared with normal tissue. The fold change of type 7 expression between tumor and normal tissue is 1.616, P-value 3E-06. The change is less than type 1 expression in EOC. In the comparison of the tumor and normal ovaries, the fold change of stage III or IV tumor is slightly decline than stage I. The fold change in stage I is 1.875, (p-value 0.029) and in stage III or IV is 1.558 (p-value 1E-06) shown in **Figure 2.2 B**.

In **Figure 2.2 C**, analysis shown that 17β-HSD5 expression is significate decreased compared with the tumor with normal ovary (fold change= -5.217, p=3E-04). The expression of 17β-HSD5 is reduced significantly with EOC development. Fold change of type 5 expression in advanced stage III or IV tumor comparing with normal ovary is -5.727 (p=0.001), and fold change of stage I is only -2.791(p=8E-04).

3.4 Effect of 17β-HSDs knockdown on cell proliferation

The knockdown effect of 17β-HSD1 or 17β-HSD7 in OVCAR-3 cells was confirmed. After 72 hours transfecting with 100 nM mixed specific 17β-HSD1, 17β-HSD7 siRNAs or control siRNA, RT-PCR had carried out and the results are shown in the photographs (**Figure 2.3 A and Figure 2.4 A**). They showed an almost complete knockdown of 17β-HSD1 or 17β-HSD7. The mRNA levels of 17β-HSD1 gene after transfection were analyzed by qRT-PCR, 2.46E+07 copies mRNA/mg total RNA in control and 6.05E+06 copies mRNA/mg total RNA after transfection with 17β-HSD1 siRNA. The siRNAs specifically silenced approximately 75% of 17β-HSD1 gene expression (**Figure 2.3 B**). The 17β-HSD1 gene's mRNA levels after transfection were analyzed by qRT-PCR, 2.04E+07 copies mRNA/mg total RNA in control and 2.90E+06 copies mRNA/mg total RNA after transfection with 17β-HSD7 siRNA. 17β-HSD7 gene expression was silenced 86% by siRNAs (**Figure 2.4 B**).

The cells were transfected with 100 nM mixed specific17β-HSD5 siRNAs after 96 h total protein was extracted from cells. Western blot measured the expression of the 17β-HSD5 in cells. In comparison with control siRNA, expression of the 17β-HSD5 in OVCAR-3 decreased 59% (**Figure 2.5 A**).

For evaluating the impact of each protein knockdown on OVCAR-3 growth, cell proliferation was measured at 72 h or 96 h after transfection with each protein specific siRNAs. The cells were cultured with hormone-free medium providing 17β-HSD types 1and 7 direct substrate E1 or DHEA as substrate. The provision of DHEA as the hormone source mimics postmenopausal steroid metabolism in cell culture[11]. The cell proliferation increased in response on a different substrate.

After transfection for 72 h with 17β-HSD1 siRNA, cell proliferation was significantly decreased compared with control siRNA: E1 0.1 nM, 19%; DHEA 100 nM,29%; DHEA 1000 nM, 23% (**Figure 2.3 C**). After transfection 96 h, cell proliferation was also dropped, but the changes were small. There were 4% with 0.1 nM E1, 3% with 100 nM DHEA and 10% with 1000 nM DHEA (**Figure 2.3 D**). Similarly in 17β-HSD7 knockdown cells, there was significant decrease on cell proliferation compared with control siRNA. At 72 h, 0.1 nM E1, 28%; 100 nM DHEA, 28%; 1000 nM DHEA, 21% (Figure 3.5C). At 96 h, 0.1 nM E1, 22%; 100 nM DHEA, 5%; 1000 nM DHEA,17% (**Figure 2.4 D**). The knockdown of 17β-HSD1 and 17β-HSD7 could inhibit OVCAR-3 cell growth.

In comparing with control siRNA, cell proliferation increased in 17β-HSD5 knockdown cells. Especially at 96 h, the increase was significant. At 72 h, 10 nM DHEA, 9%; 100 nM DHEA, 5.2%; 1000 nM DHEA, 1% (**Figure 2.5 B**). At 96 h, 10 nM DHEA, 4.2%;

100 nM DHEA, 34%; 1000 nM DHEA,18% (**Figure 2.5 C**). Knockdown 17β-HSD5 stimulated the EOC cells' growth.

4. Discussion

4.1. Expression of reductive 17β-hydroxysteroid dehydrogenases in EOC

Recent studies demonstrated reductive 17β-hydroxysteroid dehydrogenase expression in the ovarian surface epithelial cells of the post-menopausal women's ovary [9][19]. But the data about reductive 17β-HSD expression in EOC cell is limited. 17β-HSD2 and 17β-HSD5 were detected in ovarian epithelial tissue and lower expression compared with normal human surface epithelium on mRNA levels [20]. In another study, it was also proved 17β-HSD types 1, 2, 4 and 8 expressed in EOC by immunohistochemical analysis[21]. Our study demonstrated that the reductive 17β-HSD 1, 5 and 7 are all expressed in EOC cells OVCAR-3 and tissue from ovarian serous adenocarcinoma, the most frequent subtype of EOC. In OVCAR-3 cells, the mRNA level of 17β-HSD1 gene is the highest one and type 5 gene mRNA level is much lower than types 1 and 7. In clinical data analysis, we found that expression of both 17β-HSD1 and 7 are higher in EOC tissue compared with the normal ovary.

Estrogen induces ovarian cancer epithelial–mesenchymal transition (EMT) mainly through estrogen receptor α, leading to enhanced cell migratory propensity and functional alterations[2]. EMT is an important stage of cancer metastasis in which epithelial cells lose cellular adhesion and cell polarity, acquire motility and aggressiveness to become mesenchymal cells[22]. Activation of an EMT program is also related to chemoresistance, which causes cancer recurrence and metastasis after treatments such as radiation and chemotheraphy[23][24]. 17β-HSD type 5 (AKR1C3) participates in the production of prostaglandin E2 (PGE2)[17]. PGE2 is the major factor

stimulating aromatase expression. Aromatase is expressed from the CYP19 gene; it is responsible for the synthesis of E1 from the preferred substrate 4-dione and of E2 from Testo [25]. Our results showed the expression of 17β-HSD5 is significantly lower in tumors compared to normal ovary tissue. And its expression declines following EOC development, especially during the advanced stage. During the EOC progress, aromatase may rise with AKR1C3 decreasing, then estrogen (E1 and E2) levels will increase. Estrogen level increases will theoretically promote EMT stage for cancer metastasis.

4.2. Decreased 17β-HSD1 or 17β-HSD7 inhibited EOC cell growth, down-regulated 17β-HSD5 stimulated OVCAR-3 cell proliferation.

In our study, we provided E1 or DHEA at different concentrations as the hormone source for 17β-HSDs. DHEA is the unique source of hormone steroids in post-menopause women[26][27][28]. We used the upstream hormone DHEA as a source helping to mimic the postmenopausal condition in ovarian cancer cell culture. In our results, knocking down 17β-HSD types 1 or 7 inhibited EOC cell growth, but knockdown 17β-HSD5 stimulated OVCAR-3 cell proliferation.

Local estrogen metabolism is considered as an important phenomenon in EOC[20]. Reductive 17β-HSD type 1, type 5 and type 7 are key steroid-converting enzymes in estrogen synthesis. 17β-HSD1 has critical roles in regulation of E2, the most potent estrogen synthesized from E1, and in converting 4-dione to Testo[29]. Similarly, as 17β-HSD1, 17β-HSD7 plays critical roles in the regulation of E2 synthesized from E1[13]. Epidemiological studies have indicated that estrogens promote the invasion of EOC cells [30]. Down-regulation of 17β-HSD1 or 17β-HSD7 will affect the steroid pathway between E1 and E2 in cells, and may decrease intercellular E2 levels. The decreasing E2 will potentially inhibit EOC cell growth.

17β-HSD5 synthesizes 5-diol from DHEA and catalyzes the 4-dione reduction to Testo, which is further converted to E2. 17β-HSD5 also participates in the production of prostaglandin E2 (PGE2)[17]. PGE2 is the major factor stimulating aromatase expression. Aromatase is expressed from the CYP19 gene; it is responsible for the synthesis of E1 from the preferred substrate 4-dione and of E2 from Testo[25]. As we mentioned in the beginning, we suggest 17β-HSD5 expression decreases may stimulate aromatase expression and affect estrogen levels. Estrogen level increases will theoretically promote EMT and cell proliferation of EOC cells.

In conclusion, reductive 17β-HSD types 1, 5 and 7 are expressed in EOC. Due to the difficulty in early diagnosis, EOC has quite low survival rates[3][5]. The expression increases of 17β-HSD1 and 7 in EOC may prove novel targets for the development of EOC early diagnosis biomarkers. The lower expression of 17β-HSD5 in the advanced stage of epithelial ovarian tumor will help understanding development mechanisms and steroid hormone signaling in EOC. We suggest 17β-HSD1 and 7 may be involved in the protective effects against the estrogen-dependent proliferation of epithelial ovarian carcinoma. 17β-HSD1 and 7 could be studied and then may be used as potent targets for EOC treatment.

5. References

[1] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics, 2013," *CA. Cancer J. Clin.*, vol. 63, no. 1, pp. 11–30, 2013.

[2] S.-Y. Jeon, K.-A. Hwang, and K.-C. Choi, "Effect of steroid hormones, estrogen and progesterone, on epithelial mesenchymal transition in ovarian cancer development," *J. Steroid Biochem. Mol. Biol.*, vol. 158, pp. 1–8, 2016.

[3] K. R. Cho and I.-M. Shih, "Ovarian Cancer," *Annu. Rev. Pathol. Mech. Dis.*, vol. 4, no. 1, pp. 287–313, 2009.

[4] S. A. Cannistra, "Cancer of the Ovary," *N Engl J Med*, vol. 35124351, pp. 2519–29, 2004.

[5] G. C. Jayson, E. C. Kohn, H. C. Kitchener, and J. A. Ledermann, "Ovarian cancer," *Lancet*, vol. 384, no. 9951, pp. 1376–1388, 2014.

[6] M. Nourbakhsh, A. Golestani, M. Zahrai, M. H. Modarressi, Z. Malekpour, and F. Karami-Tehrani, "Androgens stimulate telomerase expression, activity and phosphorylation in ovarian adenocarcinoma cells," *Mol. Cell. Endocrinol.*, vol. 330, no. 1–2, pp. 10–16, 2010.

[7] "[Online]https://en.wikipedia.org/wiki/Telomerase," pp. 1–9, 2009.

[8] F. Modugno, R. Laskey, A. L. Smith, C. L. Andersen, P. Haluska, and S. Oesterreich, "Hormone response in ovarian cancer: Time to reconsider as a clinical target?," *Endocrine-Related Cancer*, vol. 19, no. 6. pp. R255–R279, 2012.

[9] J. C. Havelock, W. E. Rainey, K. D. Bradshaw, and B. R. Carr, "The post-menopausal ovary displays a unique pattern of steroidogenic enzyme expression.," *Hum. Reprod.*, vol. 21, no. 1, pp. 309–17, 2006.

[10] C. Y. Zhang, W. Q. Wang, J. Chen, and S. X. Lin, "Reductive 17beta-hydroxysteroid dehydrogenases which synthesize estradiol and inactivate dihydrotestosterone constitute major and concerted players in ER+ breast cancer cells," *J. Steroid Biochem. Mol. Biol.*, vol. 150, pp. 24–34, 2015.

[11] D. Xu and S. X. Lin, "Mimicking postmenopausal steroid metabolism in breast cancer cell culture: Differences in response to DHEA or other steroids as hormone sources," *J. Steroid Biochem. Mol. Biol.*, vol. 161, pp. 92–100, 2016.

[12] D. Xu, J. A. Aka, R. Wang, and S.-X. Lin, "17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation," *J. Steroid Biochem. Mol. Biol.*, 2017.

[13] X. Wang, C. Gérard, J.-F. Thériault, D. Poirier, C. J. Doillon, and S.-X. Lin, "Synergistic control of sex hormones by 17β-HSD type 7: a novel target for estrogen-dependent breast cancer," *J. Mol. Cell Biol.*, vol. 7, no. 6, pp. 568–579, 2015.

[14] C. H. Blomquist *et al.*, "Androgenic and estrogenic 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase in human ovarian epithelial tumors: evidence for the type 1, 2 and 5 isoforms," *J. Steroid Biochem. Mol. Biol.*, vol. 81,

pp. 343–351, 2002.
[15] W. He, M. Gauri, T. Li, R. Wang, and S.-X. Lin, “Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1),” *Gene*, vol. 588, no. 1, pp. 54–61, 2016.
[16] G. Moeller and J. Adamski, “Integrated view on 17beta-hydroxysteroid dehydrogenases,” *Mol. Cell. Endocrinol.*, vol. 301, no. 1–2, pp. 7–19, 2009.
[17] B. L. Dozier, K. Watanabe, and D. M. Duffy, “Two pathways for prostaglandin F2α synthesis by the primate periovulatory follicle,” *Reproduction*, vol. 136, no. 1, pp. 53–63, 2008.
[18] K. Yoshihara *et al.*, “Gene expression profiling of advanced-stage serous ovarian cancers distinguishes novel subclasses and implicates ZEB2 in tumor progression and prognosis,” *Cancer Sci.*, vol. 100, no. 8, pp. 1421–1428, 2009.
[19] A. Brodowska *et al.*, “Immunoexpression of aromatase cytochrome P450 and 17β-hydroxysteroid dehydrogenase in women’s ovaries after menopause.,” *J. Ovarian Res.*, vol. 7, p. 52, 2014.
[20] X. Ren *et al.*, “Local estrogen metabolism in epithelial ovarian cancer suggests novel targets for therapy,” *J. Steroid Biochem. Mol. Biol.*, vol. 150, pp. 54–63, 2015.
[21] K. Motohara, H. Tashiro, Y. Taura, T. Ohba, and H. Katabuchi, “Immunohistochemical analysis of 17β-hydroxysteroid dehydrogenase isozymes in human ovarian surface epithelium and epithelial ovarian carcinoma.,” *Med. Mol. Morphol.*, vol. 43, no. 4, pp. 197–203, 2010.
[22] L. Larue and A. 2005 Bellacosa, “Epithelial–mesenchymal transition in development and cancer: role of phosphatidylinositol 3 0 kinase/AKT pathways,” *Oncogene*, no. 24, pp. 7443–7454, 2005.
[23] M. Iwatsuki *et al.*, “Epithelial-mesenchymal transition in cancer development and its clinical significance,” *Cancer Sci.*, vol. 101, no. 2, pp. 293–299, 2010.
[24] L. N. Abdullah and E. K.-H. Chow, “Mechanisms of chemoresistance in cancer stem cells.,” *Clin. Transl. Med.*, vol. 2, no. 1, p. 3, 2013.
[25] E. R. Simpson *et al.*, “Aromatase—A Brief Overview,” *Annu. Rev. Physiol.*, vol. 64, no. 1, pp. 93–127, 2002.
[26] F. Labrie, “All sex steroids are made intracellularly in peripheral tissues by the mechanisms of intracrinology after menopause,” *J. Steroid Biochem. Mol. Biol.*, vol. 145, pp. 133–138, 2015.
[27] H. L. JUDD, G. E. JUDD, W. E. LUCAS, and S. S. C. YEN, “Endocrine Function of the Postmenopausal Ovary: Concentration of Androgens and Estrogens in Ovarian and Peripheral Vein Blood,” *J. Clin. Endocrinol. Metab.*, vol. 39, no. 6, pp. 1020–1024, 1974.
[28] F. Labrie, C. Martel, and J. Balser, “Wide distribution of the serum dehydroepiandrosterone and sex steroid levels in postmenopausal women: role of the ovary?,” *Menopause*, vol. 18, no. 1, pp. 30–43, 2011.
[29] P. Lukacik, K. L. Kavanagh, and U. Oppermann, “Structure and function of human

17β-hydroxysteroid dehydrogenases," *Mol. Cell. Endocrinol.*, vol. 248, no. 1–2, pp. 61–71, 2006.

[30] P. Hoffmann and P. Pujol, "Estrogens and epithelial ovarian cancer," vol. 94, pp. 25–32, 2004.

Tables

Table 2.1

Sequences of 17β-HSD types 1, 5 and 7 specific siRNAs

	siRNA	Sense sequence (5' to 3')	Anti-sense sequence (5' to 3')
17β-HSD1	siRNA 1	GCUGGACGUGAAUGUAGUA	UACUACAUUCACGUCCAGC
	siRNA 2	GCCUUUCAAUGACGUUUAU	AUAAACGUCAUUGAAAGGC
	siRNA 3	CCACAGCAAGCAAGUCUUU	AAAGACUUGCUUGCUGUGG
17β-HSD7	siRNA 1	GGUACAGCAUUGACCAAUUTT	AAUUGGUCAAUGCUGUACCTG
	siRNA 2	GCAGGGUCUCUAUUCCAAUTT	AUUGGAAUAGAGACCCUGCTG
17β-HSD5	siRNA 1	GGAACUUUCACCAACAGAUTT	AUCUGUUGGUGAAAGUUCCTT
	siRNA 2	GAAUGUCAUCCGUAUUUCATT	UGAAAUACGGAUGACAUUCTT
	siRNA 3	GGACAUGAAAGCCAUAGAUTT	AUCUAUGGCUUUCAUGUCCTT
NC		UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2.2

Primers used in RT-PCR and qRT-PCR

Name	Forward	Reverse
18s	5`-ACG GAC CAG AGC GAA AGC ATT-3`	5`-TCC GTC AAT TCC TTT AAG TTT CAG CT-3`
17β-HSD1	5`-CTT CTT TGT CCC CTG GGT CTG TGT G-3`	5`-GTC TCA CTG TGT TGC TCT GGC TGG T-3`
17β-HSD5	5`-TGG AAA ACT CAC TGA AGA AAGC-3`	5`-ACC CAT CGT TTG TCT CGT TGA-3`
17β-HSD7	5`-TCC ACC AAA AGC CTG AAT CTC TC-3`	5`-GGG CTC ACT ATG TTT CTC AGG C-3`

Table 2.3

Comparison of expression levels between 17β-HSD types 1, 5 and 7

17β-HSDs	mRNA copies number/ mg total RNA
17β-HSD1	1.79E+07± 2.34E+05
17β-HSD5	1.72E+05± 1.33E+04
17β-HSD7	7.56E+06± 7.52E+05

The data were obtained from qRT-PCR using a specific standard curve for each 17β-HSDs mRNA. The mRNA levels showed by copies number per mg of total OVCAR-3 RNA ± SD. The SDs were less than 10%.

Figures and legends

Figure 2.1

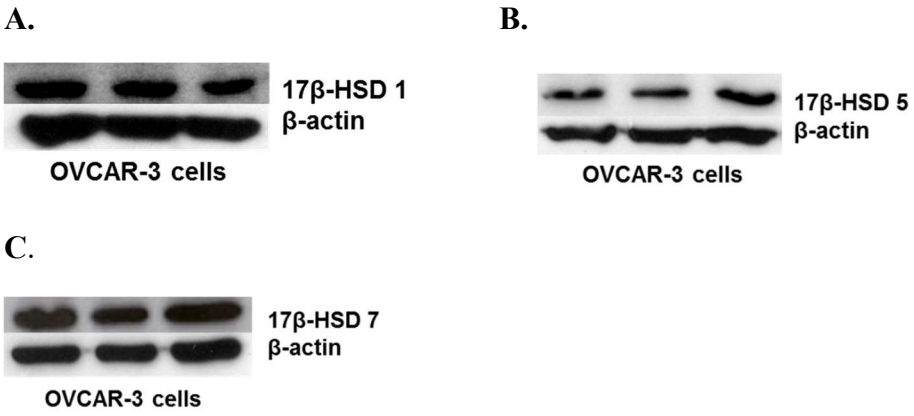
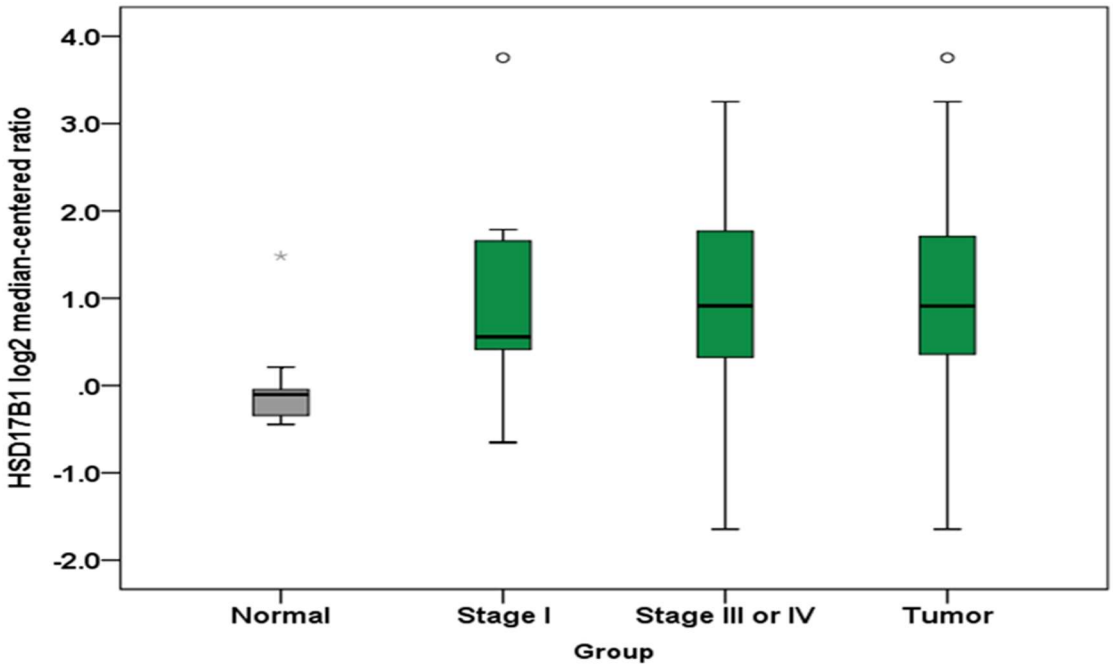


Figure 2.1. Western blot analysis of 17β-HSD types 1, 5 and 7 protein levels in OVCAR-3 cells. Total RNA (50 μg) isolated from OVCAR-3 for each lane. A. 17β-HSD1 expressed in OVCAR-3. Anti-17β-HSD1 antibody determined bands at molecular mass 36.78 kDa, anti-β-actin identified bands at molecular weight 42 kDa. B. 17β-HSD5(AKR1C3) expressed in OVCAR-3. Anti-AKR1C3 antibody determined bands at molecular mass 36 kDa, anti-β-actin identified bands at molecular weight 42 kDa. C. 17β-HSD7 expression in OVCAR-3. Anti-17β-HSD7 antibody determined bands at molecular weight 33 kDa, anti-β-actin identified bands at molecular weight 42 kDa. Each sample was performed in triplicate and was repeated in three independent experiments.

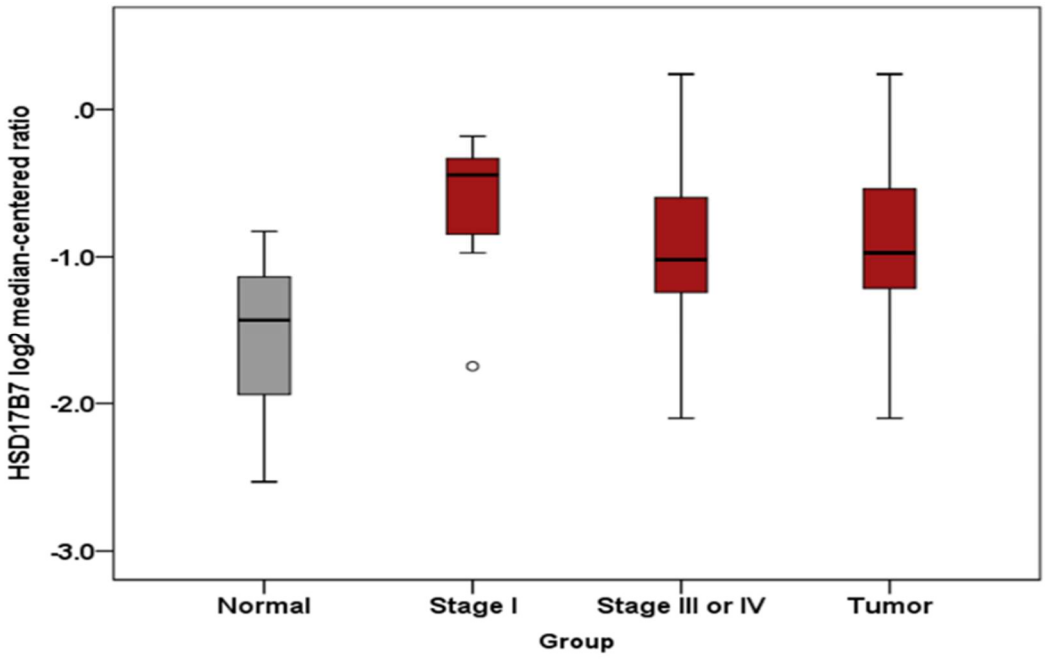
Figure 2.2

A.



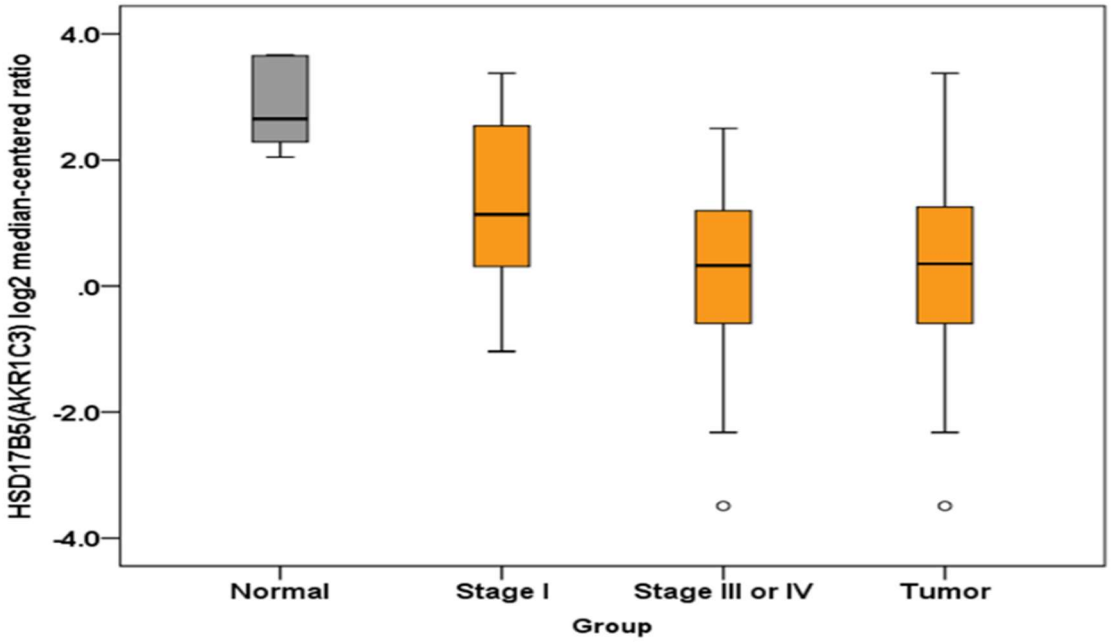
	Gene	Cases	p-value	Fold change
Normal	HSD17B1	10	/	/
Tumor (Stage I)	HSD17B1	8	0.032*	2.188
Tumor (Stage III or IV)	HSD17B1	35	0.007*	2.067
Tumor (All)	HSD17B1	43	0.005*	2.081

B.



	Gene	Cases	p-value	Fold change
Normal	HSD17B7	10	/	/
Tumor (Stage I)	HSD17B7	8	0.029*	1.875
Tumor (Stage III or IV)	HSD17B7	35	1E-06**	1.558
Tumor (All)	HSD17B7	43	3E-06**	1.616

C.



	Gene	Cases	p-value	Fold change
Normal	HSD17B5	10	/	/
Tumor (Stage I)	HSD17B5	8	8E-04**	-2.791
Tumor (Stage III or IV)	HSD17B5	35	0.001*	-5.727
Tumor (All)	HSD17B5	43	3E-04**	-5.217

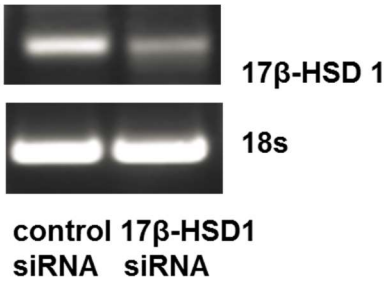
Figure 2.2

The expression status of 17β-HSD types 1, 5 and 7 in EOC with Yoshihara Ovarian dataset.

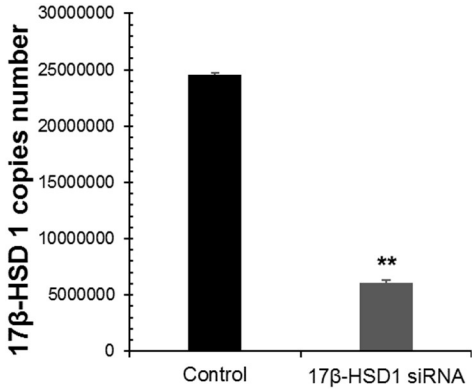
A. Data from Oncomine clinical database, expression of 17β-HSD1 in ovarian serous adenocarcinoma tissue vs. normal peritoneum tissue. The higher expression of 17β-HSD1 in tumor tissue (grade by stage I and advanced stage III or IV). B. The higher expression of 17β-HSD7 in tumor tissue (grade by stage I and advanced stage III or IV). C. The lower expression of 17β-HSD5 in ovarian serous adenocarcinoma tissue (grade by stage I and advanced stage III or IV) vs. normal peritoneum tissue. Data analysis was performed using SPSS software. *, Correlation is significant <0.05 vs. Normal 0.05 level (2-tailed); **, Correlation is significant<0.001 vs. Normal (2-tailed).

Figure 2.3

A.



B.



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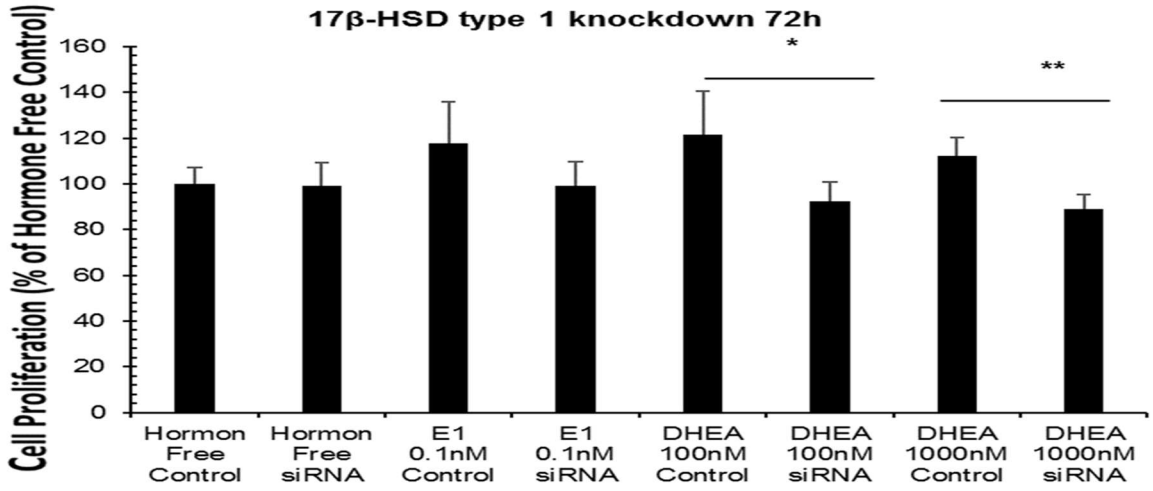


D.

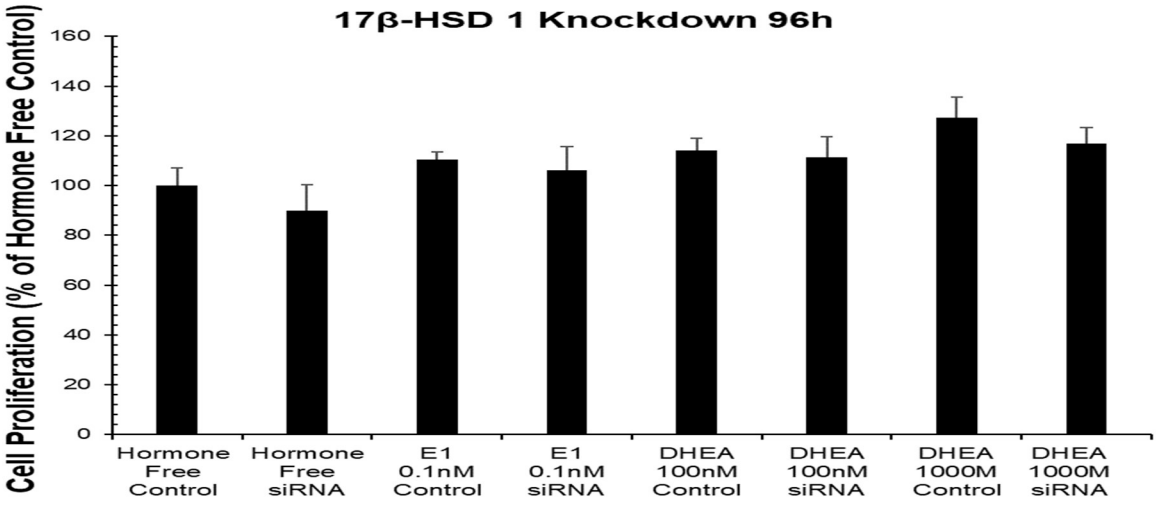


Figure 2.3

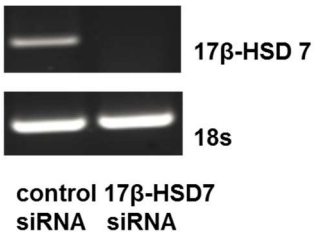
Knockdown effect by 17β-HSD1 siRNAs and cell proliferation change after siRNA transfection

Total RNA was extracted from OVCAR-3 cells. A. Rt-PCR was performed using 17β-HSD type 1 and 18S (as an internal control) primers. The 100 nM mixed 17β-HSD1-specific siRNA and control siRNA were used. B. qRT-PCR was determined the 17β-HSD1 mRNA level after siRNA transfection 72 h. Means and standard deviations are presented (N=3).*, $P< 0.05$ by Student's test.

Cell proliferation assay with the Cyquant kit for OVCAR-3 with 100 nM mixed 17β-HSD1-specific siRNA or control siRNA. The different hormone sources were proved: E1 (0.1 nM) and DHEA (100 nm and 1000 nM). Data as reported as % of DNA synthesis vs. Hormone Free Control (100%). C. After treatment with siRNA 72 h, 17β-HSD1 siRNA compared with control siRNA. D. After treatment with siRNA 96 h, 17β-HSD1 siRNA compared with control siRNA. Error bars represent SD. *, $P< 0.05$ vs.control; **, $P< 0.001$ vs. control by Student's test.

Figure 2.4

A.



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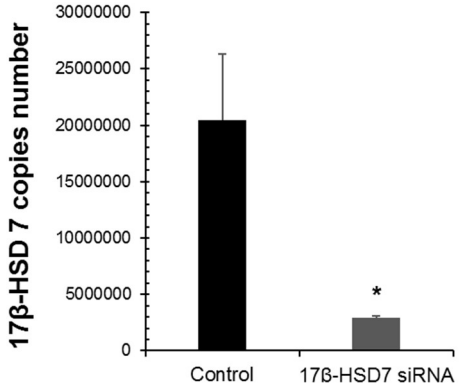

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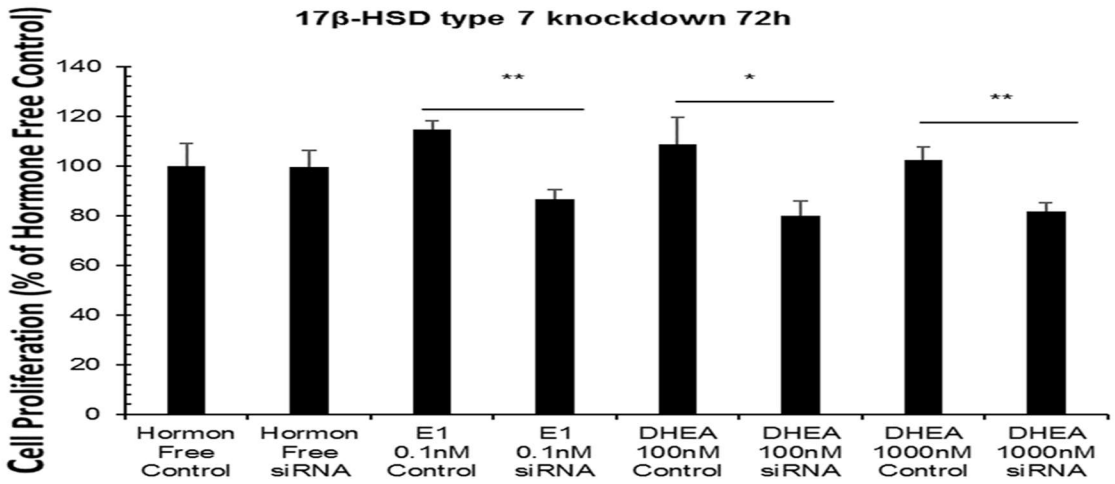

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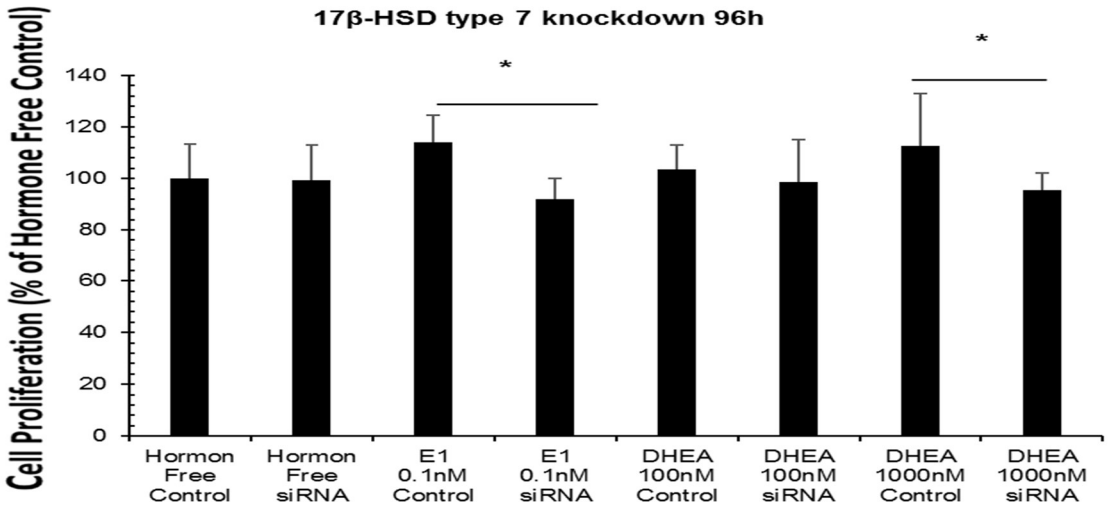


Figure 2.4

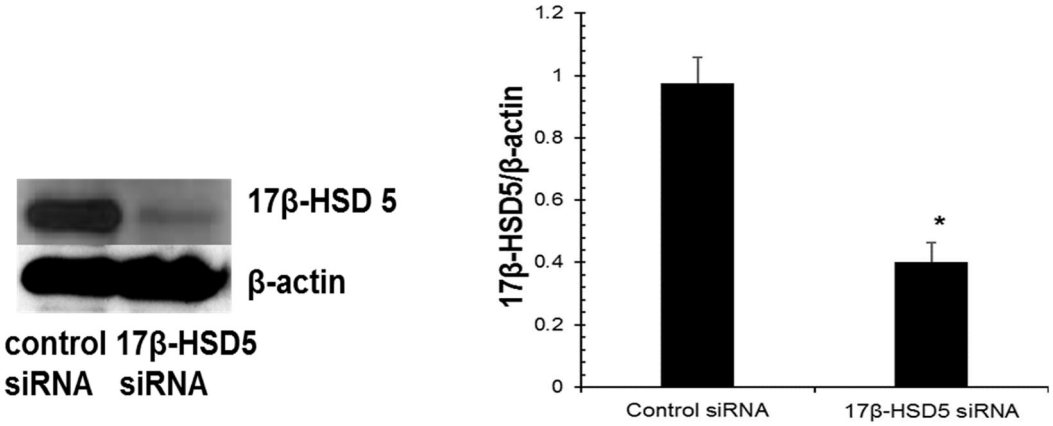
Knockdown effect by 17β-HSD7 siRNAs and cell proliferation change after siRNA transfection

Total RNA was extracted from OVCAR-3 cells. A. Rt-PCR was performed using 17β-HSD type 7 and 18S (as an internal control) primers. The 100 nM mixed 17β-HSD7-specific siRNA and control siRNA were used. B. qRT-PCR was determined the 17β-HSD7 mRNA level after siRNA transfection 72 h. Means and standard deviations are presented (N=3).*, $P< 0.05$ by Student's test.

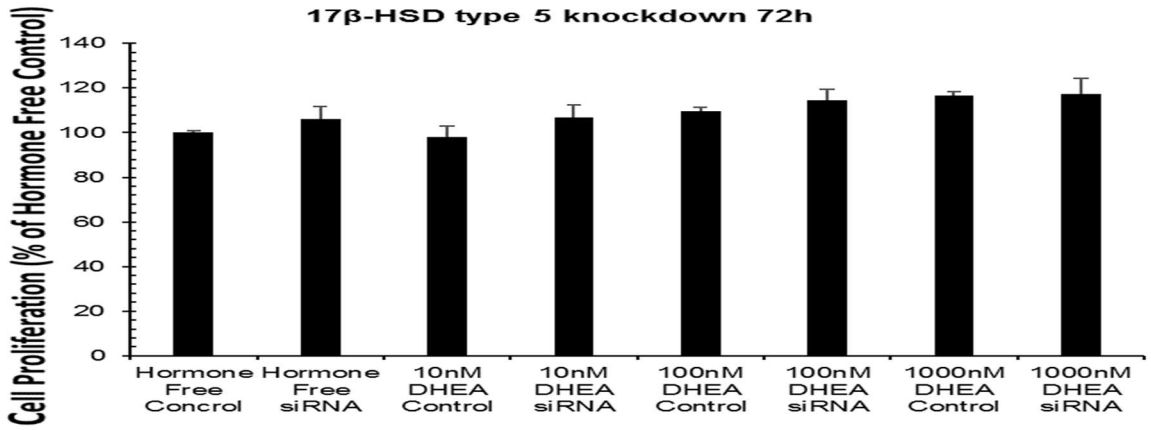
Cell proliferation assay with the Cyquant kit for OVCAR-3 with 100 nM mixed 17β-HSD7-specific siRNA or control siRNA. The different hormone sources were proved: E1 (0.1 nM) and DHEA(100 nm and 1000 nM). Data as reported as % of DNA synthesis vs. Hormone Free Control (100%). C. After treatment with siRNA 72 h, 17β-HSD7 siRNA compared with control siRNA. D. After treatment with siRNA 96 h, 17β-HSD7 siRNA compared with control siRNA. Error bars represent SD. *, $P< 0.05$ vs.control; **, $P< 0.001$ vs. control by Student's test.

Figure 2.5

A.



B.



C.

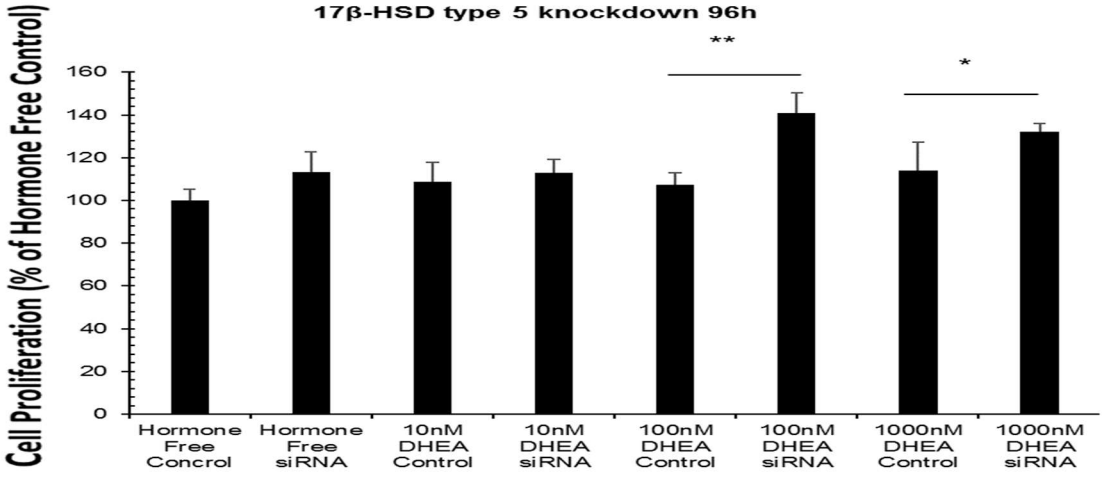


Figure 2.5

Knockdown effect by 17β-HSD5 (AKR1C3) siRNAs and cell proliferation change after siRNA transfection

Total protein was extracted from OVCAR-3 cells. A. Western blot showed the AKR1C3 protein expression after transfection with 100 nM mixed 17β-HSD5-specific siRNA and control siRNA. The bands determined by the AKR1C3 antibody. After 96 h transfection, AKR1C3 expression was silenced 59%. Error bars represent SD. *, P< 0.05 by Student's test.

Cell proliferation assay with the Cyquant kit for OVCAR-3 with 100 nM mixed 17β-HSD5-specific siRNA or control siRNA. The different hormone sources were proved: DHEA 10 nM, 100 nM and 1000 nM. Data as reported as % of DNA synthesis vs. Hormone Free Control (100%). B. After treatment with siRNA 72 h, 17β-HSD5 siRNA compared with control siRNA. C. After treatment with siRNA 96 h, 17β-HSD5 siRNA compared with control siRNA. Error bars represent SD. *, P< 0.05 vs.control; **, P< 0.001 vs. control by Student's test.

CHAPTER III

General Discussion and

Conclusions & Perspectives

3. General Discussion

3.1. Expression of 17β-hydroxysteroid dehydrogenase in epithelial ovarian cancer

Epithelial ovarian cancer derives from malignant transformation of the epithelium of the ovarian surface[57]. It is contiguous with the peritoneal mesothelium. Recent studies demonstrated 17β-hydroxysteroid dehydrogenase expression in epithelial cells of the ovarian surface in the post-menopausal ovary [48][58]. But data about 17β-HSD expression in epithelial ovarian cancer is limited. 17β-HSD2 and 17β-HSD5 were detected in ovarian epithelial tissue and lower expression compared with normal human surface epithelium on mRNA levels[59]. In another study it was also shown that 17β-HSDs is expressed in epithelial ovarian carcinoma type 2 (84.5%), type 4 (82.8%), type 8 (86.2%) and type 1 (10%) by immunohistochemical analysis in 58 cases[60]. Our study demonstrated that reductive 17β-HSD types 1, 5 and 7 are all expressed in EOC cells OVCAR-3 and tissue from ovarian serous adenocarcinoma, the biggest subtype of EOC. In OVCAR-3 cells, mRNA levels of 17β-HSD1 gene are higher than the other two, about twice that of type 7. And the type 5 gene mRNA level is much lower than type 1 and 7, around 100 times lower than type 1. In a clinical database analysis, we found that expression of both 17β-HSD types 1 and 7 are higher in EOC tissue compared with the normal ovary. But the expression of 17β-HSD5 is more than 5 times lower in tumors compared normal ovary tissue; its expression declines with the cancer progress.

Estrogen induces ovarian cancer the epithelial–mesenchymal transition (EMT) mainly through estrogen receptor α, leading to enhanced cell migration propensity and functional alterations[2]. EMT is an important stage of cancer metastasis in which epithelial cells lose cellular adhesion and cell polarity, acquire motility and aggressiveness to become mesenchymal cells [61]. Activation of an EMT program is also related to chemoresistance, which causes cancer recurrence and metastasis after

treatments such as radiation and chemotheraphy[62][63]. The advanced EOC patients after treatment often develop recurrence and evolve to chemotherapy resistance[7].

17β-HSD type 5 (AKR1C3) participates in the production of prostaglandin E2 (PGE2)[55]. PGE2 is the major factor stimulating aromatase expression. Aromatase is expressed from the CYP19 gene; it is responsible for the synthesis of E1 from the preferred substrate 4-dione and of E2 from Testo[56]. We found that decreased expression of 17β-HSD5 follows EOC development, especially during the advanced stage. During EOC progress, aromatase may rise while AKR1C3 decreases, then estrogen (E1 and E2) levels would increase. Estrogen level increases theoretically would promote EMT and cancer metastasis.

3.2. Decreased levels of 17β-HSD type 1 and type 7 inhibited epithelial ovarian cancer cell growth, down-regulated 17β-HSD type 5 stimulated OVCAR-3 cell proliferation.

In our study, we provided E1 or DHEA at different concentrations as hormone sources for 17β-HSDs. DHEA is the unique source of hormone steroids in post-menopausal women[15][16]. After menopause women, approximately 20% of circulating DHEA still released from ovaries[17]. Using the upstream hormone DHEA as a source helps to mimic the postmenopausal condition in ovarian cancer cell culture, our results showed cell proliferation changes after knocking down 17β-HSD type 1, type 5 or type 7 gene in epithelial ovarian cancer cell OVCAR-3. Knocking down 17β-HSD type 1 or type 7 inhibited epithelial ovarian cancer cell growth, but knockdown 17β-HSD type 5 stimulated OVCAR-3 cell proliferation.

Local estrogen metabolism is considered as an important study targeting epithelial ovarian cancer[59]. Reductive 17β-HSD type 1, type 5 and type 7 are key steroid-converting enzymes in estrogen synthesis. The 17β-HSD types 1 and 7 play a critical role in regulating E2, the most potent estrogen, synthesized from E1 and in regulating Testo from 4-dione[53]. Epidemiological studies have indicated that estrogens promote the invasion of epithelial ovarian cancer cells [21]. Down-regulation of 17β-HSD type 1 and type 7 will affect the regulation of E1 and E2 in cells and may decrease intercellular E2 levels and inhibit EOC cell growth.

17β-HSD type 5 synthesizes 5-diol from DHEA and catalyzes 4-dione reduction to Testo, which is further converted to E2 by aromatase. AKR1C3 also participates in the production of prostaglandin E2 (PGE2)[55]. PGE2 is the major factor stimulating aromatase expression. Aromatase is expressed from the CYP19 gene; it is responsible for the synthesis of E1 from the preferred substrate 4-dione and of E2 from Testo[56]. As we mentioned in the introduction section, we suggest 17β-HSD type 5 expression decreases may stimulate aromatase expression and affect estrogen levels. Estrogen level increases will promote EMT and cell proliferation in EOC cells.

4. Conclusion and Perspectives

Reductive enzymes 17β-HSD types 1, 5 and 7 are all expressed in epithelial OCtissue. The expression of 17β-HSD type 1 is higher than that of types 5 and type 7 in EOC cells. Most epithelial ovarian cancer patients are asymptomatic until it has been widely metastasized within the abdomen[2]. Due to the difficulty in early diagnosis, EOC has quite low survival rates[5][7]. The expression increases in 17β-HSD types 1 and 7 in EOC may reveal novel targets for the development of EOC early diagnosis biomarkers. The lower expression of 17β-HSD type 5 in advanced stage epithelial ovarian tumor will help understanding development mechanisms and steroid hormone signaling in EOC.

Our results indicate that 17β-HSD types 1 and 7 may be involved in the protective effects against the estrogen-dependent proliferation of epithelial ovarian carcinoma, but 17β-HSD type 5 has a suppressive effect on the epithelial OC cell proliferation. The results of previous endocrine therapy clinical trials showed most treatments have limited effectiveness. The study of 17β-HSD types 1 and 7 will help design clinical trials and new hormonal treatments for EOC.

REFERENCES

For Introduction and General discussion

[1] R. Siegel, D. Naishadham, and A. Jemal, "Cancer statistics, 2013," CA. Cancer J. Clin., vol. 63, no. 1, pp. 11–30, 2013.

[2] S.-Y. Jeon, K.-A. Hwang, and K.-C. Choi, "Effect of steroid hormones, estrogen and progesterone, on epithelial mesenchymal transition in ovarian cancer development," *J. Steroid Biochem. Mol. Biol.*, vol. 158, pp. 1–8, 2016.

[3] C. H. Holschneider and J. S. Berek, "Ovarian cancer: epidemiology, biology, and prognostic factors.," *Semin. Surg. Oncol.*, vol. 19, no. 1, pp. 3–10, 2000.

[4] F. Modugno, R. Laskey, A. L. Smith, C. L. Andersen, P. Haluska, and S. Oesterreich, "Hormone response in ovarian cancer: Time to reconsider as a clinical target?," *Endocrine-Related Cancer*, vol. 19, no. 6. pp. R255–R279, 2012.

[5] K. R. Cho and I.-M. Shih, "Ovarian Cancer," *Annu. Rev. Pathol. Mech. Dis.*, vol. 4, no. 1, pp. 287–313, 2009.

[6] "National Cancer Institute. 2014. https://seer.cancer.gov/statfacts/html/ovary.html (accessed April 20,2017)."

[7] G. C. Jayson, E. C. Kohn, H. C. Kitchener, and J. A. Ledermann, "Ovarian cancer," *Lancet*, vol. 384, no. 9951, pp. 1376–1388, 2014.

[8] "[Online]https:/en.wikipedia.org/wiki/Ovary."

[9] Shirish N Daftary; Sudip Chakravarti, "Manual of Obstetrics, 3rd Edition.," *Elsevier*, pp. 1–16, 2011.

[10] http://womenshealth.med.monash.edu.au, "Androgens in women."

[11] F. Labrie, "Intracrinology," *Mol. Cell. Endocrinol.*, vol. 78, pp. C113–C118, 1991.

[12] H. D. Nelson, "Menopause," *Lancet*, vol. 371, pp. 760–70, 2008.

[13] B. M. Nugent *et al.*, "Hormonal programming across the lifespan," *Hormone and Metabolic Research*, vol. 44, no. 8. pp. 577–586, 2012.

[14] F. Labrie and C. Labrie, "DHEA and intracrinology at menopause, a positive choice for evolution of the human species.," *Climacteric*, vol. 16, no. March, pp. 205–13, 2013.

[15] F. Labrie, "All sex steroids are made intracellularly in peripheral tissues by the mechanisms of intracrinology after menopause," *J. Steroid Biochem. Mol. Biol.*, vol. 145, pp. 133–138, 2015.

[16] H. L. JUDD, G. E. JUDD, W. E. LUCAS, and S. S. C. YEN, "Endocrine Function of the Postmenopausal Ovary: Concentration of Androgens and Estrogens in Ovarian and Peripheral Vein Blood," *J. Clin. Endocrinol. Metab.*, vol. 39, no. 6, pp. 1020–1024, 1974.

[17] F. Labrie, C. Martel, and J. Balser, "Wide distribution of the serum dehydroepiandrosterone and sex steroid levels in postmenopausal women: role of the ovary?," *Menopause*, vol. 18, no. 1, pp. 30–43, 2011.

[18] P. Lee, D. G. Rosen, C. Zhu, E. G. Silva, and J. Liu, "Expression of progesterone

receptor is a favorable prognostic marker in ovarian cancer," *Gynecol. Oncol.*, vol. 96, no. 3, pp. 671–677, Mar. 2005.
[19] G. P. Sutton, M. B. Senior, J. F. Strauss, and J. J. Mikuta, "Estrogen and progesterone receptors in epithelial ovarian malignancies," *Gynecol. Oncol.*, vol. 23, no. 2, pp. 176–182, 1986.
[20] R. Kühnel, J. de Graaff, B. R. Rao, and J. G. Stolk, "Androgen receptor predominance in human ovarian carcinoma.," *J. Steroid Biochem.*, vol. 26, no. 3, pp. 393–397, 1987.
[21] P. Hoffmann and P. Pujol, "Estrogens and epithelial ovarian cancer," vol. 94, pp. 25–32, 2004.
[22] H. Gharwan, K. P. Bunch, and C. M. Annunziata, "The role of reproductive hormones in epithelial ovarian carcinogenesis," *Endocr. Relat. Cancer*, vol. 22, no. 6, pp. R339–R393, 2015.
[23] H. A. Risch, "Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone.," *J. Natl. Cancer Inst.*, vol. 90, no. 23, pp. 1774–1786, 1998.
[24] S.-M. Ho, "Estrogen, progesterone and epithelial ovarian cancer.," *Reprod. Biol. Endocrinol.*, vol. 1, p. 73, 2003.
[25] M. M. Tanner, S. Grenman, A. Koul, O. Johannsson, P. Meltzer, and T. Pejovic, "Frequent Amplification of Chromosomal Region 20q12-q13 in Ovarian Cancer Frequent Amplification of Chromosomal Region 20q12-q13 in Ovarian Cancer," *Clin. Cancer Res.*, vol. 6, no. May, pp. 1833–1839, 2000.
[26] P. a Shaw, P. V. C. Rittenberg, and T. J. Brown, "Activation of Androgen Receptor-Associated Protein 70 (ARA70) mRNA Expression in Ovarian Cancer," *Gynecol. Oncol.*, vol. 80, no. 2, pp. 132–138, 2001.
[27] S. L. Anzick, "AIB1, a Steroid Receptor Coactivator Amplified in Breast and Ovarian Cancer," *Science (80-.).*, vol. 277, no. 5328, pp. 965–968, 1997.
[28] M. Nourbakhsh, A. Golestani, M. Zahrai, M. H. Modarressi, Z. Malekpour, and F. Karami-Tehrani, "Androgens stimulate telomerase expression, activity and phosphorylation in ovarian adenocarcinoma cells," *Mol. Cell. Endocrinol.*, vol. 330, no. 1–2, pp. 10–16, 2010.
[29] "[Online]https://en.wikipedia.org/wiki/Telomerase," pp. 1–9, 2009.
[30] A Kirschenbaum *et al.*, "Sex steroids have differential effects on growth and gene expression in primary human prostatic epithelial cell cultures derived from the peripheral versus transition zones," *Carcinogenesis*, vol. 27, no. 2, pp. 216–224, 2006.
[31] J. Prat, "Abridged republication of FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum," *Cancer*, vol. 121, no. 19, pp. 3452–3454, 2015.
[32] M.-C. King, "Breast and Ovarian Cancer Risks Due to Inherited Mutations in BRCA1 and BRCA2," *Science (80-.).*, vol. 302, no. 5645, pp. 643–646, 2003.

[33] C. A. Powless, G. D. Aletti, J. N. Bakkum-Gamez, and W. A. Cliby, “Risk factors for lymph node metastasis in apparent early-stage epithelial ovarian cancer: Implications for surgical staging,” *Gynecol. Oncol.*, vol. 122, no. 3, pp. 536–540, 2011.
[34] N. Colombo *et al.*, “International Collaborative Ovarian Neoplasm Trial 1: A Randomized Trial of Adjuvant Chemotherapy in Women With Early-Stage Ovarian Cancer,” *JNCI J. Natl. Cancer Inst.*, vol. 95, no. 2, pp. 125–132, 2003.
[35] B. Trimbos *et al.*, “Surgical Staging and Treatment of Early Ovarian Cancer: Long-term Analysis From a Randomized Trial,” *JNCI J. Natl. Cancer Inst.*, vol. 102, no. 13, pp. 982–987, 2010.
[36] D. K. Armstrong *et al.*, “Intraperitoneal Cisplatin and Paclitaxel in Ovarian Cancer,” *Obstet. Gynecol. Surv.*, vol. 61, no. 4, pp. 240–242, 2006.
[37] A. F. Saad, W. Hu, and A. K. Sood, “Microenvironment and Pathogenesis of Epithelial Ovarian Cancer,” *Horm. Cancer*, vol. 1, no. 6, pp. 277–290, 2010.
[38] F. Guidozzi and A. Daponte, “Estrogen replacement therapy for ovarian carcinoma survivors,” *Cancer*, vol. 86, no. 6, pp. 1013–1018, 1999.
[39] R. A. Eeles *et al.*, “Adjuvant hormone therapy may improve survival in epithelial ovarian cancer: Results of the AHT randomized trial,” *J. Clin. Oncol.*, vol. 33, no. 35, pp. 4138–4144, 2015.
[40] G. RODRIGUEZ *et al.*, “Effect of progestin on the ovarian epithelium of macaques: cancer prevention through apoptosis?, ” *J. Soc. Gynecol. Investig.*, vol. 5, no. 5, pp. 271–276, 1998.
[41] S. Cunat *et al.*, “Aromatase expression in ovarian epithelial cancers,” *J. Steroid Biochem. Mol. Biol.*, vol. 93, no. 1, pp. 15–24, 2005.
[42] D. Papadatos-Pastos, K. J. Dedes, J. S. de Bono, and S. B. Kaye, “Revisiting the Role of Antiandrogen Strategies in Ovarian Cancer,” *Oncologist*, vol. 16, no. 10, pp. 1413–1421, 2011.
[43] S. Barrie, G. Potter, P. Goddard, B. Haynes, M. Dowsett, and M. Jarman, “Pharmacology of novel steroidal inhibitors of cytochrome p450 (17)alpha (17 alpha hydroxylase/ C17-20 lyase).,” *J steroid Biochem mol biol*, vol. 50, no. 5–6, pp. 267–273, 1994.
[44] S. Marchais-Oberwinkler *et al.*, “17β-Hydroxysteroid dehydrogenases (17β-HSDs) as therapeutic targets: Protein structures, functions, and recent progress in inhibitor development,” *J. Steroid Biochem. Mol. Biol.*, vol. 125, no. 1–2, pp. 66–82, 2011.
[45] F. Labrie, “Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease,” *J. Mol. Endocrinol.*, vol. 25, no. 1, pp. 1–16, 2000.
[46] W. He, M. Gauri, T. Li, R. Wang, and S.-X. Lin, “Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1),” *Gene*, vol. 588, no. 1, pp. 54–61, 2016.
[47] G. Moeller and J. Adamski, “Integrated view on 17beta-hydroxysteroid

dehydrogenases," *Mol. Cell. Endocrinol.*, vol. 301, no. 1–2, pp. 7–19, 2009.
[48] J. C. Havelock, W. E. Rainey, K. D. Bradshaw, and B. R. Carr, "The post-menopausal ovary displays a unique pattern of steroidogenic enzyme expression.," *Hum. Reprod.*, vol. 21, no. 1, pp. 309–17, 2006.
[49] C. Y. Zhang, W. Q. Wang, J. Chen, and S. X. Lin, "Reductive 17beta-hydroxysteroid dehydrogenases which synthesize estradiol and inactivate dihydrotestosterone constitute major and concerted players in ER+ breast cancer cells," *J. Steroid Biochem. Mol. Biol.*, vol. 150, pp. 24–34, 2015.
[50] D. Xu and S. X. Lin, "Mimicking postmenopausal steroid metabolism in breast cancer cell culture: Differences in response to DHEA or other steroids as hormone sources," *J. Steroid Biochem. Mol. Biol.*, vol. 161, pp. 92–100, 2016.
[51] D. Xu, J. A. Aka, R. Wang, and S.-X. Lin, "17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation," *J. Steroid Biochem. Mol. Biol.*, 2017.
[52] X. Wang, C. Gérard, J.-F. Thériault, D. Poirier, C. J. Doillon, and S.-X. Lin, "Synergistic control of sex hormones by 17β-HSD type 7: a novel target for estrogen-dependent breast cancer," *J. Mol. Cell Biol.*, vol. 7, no. 6, pp. 568–579, 2015.
[53] P. Lukacik, K. L. Kavanagh, and U. Oppermann, "Structure and function of human 17β-hydroxysteroid dehydrogenases," *Mol. Cell. Endocrinol.*, vol. 248, no. 1–2, pp. 61–71, 2006.
[54] C. H. Blomquist *et al.*, "Androgenic and estrogenic 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase in human ovarian epithelial tumors: evidence for the type 1, 2 and 5 isoforms," *J. Steroid Biochem. Mol. Biol.*, vol. 81, pp. 343–351, 2002.
[55] B. L. Dozier, K. Watanabe, and D. M. Duffy, "Two pathways for prostaglandin F2α synthesis by the primate periovulatory follicle," *Reproduction*, vol. 136, no. 1, pp. 53–63, 2008.
[56] E. R. Simpson *et al.*, "Aromatase—A Brief Overview," *Annu. Rev. Physiol.*, vol. 64, no. 1, pp. 93–127, 2002.
[57] S. A. Cannistra, "Cancer of the Ovary," *N Engl J Med*, vol. 35124351, pp. 2519–29, 2004.
[58] A. Brodowska *et al.*, "Immunoexpression of aromatase cytochrome P450 and 17β-hydroxysteroid dehydrogenase in women's ovaries after menopause.," *J. Ovarian Res.*, vol. 7, p. 52, 2014.
[59] X. Ren *et al.*, "Local estrogen metabolism in epithelial ovarian cancer suggests novel targets for therapy," *J. Steroid Biochem. Mol. Biol.*, vol. 150, pp. 54–63, 2015.
[60] K. Motohara, H. Tashiro, Y. Taura, T. Ohba, and H. Katabuchi, "Immunohistochemical analysis of 17β-hydroxysteroid dehydrogenase isozymes in human ovarian surface epithelium and epithelial ovarian carcinoma.," *Med. Mol.*

Morphol., vol. 43, no. 4, pp. 197–203, 2010.
[61] L. Larue and A. 2005 Bellacosa, “Epithelial–mesenchymal transition in development and cancer: role of phosphatidylinositol 3 0 kinase/AKT pathways,” *Oncogene*, no. 24, pp. 7443–7454, 2005.
[62] M. Iwatsuki *et al.*, “Epithelial-mesenchymal transition in cancer development and its clinical significance,” *Cancer Sci.*, vol. 101, no. 2, pp. 293–299, 2010.
[63] L. N. Abdullah and E. K.-H. Chow, “Mechanisms of chemoresistance in cancer stem cells.,” *Clin. Transl. Med.*, vol. 2, no. 1, p. 3, 2013.

APPENDICES

ARTICLE 1.

Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1)

RÉSUME

Connaissance actuelle de la 17β-hydroxysteroide déshydrogénase type 1 (HSD17B1) multifonctionnelle

La 17β-HSD1 humaine a été la première enzyme de la famille des 17β-HSDs à être cloné, identifié, et dont la structure tridimensionnelle a été déterminée. Cette enzyme est exprimée dans le placenta humain, l'ovaire, l'endomètre et le sein. Au niveau moléculaire et dans les cellules cancéreuses du sein, il a été nappanté que cette enzyme détient une double fonction dans l'activation des œstrogènes et l'inactivation des androgènes. De plus, la 17β-HSD1 stimule de manière significative la prolifération des cellules cancéreuses. La surexpression de la 17β-HSD1 dans le cancer du sein a été démontrée à l'aide d'échantillons provenant de cliniques. L'inhibition de la 17β-HSD1 humaine a conduit à une diminution de la croissance de tumeurs dans des xénogreffes. En raison de la difficulté à éliminer l'activité œstrogénique des inhibiteurs de la 17β-HSD1, il n'existe toujours aucun médicament utilisant l'inhibition de cet l'enzyme comme approche thérapeutique. Le développement de nouveaux inhibiteurs pour cette enzyme nous donne un nouvel espoir pour un traitement contre le cancer du sein.

SUMMARY

Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1)

The human 17β-HSD1 is the first 17β-HSD that was cloned and sequenced. The three-dimensional structure of 17β-HSD1 also is the first example of human steroid-converting enzymes. It is expressed in the human placenta, ovary, endometrium and breast. A dual function of estrogen activation and androgen inactivation in 17β-HSD1 is reported in molecular and breast cancer cells. Moreover, it significantly stimulates the proliferation of such cells. The overexpression of 17β-HSD1 in breast cancer was demonstrated in clinical samples. Inhibition of human 17β-HSD1 led to xenograft tumor shrinkage. Due to the estrogenic activity associated with 17β-HSD1's inhibitors, there are no any successful 17β-HSD1 reported in literature. The development of new inhibitors for the 17β-HSD1 gives us a new hope for breast cancer treatment.

Current knowledge of the multifunctional 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1)

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Abstract

At the late 1940s, 17β-HSD1was discovered as the first member of the 17β-HSD family with its gene cloned. The three-dimensional structure of human 17β-HSD1 is the first example of any human steroid converting enzyme. The human enzyme's structure and biological function have thus been studied extensively in the last two decades. In humans, the enzyme is expressed in placenta, ovary, endometrium and breast. The high activity of estrogen activation provides the basis of 17β-HSD1's implication in estrogen-dependent diseases, such as breast cancer, endometriosis and non-small cell lung carcinomas. Its dual function in estrogen activation and androgen inactivation has been revealed in molecular and breast cancer cell levels, significantly stimulating the proliferation of such cells. The enzyme's overexpression in breast cancer was demonstrated by clinical samples. Inhibition of human 17β-HSD1 led to xenograft tumor shrinkage. Unfortunately, through decades of studies, there is still no drug using the enzyme's inhibitors available. This is due to the difficulty to get rid of the estrogenic activity of its inhibitors, which are mostly estrogen analogues. New non-steroid inhibitors for the enzyme provide new hope for non-estrogenic inhibitors of the enzyme.

Contents

1. The origin and evolution of the 17β-HSD1 gene (HSD17B1)

As early as the late 1940s and early 1950s, an enzyme regulating the balance between estrone (E1) and estradiol (E2) was discovered in human placenta (Langer and Engel, 1958). Enzyme activity in both prokaryotic and eukaryotic species was described about this first discovered

member of the 17β-hydroxysteroid dehydrogenase family, i.e. 17β-HSD1. During the course of evolution, genes encoding the 17β-HSD enzymes developed individually approximately 540 million years ago parallel to those for steroid receptors (Hartmann and Adamski, 2011; Jansson,2009). This implies an important evolutionary role for the 17β-HSD enzyme family.

Human 17β-HSD1was the first 17β-HSD to be cloned and sequence identified (Luu-The et al., 1989; Peltoketo et al., 1988). Its three dimensional structure is also the first example of any human steroid converting enzyme (Ghosh et al., 1995a, 1995b; Azzi et al., 1996; Lin et al., 1996). The 17β-HSD1 gene was determined to be located in the q.12.1 band of chromosome 17 through gene mapping by in situ hybridization. This enzyme contains 327 amino acids and exists as a homodimer with two identical subunits of 34.5 kDa (Lin et al., 1992; Peltoketo et al., 1988). 17β-HSD1 uses NADPH as a co-factor to catalyze the conversion of E1 to E2, and to a minor extent that of androgens such as 4-androstenedione (4-Adione) to testosterone (T) (Lukacik et al., 2006). 17β-HSD1 can bind to both triphosphate cofactors (NADPH) and NAD (H) at the molecular level but with much higher specificity to the former, which is rich in cells that largely governs the enzyme's catalytic direction towards estrone reduction (Karavolas et al., 1970; Lin et al., 1992; Sherbet et al., 2007). Enzyme kinetics and X-ray crystallographic studies have shown that 17β-HSD1 has the potential to bind C-19 steroids in both normal and reverse orientations resulting in the 3β-reduction of DHT into 5-androstane-3,17-diol (3β-diol) and 17β-oxidation of DHT into A-dione, both leading to the inactivation of the most potent

androgen DHT (Gangloff et al., 2003). 17β-HSD1 expression positively correlates to estrone activation, E2 levels, and proliferation of breast cancer cells (Aka et al., 2010). The multi-specificity of 17β-HSD1 is structurally based on the pseudo-symmetric structures of androgens that can accommodate the narrow enzyme substrate tunnel by both normal and alternative binding (Lin et al., 2013). Thus, 17β-HSD1 up-regulates breast cancer cell growth by a combined action on estradiol synthesis and DHT inactivation.

In primates, 17β-HSD1 is primarily expressed in the placenta and ovarian granulosa cells and to a lesser extent in the endometrium, adipose tissue and prostate. It is not expressed in the testes or adrenals (Schwabe et al., 2001; Takeyama et al., 2000). This tissue-specific expression makes 17β-HSD1 an attractive pharmaceutical target in women's diseases (Lukacik et al., 2006), particularly the breast cancer. Today, fifteen 17β-HSD enzymes have been discovered in mammals and the nomenclature of these enzymes follow their discovery order. All of these belong to the short-chain dehydrogenase/reductase family (SDR) with the exception of 17β-HSD5, which is an aldoketo-reductase (AKR). Short-chain dehydrogenase/reductase enzymes are multimeric in nature, expressed in a variety of organisms with NADPH as co-factor. Aldo-keto-reductase enzymes act as monomers and also use NADPH as co-factor. A high degree of substrate variability is shown by SDR enzymes including: steroids, retinoids, fatty acids and prostaglandins. 17β-HSD enzymes are localized in different parts of the cell across diverse tissues and show preference for a variety of substrates and co-factors. A feature common to all 17β-HSD enzymes is the ability to catalyze oxidation or reduction of the carbon at position 17 in the steroids. These enzymes have different substrate preferences such as E1, E2, T, 3β-diol and DHT. They possess distinct physiological functions (Jansson, 2009).

2. Comparison of particular gene expression in different organisms, animals and human

The sequence identities between human 17β-HSD1 and other species range from 51% (zebra fish) to 99% (chimpanzee) and homologies range from 70% to 100%, respectively. The biggest differences are located in the F/G segment (residues191–230), which lines the hydrophobic substrate binding site (SUB) and in the C-terminal region (Miyoshi et al., 2001).

Human 17β-HSD1 catalyzes the reduction of the weak estrogen E1 to the most potent, E2. This occurs in target cells where the estrogenic effect is exerted via the estrogen receptor (ER). Estrogens, especially E2, are known to stimulate the proliferation of hormone-dependent diseases (Pasqualini and Chetrite, 2005) such as breast cancer. The risk of breast cancer is positively correlated with a high level of E2 (Pasqualini et al., 1996), because this potent estrogen plays an important role in the proliferation of cancer cells (Castoria et al., 2010). It has been confirmed by microarray analysis that E2 regulates estrogen response elements (EREs), progesterone receptor (PR), pS2 and cathepsin D that affects the cell growth and differentiation (Laganière et al., 2005; Cicatiello et al., 2004).

The majority of breast cancer tumors (60–80%) express high levels of ERs, which accounts for the proliferative effect of estrogens. They tend to have a higher intratumoral estrogen concentration in comparison to normal breast tissue and plasma (Labrie et al., 2000). There is a direct relation between high [E2]/[E1] ratio and breast cancer cell proliferation. Strategies targeting the reduction of [E2]/[E1] ratio, are proposed to be an effective means of facilitating breast cancer therapy (Zhang et al., 2012). In situ synthesis and metabolism of estrogens is believed to be of great importance for the development and progression of breast cancer. In fact, 17β-HSD1 is overexpressed in many breast tumors

and as such it is an attractive target for the treatment of these diseases (Frotscher et al., 2008).

In accord with its role in sex-hormone signaling, 17β-HSD1 is expressed in placenta, endometrium and ectopic pregnancy. Immunohistochemical assays revealed that 17β-HSD1 is present in syncytiotrophoblast (ST) cells (Li et al., 2005), a large portion of extravillous cytotrophoblast (EVCT) cells and 20% of column cytotrophoblast (CCT) cells. On the other hand, no expression of 17β-HSD1 was detected in villous cytotrophoblast (VCT) cells. Localization of 17β-HSD1 was found on the surface of glandular epithelial cells when progesterone was present at typical ovulatory cycle concentrations (Mäentausta et al., 1991). It was also associated with endometrial carcinoma. In addition, 17β-HSD1 is found in epithelial cells of the fallopian tube. Interestingly, the expression level of 17β-HSD1 in the fallopian tube epithelium during tubal pregnancy is significantly higher than that found during a normal cycle. There is evidence that normal and tubal pregnancies possess identical expression of P450 aromatase and 17β-HSD1 in ST cells implicating similar E2 production in the placenta (Li et al., 2003). Furthermore, the association of 17β-HSD1 with EVCT cells indicates that 17β-HSD1 perhaps plays a role in trophoblast invasion. Increased expression of 17β-HSD1 in the epithelial cells of the fallopian tube may lead to a local E2 supply sufficient for the maintenance of tubal pregnancy (Li et al., 2003). The synthesis of estrogens was recently demonstrated in non-small cell lung carcinomas (NSCLCs) via aromatase activity. Moreover, an aromatase inhibitor (AI) did suppress estrogen receptor-positive NSCLC growth (Hershberger et al., 2005; Verma et al., 2011). Recent studies highlights the importance of 17β- HSD1 as an important prognostic factor in NSCLC patients making it an attractive target that can improve the clinical response in estrogen responsive NSCLC patients (Verma et al., 2013).

3. Structure–biological functions: dual estrogen and androgen activities and disease implications

The homogeneity and high activity of the enzyme preparation developed in early 1990s, significantly improved from former purifications, laid down the enzyme's crystallization and structural determination as the first human steroid-converting enzyme (Lin et al., 1992; Zhu et al., 1993; Ghosh et al., 1995a,b). It has been elucidated by structural and mutagenesis studies that in the Rossmann fold (Breton et al., 1996; Buehner et al., 1973) of 17β-HSD1, a positively charged amino acid is able to form a salt bridge with the 2′-phosphate group of the cofactor NADP(H), i.e. Arg37 in 17β-HSD1 (Huang et al., 2001). Structural analysis, mutagenesis studies and sequence alignment have resulted in the identification of features essential for the catalytic process namely three conserved amino acid residues, Ser142, Tyr155 and Lys159 constituting a “catalytic triad” with a water molecule (Puranen et al., 1994; Ghosh et al., 1995a,b). Further investigations showed that an additional conserved water molecule stabilized by an H-bond interaction with an Asn114 residue (together with the “catalytic triad” to form a “catalytic tetrad”) plays a critical role in the enzymatic process for HSDs (Filling et al., 2002; Hwang et al., 2005). Three catalytic mechanisms are proposed for 17β-HSD1 (Ghosh et al., 1995a, b; Ghosh and Vihko, 2001; Penning, 1997): one concerted (simultaneous transfer of hydride intermediate presence of either an oxyanion or a carbocation. (A) Firstly, the pro-S hydride of NADPH is transferred to the α-face of E1 at the planar C17 carbon resulting in an energetically favorable aromatic system; the resultant oxyanion is subsequently protonated by the acidic-OH group of Tyr155 (A2). (B) In the second proposed mechanism, initially the keto oxygen of E1 is protonated by the acidic-OH of Tyr155; the resultant carbocation then accepts the pro-S hydride of NADPH at the α-face. The proton relay is facilitated by a H-bond network involving Lys159, two water molecules and Asn114, an electrostatic interaction between the protonated side chain of Lys159 and the phenyl ring of Tyr155 (Ghosh et al., 1995a,b; Ghosh and Vihko, 2001) as well as T-stacking between Phe192 and

Tyr155 (Negri et al., 2010). Hydrogen bonds are represented by dashed lines in Fig. 1 and π–π-interactions are not depicted for the sake of clarity. Despite the availability of enormous structural information, the most likely mechanism is highly debated (Marchais-Oberwinkler et al., 2009).

To date, 20 crystal structure forms of 17β-HSD1 are available in the protein data bank (PDB) as: apo-enzyme (1bhs), holo-enzyme (1fdv, 1qyv), binary complex with E2, androgens or inhibitors (1fds, 1fdw, 1dht,3dhe, 1jtv, 1iol, 3dey, 1i5r, 3hb4, 3klm) and ternary complex with cofactor and E2 or inhibitors (1fdt, 1equ, 1fdu, 1a27, 1qyw, 1qyx,3hb5). Remarkably, no crystal structure has been determined with the E1 substrate (Marchais-Oberwinkler et al., 2009). All crystals reveal an overall identical tertiary structure: a rigid cofactor binding site (COF) and a narrow, hydrophobic SUB, which constitutes a "substrate recognition domain" delimited by the C terminal region (Alho-Richmond et al., 2006; Azzi et al., 1996). Estradiol is stabilized by hydrogen bonds between the O3 and His221/Glu282, as well as between the O17 and Tyr155/Ser142 (Azzi et al., 1996). Flexible βFαG′ loop accounts for the major differences in the structures. This loop is not resolved in twelve crystal structures and can occupy three possible orientations depending on the presence of cofactor and ligands: an opened, a semi-opened and a closed enzyme conformation (Negri et al., 2010). The binding mode is known for some steroidal inhibitors as they have been co-crystallized in complex with 17β-HSD1 (1equ, 3hb5, 1i5r). The data revealed the importance of a defined βFαG′ loop conformation for compound binding. Since no protein structure complex with non-steroidal inhibitors exists, computational studies have been performed to investigate their binding. These studies showed that the choice of the crystal structure was the determinant for the identification of a binding mode and that the latter was strongly dependent on the loop conformation (Bey et al., 2009). The multi-specificity of the enzyme has been studied and reviewed (Lin et al., 1999). The cofactor hydrogen bonding onto the enzyme main chain was found to be

conserved in 17β-HSD1 as well as in other short-chain dehydrogenase/reductase family and contributes to nicotinamide orientation (Shi and Lin, 2004).

Two principal pathways are implicated in the final steps of E2 activation in breast cancer tissue. The aromatase pathway transforms androgens into estrogens (Batzl et al., 1996), the sulfatase pathway converts DHEA sulfate into DHEA and estrone sulfate (E1S) into E1 (MacIndoe, 1988; Pasqualini et al., 1989), followed by E1 conversion into the potent E2 by the action of reductive 17β-HSDs (Aka et al., 2009; Nguyen et al., 1995; Pasqualini, 2004, Yang et al., 1992). Quantitative evaluation indicates that in human breast tumors, DHEAS and E1S via sulfatase is a much more likely precursor for E2 than androgens via aromatase (Santner et al., 1984). 17β-HSD1 remains an important enzyme for E2 production because it can use E1 as substrate for both aromatase and sulfatase pathways with NADPH as cofactor (Nguyen et al., 1995; Poutanen et al., 1995). Moreover, the expression and activity of 17β-HSD1 are significantly higher in breast cancer than in normal breast tissue (Pasqualini, 2004) and it has been suggested that this higher expression could explain the elevated E2 concentration in breast tumors. 17β-HSD1 is a major player for E1–E2 conversion and cell viability in estrogen-dependent breast cancer cells, particularly in the T47D cell line (Zhang et al., 2014). Epidemiological evidence indicates that most breast cancer risk factors are associated with prolonged exposure of the mammary gland to high levels of estradiol (E2). This potent estrogen plays a crucial role in the development and evolution of hormone-dependent breast cancer. Approximately 60% of premenopausal and 75% of postmenopausal breast cancer patients is hormone dependent (Aka et al., 2012).
17β-HSD1/DHT complex crystals were obtained by soaking the apoenzyme crystals and the complex formation was confirmed after structure determination. The complex structure was solved at 1.7 Å resolution (Aka et al., 2010). Stereo representation showed the H-bond of DHT with the residues His221 in the reverse binding mode, whereas the normal binding mode lacks this H-bond interaction. Distances between DHT, Tyr155 and the cofactor

NADP are different. In the reverse mode, the distance between the O3 of DHT and NC4 of NADP is 4.35 Å and between Tyr155 to NC4 of NADP is 5.4 Å whereas in the normal mode, the distance between the O17 of DHT and NC4 of NADP is 3.75 Å and between Tyr155 to NC4 of NADP is 5.4 Å (Fig. 2). The reduction of DHT into both 3β-diol and 3α-diol by 17β-HSD1 points towards the potential of DHT binding to the enzyme in two orientations. These results strongly support the rationale for inhibiting 17β-HSD1 in breast cancer therapy to eliminate estrogen activation via the sulfatase pathway while avoiding the deprivation of DHT (Aka et al., 2010). It was recently found that 17β-HSD1 increases breast cancer cell migration in spite of its positive regulation of the anti-metastatic gene nm23. This also correlates with its capacity to stimulate breast cancer cell growth, further confirming the necessity of targeting this enzyme in ER-positive breast cancer. These novel findings suggest several directions for future research with regard to the contribution of 17βHSD1 to breast cancer progression and related treatment (Aka et al., 2012).

4. Inhibitor design

The search for inhibitors of 17β-HSDs began in the 1970s and gradually gained momentum thereafter before culminating during the first decade of the 2000s. Significantly more inhibitors are known for the 17β-HSD1 than the other isoforms in the family. Several review articles reported structure–activity relationship studies, which are crucial for drug design and illustrate the huge diversity of 17β- HSD1 inhibitors (Brozic et al., 2008; Day et al., 2008a, b, 2010; Marchais-Oberwinkler et al., 2009; Penning, 1996; Poirier, 2003, 2009, 2010, 2011). Despite many years of research, there are no inhibitors in clinical use to date. This very likely results from the fact that 17β-HSD1 has estrogens as substrates and products such as E2 that also exhibits high affinity towards the estrogen receptor alpha (Huang et al., 2001; Jin and Lin, 1999; Lin et al., 2013). Therefore, the design of inhibitors that are analogues of estrogens makes it difficult to eliminate residual estrogenic activity (Lin et al., 2013; Mazumdar et al., 2009). With the emergence of personalized medicine

and diagnostic tests, the arrival of a potent 17β-HSD1 inhibitor in a clinical setting is highly anticipated to provide a new option for the treatment of women found to have a high expression of 17β-HSD1 and a low expression of aromatase in breast cancer tumor biopsies (Ayan et al., 2012; Maltais et al., 2011). Finally, the use of 17β-HSD1 inhibitors is also a promising approach for the treatment of other estrogen-dependent diseases, such as endometrial cancer (de Cremoux, 2011) and endometriosis (Saloniemi et al., 2010), where the enzyme has been shown to be overexpressed (Maltais et al., 2014).

Studies have shown that steroidal inhibitors preferably bind in the SUB, exhibiting interactions stabilized by hydrophobic contacts and hydrogen bonds with Tyr155/Ser142 and His221/Glu282 residues lining the pocket, whereas non-steroidal inhibitors bind partially to the SUB, but primarily to COF (Negri et al., 2010). However, competitive NMR-experiments suggested that phytoestrogens interact neither with the SUB nor with the COF. The dimer interface of 17β-HSD1 was proposed to be a possible binding site by docking studies (Michiels et al., 2009). Hybrid inhibitors interacting with both steroid and cofactor binding sites resulted in nanomolar binding affinity at the molecular level, based on the available 3D structure of 17β-HSD1 (Qiu et al., 2002; Poirier et al., 2003). Further improvement of the cell penetration is necessary.

4.1.1. Non-steroidal compounds

Inhibitor I (Fig. 3) is a non-steroidal derivative with a pyrimidinone core, which was tested by Solvay Pharmaceuticals. In their animal model, human MCF-7 cells expressing 17β HSD1 were inoculated in nude ovariectomized (OVX) mice and tumors generated in the presence of E1 (0.1 mol/kg/d) were treated for 28 days by subcutaneous (sc) injection with inhibitor I at a dose of 5 mol/kg/d (2.8 mg/kg/d). Since the estrogen-dependent MCF-7 breast cancer cells express different 17β-HSD isoforms (Laplante et al., 2009), the authors stably transfected the HEK293 cells with a plasmid expressing human 17β-HSD1.

Compared to the non-treated controls (in the presence or absence of estrone), inhibitor I reduced tumor weight by 54% and tumor area by 75%. The same group also tested five steroidal inhibitors (estrone derivatives B10721325, B10720511, B10720512, B10720440 and B10715817) in the tumor xenograft model (understanding the effect of inhibitors on estrone-stimulated human cancer cell growth in nude mice) at a dose of 5 mol/kg/d (Husen et al., 2006). Compound B10720511 was more potent than the other analogues and reduced tumor weight by 86%. This compound also showed a dose-dependent effect in this xenograft study with an estimated IC50 of 1.58 mol/kg/d (0.7 mg/kg/d). As an example, the representative compound II (B10721325) reduced tumor weight by 60%. By measuring the uterine weight, the authors also observed that such compounds produced an antiestrogenic effect.

4.1.2. Steroidal compounds

Sterix Ltd. used extensive structure-based drug design with available crystal structures of 17β-HSD1 and developed a family of steroidal inhibitors of 17β-HSD1 and selected compound III (STX1040) as a non-estrogenic candidate to be tested in a xenograft model (Day et al., 2008a; Lawrence et al., 2005). The authors inoculated estrogen dependent human T47D breast cancer cells into nude OVX mice to generate tumors that could be stimulated by E1. Although T47D cells express additional 17β-HSDs, such as types 7 and 12, it was demonstrated in vitro that 17β-HSD1 is responsible for transforming all E1 to E2 (Poirier, 2009; Laplante et al., 2009). Breast tumor growth in T47D cells was stimulated by E1 injection (0.05 or 0.1 μg E1/mouse/d) for 35 days. Subsequently after 35 days, animals that showed response to E1 dosing were provided with an additional dose of 20 mg/kg/day STX1040 daily for 28 days. STX1040 significantly inhibited E1 stimulated T47D cell proliferation and decreased tumor volumes. STX1040 also decreased the plasma concentration of E2 in the xenograft experiments and the authors determined that it did not work via ER antagonism (antiestrogen).

The last steroidal inhibitor of 17β-HSD1, compound IV (PBRM) has distinct mechanism of action, differing from the others. By replacing the phenolic-OH of E2 by a bromoethyl group and adding a characteristic carbamoyl benzyl side chain, the authors obtained a non-estrogenic compound that inhibited the enzyme (Maltais et al., 2011). This compound exhibited no binding to the ER, with no antiestrogenic function. The structure activity relationship study provided a new potent and steroidal nonestrogenic inhibitor of 17β-HSD1 named 3-{[(16β,17β)-3-(2-bromoethyl)-17-hydroxyestra-1(10),2,4-trien-16yl] methyl} benzamide (23b). This compound specifically inhibited the transformation of E1 into E2 by 17β-HSD1 in T-47D cells (IC50 =83 nM) with no effect on 17β-HSD2, 17β-HSD7, 17β-HSD12, or CYP3A4 and did not stimulate the proliferation of estrogen-sensitive MCF-7 cells. Compound 23b is a competitive and irreversible inhibitor of 17β-HSD1 (Ayan et al., 2012; Maltais et al., 2014), compound IV (10 mg/kg/d, sc) completely blocked tumor growth stimulated by E1(0.1 g/mouse/d, sc) comparable to that of the control group level (without E1) (Lin et al., 2013 A compound 6-(3-hydroxyphenyl) naphthalene-2-ol (Compound 5 in Frotscher et al., 2008) was identified as a highly active inhibitor of 17β-HSD1 showing good selectivity towards 17β-HSD2, ERα and ERβ. Furthermore it displays a medium Caco-2 permeability, reasonable metabolic stability and low inhibition of the most important hepatic CYP enzymes. This compound will be used as a primary lead in subsequent drug design process (Frotscher et al., 2008).

Recently, it is reported that 6-hydroxybenzothiazole ketones as a new class of 17β-HSD1 inhibitors with a notable activity/selectivity profile (Miralinaghi et al., 2014). They modified the benzothiazole core by a systematic bioisosteric replacement for the purpose of further optimizing parameters. Thus, they identified a new 6-hydroxybenzothiophene derivative that displayed stronger inhibition of 17β-HSD1 (IC50 =13 nM) with higher selectivity than a benzothiazole analogue. Another study focused on rational structural modifications to this compound class with the aim of gaining more insight into its

structure–activity relationship (SAR). (4-Hydroxyphenyl)-(5-(3-hydroxyphenylsulfanyl)-thiophen-2-yl) methanone was discovered as a member of a novel potent class of human 17β-HSD1 inhibitors. Computational methods were used to elucidate its interactions with the target protein. The compound also showed activity towards the murine 17β-HSD1 enzyme and is thus a starting point for the design of compounds suitable for evaluation in an animal disease model (Abdelsamie et al., 2014).

5. Some disease-related mutations

Certain mutations in the 17β-HSD family are related to disease. 17β-HSD1 polymorphisms were investigated for 16 different indications, most of which deal with breast cancer (8 studies) (Sasano et al., 2008; Subramanian et al., 2008; Suzuki et al., 2007). Three of these eight breast cancer studies have some direct associations with 17β-HSD1 SNPs (Single-nucleotide polymorphism). In multiethnic women from the US (Feigelson et al., 2001) and in Malaysian women (Wu et al., 2003) the A-allele of the SNP rs605059 (A/G: Gly312Ser) was claimed to be of high-risk. However, this observation was not repeated by the same author (Feigelson et al., 2006). In one study the AA allele in SNP rs605059 correlated with higher serum estradiol concentrations in lean women (Setiawan et al., 2004), and in another study a 12 bp deletion in the 5′ flanking area of 17β-HSD1 was only shown to influence the recurrence rate of breast cancer (Kristensen et al., 2001). AG- and AA-alleles of SNP rs605059 (A/G: S312G) in 17β-HSD1 in Chinese women seem to relate to endometrial cancer, but there is no comparable situation in US women (Setiawan et al., 2004). Conversely, the A-allele has a higher risk of endometrosis in Japanese women.

Surprisingly, 17β-HSD1 polymorphisms might play a role in prostate cancer risk prediction. In a study with a large number of multiethnic men no overall association of haplotypes of four common SNPs in 17β-HSD1 rs676387 (C/A), rs605059 (A/G), rs598126 (G/A), rs2010750 (C/T)) with prostate cancer were observed; however, two

subgroups, Latinos and Japanese Americans, with the CAGC haplotype had a lower prostate cancer risk (Kraft et al., 2005). In non-Hispanic Caucasian men the minor allele of 17β-HSD1 SNP rs605059 (A/G) was more frequent among sporadic prostate cancer cases than among controls, but no statistically significant association could be detected (Cunningham et al., 2007). An Australian ovarian cancer study with patients and controls of Caucasian origin showed no association between ovarian cancer and 17β-HSD1 or 17β-HSD4 polymorphisms (Beesley et al., 2007). In addition to cancer, 17β-HSD1 polymorphisms were found to be related to other phenotypes including vasomotor symptoms (VMS) (Crandall et al., 2006), depression and some cognitive function in Chinese women (Kravitz et al., 2006). One study analyzed the association of three 17β-HSD1 SNPs, rs2830 (A/G), rs592389 (T/G), and rs615942 (G/T), with metabolic syndrome and diabetes in a group of multiethnic women (Lo et al., 2006). The likelihood of having diabetes among Caucasian women who are homozygous for the 17β-HSD1 polymorphisms is 4- to 7-fold greater compared with women who are heterozygous for these SNPs. On the other hand, the three 17β-HSD1 gene polymorphisms were not associated with metabolic syndrome in any racial/ethnic group (Lo et al., 2006).

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References

Abdelsamie, A.S., Bey, E., Hanke, N., Empting, M., Hartmann, R.W., Frotscher, M., 2014. Inhibition of 17β-HSD1: SAR of bicyclic substituted hydroxyphenylmethanones and discovery of new potent inhibitors with thioether linker. Eur. J. Med. Chem. 82, 394–406. http://dx.doi.org/10.1016/j.ejmech.2014.05.074 (Jul 23).

Aka, J.A., Mazumdar, M., Chen, C.Q., Poirier, D., Lin, S.X., 2010. 17beta-hydroxysteroid dehydrogenase type 1 stimulates breast cancer by dihydrotestosterone inactivation in addition to estradiol production. Mol. Endocrinol. 24 (4), 832–845. http://dx.doi.org/10.1210/me.2009-0468 (Apr).

Aka, J.A.,Mazumdar, M., Lin, S.X., 2009. Reductive 17beta-hydroxysteroid dehydrogenases in the sulfatase pathway: critical in the cell proliferation of breast cancer. Mol. Cell. Endocrinol. 301 (1–2), 183–190. http://dx.doi.org/10.1016/j.mce.2008.10.042 (Mar 25).

Aka, J.A., Zerradi, M., Houle, F., Huot, J., Lin, S.X., 2012. 17beta-hydroxysteroid dehydrogenase type 1 modulates breast cancer protein profile and impacts cell migration. Breast Cancer Res. 14 (3), R92 (Jun 12).

Alho-Richmond, S., Lilienkampf, A., Wähälä, K., 2006. Active site analysis of 17beta-hydroxysteroid dehydrogenase type 1 enzyme complexes with SPROUT. Mol. Cell. Endocrinol. 248 (1–2), 208–213 (Mar 27).

Ayan, D., Maltais, R., Roy, J., Poirier, D., 2012. A new nonestrogenic steroidal inhibitor of 17β-hydroxysteroid dehydrogenase type I blocks the estrogen-dependent breast cancer tumor growth induced by estrone. Mol. Cancer Ther. 11 (10), 2096–2104. http://dx.doi.org/10.1158/1535-7163.MCT-12-0299 (Oct).

Azzi, A., Rehse, P.H., Zhu, D.W., Campbell, R.L., Labrie, F., Lin, S.X., 1996. Crystal structure of human estrogenic 17β-hydroxysteroid dehydrogenase complexed with 17β-estradiol. Nat. Struct. Biol. 3 (8), 665–668. http://dx.doi.org/10.1038/nsb0896-665.

Batzl, C., Hausler, A., Schieweck, K., et al., 1996. Pharmacology of nonsteroidal aromatase inhibitors. In: Pasqualini, J., Katzenellenbogen, B. (Eds.), Hormone-dependent Cancer. Marcel Dekker, New York, pp. 155–168.

Beesley, J., Jordan, S.J., Spurdle, A.B., Song, H., Ramus, S.J., Kjaer, S.K., Hogdall, E., DiCioccio, R.A., McGuire, V., Whittemore, A.S., Gayther, S.A., Pharoah, P.D., Webb, P.M., Chenevix-Trench, G., 2007. Australian Ovarian Cancer Study Group; Australian Cancer Study (Ovarian Cancer); Australian Breast Cancer Family Study. Association

between single-nucleotide polymorphisms in hormone metabolism and DNA repair genes and epithelial ovarian cancer: results from two Australian studies and an additional validation set. Cancer Epidemiol. Biomark. Prev. 16 (12), 2557–2565 (Dec).

Bey, E., Marchais-Oberwinkler, S., Negri, M., Kruchten, P., Oster, A., Klein, T., Spadaro, A., Werth, R., Frotscher, M., Birk, B., Hartmann, R.W., 2009. New insights into the SAR and binding modes of bis(hydroxyphenyl)thiophenes and -benzenes: influence of additional substituents on 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) inhibitory activity and selectivity. J. Med. Chem. 52 (21), 6724–6743. http://dx.doi.org/10.1021/jm901195w (Nov 12).

Breton, R., Housset, D., Mazza, C., Fontecilla-Camps, J.C., 1996. The structure of a complex of human 17beta-hydroxysteroid dehydrogenase with estradiol and NADP+ identifies two principal targets for the design of inhibitors. Structure 4, 905–915.

Brozic, P., Lanisnik Risner, T., Gobec, S., 2008. Inhibitors of 17beta-hydroxysteroid dehydrogenase type 1. Curr. Med. Chem. 15 (2), 137–150.

Buehner, M., Ford, G.C.,Moras, D., Olsen, K.W., Rossman, M.G., 1973. D-glyceraldehyde-3-phosphate dehydrogenase: three-dimensional structure and evolutionary significance.Proc. Natl. Acad. Sci. U. S. A. 70 (11), 3052–3054 (Nov).

Castoria, G., Migliaccio, A., Giovannelli, P., Auricchio, F., 2010. Cell proliferation regulated by estradiol receptor: therapeutic implications. Steroids 75 (8–9), 524–527. http://dx.doi.org/10.1016/j.steroids.2009.10.007 (Aug-Sep).

Cicatiello, L., Addeo, R., Sasso, A., Altucci, L., Petrizzi, V.B., Borgo, R., Cancemi, M., Caporali, S., Caristi, S., Scafoglio, C., Teti, D., Bresciani, F., Perillo, B., Weisz, A., 2004. Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol. Cell. Biol. 24 (16), 7260–7274 (Aug).

Crandall, C.J., Crawford, S.L., Gold, E.B., 2006. Vasomotor symptom prevalence is associated with polymorphisms in sex steroid-metabolizing enzymes and receptors. Am. J. Med. 119 (9 Suppl 1), S52–S60 (Sep).

Cunningham, J.M., Hebbring, S.J., McDonnell, S.K., Cicek, M.S., Christensen, G.B., Wang, L., Jacobsen, S.J., Cerhan, J.R., Blute, M.L., Schaid, D.J., Thibodeau, S.N., 2007. Evaluation of genetic variations in the androgen and estrogen metabolic pathways as risk

factors for sporadic and familial prostate cancer. Cancer Epidemiol. Biomark. Prev. 16 (5), 969–978 (May).

Day, J.M., Foster, P.A., Tutill, H.J., Parsons, M.F., Newman, S.P., Chander, S.K., Allan, G.M., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J., Purohit, A., 2008a. 17beta-hydroxysteroid dehydrogenase type 1, and not type 12, is a target for endocrine therapy of hormone-dependent breast cancer. Int. J. Cancer 122 (9), 1931–1940. http://dx.doi.org/10.1002/ijc.23350 (May 1).

Day, J.M., Tutill, H.J., Purohit, A., Reed, M.J., 2008b. Design and validation of specific inhibitors Miyoshi, Y., Ando, A., Shiba, E., Taguchi, T., Tamaki, Y., Noguchi, S., 2001. Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. Int. J. Cancer 94 (5), 685–689 (Dec 1).

Day, J.M., Tutill, H.J., Purohit, A., 2010. 17ß-hydroxysteroid dehydrogenase inhibitors. Minerva Endocrinol. 35 (2), 87–108.

de Cremoux, P., 2011. Hormone therapy and breast cancer. Bull. Cancer 98 (11), 1311–1319. http://dx.doi.org/10.1684/bdc.2011.1466 (Nov).

Feigelson, H.S., McKean-Cowdin, R., Coetzee, G.A., Stram, D.O., Kolonel, L.N., Henderson, B.E., 2001. Building a multigenic model of breast cancer susceptibility: CYP17 and HSD17B1 are two important candidates. Cancer Res. 61 (2), 785–789 (Jan 15).

Filling, C., Berndt, K.D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jörnvall, H., Oppermann, U., 2002. Critical residues for structure and catalysis in shortchain dehydrogenases/reductases. J. Biol. Chem. 277 (28), 25677–25684 (Jul 12).

Frotscher, M., Ziegler, E., Marchais-Oberwinkler, S., Kruchten, P., Neugebauer, A., Fetzer, L., Scherer, C.,Müller-Vieira, U., Messinger, J., Thole, H., Hartmann, R.W., 2008. Design, synthesis, and biological evaluation of (hydroxyphenyl)naphthalene and -quinoline derivatives: potent and selective nonsteroidal inhibitors of 17beta-hydroxysteroid dehydrogenase type 1 (17beta-HSD1) for the treatment of estrogen-dependent diseases. J. Med. Chem. 51 (7), 2158–2169. http://dx.doi.org/10.1021/jm701447v (Apr 10).

Gangloff, A., Shi, R., Nahoum, V., Lin, S.X., 2003. Pseudo-symmetry of C19 steroids, alternative binding orientations, and multispecificity in human estrogenic 17beta-hydroxysteroid dehydrogenase. FASEB J. 17 (2), 274–276 (Feb).

Ghosh, D., Pletnev, V.Z., Zhu, D.W., Wawrzak, Z., Duax, W.L., Pangborn, W., Labrie, F., Lin, S.X., 1995a. Structure of human estrogenic 17 beta-hydroxysteroid dehydrogenase at 2.20 A resolution. Structure 3 (5), 503–513 (May 15).

Ghosh, D., Wawrzak, Z., Pletnev, V.Z., Erman, M., Duax, W.L., Pangborn, W., Zhu, D.W. Labrie, F., Lin, S.X., 1995b. Molecular mechanism of inhibition of steroid dehydrogenases by licorice-derived steroid analogs in modulation of steroid receptor function. Ann. N. Y. Acad. Sci. 761, 341–343.

Ghosh, D., Vihko, P., 2001. Molecular mechanisms of estrogen recognition and 17-keto reduction by human 17beta-hydroxysteroid dehydrogenase 1. Chem. Biol. Interact. 130–132 (1–3), 637–650 (Jan 30).

Hartmann, R.W., Adamski, J., 2011. 17β-Hydroxysteroid dehydrogenases (17β-HSDs) as therapeutic targets: protein structures, functions, and recent progress in inhibitor development. J. Steroid Biochem. Mol. Biol. 125 (1–2), 66–82. http://dx.doi.org/10. 1016/j.jsbmb.2010.12.013 (May).

Hershberger, P.A., Vasquez, A.C., Kanterewicz, B., Land, S., Siegfried, J.M., Nichols, M., 2005. Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands. Cancer Res. 65 (4), 1598–1605 (Feb 15).

Huang, Y.W., Pineau, I., Chang, H.J., Azzi, A., Bellemare, V., Laberge, S., Lin, S.X., 2001. Critical residues for the specificity of cofactors and substrates in human estrogenic 17beta-hydroxysteroid dehydrogenase 1: variants designed from the three-dimensional structure of the enzyme. Mol. Endocrinol. 15 (11), 2010–2020 (Nov).

Husen, B., Huhtinen, K., Poutanen, M., Kangas, L., Messinger, J., Thole, H., 2006. Evaluation of inhibitors for 17beta-hydroxysteroid dehydrogenase type 1 in vivo in immunodeficient mice inoculated with MCF-7 cells stably expressing the recombinant human enzyme. Mol. Cell. Endocrinol. 248 (1–2), 109–113 (Mar 27).

Hwang, C.C., Chang, Y.H., Hsu, C.N., Hsu, H.H., Li, C.W., Pon, H.I., 2005. Mechanistic roles of Ser-114, Tyr-155, and Lys-159 in 3alpha-hydroxysteroid dehydrogenase/ carbonyl reductase from Comamonas testosteroni. J. Biol. Chem. 280 (5), 3522–3528 (Feb 4).

Jansson, A., 2009. 17Beta-hydroxysteroid dehydrogenase enzymes and breast cancer. J. Steroid Biochem. Mol. Biol. 114 (1–2), 64–67. http://dx.doi.org/10.1016/j.jsbmb. 2008.12.012 (Mar).

Jin, J.Z., Lin, S.X., 1999. Human estrogenic 17beta-hydroxysteroid dehydrogenase: predominance of estrone reduction and its induction by NADPH. Biochem. Biophys. Res. Commun. 259 (2), 489–493 (Jun 7).

Karavolas, H.J., Baedecker, M.L., Engel, L.L., 1970. Human placental 17 beta-estradiol dehydrogenase. V. Purification and partial characterization of the diphosphopyridine nucleotide (triphosphopyridine nucleotide)-linked enzyme. J. Biol. Chem. 245 (19), 4948–4952 (Oct 10).

Kraft, P., Pharoah, P., Chanock, S.J., Albanes, D., Kolonel, L.N., Hayes, R.B., Altshuler, D., Andriole, G., Berg, C., Boeing, H., Burtt, N.P., Bueno-de-Mesquita, B., Calle, E.E., Cann, H., Canzian, F., Chen, Y.C., Crawford, D.E., Dunning, A.M., Feigelson, H.S., Freedman, M.L., Gaziano, J.M., Giovannucci, E., Gonzalez, C.A., Haiman, C.A., Hallmans, G., Henderson, B.E., Hirschhorn, J.N., Hunter, D.J., Kaaks, R., Key, T., Le Marchand, L., Ma J., Overvad, K., Palli, D., Pike, M.C., Riboli, E., Rodriguez, C., Setiawan, W.V., Stampfer, M.J., Stram, D.O., Thomas, G., Thun, M.J., Travis, R., Trichopoulou, A., Virtamo, J., Wacholder, S., 2005. Genetic variation in the HSD17B1 gene and risk of prostate cancer. PLoS Genet. 1 (5), e68 (Nov).

Kravitz, H.M., Meyer, P.M., Seeman, T.E., Greendale, G.A., Sowers, M.R., 2006. Cognitive functioning and sex steroid hormone gene polymorphisms in women at midlife. Am. J. Med. 119 (9 Suppl 1), S94–S102 (Sep).

Kristensen, V.N., Kure, E.H., Erikstein, B., Harada, N., Børresen-Dale, A., 2001. Genetic susceptibility and environmental estrogen-like compounds. Mutat. Res. 482 (1–2), 77–82 (Oct 1).

Labrie, F., Luu-The, V., Lin, S.X., Simard, J., Labrie, C., El-Alfy, M., Pelletier, G., Bélanger, A., 2000. Intracrinology: role of the family of 17 beta-hydroxysteroid dehydrogenases in human physiology and disease. J. Mol. Endocrinol. 25 (1), 1–16 (Aug).

Laganière, J., Deblois, G., Lefebvre, C., Bataille, A.R., Robert, F., Giguère, V., 2005. From the cover: location analysis of estrogen receptor alpha target promoters reveals that FOXA1 defines a domain of the estrogen response. Proc. Natl. Acad. Sci. U. S. A. 102 (33), 11651–11656 (Aug 16).

Langer, L.J., Engel, L.L., 1958. Human placental estradiol-17 beta dehydrogenase. I. Concentration, characterization and assay. J. Biol. Chem. 233 (3), 583–588 (Sep).

Laplante, Y., Rancourt, C., Poirier, D., 2009. Relative involvement of three 17beta-hydroxysteroid dehydrogenases (types 1, 7 and 12) in the formation of estradiol in

various breast cancer cell lines using selective inhibitors. Mol. Cell. Endocrinol. 301 (1–2), 146–153. http://dx.doi.org/10.1016/j.mce.2008.08.026 (Mar 25).

Lawrence, H.R., Vicker, N., Allan, G.M., Smith, A., Mahon, M.F., Tutill, H.J., Purohit, A., Reed, M.J., Potter, B.V., 2005. Novel and potent 17beta-hydroxysteroid dehydrogenase type 1 inhibitors. J. Med. Chem. 48 (8), 2759–2762 (Apr 21).

Li, Y., Isomaa, V., Pulkka, A., Herva, R., Peltoketo, H., Vihko, P., 2005. Expression of 3beta-hydroxysteroid dehydrogenase type 1, P450 aromatase, and 17beta-hydroxysteroid dehydrogenase types 1, 2, 5 and 7 mRNAs in human early and mid-gestation placentas. Placenta 26 (5), 387–392 (May).

Li, Y., Qin, L., Xiao, Z.J.,Wang, Y.L., Herva, R., Leng, J.H., Lang, J.H., Isomaa, V., Piao, Y.S., 2003. Expression of P450 aromatase and 17β-hydroxysteroid dehydrogenase type 1 at fetal-maternal interface during tubal pregnancy. J. Steroid Biochem. Mol. Biol. 87, 241–246. http://dx.doi.org/10.1016/j.jsbmb.2003.09.013.

Lin, S.X., Han, Q., Azzi, A., Zhu, D.W., Gangloff, A., Campbell, R.L., 1999. 3D-structure of human estrogenic 17beta-HSD1: binding with various steroids. J. Steroid Biochem. Mol. Biol. 69, 425–429.

Lin, S.X., Poirier, D., Adamski, J., 2013. A challenge for medicinal chemistry by the 17β-hydroxysteroid dehydrogenase superfamily: an integrated biological function and inhibition study. Curr. Top. Med. Chem. 13 (10), 1164–1171.

Lin, S.X., Yang, F., Jin, J.Z., Breton, R., Zhu, D.W., Luu-The, V., Labrie, F., 1992. Subunit identity of the dimeric 17 beta-hydroxysteroid dehydrogenase from human placenta. J. Biol. Chem. 267 (23), 16182–16187 (Aug 15).

Lin, S.X., Zhu, D.W., Azzi, A., Campbell, R.L., Breton, R., Labrie, F., Ghosh, D., Pletnev, V., Duax, W.L., Pangborn, W., 1996. Studies on the three-dimensional structure of estrogenic 17beta-hydroxysteroid dehydrogenase. J. Endocrinol. 150 (Suppl), S13–S20 (Sep).

Lo, J.C., Zhao, X., Scuteri, A., Brockwell, S., Sowers, M.R., 2006. The association of genetic polymorphisms in sex hormone biosynthesis and action with insulin sensitivity and diabetes mellitus in women at midlife. Am. J. Med. 119 (9 Suppl 1), S69–S78 (Sep).

Lukacik, P., Kavanagh, K.L., Oppermann, U., 2006. Structure and function of human 17beta-hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 248 (1–2), 61–71 (Mar 27).

Luu-The, V., Labrie, C., Zhao, H.F., Couët, J., Lachance, Y., Simard, J., Leblanc, G., Côté, J., Bérubé, D., Gagné, R., Labrie, F., 1989. Characterization of cDNAs for human estradiol 17 beta-dehydrogenase and assignment of the gene to chromosome 17: evidence of two mRNA species with distinct 5′-termini in human placenta. Mol. Endocrinol. 3 (8), 1301–1309 (Aug).

MacIndoe, J.H., 1988. The hydrolysis of estrone sulfate and dehydroepiandrosterone sulfate by MCF-7 human breast cancer cells. Endocrinology 123 (3), 1281–1287 (Sep).

Mäentausta, O., Sormunen, R., Isomaa, V., Lehto, V.P., Jouppila, P., Vihko, R., 1991. Immunohistochemical localization of 17 beta-hydroxysteroid dehydrogenase in the human endometrium during the menstrual cycle. Lab. Investig. 65 (5), 582–587 (Nov).

Maltais, R., Ayan, D., Poirier, D., 2011. Crucial role of 3-bromoethyl in removing the estrogenic activity of 17β-HSD1 inhibitor 16β-(m-carbamoylbenzyl)estradiol. ACS Med. Chem. Lett. 2 (9), 678–681. http://dx.doi.org/10.1021/ml200093v (Sep 8).

Maltais, R., Ayan, D., Trottier, A., Barbeau, X., Lagüe, P., Bouchard, J.E., Poirier, D., 2014. Discovery of a non-estrogenic irreversible inhibitor of 17β-hydroxysteroid dehydrogenase type 1 from 3-substituted-16β-(m-carbamoylbenzyl)-estradiol derivatives. J. Med. Chem. 57 (1), 204–222. http://dx.doi.org/10.1021/jm401639v (Jan 9).

Marchais-Oberwinkler, S., Henn, C., Möller, G., Klein, T., Negri, M., Oster, A., Spadaro, A., Werth, R., Wetzel, M., Xu, K., Frotscher, M., Mazumdar, M., Fournier, D., Zhu, D.W., Cadot, C., Poirier, D., Lin, S.X., 2009. Binary and ternary crystal structure analyses of a novel inhibitor of 17β-HSD type1: a lead compound for breast cancer therapy. Biochem. J. 424, 357–366.

Mazumdar,M., Fournier, D., Zhu, D.W., Cadot, C., Poirier, D., Lin, S.X., 2009. Binary and ternary crystal structure analyses of a novel inhibitor of 17β-HSD type1: a lead compound for breast cancer therapy. Biochem. J. 424, 357–366.

Michiels, P.J., Ludwig, C., Stephan, M., Fischer, C., Möller, G., Messinger, J., van Dongen, M., Thole, H., Adamski, J., Günther, U.L., 2009. Ligand-based NMR spectra demonstrate an additional phytoestrogen binding site for 17beta-hydroxysteroid dehydrogenase type 1. J. Steroid Biochem. Mol. Biol. 117 (4–5), 93–98. http://dx.doi.org/10.1016/j. jsbmb.2009.07.004 (Nov).

Miralinaghi, P., Schmitt, C., Hartmann, R.W., Frotscher, M., Engel, M., 2014. 6-Hydroxybenzothiophene ketones: potent inhibitors of 17β-hydroxysteroid dehydrogenase

type 1 (17β-HSD1) owing to favorable molecule geometry and conformational preorganization. ChemMedChem 9 (10), 2294–2308. http://dx.doi.org/10.1002/cmdc.201402050 (Oct).

Miyoshi, Y., Ando, A., Shiba, E., Taguchi, T., Tamaki, Y., Noguchi, S., 2001. Involvement of up-regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high estradiol levels in postmenopausal breast cancers. Int. J. Cancer 94 (5), 685–689 (Dec 1).

Negri, M., Recanatini, M., Hartmann, R.W., 2010. Insights in 17beta-HSD1 enzyme kinetics and ligand binding by dynamicmotion investigation. PLoS ONE 5 (8), e12026. http://dx.doi.org/10.1371/journal.pone.0012026 (Aug 10).

Nguyen, B.L., Chetrite, G., Pasqualini, J.R., 1995. Transformation of estrone and estradiol in hormone-dependent and hormone-independent human breast cancer cells. Effects of the antiestrogen ICI 164,384, danazol, and promegestone (R-5020). Breast Cancer Res. Treat. 34 (2), 139–146 (May).

Pasqualini, J.R1., Chetrite, G.S., 2005. Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. J. Steroid Biochem. Mol. Biol. 93 (2–5), 221–236 (Feb).

Pasqualini, J.R., Chetrite, G., Blacker, C., Feinstein,M.C., Delalonde, L., Talbi,M., Maloche, C., 1996. Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J. Clin. Endocrinol. Metab. 81 (4), 1460–1464 (Apr).

Pasqualini, J.R., Gelly, C., Nguyen, B.L., Vella, C., 1989. Importance of estrogen sulfates in breast cancer. J. Steroid Biochem. 34 (1–6), 155–163.

Pasqualini, J.R., 2004. The selective estrogen enzyme modulators in breast cancer: a review. Biochim. Biophys. Acta 1654 (2), 123–143 (Jun 7).

Peltoketo, H., Isomaa, V., Mäentausta, O., Vihko, R., 1988. Complete amino acid sequence of human placental 17 beta-hydroxysteroid dehydrogenase deduced from cDNA. FEBS Lett. 239 (1), 73–77 (Oct 24).

Penning, T.M., 1996. 17β-hydroxysteroid dehydrogenase: inhibitors and inhibitors design. Endocr. Relat. Cancer 3, 41–56. http://dx.doi.org/10.1677/erc.0.0030041.

Penning, T.M., 1997. Molecular endocrinology of hydroxysteroid dehydrogenases. Endocr.

Rev. 18 (3), 281–305 (Jun).

Poirier, D., 2010. 17beta-hydroxysteroid dehydrogenase inhibitors: a patent review. Expert Opin. Ther. Pat. 20 (9), 1123–1145. http://dx.doi.org/10.1517/13543776.2010. 505604 (Sep).

Poirier, D., 2009. Advances in development of inhibitors of 17beta hydroxysteroid dehydrogenases. Anti Cancer Agents Med. Chem. 9 (6), 642–660 (Jul).

Poirier, D., 2011. Contribution to the development of inhibitors of 17β-hydroxysteroid dehydrogenase types 1 and 7: key tools for studying and treating estrogen-dependent diseases. J. Steroid Biochem. Mol. Biol. 125 (1–2), 83–94. http://dx.doi.org/10.1016/ j. jsbmb.2010.12.007 (May).

Poirier, D., 2003. Inhibitors of 17 beta-hydroxysteroid dehydrogenases. Curr. Med. Chem. 10 (6), 453–477 (Mar).

Poirier, D., Boivin, R.P., Bérabé, M., Lin, S.X., 2003. Synthesis of a First Estradiol-Adenosine Hybrid Compound. Synth. Commun. 33, 3183–3192.

Poutanen, M., Isomaa, V., Peltoketo, H., Vihko, R., 1995. Role of 17 beta-hydroxysteroid dehydrogenase type 1 in endocrine and intracrine estradiol biosynthesis. J. Steroid Biochem. Mol. Biol. 55 (5–6), 525–532 (Dec).

Puranen, T., Poutanen, M., Peltoketo, H., Vihko, P., Vihko, R.K., 1994. Site-directed mutagenesis of the putative active site of human 17β-hydroxysteroid dehydrogenase type 1. Biochem. J. 304 (Pt 1), 289–293 (Nov 15).

Qiu, W., Campbell, R.L., Gangloff, A., Dupuis, P., Boivin, P., Tremblay, M.R., Poirier, D., Lin, S.X., 2002. A concerted, rational design of 17beta-hydroxysteroid dehydrogenase inhibitors: complex structure with estradiol-adenosine hybrids with high affinity. FASEB J. 16, 1829–1831.

Saloniemi, T., Järvensivu, P., Koskimies, P., Jokela, H., Lamminen, T., Ghaem-Maghami, S., Dina, R., Damdimopoulou, P., Mäkelä, S., Perheentupa, A., Kujari, H., Brosens, J., Poutanen, M., 2010. Novel hydroxysteroid (17beta) dehydrogenase 1 inhibitors reverse estrogen-induced endometrial hyperplasia in transgenic mice. Am. J. Pathol. 176 (3), 1443–1451. http://dx.doi.org/10.2353/ajpath.2010.090325 (Mar).

Santner, S.J., Feil, P.D., Santen, R.J., 1984. In situ estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway. J. Clin. Endocrinol. Metab. 59 (1), 29–33 (Jul).

Sasano, H., Suzuki, T.,Miki, Y.,Moriya, T., 2008. Intracrinology of estrogens and androgens in breast carcinoma. J. Steroid Biochem. Mol. Biol. 108 (3–5), 181–185 (Feb).

Schwabe, I., Husen, B., Einspanier, A., 2001. Expression of the estradiol-synthesizing 17beta-hydroxysteroid dehydrogenases type 1 and type 7 in the nonhuman primate Callithrix jacchus. Mol. Cell. Endocrinol. 171 (1–2), 187–192 (Jan 22).

Setiawan, V.W., Hankinson, S.E., Colditz, G.A., Hunter, D.J., De Vivo, I., 2004. HSD17B1 gene polymorphisms and risk of endometrial and breast cancer. Cancer Epidemiol. Biomark. Prev. 13 (2), 213–219 (Feb).

Sherbet, D.P., Papari-Zareei, M., Khan, N., Sharma, K.K., Brandmaier, A., Rambally, S., Chattopadhyay, A., Andersson, S., Agarwal, A.K., Auchus, R.J., 2007. Cofactors, redox state, and directional preferences of hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 265–266, 83–88 (Feb).

Shi, R., Lin, S.X., 2004. Cofactor hydrogen bonding onto the protein main chain is conserved in the short-chain dehydrogenase/reductase family and contributes to nicotinamide orientation. J. Biol. Chem. 271, 16678–16685.

Subramanian, A., Salhab, M., Mokbel, K., 2008. Oestrogen producing enzymes and mammary carcinogenesis: a review. Breast Cancer Res. Treat. 111 (2), 191–202 (Sep).

Suzuki, T., Miki, Y., Moriya, T., Akahira, J., Hirakawa, H., Ohuchi, N., Sasano, H., 2007. In situ production of sex steroids in human breast carcinoma. Med. Mol. Morphol. 40 (3), 121–127 (Sep).

Takeyama, J., Suzuki, T., Hirasawa, G., Muramatsu, Y., Nagura, H., Iinuma, K., Nakamura, J., Kimura, K.I., Yoshihama, M., Harada, N., Andersson, S., Sasano, H., 2000. 17beta-hydroxysteroid dehydrogenase type 1 and 2 expression in the human fetus. J. Clin. Endocrinol. Metab. 85 (1), 410–416 (Jan).

Verma,M.K.,Miki, Y., Abe, K., Suzuki, T., Niikawa, H., Suzuki, S., Kondo, T., Sasano, H., 2013. Intratumoral localization and activity of 17β-hydroxysteroid dehydrogenase type 1 in non-small cell lung cancer: a potent prognostic factor. J. Transl. Med. 11, 167. http://dx.doi.org/10.1186/1479-5876-11-167 (Jul 9).

Verma, M.K., Miki, Y., Sasano, H., 2011. Sex steroid receptors in human lung diseases. J. Steroid Biochem. Mol. Biol. 127 (3–5), 216–222. http://dx.doi.org/10.1016/j.jsbmb.2011.07.013 (Nov).

Wu, A.H., Seow, A., Arakawa, K., Van Den Berg, D., Lee, H.P., Yu, M.C., 2003. HSD17B1 and CYP17 polymorphisms and breast cancer risk among Chinese women in Singapore. Int. J. Cancer 104 (4), 450–457 (Apr 20).

Yang, F., Zhu, D.W., Wang, J.Y., Lin, S.X., 1992. Rapid purification yielding highly active 17beta-hydroxysteroid dehydrogenase: application of hydrophobic interaction and affinity fast protein liquid chromatography. J. Chromatogr. 582, 71–76.

Zhang, C.Y., Chen, J., Yin, D.C., Lin, S.X., 2012. The contribution of 17beta-hydroxysteroid dehydrogenase type 1 to the estradiol-estrone ratio in estrogen-sensitive breast cancer cells. PLoS ONE 7 (1), e29835. http://dx.doi.org/10.1371/journal.pone.0029835.

Zhang, C.Y., Wang, W.Q., Chen, J., Lin, S.X., 2014. Reductive 17beta-hydroxysteroid dehydrogenases which synthesize estradiol and inactivate dihydrotestosterone constitute major and concerted players in ER+ breast cancer cells. J. Steroid Biochem. Mol. Biol. 150, 24–34. http://dx.doi.org/10.1016/j.jsbmb.2014.09.017.

Zhu, D.W., Lee, X., Breton, R., Ghosh, D., Pangborn,W., Duax,W.L., Lin, S.X., 1993. Crystallization and preliminary X-ray diffraction analysis of the complex of human placental 17beta-hydroxysteroid dehydrogenase with NADP+. J. Mol. Biol. 234, 242–244.

Figures.

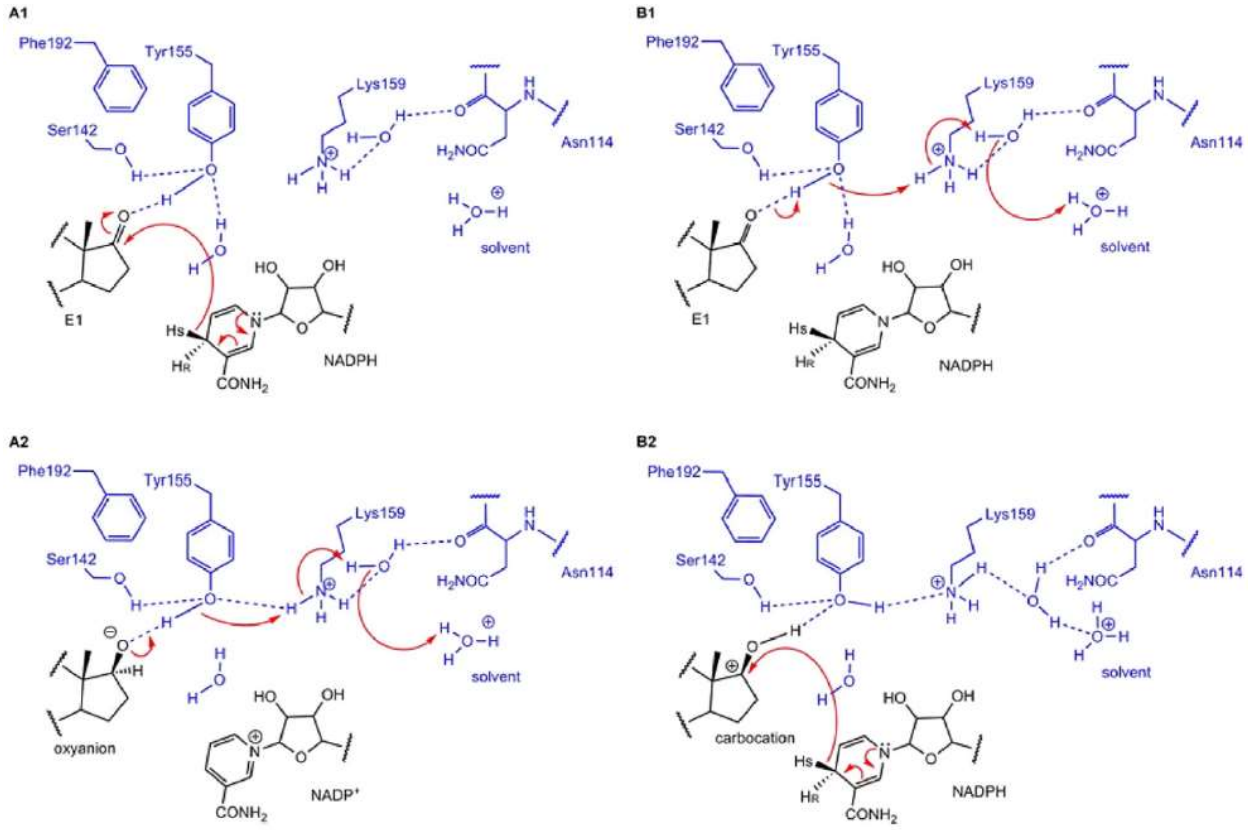

Fig. 1. Two possible stepwise catalytic mechanisms for 17β-HSD1. (A) In the first step the pro-S hydride of NADPH is transferred to theα-face of E1 at the planar C17 carbon (A1), resulting in an energetically favorable aromatic system; the resultant oxyanion is subsequently protonated by the acidic\\OH group of Tyr155 (A2). (B) In the first step the keto oxygen of E1 is protonated by the acidic\\OH of Tyr155 (B1); the resultant carbocation then accepts the pro-S hydride of NADPH at the α-face (B2). The proton relay is facilitated by a H-bond network involving Lys159, two water molecules and Asn114, an electrostatic interaction between the protonated side chain of Lys159 and the phenyl ring of Tyr155 (Ghosh and Vihko, 2001) as well as T-stacking between Phe192 and Tyr155 (Negri et al., 2010). Hydrogen bonds are represented by dashed lines. For the sake of clarity π–π-interactions are not depicted.

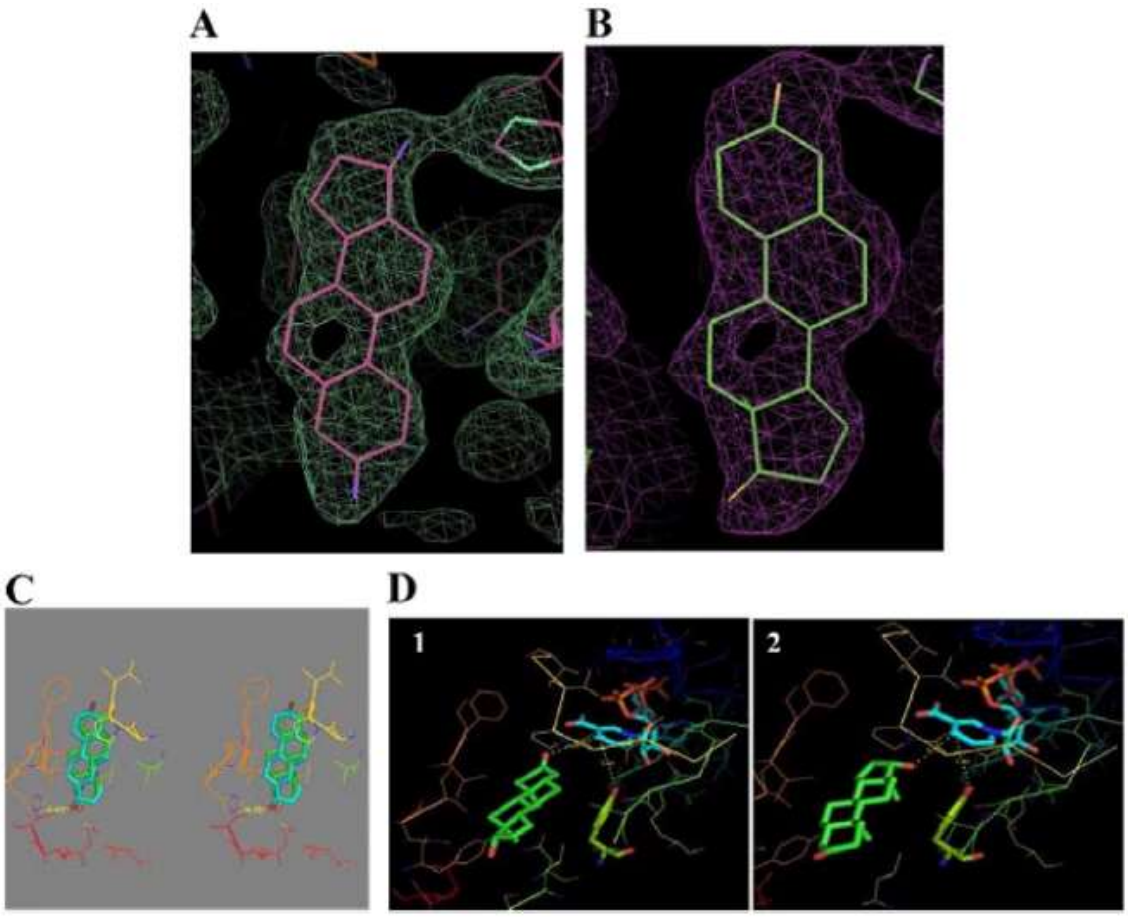

Fig. 2. Crystal complex structure of 17β-HSD1/DHT. A and B, Electronic density of DHT for 2Fo-Fc map seen at 0.8σ cutoff in reverse binding mode (A) and normal binding mode (B). C, Stereo representation showing the H-bond of DHT with the residue His221 in the reverse binding mode (DHT represented in blue), whereas in the normal binding mode, there is no H-bond interaction present (DHT in green). D, Distances between DHT, Tyr155, and the cofactor NADP in 1) reverse mode (the distance between the O3 of DHT with NC4 of NADP is 4.35 Å and between Tyr155 toNC4 of NADP is 5.4 Å) and in 2) normal mode (the distance between theO17 of DHT with NC4 of NADP is 3.75 Å and between Tyr155 to NC4 of NADP is 5.4 Å). Note that the final model of the complex has been submitted to the protein data bank with PDB code 3KLM (Aka et al., 2010).

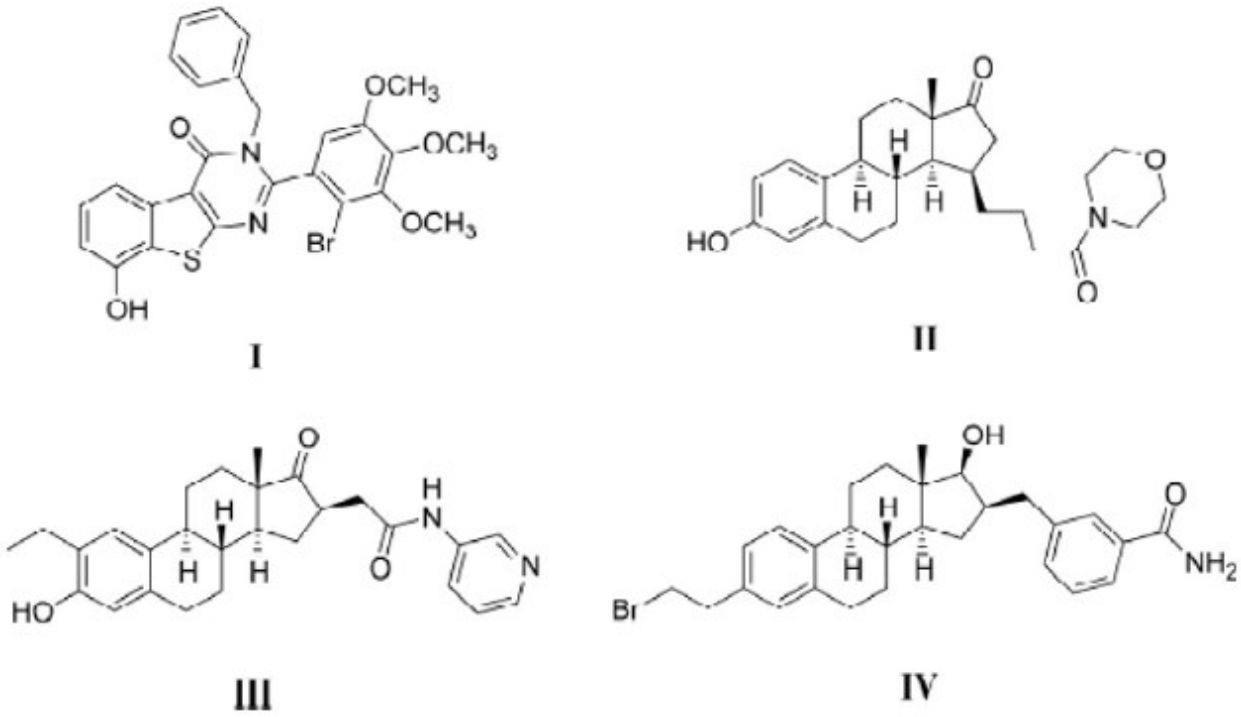

Fig. 3. Representative inhibitors of 17β-HSD1, which demonstrated efficacy in reducing estrogen-dependent breast tumors in vivo (animal models). Cited from Lin et al., 2013.

ARTICLE 2.

17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation

RÉSUMÉ

La 17beta-hydroxystéroïde déshydrogénase type 5 est corrélée négativement à l'inhibiteur d'apoptose GRP78 et à la protéine sécrétée par une tumeur PGK1, et module la viabilité et la prolifération des cellules cancéreuses du sein

La 17beta-hydroxystéroïde déshydrogénase de type 5 (17β-HSD5) est une enzyme cruciale associée au métabolisme des stéroïdes sexuels. Dans la littérature, ses niveaux d'expression et sa valeur pronostique pour le cancer du sein (BC) sont incohérents. Nous avons démontré une plus faible expression de la 17β-HSD5 dans les tissus de BC en comparaison avec des tissus normaux. Les profils de protéome des cellules de BC ER+ de 17β-HSD5-knockdown dans les cellules MCF-7 ont été comparés à ceux des cellules MCF-7 normales. Nous avons identifié des protéines régulées vers le haut dans des cellules MCF-7 -knockdown pour la 17β-HSD5, ces protéines étant impliquées dans 2 réseaux et à une voie d'ubiquitination. Les fonctions des protéines régulées vers le haut augmentant le développement de BC, comme l'inhibiteur de l'apoptose GRP78. L'augmentation de la régulation du GRP78 inhibe l'apoptose et augmente la prolifération cellulaire. Ceci est cohérent avec l'augmentation de la prolifération cellulaire après le knockdown de la 17β-HSD5. La 17β-HSD5 ne peut donc pas être une cible pour le traitement du cancer du sein, mais pourrait représenter un faible facteur de pronostic lorsque des niveaux d'enzymes inférieurs sont détectés.

SUMMARY

17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation

17β -hydroxysteroid dehydrogenase type 5 (17β-HSD5) is a crucial enzyme associated with sex steroid metabolism. In the literature, expression and prognostic value of 17β-HSD5 in breast cancer (BC) are inconsistent. We demonstrated lower expression of 17β-HSD5 in BC tissue comparing normal tissue. The proteome profiles of the 17β-HSD5-knockdown ER+ breast cancer cells MCF-7 was compared to that of MCF-7 cells. We identified proteins up-regulated in 17β-HSD5- knockdown MCF-7 cells are being involved in 2 networks and ubiquitination pathway. The functions of the up-regulated proteins, such as apoptosis inhibitor GRP78, enhance BC development. The up-regulation of GRP78 inhibits apoptosis and increases cell proliferation. This is consistent with the increase in cell proliferation after 17β-HSD5 knockdown. 17β-HSD5 may not be a target for breast cancer treatment but could represent a poor prognosis factor in lower enzyme levels.

17beta-hydroxysteroid dehydrogenase type 5 is negatively correlated to apoptosis inhibitor GRP78 and tumor-secreted protein PGK1, and modulates breast cancer cell viability and proliferation

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Abstract

17beta-hydroxysteroid dehydrogenase type 5 (17β-HSD5) is an important enzyme associated with sex steroid metabolism in hormone-dependent cancer. However, reports on its expression and its prognostic value in breast cancer are inconsistent. Here, we demonstrate the impact of 17β-HSD5 expression modulation on the proteome of estrogen receptor-positive (ER+) breast cancer cells. RNA interference technique (siRNA) was used to knock down 17β-HSD5 gene expression in the ER+ breast cancer cell line MCF-7 and the proteome of the 17β-HSD5-knockdown cells was compared to that of MCF-7 cells using two-dimensional (2-D) gel electrophoresis followed by mass spectrometry analysis. Ingenuity pathway analysis (IPA) was additionally used to assess functional enrichment analyses of the proteomic dataset, including protein network and canonical pathways. Our proteomic analysis revealed only four differentially expressed protein spots (fold change > 2, $p < 0.05$) between the two cell lines. The four spots were up-regulated in 17β-HSD5-knockdown MCF-7 cells, and comprised 21 proteins involved in two networks and in functions that include apoptosis inhibition, regulation of cell growth and differentiation, signal transduction and tumor metastasis. Among the proteins are nucleoside diphosphate kinase A (NME1), 78 kDa glucose-regulated protein (GRP78) and phosphoglycerate kinase 1 (PGK1). We also showed that expression of 17β-HSD5 and that of the apoptosis inhibitor GRP78 are strongly but negatively correlated. Consistent with their opposite regulation, GRP78 knockdown decreased MCF-7 cell viability whereas 17β-HSD5 knockdown or inhibition increased cell viability and proliferation. Besides, IPA analysis revealed that ubiquitination pathway is significantly affected by 17β-HSD5 knockdown. Furthermore, IPA predicted the proto-oncogene c-Myc as an upstream regulator linked to the tumor-secreted protein PGK1. The latter is over-expressed in invasive ductal breast carcinoma as compared with normal breast tissue and its expression increased following 17β-HSD5 knockdown. Our present results indicate a 17β-HSD5 role in down-regulating breast cancer development.

We thus propose that 17β-HSD5 may not be a potent target for breast cancer treatment but its low expression could represent a poor prognosis factor.

1. INTRODUCTION

Breast cancer is a common cancer diagnosed among women. In North America (The United States and Canada), it is the second leading cause of cancer death in women, after lung cancer [1]. Estrogens have a significant role in the development and progression of breast cancer. 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) is an important enzyme associated with sex steroid metabolism. It synthesizes 5-androstene-3β,17β-diol (5-DIOL) from dehydroepiandrosterone (DHEA) and catalyzes 4-androstenedione (4-DIONE) reduction to testosterone (T). The latter can then be aromatised by CYP19 aromatase providing a route for estradiol (E2) biosynthesis independent of 17β-HSD type 1 (17β-HSD1) especially after menopause [2, 3, 4]. By carrying out multiple catalyses, 17β-HSD5 regulates the formation of both estrogens and androgens in hormone-dependent cancer cells, leading to the modulation of the cell proliferation [5, 6]. 17β-HSD5 is the only enzyme of the 17β-HSD family which is structurally a member of the aldo-keto reductase (AKR) superfamily [7, 8]. It is also expressed in various human tissues including prostate, endometrium and mammary gland [9]. 17β-HSD5 expression has been shown to be significantly higher in breast tumor specimens than in normal tissues and patients with 17β-HSD5 overexpression had a worse prognosis than patients with low expression [10]. In addition, patients with estrogen receptor positive (ER+) breast tumor and with high levels of 17β-HSD5 expression showed a greater risk of developing recurrence in breast cancer after five years diagnosis than patients with low and intermediate 17β-HSD5 levels [11]. However, the relationship of 17β-HSD5 with the disease recurrence was not confirmed by multivariate analysis of breast cancer [11]. It has been revealed that inhibition of 17β-HSD type 1 (17β-HSD1) was suitable for the treatment of estrogen-dependent diseases, such as breast cancer, but the roles of other 17β-HSDs, including 17β-HSD5, are still controversial [12]. All these observations revealed the need for further research to clarify the importance of 17β-HSD5 expression in breast cancer development.

The purpose of the present study was to investigate the impact of 17β-HSD5 knockdown on breast cancer cell protein profile. MCF-7 cell line is widely used in breast cancer research because it expresses both estrogen and androgen receptors and has high 17β-HSD5 expression level [13-15]. We used small interfering RNAs (siRNAs) to silence 17β-HSD5

expression in MCF-7 and then carried out a proteomic study using two-dimensional (2-D) electrophoresis and mass spectrometry (MS) analyses.

2. MATERIALS AND METHODS

2.1. Cell Culture

MCF-7 cells were from the American Type Culture Collection (ATCC) and were maintained in phenol red-free DMEM low glucose medium supplemented with 10% fetal bovine serum (FBS) at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. For experiments, exogenous hormones in the serum was eliminated by treating FBS overnight at 4ºC with 2% dextran-coated-charcoal (Sigma, St. Louis, MO, USA) before adding to the cell culture media. Cell medium was supplemented with 1 µM DHEA to mimic the physiological conditions of post-menopausal women.

2.2. siRNA transfection

For proteomic study, MCF-7 cells were seeded in 10-cm-diameter dishes with 1×10^6 cells/dish. The next day, cells were transfected with 200 nM mixed 17β-HSD5 specific siRNAs (see Table 1 for siRNA sequences of 17β-HSD5, AKR1C3 Genebank accession # NM_003739) or with negative control siRNA in the 10-cm-diameter dishes using Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada). Transfected cells were incubated for four days before protein extraction for proteomics analysis. Each condition included four independent biological replicates, coming from four independent cell culture experiments. The GRP78 siRNAs were used at 100 nM (see Table 1 for siRNA sequences).

2.3. Protein extracts for proteomics analysis

Four days after transfection, total proteins of siRNA-transfected MCF-7 cells were extracted from the 10 cm^2 dishes as follows. Cells were first washed twice with 5 ml cold phosphate buffered saline (PBS, 1X). Second, 300 µl lysis buffer T8 (7 M urea, 2 M thiourea, 3% CHAPS, 20 mM DTT, 5 mM TCEP, 0.5% IPG pH 4-7, 0,25% IPG pH 3-10) were added and cells were scraped with a rubber policeman, and collected in eppendorf tube. 50 mM Tris-HCL pH 8.8 and 1% protease inhibitors cocktail (EMD Chemical, Gibbs-

town, NJ, USA) were added and protein samples were mixed gently for 2 hours at room temperature, then centrifuged at 16,000 x g for 5 minutes and the supernatant was collected. Protein samples were precipitated using the two-dimensional Clean-Up kit (GE Healthcare, Piscataway, NJ, USA) and resolubilized in T8 buffer. The protein concentrations were determined using the 2D Quant kit (GE Healthcare).

2.4. Two-dimensional gel electrophoresis and gel image analysis

Two-dimensional gel electrophoresis and gel image analysis were carried out as previously described [16]. Briefly, 200 µg protein were loaded onto 24 cm Immobiline Dry Strip (GE Healthcare) pH 4-7 on IPGPhor isoelectric focusing system (GE Healthcare) for first gel dimension as recommended by the manufacturer. Then, strips were equilibrated in equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, trace of bromphenol blue) which contained 10 mg/ml dithiothreitol for 15 min and then in equilibration buffer containing 25 mg/ml iodoacetamide for 15 min. The second dimension was run on 2D gel 12% acrylamide gel using Ettan Dalt twelve (GE Healthcare). Four independent protein samples coming from four independent cell culture experiments were run for each condition. Gels were stained with Sypro Ruby (Invitrogen) and scanned with the ProXpress scanner (PerkinElmer, Walthan, MA, USA). The 2-D gel electrophoresis was performed on the Proteomic Platform of the Infectious Disease Research Center (Québec, Canada).

Comparative analysis of the combination of four replicates of control-siRNA-transfected MCF-7 cells and four replicates of 17β-HSD5-siRNA-transfected MCF-7 was done using Progenesis Same Spots software (Nonlinear Dynamics, Durham, NC, USA). Protein spots with differences in expression more than 2-fold ($p < 0.05$) were selected, excised from the gel using a ProXcision_Spot cutter (Perkin Elmer) and sent for mass spectrometry (MS) analysis.

2.5. Mass spectrometry and protein identification

Mass spectrometry analysis was performed by the Proteomics Platform of the Quebec Genomic Centre (Quebec City, Quebec, Canada). Protein spots were conserved in 1% acetic acid and submitted to trypsin digestion before mass spectrometry analysis. The tryptic digestion was performed on a MassPrep liquid handling robot (Waters, Milford, USA) following the manufacturer's specifications and the protocol of *Shevchenko et al* [17] with the modifications suggested by *Havlis et al* [18]. Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES MS/MS). The experiments were carried out with an Agilent 1200 nano pump connected to a 5600 mass spectrometer (AB Sciex, Framingham, MA, USA) furnished with a nanoelectrospray ion source. Peptide separation took place on a self-packed PicoFrit column (New Objective, Woburn, MA) packed with Jupiter (Phenomenex) 5u, 300A C18, 15 cm x 0.075 mm internal diameter. Peptides were eluted with a linear gradient from 2-50% solvent B (acetonitrile, 0.1% formic acid) in 30 minutes, at 300 nL/min. Mass spectra were acquired using a data-dependent acquisition mode using Analyst software version 1.6. Each full scan mass spectrum (400 to 1250 m/z) was followed by collision-induced dissociation of the twenty most intense ions. Dynamic exclusion was fixed for 3 sec and a tolerance of 100 ppm.

All MS/MS peak list were generated with Protein Pilot (AB Sciex, Framingham, MA, USA, Version 4,5) and samples MGF were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0). Mascot was set up to search the Uniref100-Homo sapiens database (release 13-03) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.10 Da and a parent ion tolerance of 0.10 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modification. Two missed cleavages were allowed.

Scaffold (version 4, Proteome Software Inc, Portland, OR, USA) was used to validate MS/MS-based peptide and protein identification. The protein identification cut off was set at a confidence level of 95% (Mascot score > 33) with at least 2 peptides matching to a protein. For each spot, only the proteins identified with a confidence level higher than 95%

and with at least 2 peptides matching to the protein were selected for functional analysis. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.6. Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) (www.ingenuity.com) was used to gain insights into the protein networks, biological pathways and upstream regulators affected by 17β-HSD5 gene knockdown in MCF-7 cells. Analyzes were performed by the Proteomics Platform of the Quebec Genomic Centre (Quebec City, Quebec, Canada). The networks and pathways were represented graphically. The nodes represented proteins, and the biological interaction between two nodes was represented as lines. We selected networks and upstream regulatory scoring ≥ 2.

2.7. Western blot

Total proteins were extracted from cells with RIPA buffer (Invitrogen) supplemented with 1% protease inhibitors cocktail (EMD Chemicals), and quantify by Bradford method. 30 µg total proteins from each sample were separated on a 12% SDS-PAGE gel. Membranes were incubated 1.5-2 hours at room temperature with primary antibodies directed against AKR1C3 (ab84327 from Abcam, used at dilution 1:1000), GRP78 (ab21685 from Abcam, used at dilution 1:500) and phosphoglycerate kinase 1 (PGK1, from Santa Cruz Biotechnology used at 1:500 dilution). Monoclonal β-actin antibody produced in mouse was used a 1:5000 dilution for internal control. Goat anti-rabbit IgG peroxidase-conjugated (Santa Cruz Biotechnology) diluted 1:10,000 times and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) diluted 1: 10,000 times were used as secondary antibody. Membranes were washed by TBST, and proteins were visualized using the western lighting™ Plus ECL (Perkin Elmer). The radiographic films were scanned and the Image program (Molecular Dynamics, Sunnyvale, CA) was used to quantify band intensities.

2.8. Cell proliferation

Cell proliferation was determined by CyQuant cell proliferation kit (Molecular Probe, Eugene, OR, USA). MCF-7 cells (3×10^3) were plated onto 96-well plates containing 100 µl charcoal-treated hormone-free culture medium. After 24h, cells were transfected with 100 nM 17β-HSD5 or control siRNAs. The control group and 17β-HSD5 siRNA groups were supplemented with 1 µM DHEA five hours after transfection. Untransfected cells left without DHEA were identified as hormone free group. Cell culture medium was half changed every two days. At 72h, 84h, 96h and 108h post-transfection, cells were washed with PBS 1x and frozen in 96-well plates at −80°C until time of analyses. Cell proliferation assay was determined by CyQuant cell proliferation kit (Molecular Probe, Eugene, OR, USA). The plates were thawed at room temperature for 15 min, then 200 µl of CyQuant GR dye/cell-lysis buffer was added to each well according to manufacturer's instructions. Sample fluorescence was measured using a fluorescence microplate reader at 480 nm excitation and 520 nm emission. Hormone free group proliferation was fixed at 100% and data are reported as percentage of hormone free group proliferation.

2.9. Cell viability assay

Cell viability was evaluated by using MTT test. 3×10^3 cells were seeded in 96-well plates and incubate for 24 hours. Cells were then transfected with GRP78 specific siRNAs or control siRNA, and also incubated with a 17β-HSD5 steroidal inhibitor EM1404 (IC50 = 3.2 ± 1.5 nM). Four days after transfection and inhibitor treatment, 10 µl MTT reagent was added to each well, and cells were incubated at 37^OC for 2-4 hours until purple precipitates were visible before 100 ul detergent reagent were added. The plate was left in the dark overnight at room temperature, and absorbance was recorded at 570 nm using a plate reader.

2.10. Measurement of estradiol concentration

Cells were seeded into 24-well plates at a density of 5×10^4cells/well in 500 µl hormone-free culture medium. After 24-hour incubation, cells were transfected with 100 nM GRP78 specific siRNAs or control siRNA as the negative control. Each condition was performed in duplicate. The culture medium was replaced with hormone-free medium containing 1 µM DHEA five hours after transfection. The medium was collected from wells 4 days after

transfection and immediately frozen at −80 ºC until analysis. The levels of E2 in the medium were determined as previously described [19] using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Duplicate wells were prepared for each condition to be measured. E2 plates were read at 420 nm in a plate reader (Spectra Max 340PC; Molecular Devices, Sunnyvale, CA).

3. RESULTS

3.1. 17β-HSD5 expression leads to a proteomic modification of breast cancer cells

To investigate the proteomic modifications of MCF-7 cells in response to 17β-HSD5 knockdown, we performed 2-D gel analysis using total protein lysates of the MCF-7 cells (control cells) and MCF-7 cells transfected with 17β-HSD5-specific siRNA cultured four days in medium containing 1 μM DHEA. Before 2-D gel analysis, Western blot was carried out to make sure 17β-HSD5 was reliably knocked down (**Figure 1A**). Proteomic analysis was performed on eight 2-D electrophoresis gel made from four independent biological repetitions of protein samples from control cells and 17β-HSD5-siRNA-transfected cells. As shown in **Figure 1A**, 17β-HSD5 expression was significantly downregulated in the latter cells in all the four independent biological repetitions. The 2-D gel image analysis showed that control and 17β-HSD5-siRNA-transfected cells displayed similar protein spot patterns (**Figure 1B**), which allowed a good spot alignment for the proteome comparison. Progenesis software and a *t*-test with a *P*-value < 0.05 were used for the proteomic analysis. Only four significant differential protein spots were identified between control and 17β-HSD5-siRNA-transfected cells, and all the four spots were up-regulated in the 17β-HSD5-siRNA-transfected cells (**Figure 1B, right**). The spots were selected for MS analysis which allowed the identification of a total of 21 proteins with known UniProt accession numbers among all the four spots (**Table 2**). Using the Uniprot database [20] and Scaffold software, we determined the functions or biological processes of each of the identified 21 proteins (**Table 2**). From the results, we observed that proteins involved in cell cycle, cell proliferation and metastasis were up-regulated after knocking down 17β-HSD5 in MCF-7 cells. The largest proportion of functional category was metabolic process (28% of the proteins). The other functional categories include stress response (12% of the proteins), signal transduction (11%), transport (11%), cell cycle (8%), biosynthesis process (7%),

mRNA processing (5%), apoptosis (4%) and cell proliferation (3%). (**Figure 1C**). These results reveal that 17β-HSD5 plays an important role in MCF-7 breast cancer cells and that expression of proteins involved in metabolic pathways are modified by 17β-HSD5 knockdown in MCF-7 cells.

3.2. Protein network and pathway analyses by IPA

IPA Network analysis was used to study the proteomic data (the 21 proteins revealed by mass spectrometry). The 21 identified proteins in Table 2 were associated with two networks by IPA (**Figure 2A and 2B**). The first and the highest score (score 34) corresponds to the Network 1 which comprise a list of 13 proteins from the proteomic dataset and 22 other partner proteins added by IPA for the network completion (**Table 3, ID # 1, see also Table 2 and Figure 2A**). The 13 proteins include Annexin A7 (ANXA7), CAND1, Cofilin-1 (CFL1), HNRNPH1, HSCB, HSPA5, HSPB1, KRT19, MCM7, NME1, PCBP2, PGK1, PSMB4 (**Table 2 and Figure 2A, Network 1**). Three functions are associated to the network, which are signaling and interaction between cells (cell to cell signaling and communication), tissue development and certain hereditary disorders **(Table 3).** Of the partner proteins in the Network 1, the most interesting interactions are the ERK1/2, JnK and NF-kB complex (**Figure 2A**). The second network, Network 2, consists of eight proteins from the list of the proteomic dataset and 27 partner proteins **(Table 3, ID # 2, see also Table 2 and Figure 2B)**. The eight proteins from the list are ATIC, DDX39B, OBFC1, PCYT1A, PPME1, PSMC6, RAB11A, SEPHS1. The three functions associated with that network are molecular transport, RNA trafficking and developmental disorder **(Table 3)**. An important part of the interactions in the Network 2 is done mainly with the polyubiquitin-C (UBC, **Figure 2B**). Note that ubiquitin is also present in Network 1 (**Figure 2A**).

Pathway analyses by IPA revealed that the most significant affected pathway is the protein ubiquitination pathway, with five proteins (HSPA5, HSPB1, PSMB4, PSMC6 and HSCB) of the list (the 21 proteins revealed by the proteomic study) implicated (**Table 4 and Supplemental Figure 1**). The five proteins are involved in two successive steps of the ubiquitination. Two HSPs, HSPA5 and HSPB1, are part of the protein folding control

process and the polyubiquitination (**Supplemental Figure 1**). Both mechanisms are associated with cellular stress mechanisms. The second most significant affected pathway is the aldosterone signaling in epithelial cells (see **Table 4**), with three proteins (HSPA5, HSPB1 and HSCB). As already mentioned, all three proteins are also involved in ubiquitination pathways.

IPA analysis for identification of biological functions was also performed for the 21 proteins revealed by the proteomic study. The 21 proteins were significantly ($p < 0.05$) associated with 262 functional categories (data not shown). However, only one functional category (organismal death) showed a highly significant z-score (z-score > 2), with eight proteins of the list involved (ANXA7, CFL1, HSPA5/GRP78, KRT19, NME1, PCYT1A, PPME1, PSMB4) (see **Supplemental Figure 3**).

3.3. Correlation between 17β-HSD5 and HSPA5 expression

From the above proteomics results, we could observe that after knocking down 17β-HSD5, HSPA5 (GRP78) expression was up-regulated, which represents a critical protein in ubiquitination pathways and apoptosis. Therefore, Western blot was carried out to specifically verify if GRP78 expression was up-regulated when 17β-HSD5 was depleted. Results show that GRP78 expression increased (37.6%) after knocking down 17β-HSD5 in MCF7 (**Figure 3A**). Following this result, we were interested to know the effect of GRP78 expression modulation on the expression of 17β-HSD5. We thus investigate whether GRP78 gene knockdown would increase 17β-HSD5 expression. GRP78 specific siRNAs were designed and used in the transfection experiments. MCF7 cells were transfected with mixed GRP78 specific siRNAs or with control siRNA (control cells). Total proteins were extracted after 48-hour post-transfection for Western blot analysis. As shown in **Figure 3B**, GRP78 protein expression was significantly down-regulated (by 78%) after GRP78-siRNA-transfection in cells compared to the control cells. Meanwhile, 17β-HSD5 protein expression level was significantly and strongly up-regulated (3.3-fold increase) by GRP78 knockdown (**Figure 3B**). Thus, GRP78 and 17β-HSD5 exert a negative regulation on each other in MCF-7 cells.

3.4. 17β-HSD5 and GRP78 exert opposite effects on breast cancer cell viability

Due to the negative correlation between 17β-HSD5 and GRP78 expression, and as GRP78 has an anti-apoptotic function [21], we were interested to know the effects of the expression modulation of the two proteins on breast cancer cell growth and viability. After knocking down 17β-HSD5 expression with its specific siRNAs, MCF-7 cell growth was determined by measuring cell proliferation using the Cyquant cell proliferation kit (Molecular Probe). 84h, 96h and 108h after transfection, cell proliferation significantly increased by 19, 20 and 13%, respectively ($P < 0.05$), in 17β-HSD5-silenced cells compared to control cells (**Figure 4A**). The effect of 17β-HSD5 activity inhibition by its specific steroidal inhibitor EM1404 (IC50 = 3.2 ± 1.5 nM) [22] were evaluated. MCF-7 cells treated with 6.4 nM (2IC50) 17β-HSD5 inhibitor EM1404 for 4 days showed 26% ($p = 0.01$) increased cell viability when compared with control cells treated with vehicle (**Figure 4B**). To determine the role that GRP78 silence plays in cell viability and hormone steroid changes in MCF-7 cells, MTT test and ELISA measurement were performed after cell transfection with GRP78 siRNA. Results showed that, cell viability significantly decreased (by 27%, $p = 0.003$) in the GRP78-knockdown cells when compared with control cells (**Figure 4C**). E2 average levels decreased from 229.55 pg/ml in control siRNA to 132.9pg/ml ($p = 0.01$) in the GRP78-siRNA-transfeted cells (**Figure 4D**).

3.5. MYC was predicted as an upstream regulator that leads to PGK1 activation.

Upstream regulators of proteomic dataset (the 21 proteins of Table 2) were analyzed by IPA. Only three regulators (MYC, miR-4651 and miR-495-3p) showed a significant z-score (z-score ≥ 2, *p*-value of overlap < 0.05) (**Table 5, Figure 5A, Supplemental Table 1 and Supplemental Figure 2**). Due to its important role in cell transcription regulation, we did further investigation linked to the upstream regulator MYC (z score = 2, *p*-value of overlap = 1.64E-03). Target molecules from the proteomic dataset linked to MYC are HSPB1, DDX39B, MCM7, NME1 and PGK1 (**Figure 5A)**, with the latter four predicted to be activated by MYC. PGK1 (phosphoglycerate kinase 1) is an ATP-generating glycolytic enzyme that is associated with hypoxia of many solid tumors [23]. Therefore, we were interested to know the relationship between expression 17β-HSD5 and PGK1 expression. Western blot was carried out to verify if PGK1 expression was up-regulated when 17β-

HSD5 was depleted. Results showed that PGK1 expression increased 2.01 fold ($p < 0.05$) in the 17β-HSD-knockdown cells when compared with control cells (**Figure 5B**). We then used the Oncomine database [24] to compare PGK1 gene expression in normal breast and invasive ductal breast carcinoma tissue. The database showed that invasive ductal breast carcinoma tissue significantly (2 fold changes, p = 1E-4) over-expressed PGK1 gene when compared with the normal breast (**Figure 5C**).

4. DISCUSSION

17β-HSD5 participates in estradiol synthesis of hormone steroid pathway [3-5]. However, its role in breast cancer is still controversial [11]. Due to proteins being the actual effectors driving cell behavior and proteomics technology advance [25], we used proteomics study to clarify 17β-HSD5 role in breast cancer. MCF-7 cell line is an ideal model and it has been extensively used to study ER-positive (ER+) breast cancer. MCF-7 cells show estrogen-dependent growth and ERα activation and regulation, as well as an accurate response to hormone treatment observed in mono- and co-culture [26]. Additionally, MCF-7 cell line was found to have high expression level of 17β-HSD5 as determined by the mRNA copy number revealed by reverse transcription quantitative real-time PCR (RT-qPCR) [15-16]. Therefore, we chose MCF-7 cell line to knock down 17β-HSD5 and to perform proteomic analysis in order to better understand the role of 17β-HSD5 in breast cancer. The 2-D gel images of wild type MCF-7 and 17β-HSD5-knockdown-MCF-7 cells showed only four significantly different spots (fold change > 2, $p < 0.05$). MS analysis showed that 21 proteins were present in these four spots. After classifying all the proteins by function, we found the largest proportion of the proteins fell to the metabolism processing functional category (28%), indicating that 17β-HSD5 is mainly involved in cell metabolism processing. The other important function categories are response to stress (with 12% of modulated proteins), signal transduction (11%) and cell cycle (8%). This suggests a role of the enzyme in these cellular functions.

IPA network analysis showed that two networks were associated with proteins regulated by 17β-HSD5. The proteins involved in this pathway include GRP78, a member of the heat shock protein 70 (HSP70) family [27]. Recent research on GRP78 has improved our understanding of the protein's role. GRP78 is implicated in genomic instability and gene mutation, cancer-associated inflammation, tumor immune escape, tumor cell growth and death resistance, regulation of cell metabolism, tumor angiogenesis, tumor cell invasion and metastasis, tumor cell replicative immortality, and has implications for cancer treatment [28]. GRP78 regulates the apoptosis regulator Bcl-2 (BCL2) sequestered by BCL-2-interacting killer (Bik) at endoplasmic reticulum, thus uncovering a new mechanism by which GRP78 confers endocrine resistance in breast cancer [21, 29]. Apoptosis is a programmed cell death, and several mechanisms are involved in its regulation. GRP78 was shown to have a regulatory role in some of these mechanisms [29]. In the present study, we showed for the first time that GRP78 and 17β-HSD5 expression are negatively correlated. GRP78 expression was up-regulated when 17β-HSD5 was knocked down while 17β-HSD5 expression significantly increased after GRP78 knockdown. Furthermore, we measured cell viability and cell proliferation after 17β-HSD5 expression inhibition, and revealed that both were significantly increased. One reason for the cell growth increase in response to the 17β-HSD5 knockdown may be due to the up-regulation of GRP78; the later reduced apoptosis thus promoting cell growth. On the contrary, cell viability and E2 concentration significantly decreased after GRP78 knockdown. These results can be explained by the fact that GRP78 knockdown induces cell apoptosis, based on the function of GRP78 and apoptosis mechanism [28-31]. Elevated GRP78 level correlated with higher pathologic grade, recurrence, and poor patient survival in breast cancer [32], and GRP78 may be a target for breast cancer treatment. In regard to our data, we propose that 17β-HSD5 should not be inhibited for breast cancer treatment, as low expression of 17β-HSD5 can enhance the cancer development, one reason being the negative correlation between GRP78 and 17β-HSD5 expression (GRP78 level would increase with low expression of 17β-HSD5 in breast cancer cells).

Ubiquitin was present in the two protein networks associated with our proteomic dataset and, as revealed by IPA analysis, the protein ubiquitination pathway is the most significant

pathway affected by 17β-HSD5 knockdown, with five proteins from the dataset involved. In addition to HSPA5/GRP78, these proteins include HSPB1, PSMB4, PSMC6 and HSCB. HSPA5/GRP78 and the protein Hsp27 (whose gene is HSPB1) are chaperones belonging to the HSP70 and sHsp family, respectively. Functions of these chaperones include apoptosis inhibition and protein folding [33]. The protein PSMC6 is a member of ATPase subunit and involved in chaperone activity. PSMB4 is involved in the 26S assembling, interacting with the oncoprotein PSMD10 [34], a chaperone of the 26S complex that is overexpressed in breast cancer. Three of the ubiquitin pathway proteins (HSPB1, HSCB and HSPA5/GRP78) were associated with the aldosterone signaling in epithelial cells. Ubiquitination results in the degradation of unwanted proteins. The association between 17β-HSD5 and these five proteins suggests an implication of 17β-HSD5 in protein degradation via ubiquitination and in cellular apoptosis, and further reinforce our hypothesis that 17β-HSD5 may be a target for breast cancer treatment, but should not be inhibited.

IPA analysis revealed that MYC (c-Myc) is an upstream regulator of proteins changed by 17β-HSD5 knockdown in MCF-7 cells. c-Myc is a transcriptional regulator that participates in important cellular function such as replication, growth and differentiation [35]. Many studies have shown that c-Myc protein is increased in most breast cancer cases [36-40]. It has been well demonstrated that estrogens stimulate expression of the c-Myc gene thus up-regulates c-Myc mRNA level [41-43], making it a well-known estrogen-responsive gene. In the IPA analysis, our results showed that c-Myc leads to PGK1 activation. PGK1 was up-regulated 2.13-fold in 17β-HSD5-knockdown MCF-7 cells compared to control MCF-7 cells. PGK1 is a glycolytic enzyme, generating ATP from the glycolytic pathway. Solid tumor cells employ glycolytic enzymes such as PGK1 to produce ATP when tumor cells are in hypoxia [44]. PGK1 is secreted extracellularly by tumors [23], and has been linked to various cancers [23; 45-47], including prostate cancer, where it regulates angiogenesis [23]. In gastric cancer, PGK1 is a promoting enzyme in the process of peritoneal dissemination. Moreover, PGK1 mRNA and protein expression were significantly higher in breast cancer tissues than in normal breast tissues and have been linked to poor survival and to the prognostic of chemoresistance to paclitaxel treatment in breast cancer [47]. Similarly, our

Oncomine analyses showed that the PGK1 gene had a higher expression (2.03-fold) in invasive ductal breast carcinoma compared with normal breast tissues. Our siRNA knockdown analysis revealed that 17β-HSD5 knockdown up-regulated PGK1 protein expression level. Meanwhile, we showed that 17β-HSD5 knockdown or activity inhibition promote breast cancer growth and viability. Taking together, these results show an expression and/or function relationship between the two proteins. One could thus postulate that PGK1 may be a potential target to be inhibited for breast cancer treatment and 17β-HSD5 expression as a prognostic marker.

5. CONCLUSION

The present study reveals that 17β-HSD5 knockdown modulated (up-regulated) proteins, involved in two networks and in ubiquitination pathways in breast cancer cells. The functions of these 17β-HSD5-up-regulated proteins can lead to the enhancement of breast cancer development. For example, GRP78 which is an apoptosis inhibitor is up-regulated by 17β-HSD5 knockdown, and this will counteract apoptosis, leading to an increase in the cell proliferation. In addition, 17β-HSD5 knockdown increased PGK1 expression, the proliferation and viability of breast cancer cells, and this also can enhance breast cancer development. We thus conclude that 17β-HSD5 may not be a potent target for breast cancer treatment, but a low-level expression could serve as a poor prognosis factor.

Conflict of interest

The authors have no conflict of interest to declare.

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REFERENCES

[1] Breast Cancer statistics. Canadian Cancer Society (2013). [http:// www.cancer.ca/cancer].

[2] Luu-The V, Labrie F (2010) The intracrine sex steroid biosynthesis pathways. *Program Brain. Res*. **181**:177–192.

[3] Penning TM, Jin Y, Steckelbroeck S, Lanisnik Rizner T, Lewis M (2004) Structure-function of human 3α-hydroxysteroid dehydrogenases: genes and proteins. *Mol. Cell. Endocrinol.* **215**: 63–72.

[4] Labrie F (1991) Intracrinology. *Mol. Cell. Endocrinol* **78**:113–118.

[5] Lin SX, Poirier D, Adamski J (2013) A challenge for medicinal chemistry by the 17β-hydroxysteroid dehydrogenase superfamily: an integrated biological function and inhibition study. *Curr Top Med Chem.* **13**:1164–1171.

[6] Byrns MC, Jin Y, Penning TM (2011) Inhibitors of type 5 17β-hydroxysteroid dehydrogenase (AKR1C3): overview and structural insights. *J Steroid Biochem Mol Biol*. **125**:95–104.

[7] Jez JM, Bennett MJ, Schlegel BP, Lewis M and Penning TM (1997) Comparative anatomy of the aldo-keto reductase superfamily. *Biochem. J* **326**:625–636.

[8] Jez JM, Flynn TG, Penning TM (1997) A new nomenclature for the aldo-keto reductase superfamily. *Biochem. Pharmacol.* **54**:639–647.

[9] Penning TM, Burczynski ME, Jez JM, Hung CF, Lin HK, Ma H, Moore M, Palackal N, Ratnam K (2000) Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reducatse superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem. J.* **351**: 67–77.

[10] Oduwole OO, Li Y, Isomaa VV, Mäntyniemi A, Pulkka AE, Soini Y, Vihko PT (2004) 17β-Hydroxysteroid Dehydrogenase type 1 is an independent prognostic marker in breast cancer. *Cancer Res.* **64**:7604–7609.

[11] Jansson AK, Gunnarsson C, Cohen M, Sivik T, Stål O (2006) 17β-Hydroxysteroid dehydrogenase 14 affects estradiol levels in Breast Cancer and Is a prognostic maker in estrogen receptor-Positive Breast Cancer. *Cancer Res.* **66**:11471–11477.

[12] Poirier D (2010) 17beta-Hydroxysteroid dehydrogenase inhibitors: a patent review. *Expert Opin. Ther. Pat.* **20**:1123–1145.

[13] Neve RM , Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* **10**:515–527.

[14] Aka JA, Lin SX (2012) Comparison of functional proteomic analyses of human breast cancer cell lines T47D and MCF-7. *PLOS ONE* **7**:e31532.

[15] Laplante Y, Rancourt C, Poirier D (2009) Relative involvement of three 17β-HSDs (types 1, 7 and 12) in the formation of estradiol in various breast cancer cell lines using selective inhibitors. *Mol Cell Endocrinol.* **25**:146–153.

[16] Aka JA, Zerradi M, Houle F, Huot J, Lin SX (2012) 17beta-hydroxysteroid dehydrogenase type 1 modulates breast cancer protein profile and impacts cell migration. *Breast Cancer Res.* **14**:R92.

[17] Shevchenko M, Wilm O, Vorm, and Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**:850–858.

[18] Havlis J, Thomas H, Sebela M, Shevchenko A (2003) Fast-Response Proteomics by Accelerated In-Gel Digestion of Proteins. *Anal. Chem.* **75**:1300–1306.

[19] Xu D, Lin SX (2016) Mimicking postmenopausal steroid metabolism in breast cancer cell culture: Differences in response to DHEA or other steroids as hormone sources. *J Steroid Biochem Mol Biol.* **161**:92–100.

[20] Uniprot database. [http://www.uniprot.org].

[21] Zhou H, Zhang Y, Fu Y, Chan L, Lee AS (2011) Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). *J Biol Chem.* **286**:25687–25696.

[22] Qiu W, Zhou M, Mazumdar M, Azzi A, Ghanmi D, Luu-The V, Labrie F, Lin SX (2007) Structure-based inhibitor design for an enzyme that binds different steroids: a potent inhibitor for human type 5 17beta-hydroxysteroid dehydrogenase. *J Biol Chem.* **282**:8368–8379.

[23] Wang J, Wang J, Dai J, Jung Y, Wei CL, Wang Y, Havens AM, Hogg PJ, Keller ET, Pienta KJ, Nor JE, Wang CY, Taichman RS (2007) A glycolytic mechanism regulating an angiogenic switch in prostate cancer. *Cancer Res.* **67**:149–159.

[24] Oncomine [https://www.oncomine.org].

[25] Weston AD, Hood L (2004) Systems Biology, Proteomics, and the Future of Health Care: Toward Predictive, Preventative, and Personalized Medicine". *J. Proteome Res* **3**:179–196.

[26] Jessica DL, Scott MB, Ginny LP, David JB and Elaine TA (2013) Hormonally responsive breast cancer cells in a microfluidic coculture model as a sensor of microenvironmental activity. *Integr. Biol. (Camb.)* **5**:807–816.

[27] Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett* **581**:3702–3710.

[28] Li Z and Li Z (2012) Glucose regulated protein 78: A critical link between tumor microenvironment and cancer hallmarks. *Biochim. Biophys. Acta.* **1826**:13–22.

[29] Cook KL, Clarke PA, Clarke R (2013) Targeting GRP78 and antiestrogen resistance in breast cancer. *Future Med. Chem*. **5**:1047–1057.

[30] Baumeister P, Dong D, Fu Y, Lee AS (2009) Transcriptional induction of GRP78/BiP by histone deacetylase inhibitors and resistance to histone deacetylase inhibitor–induced apoptosis. *Mol. Cancer Ther.* **8**:1086–1094.

[31] Rao RV, Peel A, Logvinova A, del Rio G, Hermel E, Yokota T, Goldsmith PC, Ellerby LM, Ellerby HM, Bredesen DE (2002) Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78. *FEBS Lett* **514**:122–128.

[32] Amy SL (2007) GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res.* **67**:3496–3499.

[33] Rappa F, Farina F, Zummo G, David S, Campanella C, Carini F, Tomasello G, Damiani P, Cappello F, DE Macario EC, Macario AJ (2012) HSP-molecular chaperones in cancer biogenesis and tumor therapy: an overview. *Anticancer Res.* **32**:5139–5150.

[34] Tai HC, Besche H, Goldberg AL, Schuman EM (2010) Characterization of the Brain 26S Proteasome and its Interacting Proteins. *Front Mol Neurosci*. **3**:12.

[35] Liao DJ and Dickson RB (2007) c-Myc in breast cancer. *Endocrine-related Cancer* **7**:143–164.

[36] Agnantis NJ, Mahera H, Maounis N and Spandidos DA (1992) Immunohistochemical study of ras and myc oncoproteins in apocrine breast lesions with and without papillomatosis. *Eur. J. Gynaecol. Oncol*. **13**:309–315.

[37] Pavelic ZP, Pavelic L, Lower EE, Gapany M, Gapany S, Barker EA and Preisler HD (1992) c-myc, c-erbB–2, and Ki–67 expression in normal breast tissue and in invasive and noninvasive breast carcinoma. *Cancer Res.* **52**:2597–2602.

[38] Saccani JG, Fontanesi M, Bombardieri E, Gabrielli M, Veronesi P, Bianchi M, Becchi G, Bogni A and Tardini A (1992) Preliminary study on oncogene product immunohistochemistry (c-erbB–2, c-myc, ras p21, EGFR) in breast pathology. *Int. J. Biol. Markers* **7**:35–42.

[39] Hehir DJ, McGreal G, Kirwan WO, Kealy W and Brady MP (1993) c-myc oncogene expression: a marker for females at risk of breast carcinoma. *J. Surg. Oncol*. **54**:207–209.

[40] Pietilainen T, Lipponen P, Aaltomaa S, Eskelinen M, Kosma VM and Syrjanen K (1995) Expression of c-myc proteins in breast cancer as related to established prognostic factors and survival. *Anticancer Res.* **15**:959–964.

[41] Schuchard M, Landers JP, Sandhu NP and Spelsberg TC (1993) Steroid hormone regulation of nuclear proto-oncogenes. *Endocr. Rev*. **14**:659–669.

[42] Shiu RP, Watson PH and Dubik D (1993) c-myc oncogene expression in estrogen-dependent and -independent breast cancer. *Clin. Chem*. **39**:353–355.

[43] Hyder SM, Stancel GM and Loose-Mitchell DS (1994) Steroid hormone-induced expression of oncogene encoded nuclear proteins. *Crit. Rev. Eukaryot. Gene Expr*. **4**:55–116.

[44] Daly EB, Wind T, Jian XM, Sun L, Hong PJ (2004) Secretion of phosphoglycerate kinase from tumour cells is controlled by oxygensensing hydroxylases. *Biochim. Biophys. Acta.* **1691**:17–22.

[45] Zieker D, Königsrainer I, Tritschler I, Löffler M, Beckert S, Traub F, Nieselt K, Bühler S, Weller M, Gaedcke J, Taichman RS, Northoff H, Brücher BL, Königsrainer A (2010) Phosphoglycerate kinase 1 a promoting enzyme for peritoneal dissemination in gastric cancer. *Int. J. Cancer* **126**:1513–1520.

[46] Li X, Jiang Y, Meisenhelder J, Yang W, Hawke DH, Zheng Y, Xia Y, Aldape K, He J, Hunter T, Wang L, Lu Z (2016) Mitochondria-Translocated PGK1 Functions as a Protein Kinase to Coordinate Glycolysis and the TCA Cycle in Tumorigenesis. *Mol Cell* **61**:705–719.

[47] Sun S, Liang X, Zhang X, Liu T, Shi Q, Song Y, Jiang Y, Wu H, Jiang Y, Lu X, Pang D (2015) Phosphoglycerate kinase-1 is a predictor of poor survival and a novel prognostic biomarker of chemoresistance to paclitaxel treatment in breast cancer. *Br J Cancer* **112**:1332–1339.

TABLES

Table 1. Sequences of 17β-HSD5 and GRP78 specific siRNAs

Gene	siRNA name	Sense sequence (5’ to 3’)	Anti-sense sequence (5’ to3’)
17β-HSD5	siRNA 1	GGAACUUUCACCAACAGAUTT	AUCUGUUGGUGAAAGUUCCTT
	siRNA 2	GAAUGUCAUCCGUAUUUCATT	UGAAAUACGGAUGACAUUCTT
	siRNA 3	GGACAUGAAAGCCAUAGAUTT	AUCUAUGGCUUUCAUGUCCTT
GRP78	siRNA 1	GGUUACCCAUGCAGUUGUUTT	AACAACUGCAUGGGUAACCTT
	siRNA 2	GGAGCGCAUUGAUACUAGATT	UCUAGUAUCAAUGCGCUCCTT
	siRNA 3	GGGCAAAGAUGUCAGGAAATT	UUUCCUGACAUCUUUGCCCTT
	Control siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2. Mass spectrometry identification of protein spots up-regulated in MCF-7-17β-HSD5 siRNA cells as compared to MCF-7 control siRNA cells. Spot, spot number; FC, fold change; MW (exp/pred), molecular weight as determined from the 2-D gel experiments (exp) and predicted (pred); Pep, number of unique peptides; Description, the name of the protein, the symbol in the brackets.

Spot	FC	Description (Gene symbol)[a]	Uniprot number	MW (exp/pred)	Pep	Function and/or biological process[b]
2119	2	Heterogeneous nuclear ribonucleoprotein H (HNRNPH1)	P31943	49/35	4	mRNA metabolism and transport
		Spliceosome RNA helicase DDX39B (*DDX39B*)	Q13838	49/35	4	Nuclear export of spliced and unspliced mRNA
2860	2.6	Nucleoside diphosphate kinase A (NME1/NM23)	P15531	17/20	3	Cell proliferation, differentiation and development, signal transduction, G protein-coupled receptor endocytosis, and gene expression. Associate with tumor metastasis
		Cofilin-1 (CFL1)	P23528	19/20	2	Normal progress through mitosis and normal cytokinesis
2497	2.1	Phosphoglycerate kinase 1 (PGK1)	P00558	45/28	11	Over-expression in many cancer, down regulation PGK1 initiating apoptosis and suppressing cancer metabolism
		Heat shock protein beta-1 (HSPB1/HSP27)	P04792	23/28	5	Phosphorylated in MCF-7 cells on exposure to protein kinase C activators and heat shock
		Proteasome subunit beta type-4 (PSMB4)	P28070	29/28	4	BRCA1 up-regulated genes in MCF-7 breast carcinoma cells
		Ras-related protein Rab-11A (*RAB11A*)	P62491	27/28	2	EGFR recycling, enhances proliferation, and prevents motility of an immortal breast cell line (MCF 10A)
		Iron−Sulfur Cluster Cochaperone HscB (HSCB)	Q8IWL3	24/28	2	A co-chaperone in iron-sulfur cluster assembly in mitochondria
3326	2.5	Bifunctional purine biosythesis protein PURH (*ATIC*)	P31939	65/45	12	Bifunctional enzyme that catalyzes 2 steps in purine biosynthesis
		CST complex subunit STN1 (*OBFC1*)	Q9H668	42/45	7	Binds to single-stranded DNA and is required to protect telomeres from DNA degradation
		Protein phosphatase methylesterase 1 (*PPME1*)	Q9Y570	42/45	7	Demethylates proteins
		DNA replation licensing factor MCM7 (MCM7)	P33993	81/45	5	Cell proliferation, DNA replication initiation and elongation
		Poly(rC)-binding protein 2 (PCBP2)	Q15366	39/45	5	Binds to oligo dC
		Keratin, type1 cytoskeletal 19 (KRT19)	P08727	44/45	5	Myofibers organization
		26S protease regulatory subunit 10B (*PSMC6*)	P62333	44/45	4	ATP-dependent degradation of ubiquitinated proteins
		Choline-phosphate cytidylytransferase A (*PCYT1A*)	P49585	42/45	4	Controls phosphatidylcholine synthesis
		78kDa glucose-regulated protein (HSPA5/GRP78)	P11021	72/45	3	Overexpression of GRP78 suppresses apoptosis
		Cullin-associated NEDD8-dissociated protein 1 (CAND1)	Q86VP6	136/45	2	Assembly factor of SCF E3 ubiquitin ligase complexes
		Selenide, water dikinase 1 (*SEPHS1*)	P49903	43/45	2	Synthesizes selenophosphate from selenide and ATP
		Annexin A7 (ANXA7)	P20073	53/45	2	Membrane fusion and exocytosis

[a]Protein in bold were associated to the IPA Network 1. Protein in italic were associated to the IPA Network 2.
[b]The function description and/or biological process were quoted from the Scaffold 4 software functionally classification.

Table 3. Data summary of the two networks revealed by the IPA analysis of the proteomic datasets.

ID	MOLECULES IN NETWORK	SCORE	FOCUS MOLECULES	TOP DISEASES AND FUNCTIONS
1	Adaptor protein 1, ANXA7, CAND1, CD3, CFL1, DAD1, DAJC1, DNAJC9, DOHH, ERK1/2, GNL2, GPR37, HERC5, HNRNPH1, HSCB, HSP, HSPA5, HSPB1, HSPB3, Insulin, Jnk, KATNA1, KRT19, LGALS4, MCM7, NFkB(complex), NME1, NUDT1, PCBP2, PDGFBB, PGK1, Pif, PSMB4, TCR, Ubiquitin	34	13	Cell-To-Cell Signaling and Interaction, Tissue Development, Hereditary Disorder
2	19s proteasome, ADSL, ATIC, C9orf9, CD320, CWC22, DDX39B, DHX16, EIF3D, EXOSC9, HNRNPLL, JKAMP, KATNA1, KIFC3, KIN, MED25, NUP88, OBFC1, PAAF1, PCYT1A, PPME1, PSMC6, RAB11A, RPS15A, SARNP, SEPHS1, TDRD1, THOC1, THOC3, THOC6, THOC7, UBC, VPS33B, WDR62, ZBTB25	18	8	Molecular Transport, RNA Trafficking, Developmental Disorder

Table 4. IPA pathway analysis of the proteomic dataset.

Ingenuity Canonical Pathways	-log (p-value)	Ratio	Molecules
Protein Ubiquitination Pathway	5.25E+00	1.96E-02	HSPB1, PSMB4, HSCB, HSPA5, PSMC6
Aldosterone Signaling in Epithelial Cells	3.28E+00	1.97E-02	HSPB1, HSCB, HSPA5
Inosine-5'-phosphate Biosynthesis II	2.50E+00	3.33E-01	ATIC
Selenocysteine Biosynthesis II (Archaea and Eukaryotes)	2.20E+00	1.67E-01	SEPHS1
Phosphatidylcholine Biosynthesis I	2.13E+00	1.43E-01	PCYT1A
Aryl Hydrocarbon Receptor Signaling	2.03E+00	1.43E-02	HSPB1, MCM7
Purine Nucleotides De Novo Biosynthesis II	1.90E+00	8.33E-02	ATIC
Choline Biosynthesis III	1.87E+00	7.69E-02	PCYT1A
Granzyme A Signaling	1.68E+00	5.00E-02	NME1
Endoplasmic Reticulum Stress Pathway	1.66E+00	4.76E-02	HSPA5

Table 5. IPA upstream regulator analysis of the proteomic dataset. There were only three regulators that regulate three or more proteins of the list with a significant z-score (Z-score > 2).

Upstream Regulator	Molecule type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
MYC	Transcription regulator		2.0	1.64E-03	DDX39B, HSPB1, MCM7, NME1, PGK1
miR-4651 (and other miRNAs w/seed GGGGUGG)	Mature microRNA	Inhibited	-2.2	4.64E-02	CFL1, DDX39B, OBFC1, PCBP2, PPME1
miR-495-3p (and other miRNAs w/seed AACAAAC)	Mature microRNA	Inhibited	-2.2	2.58E-03	HNRNPH1, HSPA5, PGK1, PPME1, PSMC6

FIGURES AND FIGURES LEGENDS

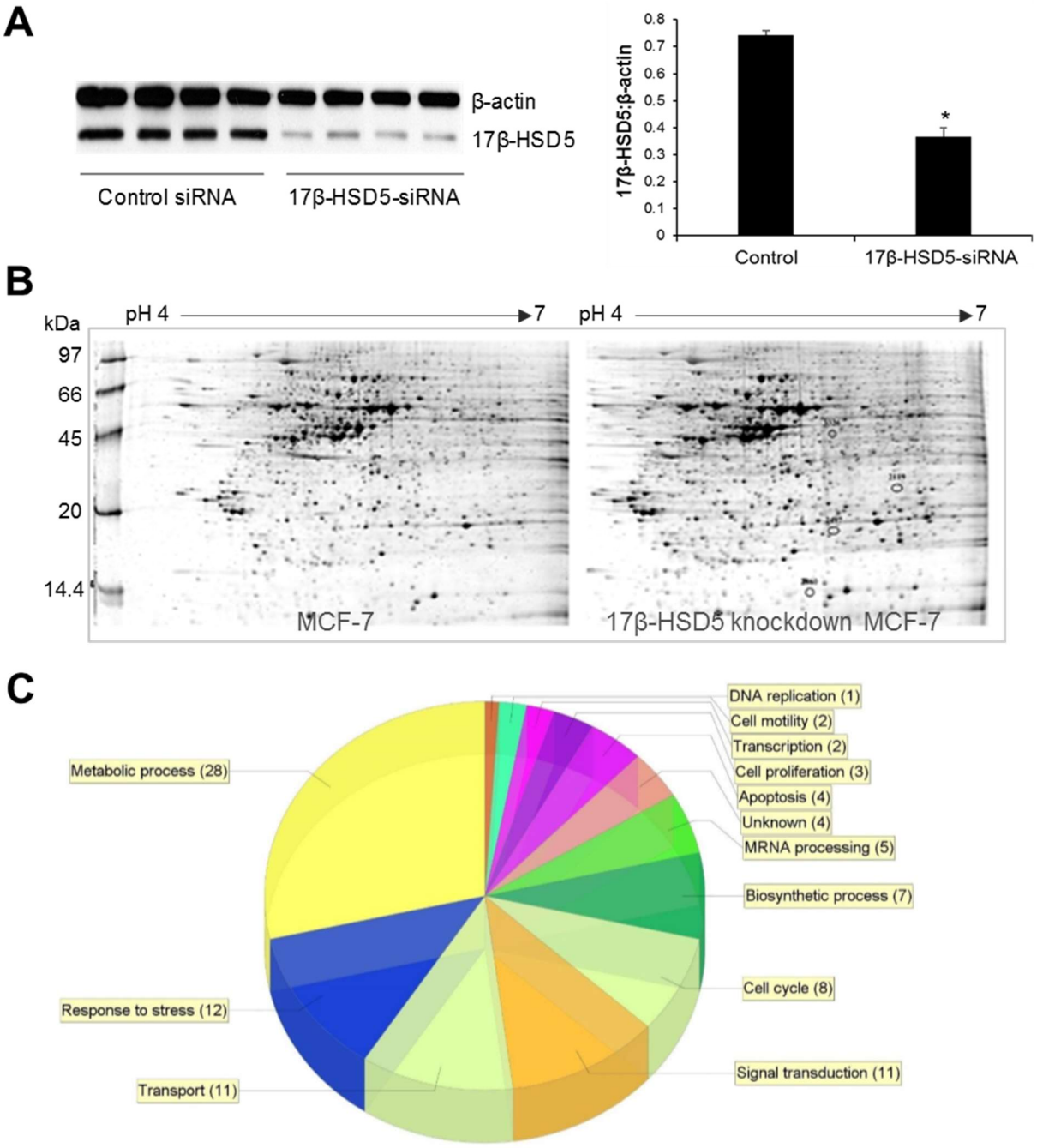


Figure 1. Proteomic analysis of MCF-7 cells transfected with control and 17β-HSD5 siRNAs. A) Western blot carried out before the proteomic analysis showed a successful 17β-HSD5 knockdown in all the four different protein samples extracted from cells transfected with 17β-HSD5 siRNAs. **B**) Representative 2-D gel images for MCF-7 cells and 17β-HSD5 knockdown MCF-7 cells showing the positions of the four differentially

expressed protein spots (2-fold or higher, $p < 0.05$). The four spots were picked for mass spectrometry (MS) analysis. **C**) Functional category of the 21 differentially expressed proteins (revealed by the MS analysis) between 17β-HSD5-knockdown and the parental MCF-7 cells.

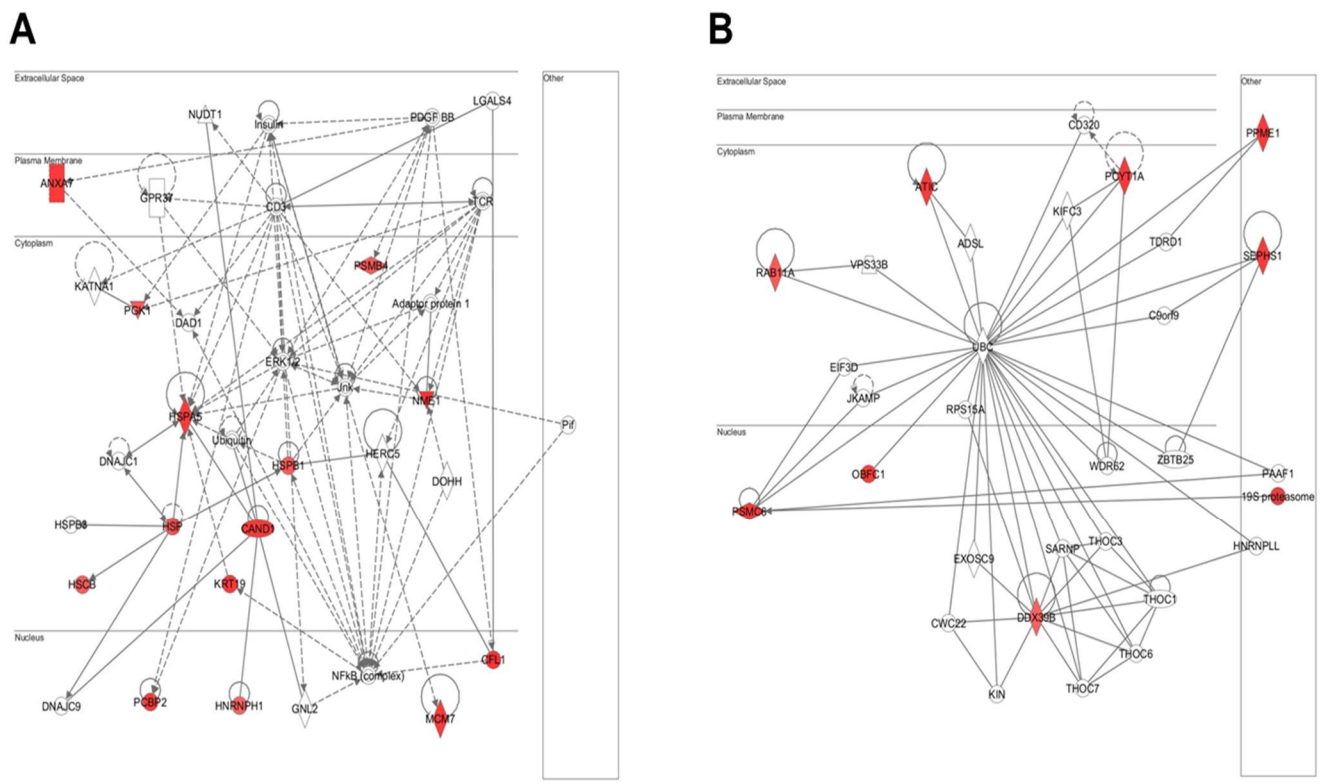


Figure 2. IPA network analysis. A) The first network: IPA highlights interaction between several proteins functionally associated directly and indirectly to 13 proteins (ANXA7, CAND1, CFL1, HNRNPH1, HSCB, HSPA5, HSPB1, KRT19, MCM7, NME1, PCBP2, PGK1, PSMB4). **B)** The second interaction network generated by IPA analysis consists of eight proteins from the list (ATIC, DDX39B, OBFC1, PCYT1A, PPME1, PSMC6, RAB11A, SEPHS1). A crucial part of the interactions network is the ubiquitin C (UBC) node.

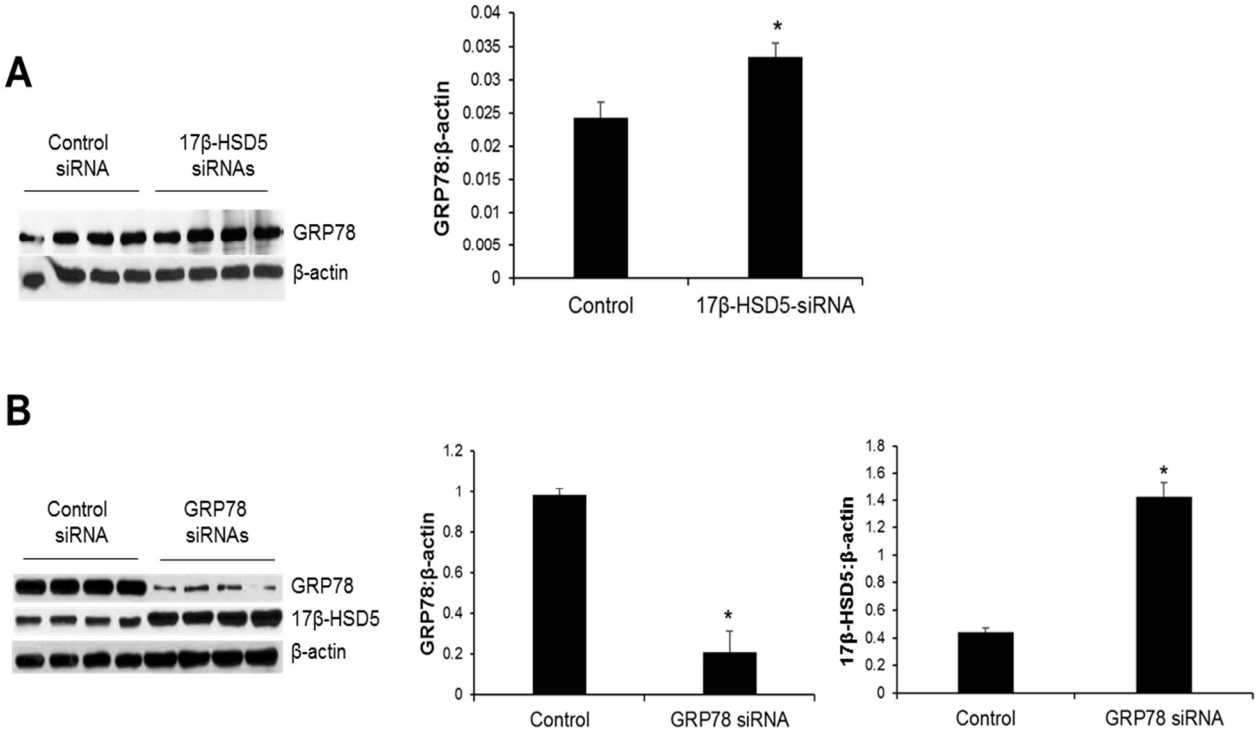


Figure 3. Negative crosstalk between 17β-HSD5 and GRP78 expression. A) The GRP78 expression was measured by Western blot after 17β-HSD5 knockdown. The Western blot image analysis showed that GRP78 expression was up-regulated by 37.6%. **B)** Western blot showed 17β-HSD5 protein up-regulation by GRP78 knockdown.

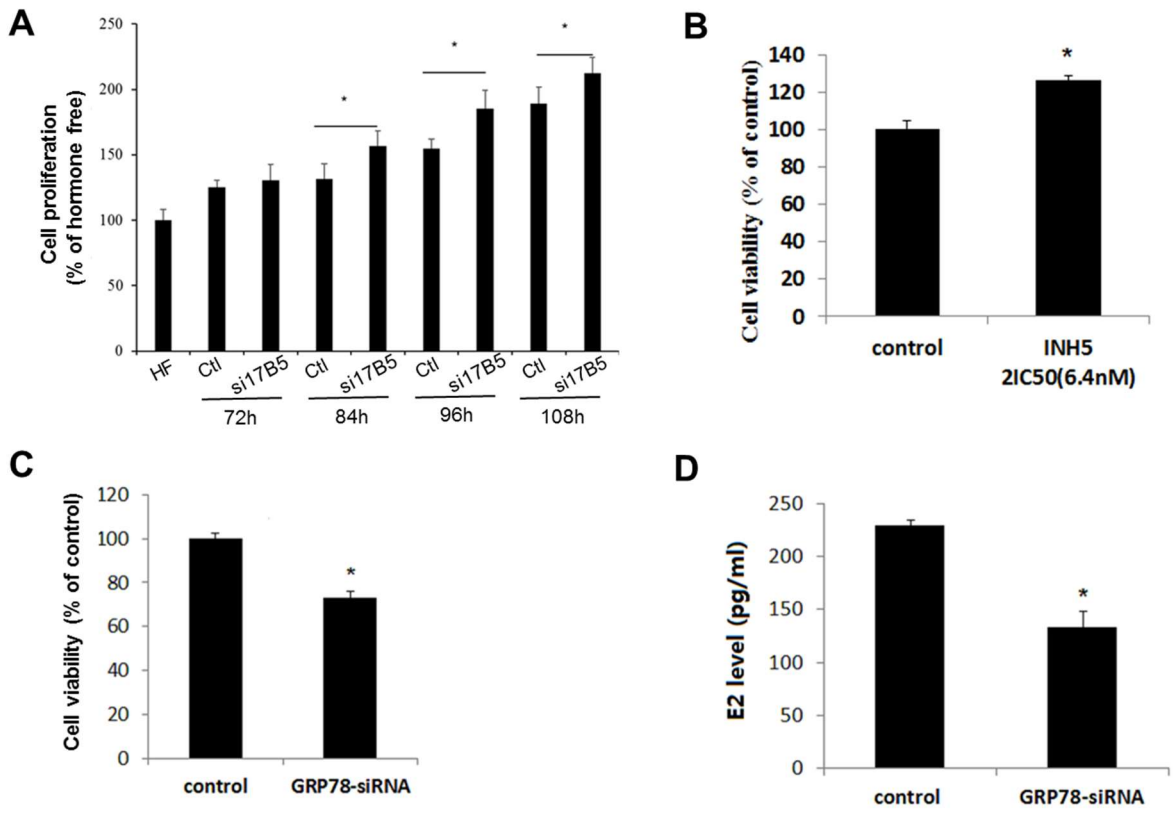


Figure 4. MCF-7 cell growth and estradiol production. A) Cell proliferation significantly increased after 17β-HSD5 knockdown. MCF-7 cell were transfected in 96-well plates with 100 nM control or mixed 17β-HSD5 siRNAs then supplement with 1μM DHEA. Hormone free group was untransted cells without DHEA. Cell proliferation assay was determined by CyQuant cell proliferation kit 72h, 84h, 96h and 108h after transfection. Hormone free group proliferation was fixed at 100% and data are reported as percentage of hormone free group proliferation. Each point represents the mean of experiments carried out in quadruplicate (mean±SD). Statistical significance by T-test:* $P < 0.05$ vs. control of each time point. HF, Hormone free; Ctl, Control; si17B5, 17β-HSD5 siRNAs. **B**) Cell viability significantly increased after 17β-HSD5 inhibition. **C** and **D**) Cell viability (C) and estradiol level (D) significantly decreased after GRP78 knockdown.

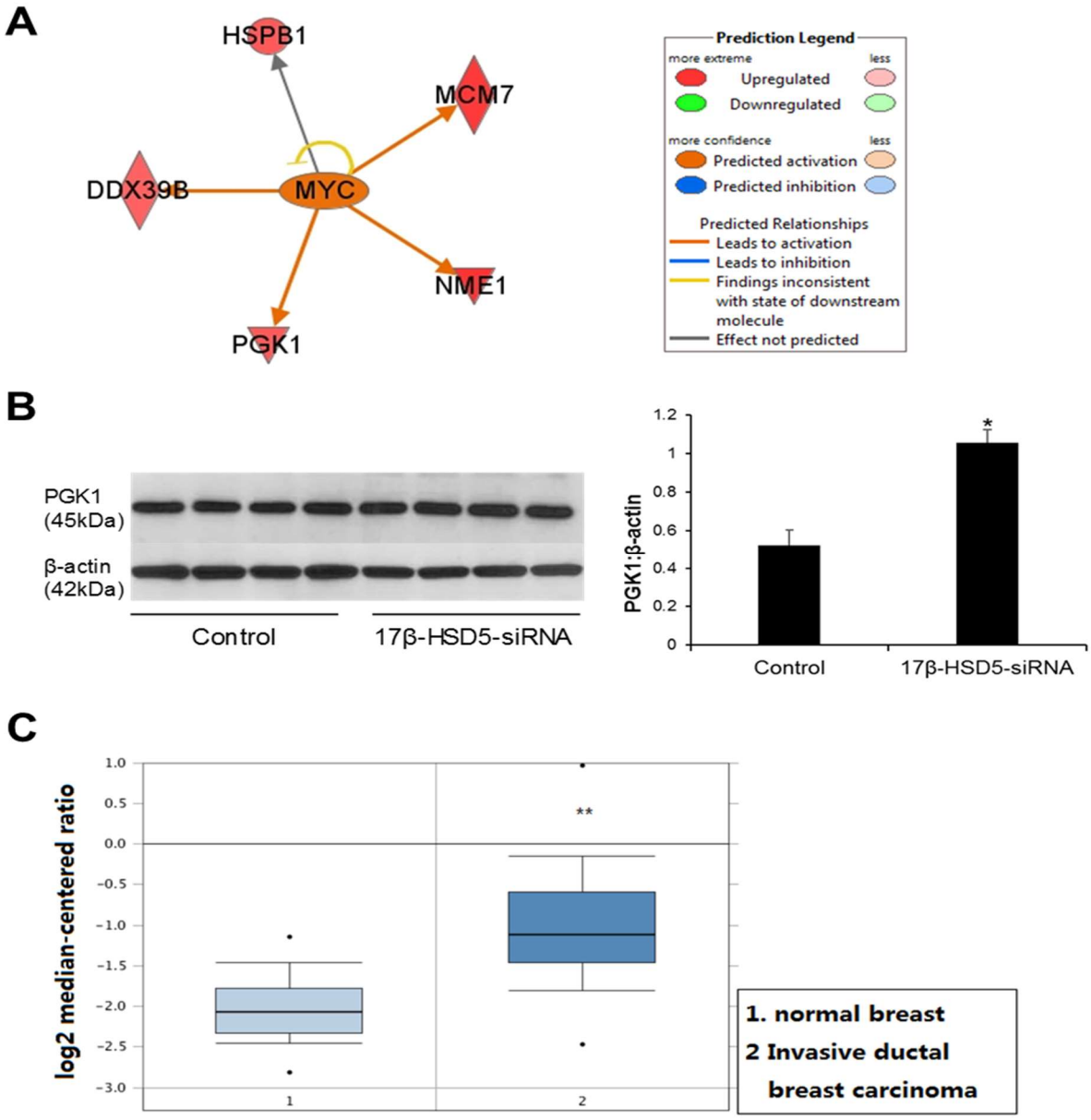


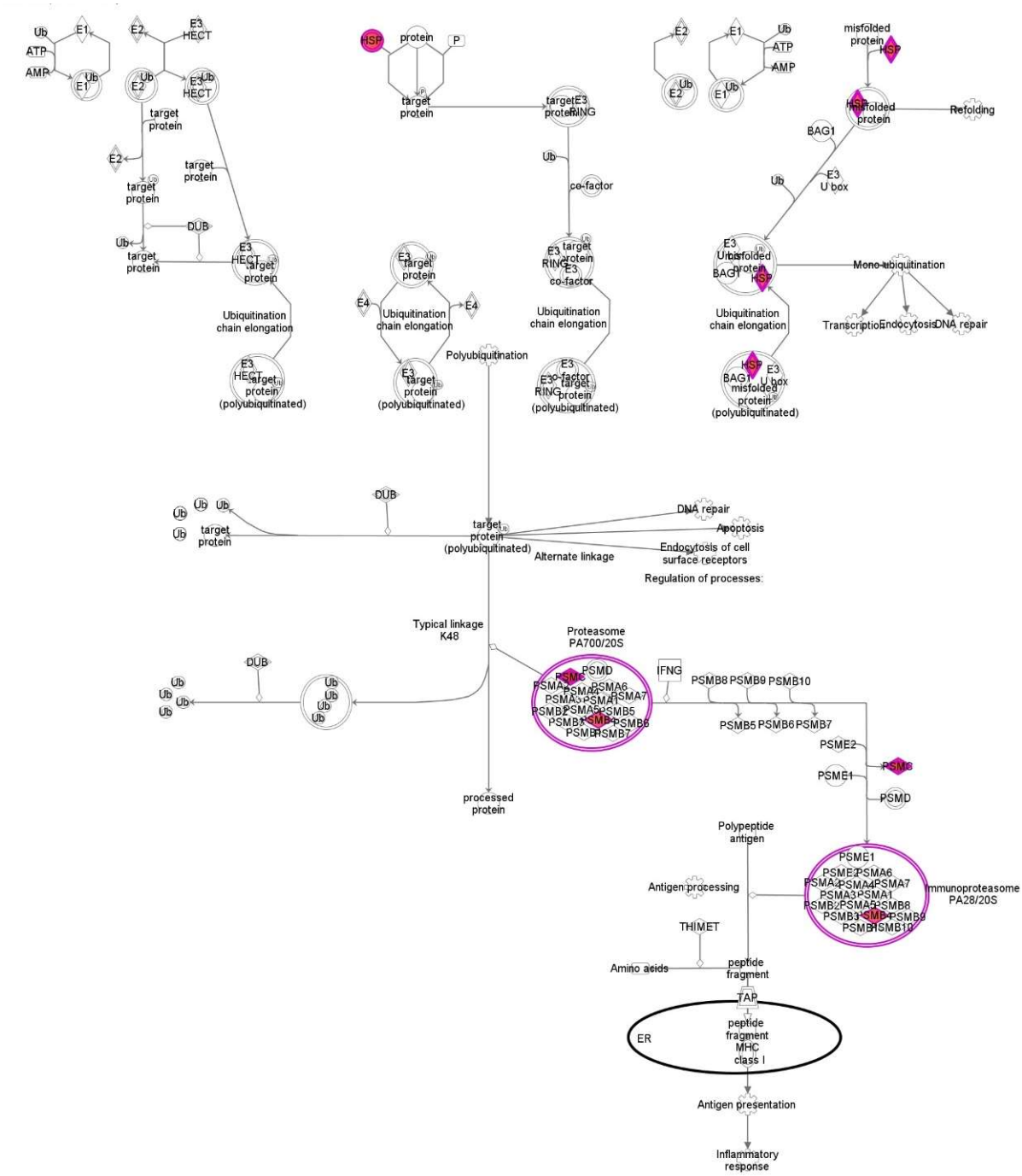
Figure 5. PGK1 expression was up-regulated in 17β-HSD5-knockdown MCF-7 cells. **A**) IPA analysis predicted activation of upstream regulator MYC. Target molecules in the dataset include DDX39B, HSPB1, MCMF, NME1 and PGK1. **B**) Western blot image and analysis showing PGK1 up-regulation after 17β-HSD5 knockdown in MCF-7 cells. **C**). Oncomine clinical database analysis of samples from the TCGA cohort showed that PGK1 was more highly expressed in invasive ductal breast carcinoma compared to normal breast tissue.

SUPPLEMENTAL FILES

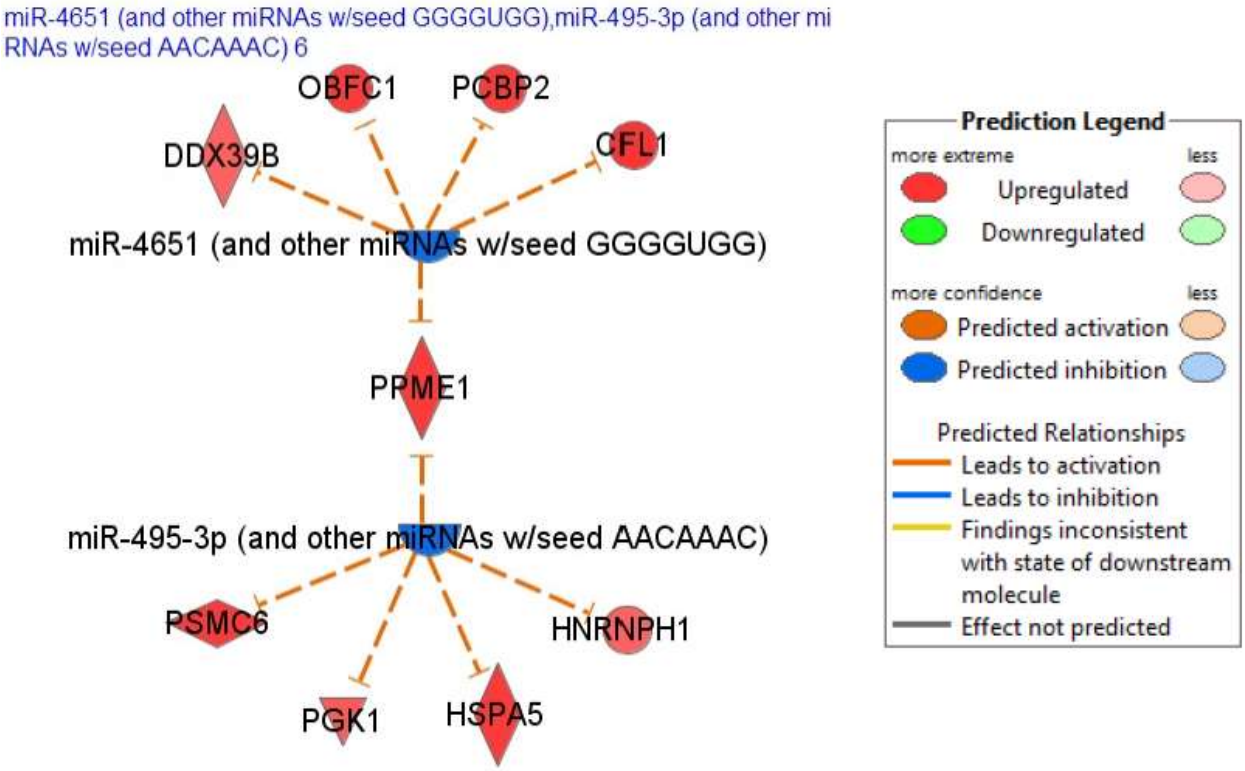
Supplemental Table 1. IPA upstream regulator analysis of the proteomic dataset with three or more regulated proteins. There were 23 regulators but only three (MYC, miR-4651 and miR-495-3p) showed a significant z-score (Z-score> 2).

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
MYC	transcription regulator		2.0	1.64E-03	DDX39B,HSPB1,MCM7,NME1,PGK1
TGFB1	growth factor		0.9	4.09E-04	CFL1,HNRNPH1,HSPA5,HSPB1,KRT19,NME1,PGK1
TP53	transcription regulator		0.1	3.16E-02	CAND1,HSPB1,MCM7,NME1
OSM	cytokine		-0.6	1.19E-03	HSPA5,KRT19,MCM7,PGK1
miR-344a-5p	mature microrna		-1.9	1.17E-02	CFL1,PCBP2,PPME1,SEPHS1
miR-4758-3p	mature microrna		-1.9	4.29E-02	HSPA5,PCBP2,PGK1,RAB11A
miR-4651	mature microrna	Inhibited	-2.2	4.64E-02	CFL1,DDX39B,OBFC1,PCBP2,PPME1
miR-495-3p	mature microrna	Inhibited	-2.2	2.58E-03	HNRNPH1,HSPA5,PGK1,PPME1,PSMC6
PML	transcription regulator			1.36E-05	CFL1,HSPB1,PCBP2
IGF1R	transmembrane receptor			3.57E-05	HSPA5,KRT19,NME1,PSMC6
MAPT	other			7.08E-05	CFL1,HSPA5,NME1,PGK1
PSEN1	peptidase			3.64E-04	CFL1,HSPA5,NME1,PGK1
APP	other			4.41E-04	CFL1,HSPA5,HSPB1,NME1,PGK1
SP3	transcription regulator			7.21E-04	HSPA5,PCYT1A,PGK1
SP1	transcription regulator			9.81E-04	HSPA5,KRT19,PCYT1A,PGK1
TCR	complex			2.05E-03	HSPA5,NME1,PGK1
HIF1A	transcription regulator			3.77E-03	HSPA5,KRT19,PGK1
PTEN	phosphatase			6.37E-03	KRT19,PCBP2,PGK1
HNF1A	transcription regulator			7.02E-03	HSPA5,OBFC1,PGK1
HNF4A	transcription regulator			7.83E-03	DDX39B,HSPA5,NME1,PCYT1A,PPME1,RAB11A
HRAS	enzyme			1.38E-02	HSPB1,KRT19,PCYT1A
CD3	complex			1.95E-02	HSPA5,NME1,PCBP2
HTT	transcription regulator			2.51E-02	HSPA5,PSMB4,SEPHS1

Supplemental Figure 1. Protein ubiquitination pathway generated by the ingenuity pathway analysis (IPA) software. Protein ubiquitination is associated with apoptosis, DNA repair and endocytosis of cell surface receptors regulation of the process. Proteins in shaded nodes were found to be highly expressed in 17β-HSD5-knockdown MCF-7 cells, as compared to MCF-7 cells.



Supplemental Figure 2. IPA analysis predicts activation of upstream regulator miR-4651 and miR-495-3p after 17β-HSD5 knockdown in MCF-7 cells. Target molecules in the dataset are shown.



Supplemental Figure 3. IPA biological function analysis. Only the organismal survival/death functional category showed a highly significant z-score (z-score > 2, $p < 0.05$), with eight proteins from the proteomic dataset involved.