Tampereen yliopisto

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CHARACTERIZATION OF Wnt5a-ROR PATHWAY IN CISPLATIN-RESISTANT OVARIAN CANCER CELL LINES

Faculty of Medicine and Health Technology
Master's Thesis
November 2020

Pro gradu -tutkielma

Paikka:	TAMPEREEN YLIOPISTO, Lääketieteen ja terveysteknologian tiedekunta
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Otsikko:	Wnt5a-ROR-signalointireitin karakterisointi sisplatiiniresistenteissä munasarjasyöpäsolulinjoissa
Sivumäärä:	58
Ohjaajat:	FT, dosentti Daniela Ungureanu
Tarkastajat:	FT, dosentti Daniela Ungureanu ja professori Vesa Hytönen
Päiväys:	5.11.2020

Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Epiteeliset munasarjasyövät (OC), joista 70–80% on alatyypiltään huonosti erilaistunutta seroosia karsinoomaa (HGSOC), ovat kehittyneiden maiden johtava gynekologisten syöpäkuolemien aiheuttaja. Tällä hetkellä standardihoitona on sytoreduktiivinen leikkaus ja sitä seuraava platinapohjainen kemoterapia yhdistettynä taksaaneihin kuten paklitakseliin. HGSOC:ta sairastavista potilaista alle 40% on elossa viiden vuoden kuluttua diagnoosista. Kemoresistenssi on merkittävä huonoon ennusteeseen johtava tekijä, minkä vuoksi uusien hoitokeinojen löytäminen on tarpeen potilaiden ennusteiden parantamiseksi.

ROR-proteiiniperhe (Receptor tyrosine kinase-like Orphan Receptor) on osa ei-kanoonista Wnt-signalointia ja koostuu ROR1- ja ROR2-reseptoreista, jotka pystyvät sitomaan Wnt5a-ligandin solunulkoisella domeenillaan. OC:ssa molemmat reseptorit ovat tärkeitä solun kasvun, migraation ja invaasion kannalta ja lisäksi ROR2:n korkea määrä korreloi platinaresistenssin kanssa. ROR1 puolestaan on huonon ennusteen markkeri OC:ssa.

Tässä tutkimuksessa pyrimme ymmärtämään ROR1:n ja ROR2:n ilmentymisen vaikutusta sisplatiiniresistenssiin ja selvittämään, kuinka ROR1:n tai ROR2:n hiljentäminen vaikuttaa kemoresistenttien OC-solujen selviämiseen.

Menetelmät: Kokeisiin käytettiin kaupallisia OC-solulinjamalleja. Sisplatiinisensitiivisyyttä tutkittiin solujen jakautumisnopeuden perusteella. Solulinjoja karakterisoitiin Western-blottauksella eri signalointireittien osalta; näihin lukeutuivat Wnt5a-ROR1/ROR2/PTK7-, AKT/PI3K-, EMT- ja Wnt/Ca^{2+}-reitit sekä kantasolusignalointi. ROR1:n ja ROR2:n hiljentämisen vaikutuksia tutkittiin käyttämällä shRNA:ta. Wnt5a-stimulaatiolla tutkittiin muutoksia AKT- ja ERK-proteiinien fosforylaatiossa.

Tutkimustulokset: Analysoimme Wnt5a-ROR1/ROR2-reitin ilmentymistä OC-solulinjoissa niiden sisplatiinisensitiivisyyden perusteella. Wnt5a/ROR2:n ilmentyminen kasvoi OVCAR3cis-soluissa ja ROR2:n ilmentyminen kasvoi A2780cis-soluissa verrattuna parentaalisolulinjoihin. ROR1:n hiljentäminen vähensi STAT3:n ilmentymistä A2780cis-soluissa. Wnt5a-stimulaatio kasvatti ERK-fosforylaatiota sisplatiiniresistenteissä Kuramochi-soluissa.

Johtopäätökset: Havaitsimme Western-blottauksen avulla, että Wnt5-ROR-signaloinnin aktiivisuus kasvoi OVCAR3cis- ja A2780cis-soluissa verrattuna parentaalisolulinjoihin. Tämän perusteella sisplatiiniresistenssillä ja ei-kaanonisella Wnt-ROR-signaloinnilla saattaa olla yhteys. Näihin reseptoreihin kohdistuvia hoitoja tulisi siksi pitää potentiaalisena lähestymistapana sisplatiiniresistenttien syöpäsolujen hoidossa.

Master's thesis

Place:	TAMPERE UNIVERSITY, Faculty of Medicine and Health Technology
Author:	Kaleva, Laura Kukka Elina
Title:	Characterization of Wnt5a-ROR Pathway in Cisplatin-resistant Ovarian Cancer Cell Lines
Pages:	58
Supervisors:	PhD, docent Daniela Ungureanu
Reviewers:	PhD, docent Daniela Ungureanu and Professor Vesa Hytönen
Date:	5.11.2020

Abstract

Background and aims: Epithelial ovarian cancers (OC), of which 70–80% are high-grade serous ovarian cancer (HGSOC), are the leading cause of gynecological cancer deaths in developed countries. Currently, the standard OC treatment is cytoreductive surgery followed by platinum-based chemotherapy and taxane agents such as paclitaxel. Chemoresistance contributes greatly to the poor prognosis of OC patients and the five-year survival rate in HGSOC is less than 40%. Therefore, novel therapeutic approaches are highly needed to improve patient outcomes.

ROR (Receptor tyrosine kinase-like Orphan Receptor) family of proteins belongs to the non-canonical Wnt pathway and comprises of two receptors, ROR1 and ROR2, that are able to bind Wnt5a ligand via their extracellular domain. In OC, both ROR1 and ROR2 are important for cell growth, migration, and invasion, while high levels of ROR2 correlated with platinum resistance. ROR1 is a prognostic marker for shorter overall survival of OC patients.

In this study, we aimed to understand how expression of ROR1 and ROR2 is related to cisplatin resistance and how targeting ROR1 or ROR2 expression affects OC cell survival.

Methods: Experiments were performed on commercially available OC cell lines. Cisplatin sensitivity was investigated via cell proliferation. Cell lines were characterized by Western blotting for the expression of signaling pathways such as Wnt5a-ROR1/ROR2/PTK7, AKT/PI3K, EMT, Wnt/Ca^{2+} and stem cell pathways. The effects of silencing ROR1 and ROR2 expression were investigated with shRNA. Wnt5a stimulation was done to investigate changes in phosphoprotein expression of AKT and ERK pathway.

Results: We analyzed the expression of Wnt5a-ROR1/ROR2 in OC cell lines based on their cisplatin sensitivity. Wnt5a/ROR2 was upregulated in OVCAR3cis compared to parental cell line. ROR2 was also upregulated in A2780cis compared to A2780 parental cell line. Targeting ROR1 downregulated STAT3 in A2780cis. pERK was upregulated in cisplatin-resistant Kuramochi cells after Wnt5a stimulation.

Conclusions: We observed upregulation of Wnt5a-ROR pathway by Western blotting in OVCAR3cis and A2780cis compared to parental cell lines, suggesting that acquired cisplatin resistance could be involved in changes in non-canonical Wnt signaling via ROR receptors. This will also mean that targeting these receptors in cisplatin resistant cells should be considered as a possible therapeutic approach.

Acknowledgements

This thesis project was carried out in Cancer Signaling group lead by PhD, docent Daniela Ungureanu, Tampere University, Tampere. I would like to thank my supervisor Daniela Ungureanu for her guidance, support, and patience throughout and beyond this project. I would like to thank Hanna Karvonen and Wilhelmiina Niininen for their guidance and help in and outside the lab. Working in Cancer Signaling group has given me invaluable experiences, lessons, and opportunities for which I am incredibly grateful. I would also like to thank my amazing support team at home.

Tampere 5.11.2020

Laura Kaleva

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Abbreviations

CTG	CellTiter Glo®
CSC	Cancer stem cell
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
HGSOC	High-grade serous ovarian cancer
HR	Homologous recombination
LGSOC	Low-grade serous ovarian cancer
LRP	Low-density lipoprotein receptor-related protein
OC	Ovarian cancer
PARP	Poly (ADP-ribose) polymerase
PCP	Planar cell polarity pathway
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1/2
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
Wnt	Wingless related integration site

1 Introduction

1.1 Ovarian cancer in brief

Ovarian cancer (OC) is one the most lethal cancers among women (Slaughter et al., 2016). Every year around 295,000 women around the world are diagnosed with this disease (Ferlay et al., 2018). 185,000 related deaths were estimated in 2018 (Ferlay et al., 2019). Globally, it is the 7th most common cancer and the 8th most common cause for a cancer-related death among women (Webb & Jordan, 2017; Lisio et al., 2019).

The survival rates have not improved significantly over the past four decades (Lisio et al., 2019) and the current overall five-year survival rate is less than 45% (Webb & Jordan, 2017). 70% of patients are diagnosed at advanced stage (III or IV) (Al-Alem et al., 2019; Pieterse et al., 2019) because the disease is often asymptomatic (Lheureux et al., 2019). For these patients, the 5-year survival rate is only 25% (Lheureux et al., 2019; Pieterse et al., 2019). By contrast, patients that are diagnosed at an early stage have a survival rate of 92% (Lheureux et al., 2019).

1.1.1 Origin of ovarian cancer

OC is a remarkably heterogeneous group of diseases and has several distinct sites of origin (Testa et al., 2017). It has been traditionally thought that most ovarian malignancies originate from ovarian surface epithelium (Nezhat et al., 2015) but OCs can also originate from endometrial tissue, the fallopian tube, or germ cells (Kurman & Shih, 2016). It is currently thought that most high-grade serous ovarian cancers (HGSOC) originate from secretory cells in the distal end of the fallopian tube (Kurman & Shih, 2016; Lisio et al., 2019).

Although some obscurity remains about the etiology of OC, some factors that could increase the risk of developing OC have been discovered (Ottevanger, 2017). Mutations in tumor suppressor genes *BRCA1* and *BRCA2* inflict a risk for developing OC and they can be hereditary (Ottevanger, 2017). Mutations associated with OC are further discussed in section 1.1.2. Higher number of lifetime ovulations has been acknowledged as a risk factor for ovarian cancer (Suh et al., 2017). Thus, ovulation-inducing agents also increase the risk of ovarian cancer (Ottevanger, 2017). Age at menarche and menopause and number of pregnancies can also affect the risk (Testa et al., 2017). There are two hypotheses for the mechanism of this phenomenon: Firstly, gonadotropins may have a stimulatory role in ovarian oncogenesis; secondly, higher

number of ovulation-related rupture and repair of ovarian surface epithelium increases the risk of spontaneous somatic mutations (Nezhat et al., 2015; Testa et al., 2017). Inflammatory effects after follicle rupture may also contribute to the incidence of ovarian cancer (Nezhat et al., 2015; Ottevanger, 2017).

Endometriosis may be a risk factor for developing certain subtypes of OC. Low-grade serous ovarian cancer (LGSOC), endometrioid, and clear cell carcinomas originate from borderline serous carcinomas or endometriosis. Different genetic, hormonal, and immunological factors have been also proposed as driving causes for malignant transformation of endometriosis (Nezhat et al., 2015).

1.1.2 Subtypes of epithelial ovarian cancer

OCs are divided into epithelial and non-epithelial cancers, as depicted in **Figure 1.** Epithelial subtypes include high-grade serous, low-grade serous, mucinous, clear cell, and endometrioid; non-epithelial subtype comprises sex cord-stromal subtype and other ovarian cancers. However, it is not unusual that a neoplasm consists of a mixture of different subtypes (Rojas et al., 2016; Testa et al., 2017). EOC tumors are highly heterogeneous both morphologically and molecularly and they have differential responses to therapy (Lheureux et al., 2019).

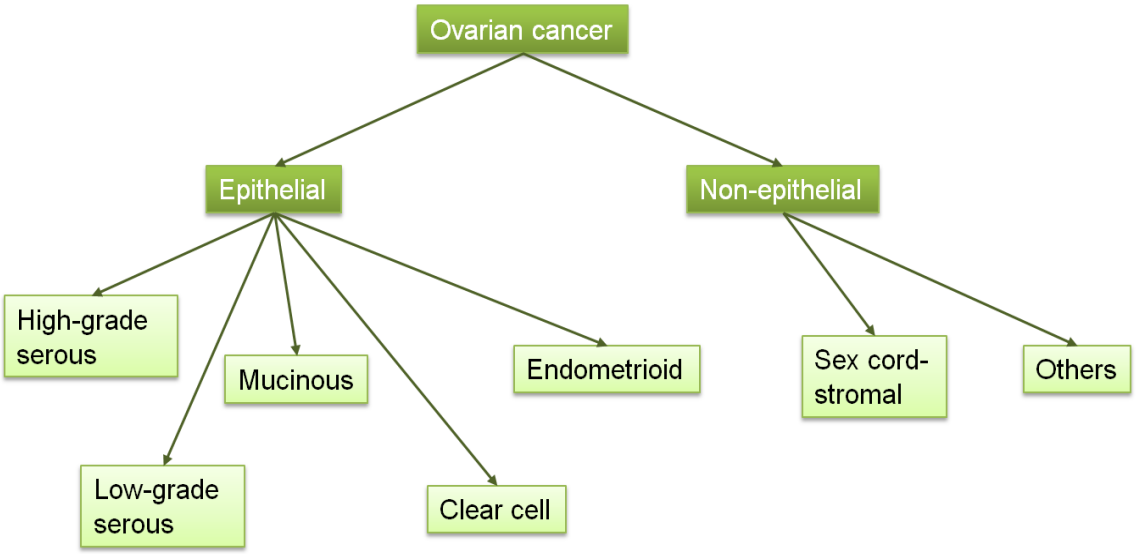

Figure 1. Subtypes of ovarian cancer. Ovarian cancer is classified into epithelial and non-epithelial subtypes. The epithelial subtype covers high-grade serous, low-grade serous, mucinous, clear cell, and endometrioid subtypes. Non-epithelial subtype covers sex cord-stromal subtype and other ovarian cancers that are not classified as any of the previous subtypes.

High-grade serous

The vast majority (75%) of EOC cases are high-grade serous ovarian cancer (HGSOC) (Lheureux et al., 2019). It is the most aggressive form of OC (Pieterse et al., 2019) and the main cause of mortality in gynecological malignancies (Testa et al., 2017). Today, the 5-year survival rate is approximately 35–40% (Al-Alem et al., 2019). Initially, HGSOC responds well to chemotherapy but will eventually acquire chemoresistance due to genetic instability (Cooke et al., 2010; Lheureux et al., 2019).

HGSOC can be further divided into different subtypes (Lisio et al., 2019) with different gene expression signatures (Kurman & Shih, 2016): differentiated, immunoreactive, mesenchymal, and proliferative. Among these HGSOC subtypes, the mesenchymal one has the poorest prognosis (Basu et al., 2018). HGSOC is thought to develop rapidly (Vang et al., 2009).

Over 95% of HGSOC cases involve *TP53* mutations (Kurman & Shih, 2011; Nezhat et al., 2015). Mutations in *BRCA1/2* are present in 15% of all HGSOC cases and it is estimated that any mutations leading to homologous repair deficiency are present in nearly 50% of all HGSOC

cases (The Cancer Genome Atlas Research Network, 2011; Grunewald & Ledermann, 2017; Basu et al., 2018). Homologous repair deficiency is usually associated with platinum sensitivity (Lheureux et al., 2019). Patients with mutations in *BRCA1* have a 40–50% risk of developing ovarian cancer and the equivalent risk for *BRCA2* is 10–20% (Suh et al., 2017; Webb & Jordan, 2017). Specifically, most of the hereditary *BRCA1/2*-associated OCs are of the HGSOC subtype (Vang et al., 2009).

Low-grade serous

Low-grade serous ovarian cancers (LGSOC) can be either invasive or non-invasive (Kurman & Shih, 2016) but they are non-aggressive (Lheureux et al., 2019). Majority of LGSOC tumors derive from Müllerian epithelium, ovarian surface epithelium, distal end of the fallopian tube or ovarian cancer-like stem cells (Ottevanger, 2017). LGSOC is characterized by low proliferative rate and relative chemoresistance (Lheureux et al., 2019). LGSOC is more common in middle-age women (Matz et al., 2017); the onset age is 45–57 years (Vang et al., 2009). The 5-year survival rate is 40–56% (Vang et al., 2009). *TP53* mutations are not common in LGSOC (Vang et al., 2009). Commonly mutated genes in LGSOC include *BRAF*, *KRAS*, *NRAS* and *PIK3CA* (Vang et al., 2009; Lheureux et al., 2019).

Endometrioid

Endometrioid OC accounts for 10% of EOCs (Kim J. et al., 2018). It can arise from secretory epithelial cells and basal cells of the fallopian tube, or the endometrium (Ottevanger, 2017). It is often associated with endometriosis (Nezhat et al., 2015; Lheureux et al., 2019). Endometrioid carcinomas are usually well-differentiated (Kurman & Shih, 2016). Key target genes of the endometrioid subtype include *PIK3CA*, *PTEN*, *ARID1A*, and *POLE* (Lheureux et al., 2019).

Clear cell

Clear cell ovarian carcinoma accounts for 10% of EOCs (Kim J. et al., 2018). The tumors can arise from secretory epithelial cells, basal cells, the proximal end of the fallopian tube, and endometrium (Ottevanger, 2017). Clear cells tumors often have large intratumoral heterogeneity (Testa et al., 2017). Key targets in treating clear cell carcinoma with targeted therapy include

PIK3CA, *ARID1A* and *PTEN* (Lheureux et al., 2019). Clear cell ovarian carcinoma is an aggressive cancer (Chan et al., 2008) and also resistant to chemotherapeutic drugs and has poor prognosis (Testa et al., 2017; Lheureux et al., 2019).

Mucinous

There have been inconsistencies in the reported frequencies of mucinous EOC; while several sources have previously reported it to be 10%, it is now thought that in fact, many metastatic sites of cancers from the gastrointestinal tract have mistakenly been presumed to be primary mucinous EOCs, and thus the actual frequency of mucinous EOC is closer to 3% (McCluggage, 2011; Rojas et al., 2016; Matz et al., 2017). Mucinous subtype arises from germ cells (Kurman & Shih, 2016). Mucinous EOC is well differentiated (Kurman & Shih, 2016). Alterations in RAS/RAF pathway are often present in mucinous EOC (Testa et al., 2017). Key target genes include *PIK3CA*, *KRAS*, and *HER2* (Lheureux et al., 2019). The mucinous subtype is resistant to platinum-based treatment (Testa et al., 2017; Lheureux et al., 2019).

Mixed type

Several subtypes can exist within the same tumor. Although this is usual, a tumor is only considered mixed subtype if the minor components account for at least 10% of the neoplasm (McCluggage, 2011). Seromucinous is one of the mixed subtypes. The seromucinous subtype is often non-invasive. The seromucinous tumors are composed of endometrioid, squamous, endocervical-type mucinous cells (Kurman & Shih, 2016). The relative frequency of mixed subtype is 6% (McCluggage, 2011).

1.1.3 Therapeutic approaches

Standard treatment and resistance

The current standard treatment of advanced-stage EOC involves primary debulking surgery followed by chemotherapy with platinum-based and taxane-based drugs to interfere with DNA repair mechanisms. An alternative option for patients with a more advanced disease is neoadjuvant chemotherapy followed by interval debulking surgery (Kim S. et al., 2018). Cisplatin, a commonly used platinum-based agent, forms crosslinks in DNA, thus interfering with normal DNA functions (Henry et al., 2016). The standard treatment guidelines have been constructed

largely based on the requirements of HGSOC, even though it has been suggested that treatment should be chosen in consideration of each subtype's genomic aberrations (Cooke et al., 2010; Lheureux et al., 2019).

The greatest challenge in treating OC is overcoming its recurrence and chemoresistance (Al-Alem et al., 2019). 70% of all EOC patients and 80% of advanced EOC patients are initially responsive to the treatment (Zhang S. et al., 2014; Ricci et al., 2018). Almost 25% of HGSOC cases will progress on primary platinum treatment or within six months after completion of therapy – these cases are defined as platinum resistant. For the rest of patients, relapses occur more than six months after primary treatment and these cases are considered platinum sensitive. Overall, almost 80% of HGSOC patients will eventually relapse, leading to the development of chemoresistance that is difficult to treat (Testa et al., 2017; Lisio et al., 2019). Longer platinum-free interval between treatment cycles is associated with better prognosis. However, extending the interval with other single-agent chemotherapies has been found to deteriorate overall survival (Suh et al., 2017).

Genetic heterogeneity of the tumor is a key factor in determining resistance and relapses (Cooke et al., 2010). Genetic heterogeneity and varying microenvironments of EOC lead to different responses to therapeutic agents (Basu et al., 2018; Pieterse et al., 2019). One reason for the poor survival rate of advanced-stage disease is that current treatment strategies are not able to eliminate the tumors to a full extent (Al-Alem et al., 2019).

Although not yet fully elucidated, it is known that several mechanisms can cause platinum resistance in OC cells; for example, regulation of transmembrane proteins directly affects the efflux of platinum (Ottevanger, 2017). Resistance can also be induced by mutations caused by exposure to different therapies (Lheureux et al., 2019). Upregulation of repair pathways such as PARP (Poly (ADP-ribose) polymerase) can be activated upon DNA damage (Ottevanger, 2017). Mesenchymal cells in the stromal microenvironment may promote chemoresistance by secreting specific signal molecules (Kim S. et al., 2018). Relapses can also occur if the chemotherapeutic agent is unable to access some parts of the non-resistant tumors for an environmental reason (Cooke et al., 2010). A patient may also have multiple mechanisms for chemoresistance (Lheureux et al., 2019).

Other approaches

Recently, interest has been shifted towards developing targeted agents instead of cytotoxic chemotherapy (Slaughter et al., 2016). The overall current trend is moving towards more personalized therapies (Kim S. et al., 2018). Non-selective cytotoxic chemotherapy results typically in toxicity while it provides only short-term anti-tumor responses (Grunewald & Ledermann, 2017). It is believed that targeted therapies could be less detrimental to normal cells (Cooke et al., 2010). It has also been suggested that different therapeutic approaches should be combined to target different subpopulations of cancer cells (Cooke et al., 2010; Lheureux et al., 2019).

PARP inhibitors. Poly (ADP-ribose) polymerase (PARP) inhibitors are currently one of the key targeted therapies of interest for treating ovarian cancer (Basu et al., 2018). PARP enzymes act in repair of DNA double-stranded breaks. When the homologous recombination (HR) repair pathway is deficient, the importance of PARP is emphasized (Basu et al., 2018). Mutations in *BRCA1/2* are the most common inducer of HR deficiency (Grunewald & Ledermann, 2017). It is estimated that PARP inhibitors, such as olaparib, could be effective against nearly 50% of HR-deficient HGSOC tumors (Basu et al., 2018; Veskimäe et al., 2018). PARP inhibitors have been shown to be effective against platinum-resistant OCs (Suh et al., 2017). However, several mechanisms, such as reverting mutations, allow cancer cells to gain PARP inhibitor resistance (Basu et al., 2018).

VEGF inhibitors. Vascular endothelial growth factor (VEGF) is needed in tumor blood vessels in greater quantities than in normal blood vessels and indeed, VEGF receptors are expressed in high amounts in cancer cells, as angiogenesis contributes to metastasis and tumor growth (Grunewald & Ledermann, 2017). Several genes that are involved in angiogenesis have been shown to have differential expression in HGSOC (Siamakpour-Reihani et al., 2015). Anti-angiogenic drugs or VEGF inhibitors, such as bevacizumab, normalize the structure and function of tumor blood vessels, which enhances the delivery of chemotherapeutic agents (Grunewald & Ledermann, 2017). However, VEGF inhibitors have been shown to have significant side effects on patients (Siamakpour-Reihani et al., 2015).

PI3K inhibitors. The PI3K/AKT/mTOR pathway mediates cell growth and proliferation and it is pivotal in tumorigenesis (Basu et al., 2018). The pathway is hyperactive in up to 70% of OC

tumors (Siamakpour-Reihani et al., 2015; Al-Alem et al., 2019). To date, however, agents targeting the pathway have shown little success in treating OC (Grunewald & Ledermann, 2017).

Tyrosine kinase inhibitors. Tyrosine kinase inhibitors can inhibit abnormal phosphorylation of tyrosine kinases, thus preventing different events of tumorigenesis such as increased proliferation, angiogenesis, and averted apoptosis. They can also target epidermal growth factor receptors (Basu et al., 2018).

Monoclonal antibodies. Monoclonal antibodies can be used to target ligands and receptors associated with cancer cells and deliver cytotoxic drugs and radioisotopes. The benefit is that toxicity to non-cancer cells can be minimized (Basu et al., 2018).

Immunoconjugates/immunotherapy. Immunoconjugates are monoclonal antibodies that recognize surface proteins on tumor cells and can be used to deliver cytotoxic drugs, cytokines, and radioisotopes to tumor cells (Basu et al., 2018). As an example, PDL-1 and PD1 have been targeted in such approaches; many tumor cells express PDL-1 (Basu et al., 2018), a ligand for programmed cell death 1 (PD-1) that is mainly expressed in T-cells. Along with other mechanisms, expression of PDL-1 allows the cancer cells to escape the immune system (Grunewald & Ledermann, 2017). Studies have investigated the use of anti-PD1 or anti-PDL-1 antibodies as treatment for ovarian cancer (Grunewald & Ledermann, 2017; Basu et al., 2018). In clinical trials, an anti-PD-1 antibody (nivolumab) has shown an overall response rate of 15% in platinum-resistant OC, whereas an anti-PDL-1 antibody (avelumab) showed an overall response of 11% in recurrent OC (Basu et al., 2018).

1.1.4 Ovarian cancer stem cells

Chemotherapy-resistant cancer stem cells (CSCs) are found in tumors in small numbers (<5%) (Li Y et al., 2018). They have lost the ability to maintain the homeostatic balance of self-renewal and their differentiation is dysregulated (Al-Alem et al., 2019). These cells can arise from dysregulation of proliferation and differentiation in normal stem cells or progenitor cells or from acquired expression of stem cell-related genes in normal cells (Katoh & Katoh, 2007). The tumor microenvironment can regulate the activation of CSC, supporting their chemoresistance, and maintaining their stemness (Pieterse et al., 2019).

CSCs are responsible for tumor initiation, dissemination, metastasis, and recurrence (Al-Alem et al., 2019; Pieterse et al., 2019). The proportion of CSCs in the tumor correlates with cancer

aggressiveness (Burgos-Ojeda et al., 2012). CSCs within tumors are thought to be intrinsically chemoresistant and their subpopulation can contribute to chemoresistance of the tumor (Testa et al., 2017). CSCs can persist after chemotherapy treatment and have then the ability to repopulate the tumor as a result of the selective pressure (Zhang S. et al., 2014; Rojas et al., 2016; Al-Alem et al., 2019; Pieterse et al., 2019). Multiple rounds of chemotherapy treatment can enrich the CSC population within the tumor by eradicating chemosensitive non-stem cells from the tumor (Pieterse et al., 2019). Indeed, the number of CSCs is low in pre-chemotherapy OCs, but it has been shown to increase after chemotherapy treatment (Ottevanger, 2017).

Targeting CSCs is therefore important in order to have better clinical response to therapy in OC. New therapies could be developed to target CSCs but characterizing the mechanism of action for regulating CSCs in OC is required (Pieterse et al., 2019).

1.2 Wnt5a-ROR signaling and its prognostic values in ovarian cancer

The Wnt signaling pathway is involved in many cellular processes such as proliferation, differentiation, polarity, adhesion, and motility (Asem et al., 2016). The Wnt signaling pathway can be divided to canonical (or β-catenin dependent) and non-canonical (or β-catenin independent) pathways (Ford et al., 2014) as shown in **Figure 2**. Both are involved in embryogenesis (Qi et al., 2014). There are 19 identified Wnt ligands in humans and these ligands can bind to several receptors such as Frizzled receptors, low-density lipoprotein receptor-related protein co-receptors (LRP), receptor tyrosine kinase-like orphan receptor family (ROR1 and ROR2), receptor tyrosine kinase (Ryk) receptor, and protein tyrosine kinase 7 (PTK7) receptor. The diversity of Wnt ligands, receptors, and co-receptors makes it possible to have many different downstream effects (Ford et al., 2014; Asem et al., 2016; Berger et al., 2017).

Canonical Wnt signaling is activated upon binding of Wnt molecule to Frizzled and co-receptor LRP5/6. This leads to the destruction of β-catenin destruction complex which, in turn, allows the accumulation of β-catenin, its translocation into the nucleus, and subsequent expression of target genes (Ford et al., 2016). This is illustrated in **Figure 2**. Subsequently activated signaling pathways include T-cell factor/lymphoid enhancer factor (TCF/LEF) pathway (Li X et al. 2018).

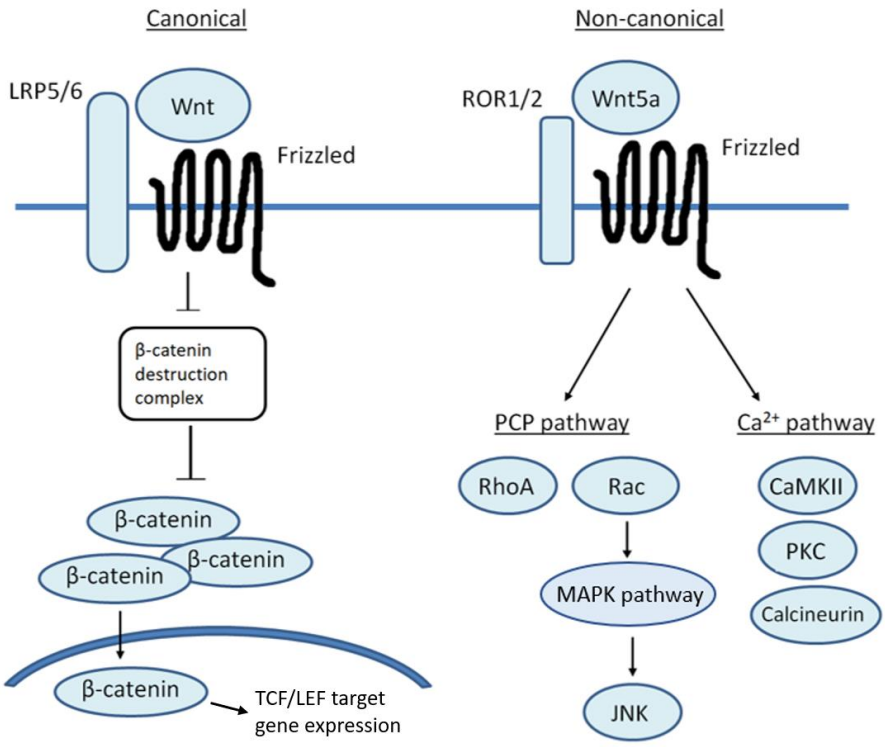

Figure 2. Canonical and non-canonical Wnt signaling pathways. Activation of the canonical Wnt signaling pathway leads to disassembly of β-catenin destruction complex which allows β-catenin to accumulate in the cell. *β*-catenin is translocated into the nucleus where it promotes the expression of TCF/LEF target genes. Non-canonical signaling consists of the planar cell polarity (PCP) and Ca^{2+} signaling pathways. Modified from Kobayashi et al., 2018. and Ford et al., 2016.

Non-canonical Wnt signaling is further described in the following sections.

1.2.1 ROR1 and ROR2 as receptors for Wnt5a in the non-canonical Wnt signaling pathway

RORs are type one (single-pass) transmembrane proteins. Their domain structure (**Figure 3**) is evolutionarily conserved (Zhang H. et al., 2014; Endo et al., 2015). RORs have an extracellular part that consists of an immunoglobulin-like domain, cysteine-rich domain (CRD), and Kringle domain. The cytoplasmic part consists of Tyrosine kinase-like domain, serine/threonine-rich domain (S/TRD), and a proline-rich domain (PRD). The CRD domain shares high homology with Frizzled CRD and has been identified as the domain that binds Wnt ligands (Stricker et al., 2017). ROR1 and ROR2 share a higher homology in S/TRD, 60% identical amino acid sequence, but their PRDs only share 30% (Endo et al., 2015).

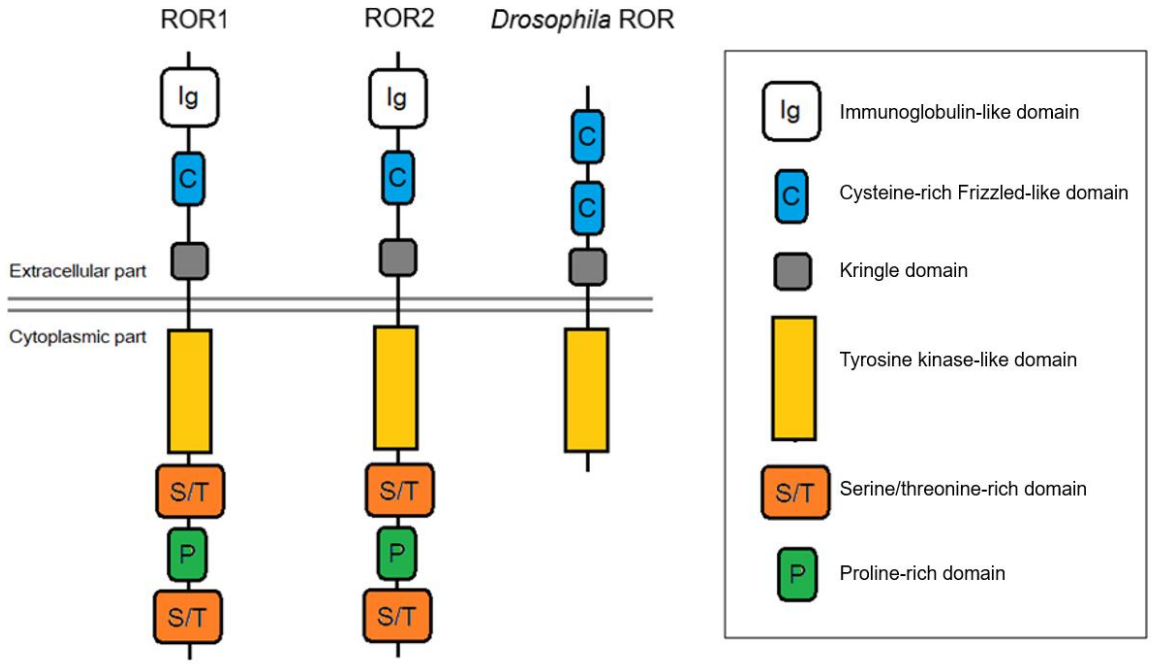

Figure 3. Schematic image of structures of ROR family receptors in human and *Drosophila*. The extracellular part of ROR1 and ROR2 consist of immunoglobulin-like domain, cysteine-rich Frizzled-like domain, and Kringle domain, while the intracellular part consists of tyrosine kinase-like domain, serine/threonine-rich domain, and proline-rich domain, as indicated in the image. The structure is conserved but the *Drosophila* ROR lacks some of the domains. Modified from Minami et al., 2010.

ROR1 and ROR2 are primarily expressed in cells during embryonic development, where they participate for example in gastrulation movements and formation of body axes (Stricker et al., 2017) as well as regulation of tissue and organ development (Minami et al., 2010). Their expression decreases in adult tissues (Asem et al., 2016), remaining detectable only in some adult tissue types (Liu et al., 2015). ROR1 and ROR2 are also involved in tissue repair and inflammation (Kamizaki et al., 2017). However, abnormal expression of ROR1 and ROR2 has been detected in many different cancers (Henry et al., 2016).

1.2.2 Main pathways activated by Wnt5a-ROR1/ROR2 signaling

Planar cell polarity pathway

The planar cell polarity (PCP) signaling pathway is involved in embryonic development (Mayor & Theneveau, 2014). PCP pathway participates in regulating epithelial cell polarity, motility, and migration (Ford et al., 2014; Henry et al., 2015). Cell orientation in the epithelial plane is

determined by this pathway (Berger et al., 2017). Activation of this pathway leads to rearrangements in the cell cytoskeleton (Mayor & Theveneau, 2014).

PCP pathway can be activated when Wnt5a binds to a ROR2-Frizzled complex (Stricker et al., 2017). PTK7 can also act as a regulatory receptor in the pathway (Berger et al., 2017). Downstream signaling molecules include RhoA, Rac, and JNK (Ford et al., 2016), as illustrated in **Figure 2**.

Wnt/Ca^{2+} pathway

The Wnt/Ca^{2+} pathway drives mobilization of intracellular Ca^{2+} (Asem et al., 2016). The pathway can be activated by Wnt5a-ROR2 signaling (Asem et al., 2016) but involvement of ROR1 has also been indicated (Katoh, 2017). Changes in the intracellular calcium level influence several cellular processes (Mayor & Thevenau, 2014) and can lead to changes in cell adhesion, such as actin cytoskeleton remodeling (Asem et al., 2016), and gene expression (Ford et al., 2016). Increase of intracellular Ca^{2+} results in activation of multiple transcription factors (Ford et al., 2014) and calcium-dependent signaling molecules (Asem et al., 2016). The Wnt/Ca^{2+} pathway is involved in cell proliferation, differentiation, polarity, and migration (Kühl et al., 2000). In ovarian cancer, specifically, the pathway is involved in tumor cell motility, migration, and actin cytoskeleton remodeling (Qi et al., 2014; Asem et al., 2016).

ROR1 and downstream signaling proteins

ROR1 and YAP/TAZ. Activity of Yes-associated protein (YAP) and its transcriptional co-activator PDZ-binding motif (TAZ) is often regulated by the Hippo pathway (Warren et al., 2018). Non-canonical Wnt signaling via ROR1 and Frizzled receptors can mediate the activation of YAP/TAZ signaling (Park H. et al., 2015). When activated via dephosphorylation, YAP translocates into the nucleus and induces the expression of target genes (Snigdha et al., 2019) via interaction with a diversity of transcription factors such as transcriptional enhanced associate domain (TEAD) family transcription factors and early growth response protein 1 (EGR-1) (Kim M. et al., 2018). YAP is involved in embryogenesis and in adult cells in tissue repair, but its overexpression in adult cells is associated with cancer (Warren et al., 2018; Snigdha et al., 2019). YAP/TAZ signaling also mediates cell migration and metastasis. High YAP/TAZ expression promotes formation of tumors (Warren et al. 2018). YAP/TAZ signaling has an antagonistic effect on canonical Wnt signaling (Park et al., 2015).

ROR1 and STAT3. Signal transducer and activator of transcription 3 signaling (STAT3) is involved in regulation of embryonic stem cells during development as well as adult stem cell maintenance (Galoczova et al., 2018). Constitutive activation of STAT3 in adult cells has been associated with many tumors (Galoczova et al., 2018). Furthermore, involvement of STAT3 in promoting OC pathogenesis has been documented (Al-Alem et al., 2019). STAT3 is part of the signaling pathway which can be activated by ROR1 (Katoh, 2017). Additionally, JAK-STAT3 signaling plays a role in upregulation of *WNT5A* expression (Katoh & Katoh, 2009).

ROR1 and NF-κB. Nuclear factor kappa B (NF-κB) is a transcription factor involved in many normal functions in the cell (Kamizaki et al. 2017; Al-Alem et al., 2019). It is a regulator of immune and inflammatory responses and it has a carcinogenesis-promoting role (Wang et al., 2015). High levels of NF-κB activation has been seen in many cancers, including OC (Al-Alem et al., 2019). Studies have shown that NF-κB can be activated by Wnt5a-ROR1 signaling in leukemia B cells (Kamizaki et al., 2017). NF-κB has been shown to be involved in OC invasion (Al-Alem et al., 2019) and EMT (Galoczova et al., 2018). It has also been suggested that NF-κB can mediate ROR1 expression via a feedback regulatory loop (Kamizaki et al., 2017).

1.2.3 Wnt5a-ROR expression and prognostic value in ovarian cancer

While usually absent in most adult tissues (Al-Alem et al., 2019), ROR1 and ROR2 are often overexpressed in cancers. ROR1 has been found to have an increased expression in high-grade, less-differentiated OCs, and it is considered a prognostic factor for poorer survival and stem cell-like gene expression in OC (Zhang S. et al., 2014; Henry et al., 2015; Asem et al., 2016).

In a study conducted by Zhang H. et al (2014), the quantity of ROR1 mRNA was found significantly elevated in both low and high-grade OCs in comparison to non-cancerous samples. The study also found that the expression was higher in advanced-stage (68%) and high-grade cancers in comparison to early-stage (45%) and low-grade. High ROR1 expression was present in 71% of OCs with metastases in lymph nodes as opposed to 46% in those without. ROR signaling activities in the tumor and stroma have differences across different OC subtypes (Zhang S. et al., 2014). ROR1 expression in the stroma is lower than in the tumor in clear cell, endometrioid, and mucinous OC, while the expressions are equal in serous OC. Presence of both ROR1 and ROR2 in the stroma surrounding tumors is heavily associated with poor survival (Henry et al., 2017).

ROR2 expression is similar between the distinct subtypes of EOC (Henry et al., 2015). ROR2 is overexpressed in OC and borderline tumors and in general, at high levels in cancer cells (Endo et al., 2015; Henry et al., 2015). In a study conducted by Henry, OC patients with ROR2 expression had better overall survival during a 20-year follow-up than those without, which may be due to ROR2 having a different function in ovarian oncogenesis.

Wnt5a expression has been discovered to be high in EOC patients, but there is no major difference between the four subtypes (Ford et al., 2014). Although a correlation has been found between high Wnt5a expression and reduced survival in OC, Wnt5a has also been seen to act as a tumor suppressor in several cancers (Qi et al., 2014).

ROR expression in EMT and metastasis

During epithelial-to-mesenchymal transition (EMT), epithelial cells are dedifferentiated and gain mesenchymal features. Cancer cells gain migratory and invasive properties while losing apical-basal polarity and cell-cell adhesions (Tan H et al., 2016; Endo et al., 2015). EMT is an important feature of CSCs (Liao et al., 2018).

Both canonical and non-canonical Wnt signaling pathways can activate EMT (Asem et al., 2016). It has been studied that Wnt5a signaling through ROR1 and ROR2 receptors can induce EMT in OC (Asem et al., 2016). Wnt5a expression has been found to correlate with tumor metastasis (Qi et al., 2014). It regulates migration and invasion of ovarian cancer cells (Henry et al., 2015).

ROR1 plays a role in invasion and migration (Henry et al., 2015; Testa et al., 2017). ROR1 is known to regulate both EMT and metastasis in breast cancer (Al-Alem et al., 2019). Enrichment of EMT-induced genes has been observed in tumor samples with high ROR1 expression. It has also been observed that OC cells with high ROR1 expression have a greater ability to invade extracellular matrix and engraft mice with immunodeficiency (Zhang S. et al., 2014). ROR1 overexpression changes expression of certain adhesion molecules – it namely reduces expression of epithelial type-related E-cadherin expression and increases mesenchymal type-related N-cadherin – and it induces changes in expression of mesenchymal-type related vimentin and transcription factor Snai1, which together suggests EMT occur (Tan H et al., 2016). Knockdown of ROR1, conversely, results in reduced expression of EMT markers (Henry et al., 2015).

ROR2 can increase in tumor cells at metastatic sites (Henry et al., 2017). ROR2 is a regulator of migration and invasion in OC cells (Henry et al., 2015).

In vitro studies with OC cell models have shown that while knockdown of either ROR1 or ROR2 decreases capacity for migration and invasion, knockdown of both has a more prominent effect (Al-Alem et al., 2019). ROR1 and ROR2 also have a synergistic effect on OC migration and invasion (Henry et al., 2015). It has previously been suggested that targeting ROR1 and ROR2 could provide a more efficient treatment for OC (e.g. Henry et al., 2016). Encouraging results have been seen in studies with preclinical and clinical models (Veskimäe et al., 2018).

ROR expression and chemoresistance

Upregulation of ROR1 and ROR2 leads to chemoresistance in OC (Henry et al., 2016; Veskimäe et al., 2018). ROR1 and ROR2 expressions have been found to be increased in patients after neoadjuvant chemotherapy (Henry et al., 2017). High Wnt5a expression is associated with lower sensitivity to many chemotherapeutic drugs (Veskimäe et al., 2018). Wnt5a/ROR2 has been proposed as a pathway responsible for chemoresistance modulation (Veskimäe et al., 2018).

1.2.4 Stem cell markers

CSC identification in EOC is still open to discussion even though there are several markers that are associated with stem-like properties (Li Y et al., 2018). Combinations of different stem cell markers are expressed in different types of OC (Al-Alem et al., 2019). Below we discuss some of these markers.

ALDH1

Ovarian CSCs express aldehyde dehydrogenase 1 (ALDH1) in a relatively high quantity (Zhang S. et al., 2014; Asem et al., 2016). It is expressed in the majority of OC patients in comparison to other stem cell markers (including CD24, CD44, CD117, and CD133) (Pieterse et al., 2019). High expression of ALDH1 has been associated with self-renewal and stress resistance in many cancers (Li Y et al., 2018). In OC cells, high ALDH1 correlates with higher pluripotency, stronger aggressiveness, EMT, and apoptosis resistance. ALDH1-positive cells have higher capacity of initiating tumors (Ottevanger et al., 2017). ALDH1 expression is associated with resistance to many cancer drugs such as taxanes and platinum; ALDH1 activity may also detoxify cytotoxic drugs (Zhang S. et al., 2014; Al-Alem et al., 2019). ALDH1 expression has also been shown to increase during each round of cisplatin treatment (Pieterse et al., 2019). ALDH1 expression has a positive correlation with increased ROR1 expression (Zhang S et al., 2014).

Bmi1

Polycomb complex protein BMI-1 (Bmi1) has an important function in regulation of normal stem cell and progenitor cell proliferation (Bhattacharya et al., 2009). It represses the transcription of genes that are involved in cell death and senescence (Park et al., 2004). It also plays an oncogenic role in OC as well as many other solid cancers (Testa et al., 2017). Bmi1 activity results in self-renewal of CSCs (Zhang S et al., 2014). OC cells express high levels of Bmi1, and that expression correlates with high-grade OC and advanced disease phase (Bhattacharya et al., 2009).

Cluster of differentiation proteins

Different surface antigens expressed in CSCs may serve as stem cell markers; these include CD24, CD44, CD117, and CD133 (Zhang S. et al., 2014; Pieterse et al., 2019). Any marker alone is not sufficient to identify CSCs, rather a combination of several is required (Al-Alem et al., 2019).

CD24. Ovarian CSCs with CD24 expression have an increased potential for tumor initiation (Burgos-Ojeda et al., 2012). CD24 is believed to be a metastasis-promoting factor. CD24-positive OC cells are associated with expression of stemness-associated genes and increased chemoresistance. However, it has a low specificity and cannot be used in identifying a specific

OC type or subtype. Overall, CD24 overexpression is connected to poor prognosis (Pieterse et al., 2019).

CD44. CD44 is a surface protein that is involved in migration, cell-cell interactions, cell adhesion, and invasion (Siamakpour-Reihani et al., 2015; Li Y et al., 2018). Expression of CD44 correlates with the HGSOC type and an advanced stage (Pieterse et al., 2019). CD44 expression has shown correlation with chemoresistance (Ottevanger et al., 2017). There have, however, been reports stating that CD44 expression alone does not suffice as a marker for ovarian CSCs due to lack of correlation between primary and recurrent OC (Pieterse et al., 2019). Combined expression of CD44 and CD117 has been shown to increase resistance to cisplatin, carboplatin, and paclitaxel in ovarian serous adenocarcinoma (Al-Alem et al., 2019; Pieterse et al., 2019). Cells that express CD44 and CD117 can also give rise to highly neoplastic EOC progenitor cells (Siamakpour-Reihani et al., 2015).

CD117. High CD117 expression in OC correlates significantly with chemoresistance (Ottevanger, 2017). HGSOC cells have high CD117 expression. CD117 is associated with increased tumor development; it promotes proliferation and evasion of tumor cell death in tumor cells (Al-Alem et al., 2019). CD117-positive cells have increased activity of migration and invasion (Li Y et al., 2018). Additionally, it has been found that CD117 promotes the ability to evade tumor cell death (Al-Alem et al., 2019). CD117 expression is connected to poor clinical outcome (Al-Alem et al., 2019).

CD133. CD133 has been recognized as a CSC marker in many solid cancers (Al-Alem et al., 2019). CD133 has been associated with HGSOC and tumors without response to cisplatin therapy (Testa et al., 2017; Pieterse et al., 2019). It is believed that CD133 suppresses differentiation, thus maintaining cell stemness (Pieterse et al., 2019). Studies have found CD133 to have an association with tumor development in OC, however, inconsistently to this, contradicting results have also been reported where CD133 expression did not affect OC cell tumorigenicity (Burgos-Ojeda et al., 2012; Al-Alem et al., 2019). CD133 expression correlates with high level of migration and invasion and high proliferation potential (Ottevanger et al., 2017; Li Y et al., 2018).

ROR1

OC CSCs expressing ROR1 are more commonly high-grade and less differentiated (Henry et al., 2015). Increased expression of ROR1 has been detected in OC cells and especially those that have CSC gene expression signatures (Zhang S. et al., 2014; Asem et al., 2016). For example, a correlation has been found between the expressions of ROR1 and ALDH1, as stated earlier (Asem et al., 2016). It has been proposed that ROR1 could be targeted for anti-CSC therapy (Henry et al., 2015). Recently, we have also shown that treatment with glucocorticoids like dexamethasone increases ROR1-associated stemness signature in OC cells. Dexamethasone treatment increased ROR1, ALDH1, YAP/TAZ and Bmi1 levels in OC cells and ex vivo patient samples (Karvonen et al., 2020).

2 Aim of the research

The aim of this research is to find improvements for the treatment of ovarian cancer by targeting ROR1 and ROR2 receptors. We wanted to see if the expression of ROR1/ROR2 is correlated with cisplatin resistance in ovarian cancer cell lines and how the cell viability is affected by targeting ROR1 or ROR2 expression and what intracellular pathways are affected then.

3 Materials and methods

3.1 Cell lines and culture conditions

Several different OC commercial cell lines were used for the experiments. The cell lines as well as their descriptions and growth media are listed in **Table 1**. Ascites represents the accumulation of inflammatory liquid in the peritoneal cavity of OC patients. Ascites samples are usually rich in tumor spheroids that are shedding from the primary OC tumor and is a rich source for OC cell line development.

Table 1. List of used cell lines and their respective growth media.

Cell line	Description/Source	Growth medium
A2780	Endometrioid adenocarcinoma; derived from an untreated patient; SIGMA	RPMI-1640 (Lonza, Basel, Switzerland), 10% FBS, 1% L-Glutamine (Lonza), antibiotic
A2780cis	Derived from A2780, cisplatin-resistant; SIGMA	RPMI-1640 RPMI-1640, 10% FBS, 1% L-Glutamine, antibiotic
JHOS2	HGSOC-like serous; useful model for BRCA-associated EOC; ATCC	DMEM/F-12 (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), 10% FBS
SKOV3	From ascites; no *TP53* mutations; ATCC	McCoy's 5a (Gibco™, Thermo Fisher Scientific), 10% FBS, antibiotic
Kuramochi	HGSOC-like; from ascites; ATCC	RPMI-1640, 10% FBS, 1% L-Glutamine, antibiotic
Ovsaho	HGSOC, metastasis from abdomen; ATCC	RPMI-1640, 10% FBS, 1% L-Glutamine, antibiotic
OVCAR3	From ascites, cisplatin-treated patient; ATCC	RPMI-1640, 10% FBS, 1% L-Glutamine, antibiotic
OVCAR3cis	Developed from OVCAR3 in our lab, cisplatin-resistant	RPMI-1640, 10% FBS, 1% L-Glutamine, antibiotic

Cell counts

Cells were counted with Countess™ II Automated Cell Counter (Thermo Fisher Scientific) using Trypan Blue Stain (0.4%, Invitrogen™, Thermo Fisher Scientific). The mean of 3 measurements was used.

Cell culture conditions

Culture conditions were kept at 37°C and 5% CO_2. Cells were cultured on Petri dishes in 8 mL growth medium (**Table 1**). Cisplatin resistance was maintained in A2780cis and OVCAR3cis by adding 1µM cisplatin (Sigma-Aldrich, Merck, Darmstadt, Germany) to the growth medium. Maintenance plates were split twice per week to 1/5. Cells were detached with TrypLE™ Express Enzyme (1X) with phenol red (Life Technologies, Thermo Fisher Scientific).

shRNA-induced cell lines

shRNA cell lines were used to study the effects of silencing ROR1 and ROR2. The following shRNA-induced cell lines were used: shCtrl, shROR1, and shROR2 for A2780, A2780cis, and JHOS2; and shCtrl and shROR2 for OVCAR3 and OVCAR3cis. The stable shRNA cell lines were produced in the Cancer Signaling laboratory. My task was to analyze the expression of signaling pathways after targeted shRNA KO of ROR1 or ROR2 receptors.

3.2 Determining cisplatin chemosensitivity of cell lines

Cisplatin chemosensitivity was determined with CellTiter Glo® 2.0 (CTG) (Promega, Madison, WI, USA) viability assays. All experiments were performed in triplicates.

All cell lines were cultured on flat-bottom 96-well plates at the density of 50,000 cells/mL. Cisplatin was added at the following concentrations: 1µM, 4µM, 6µM, 8µM, 10µM, 20µM, 30µM, 50µM, and 60µM. Untreated cells were used as control. Plates were incubated at 37°C for 72h. Additional assays were performed similarly for A2780, A2780cis, OVCAR3, and OVCAR3cis cell lines with concentrations of 250nM, 500nM, 1µM, 5µM, 10µM, and 100µM.

12µL of CTG reagent was pipetted into each well after the 72h incubation. Plates were centrifuged for 1min at 200G. The plates were incubated 30min at room temperature in darkness. Luminescence was measured with EnVision® Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

Data analysis

Data was analyzed with PRISM (GraphPad, version 5.02). The mean value of control samples was used to normalize all measured values. The relative survival curves were constructed by using $\log_{10}$ µM values of the concentrations. The plots were created using the following settings: Plot Mean and Error; SD → XY analyses. Analyze → Nonlinear regression (curve fit) → Dose-response – Inhibition: log(inhibitor) vs. response – Variable slope (four parameters). Constrain → HillSlope: must be less than 1.5.

3.3 Lysis and Western blotting

Cells were kept on ice during all steps of lysis. Media was rinsed with 4°C PBS. 100µL of Triton X lysis buffer (see **Table 2**) was used for every 6-well plate and 400µL for every Petri plate. Cells were scraped in the presence of the buffer and the suspensions were homogenized by pipetting up and down. The suspensions were collected and incubated on ice for 15min. The suspensions were centrifuged for 20min at 20,000G at 4°C. Supernatants were collected.

The lysates were balanced for protein concentrations with Bradford method and β-tubulin expression from the Western blot within the same cell line.

All samples were prepared by combining 25% 4x Laemmli loading buffer (see **Table 2**) and 75% lysate and boiling (95°C) the mixture on a heating block for 5 min to denature the proteins.

Table 2. Reagents used for cell lysis.

Reagent	Content	Final concentration
Lysis buffer	Triton X-100 (Sigma, #T8787-50ML)	1%
	Glycerol	10%
	NaCl	150mM
	EDTA	1mM
	NaF	50mM
	Tris-HCl pH 7.4	50mM
	Phosphatase inhibitor cocktail A (Bimake #B15001)	1%
	Phosphatase inhibitor cocktail B (Bimake #B15001)	1%
	Protease inhibitor cocktail (Bimake #B14001)	1%
	Potassium vanadine	1%
Laemmli sample buffer	Laemmli sample buffer 4x (Bio-Rad, #1610747)	

Proteins were separated from lysates with SDS-PAGE (125V, 400mA, 1h) on 7.5% and 10% gels and transferred (12V, 100mM, 50min) from the gels onto nitrocellulose blotting membranes (Amersham™ Protran™ 0.45µm NC, GE Healthcare Life science). After the transfer, the membranes were blocked in blocking buffer on a shaker for 1h at room temperature. The membranes were rinsed with Tris-buffer saline (TBS) and incubated in primary antibody dilutions (1:1000) overnight at 4°C with agitation.

The membranes were washed with each washing buffer, with three different Tween20 concentration (0.5%, 0.1%, and 0.05%), for 10 min each. The membranes were incubated in secondary antibody dilutions (à 1.5µL in 30mL of 0.05% Tween20 in TBS) protected from light while shaking for 1h in room temperature. Washings were repeated after this. Reagents used for both SDS-page and Western blotting are specified in **Table 3**.

The blots were scanned with Odyssey® CLx Imaging System (LI-COR) and analyzed with Image Studio Lite software (LI-COR). Protein bands were quantified by using the built-in "Add Rectangle" analysis tool.

Used primary antibodies are listed in the following sections according to the pathway that was studied. The used secondary antibodies were IRDye® 800 CW Donkey anti-mouse (Li-COR #926-32212) and IRDye® 700 CW Donkey anti-rabbit (Li-COR #926-68073).

Table 3. Reagents used in SDS-PAGE and Western blotting.

Step	**Reagents**	**Composition**
SDS-PAGE	Gel	30% Acrylamide/Bis Solution (161-0156 BioRad) 1.5M Tris, pH 8.8, with 0.4% SDS 1.5M Tris, pH 6.8, with 0.4% SDS 10% Ammonium persulfate 10% TEMED
	Running buffer	25mM Tris 192mM glycine 0.1% SDS
	2x Laemmli sample buffer	2x Laemmli sample buffer (Bio-Rad) β-mercaptoethanol
	Marker	PageRuler™ Plus Prestained protein ladder (Thermo Scientific)
Transfer	Transfer buffer	30.8mM Tris 0.24mM glycine 20% MetOH
Blocking	Blocking buffer	4% BSA (Sigma-Aldrich) in 0.05% Tween 20 (BioTop, Thermo Scientific)/ TBS solution
Antibody dilutions	Antibody dilution buffer	0.05% Tween 20 in TBS 0.5% BSA (Sigma-Aldrich) 0.005% NaN_3
Washing	Tween 20 / TBS buffers	0.5% Tween 20/TBS 0.1% Tween 20/TBS 0.05% Tween 20/TBS
	10X TBS (Tris-buffered saline)	250mM Tris 1.37M NaCl 27mM KCl pH 7.4 with HCl

3.3.1 Primary antibodies used in Western blotting

Cell lines were characterized for expression of Wnt signaling-associated proteins by Western blotting. The used antibodies are listed in **Table 4**.

Table 4. Antibodies for studying ROR1/2-Wnt5a signaling.

Antibody	**Size (kDa)**	**Isotype**	**Manufacturer**
ROR1	130	Mouse	BD Pharmingen #564464
ROR2	100-150	Mouse	BD Pharmingen #565550
LRP6	180, 210	Rabbit	Cell Signaling Technology #3395
PTK7	160	Rabbit	Cell Signaling Technology #25618
Wnt5a	45	Rabbit	Cell Signaling Technology #2530
β-catenin	92	Rabbit	Cell Signaling Technology #8480
RhoA	21	Rabbit	Cell Signaling Technology #2117

Activity of PI3K/AKT cell survival pathway was studied by determining protein expressions. The used primary antibodies are listed in **Table 5**.

Table 5. Antibodies for studying PI3K/AKT pathway.

Antibody	**Size (kDa)**	**Isotype**	**Manufacturer**
AKT	60	Mouse	Santa Cruz #sc-5298
pAKT (S473)	60	Rabbit	Cell Signaling Technology #4060
ERK	42, 44	Mouse	Santa Cruz #sc-135900
pERK	42, 44	Rabbit	Cell Signaling Technology #9101
MEK	45	Mouse	Santa Cruz #sc-6250
pMEK	45	Rabbit	Cell Signaling Technology #9121
NF-κB	65	Mouse	Cell Signaling Technology #6956
pNF-κB (S536)	65	Rabbit	Cell Signaling Technology #3033
STAT3	79, 86	Mouse	Cell Signaling Technology #9139
pSTAT3 (Y705)	79, 86	Rabbit	Cell Signaling Technology #9145

Calcium ion regulation pathway

The effects of Wnt/Ca^{2+} signaling on the calcium ion regulation pathway were studied using a calcium ion regulation antibody kit. The used antibodies are listed in **Table 6**. shRNA-induced JHOS2, A2780, and A2780cis cell lines were used in addition to parental cell lines to study the effects of silencing ROR1 and ROR2 on the activity of the pathway.

Table 6. Antibodies used for studying calcium ion regulation.

Antibody	**Size (kDa)**	**Isotype**	**Manufacturer**
ATP2A1 (Sarcoplasmic/endo-plasmic reticulum calcium ATPase 1)	100	Rabbit	Cell Signal Technology #12293
ATP2A2	114, 140	Rabbit	Cell Signal Technology #9580S
PKA C-α	42	Rabbit	Cell Signal Technology #5842
pPKA C	42	Rabbit	Cell Signal Technology #5661
Phospholamban	12, 24	Rabbit	Cell Signal Technology #14562
p-Phospholamban	12, 24	Rabbit	Cell Signal Technology #8496

Epithelial-to-mesenchymal transition pathway

Expression of EMT-associated proteins was studied with antibodies listed in **Table 7**. The analysis was done for shRNA-induced A2780 and A2780cis cell lines to see how silencing ROR1 and ROR2 affects the activity of the signaling pathway.

Table 7. Used antibodies related to epithelial-to-mesenchymal transition pathway.

Antibody	**Size (kDa)**	**Isotype**	**Manufacturer**
E-cadherin	135	Rabbit	Cell Signaling Technology #3195
N-cadherin	140	Rabbit	Cell Signaling Technology #13116
Oct-4	45	Rabbit	Cell Signaling Technology #2750
Snai1	29	Rabbit	Cell Signaling Technology #3879
TCF8	200	Rabbit	Cell Signaling Technology #3396
Vimentin	57	Rabbit	Cell Signaling Technology #5741
ZO-1	220	Rabbit	Cell Signaling Technology #8193

Stem cell pathway

Cancer cell stemness of the OC model cell lines was studied by determining the expression levels of the following stem cell markers listed in **Table 8**.

Table 8. Antibodies used for studying cancer cell stemness.

Antibody	**Size (kDa)**	**Isotype**	**Manufacturer**
Bmi1	41, 43	Rabbit	Cell Signaling Technology #5856
Snai1	29	Rabbit	Cell Signaling Technology #3879
YAP/TAZ	50, 70	Rabbit	Cell Signaling Technology #8418

3.4 Wnt5a stimulation

Kuramochi, Ovsaho, and SKOV3 cell cultures were set up on 6-well plates: 125,000 cells/mL were plated for Kuramochi and Ovsaho; 100,000 cells/mL were plated for SKOV3. After 24h of culturing, the cells were rinsed with PBS and then put in starvation media (0% FBS) for 24h. Cells were stimulated with 50nM Wnt5a (BioTechne, Minneapolis, MN, USA; 100µg/mL stock was diluted in PBS) for the durations of 2h and 30min and then lysed. Cells without Wnt5a stimulation were used as control. Protein expressions of unphosphorylated and phosphorylated AKT and ERK (see **Table 5**) were determined with a Western blot analysis to investigate how the stimulation affects their signaling activities.

4 Results

4.1 Analysis of cisplatin sensitivity of ovarian cancer cell lines

We wanted to investigate the cisplatin sensitivity of OC cell lines A2780, A2780cis, OVCAR3, OVCAR3cis, SKOV3, JHOS2, Kuramochi, and Ovsaho. We developed in our lab the cisplatin-resistant variant of OVCAR3, by incubating the cells with increased concentrations of cisplatin for a longer period of time (3-4 months). For the cisplatin sensitivity assay, cells were cultured in 96-well plates and treated with increased amounts of cisplatin for 72h. Untreated cells were used as control. Cell viability was measured by CTG assays, which is a fluorescent-based assay that measures the amount of ATP in live cells. Additional assays to further highlight the differences between A2780 and A2780cis as well as OVCAR3 and OVCAR3cis survival were done separately with increasing cisplatin concentrations. OV90 cell line, marked with red in **Figure 4**, was not part of this thesis project and thus will not be discussed further.

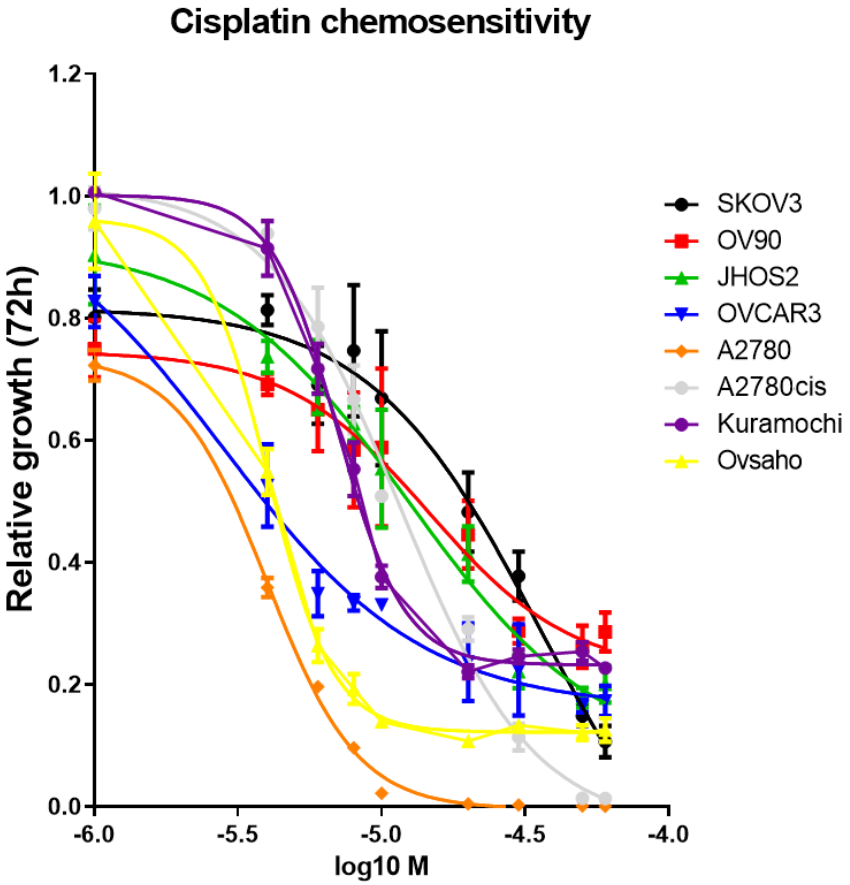

Figure 4. Relative survival of the cell lines as function of logarithmic concentration of cisplatin after 72h. The graph illustrates how the viability of different cell lines decreased when cisplatin concentration was increased. The bars represent mean value ± SD. The data points from left to right are 1µM, 4µM, 6µM, 8µM, 10µM, 20µM, 30µM, 50µM, and 60µM.

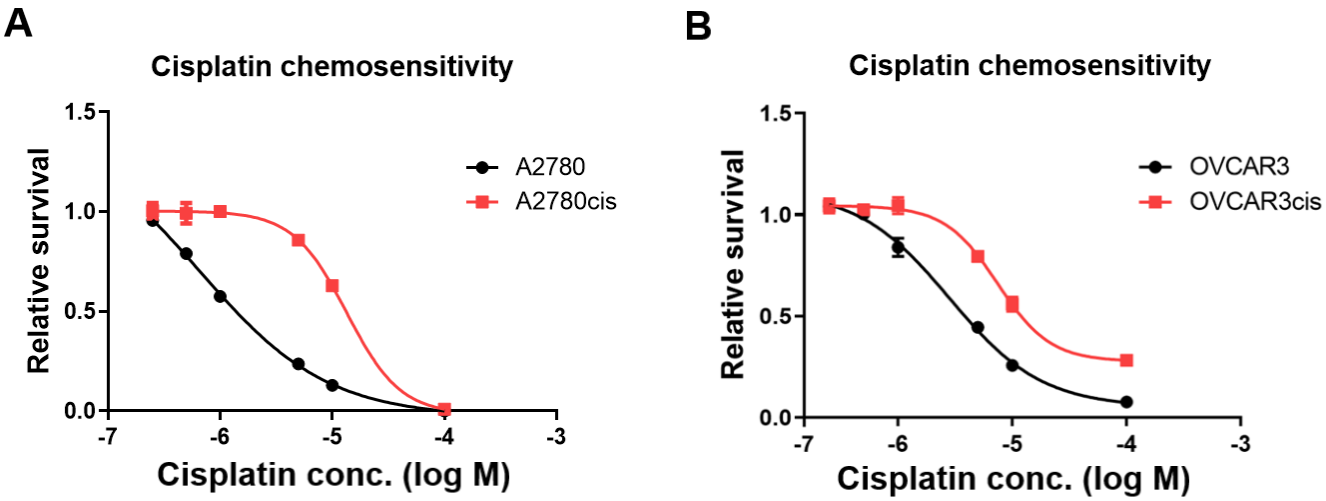

Figure 5. Relative viability of **A.** A2780 and A2780cis, **B.** OVCAR3 and OVCAR3cis after 72h in various cisplatin concentrations. The data points from left to right are 250nM, 500nM, 1µM, 5µM, 10µM, and 100µM. The survival is presented as a function of logarithmic cisplatin concentration.

Cisplatin chemosensitivity assay (**Figure 4**) showed how the different cell lines responded to cisplatin in terms of relative growth at increasing drug concentrations.

A2780, OVCAR3, and SKOV3 had the lowest relative survival values at the lowest studied cisplatin concentration (1µM) in the assay presented in **Figure 4**, whereas Kuramochi and A2780cis had the highest relative survival values. However, the relative growth rate of SKOV3 started decreasing after 10µM concentration which was higher compared to the other cell lines. In addition to A2780 and A2780cis, Kuramochi and Ovsaho also showed drastic decreases of relative growth as the cisplatin concentration increased. Ovsaho and Kuramochi both reached a clear plateau after which the relative growth rates did not decrease (10µM and 20µM, respectively). For Ovsaho, the decrease was more drastic and the eventual survival rate at the plateau was lower. SKOV3 and JHOS2 did not reach a plateau within the used concentration range.

Comparison of IC50 values, i.e. cisplatin concentrations at which the relative survival of each cell line was reduced to half, showed that the most sensitive cell lines were A2780 and OVCAR3 and the most platinum-resistant cell lines were SKOV3 and JHOS2. Interpolation of the curves at the relative survival of 0.5 shows that the relative survival of A2780cis was reduced to half at a cisplatin concentration that was approximately 10-fold higher than the required concentration for A2780 (**Figure 5A**). Similarly, OVCAR3cis required a concentration approximately 3-fold higher than OVCAR3 (**Figure 5B**). A2780cis and OVCAR3cis are clearly more resistant to cisplatin than their parental cell lines. The comparison between cell line pairs OVCAR3 and OVCAR3cis (**Figure 5B**) showed that the cisplatin-sensitive cell lines started responding to cisplatin treatment at lower concentrations, whereas cisplatin-resistant cell line started responding only at higher concentrations. The difference between OVCAR3 and OVCAR3cis was similar as the difference between A2780 and A2780cis in sense that OVCAR3cis survived at the higher cisplatin concentration compared to parental cell line.

4.2 Analysis of Wnt5a-ROR pathway expression and its downstream signaling in ovarian cancer cell lines

4.2.1 Wnt pathway

We wanted to investigate the expression of non-canonical Wnt signaling pathway-associated receptors such as ROR1 and ROR2 and Wnt5a ligand by Western blot analysis on the studied OC cell lines. Cells were cultured according to manufacturers' instructions and were lysed after 48h culture and subjected to SDS-PAGE gel run and Western blotting with various antibodies. The results of the Western blot analyses are presented in **Figure 6A**.

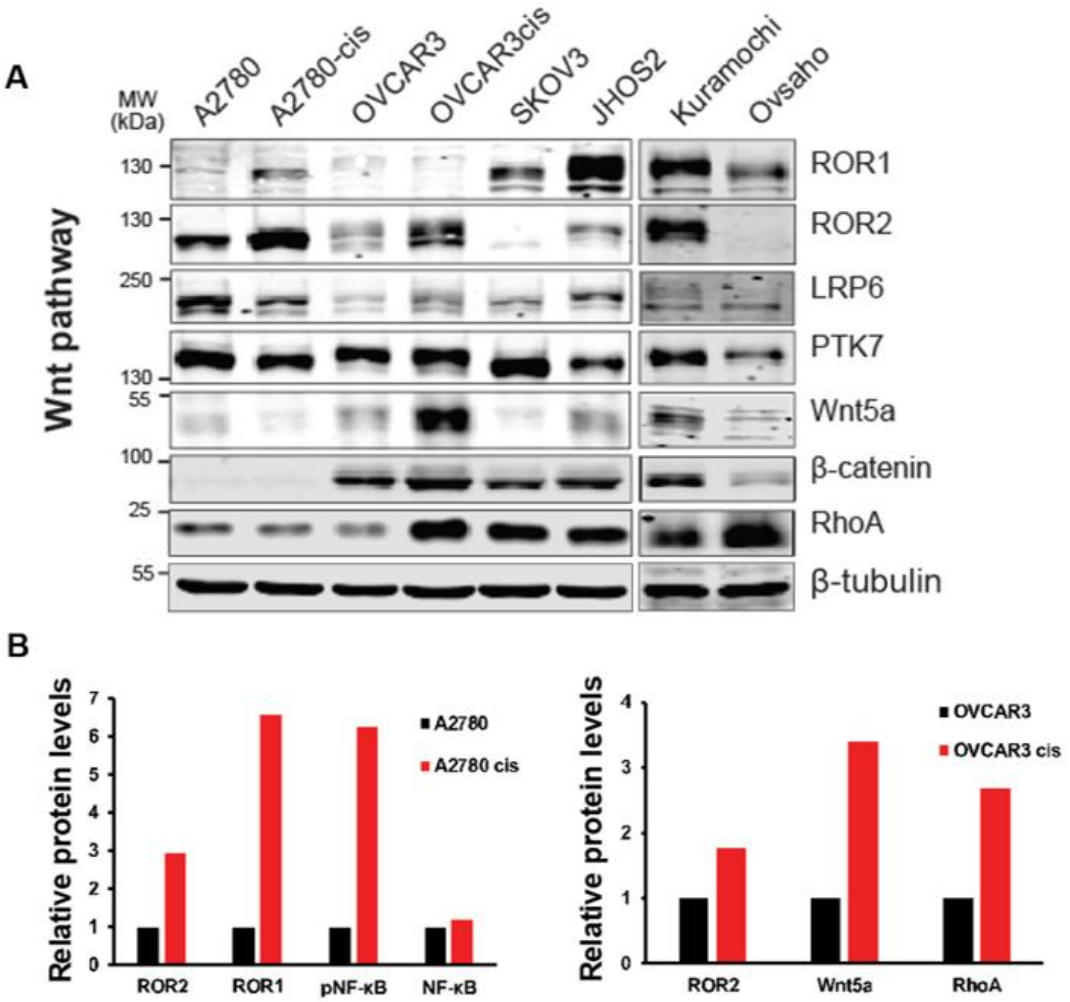

Figure 6. **A.** Expressions of Wnt pathway-associated proteins in OC cell lines were studied with Western blot analyses on lysed cells. **B.** Quantification of proteins that were the most differentially expressed between parental and cisplatin-resistant cell lines as indicated. Quantifications were based on Western blot analyses in **Figure 6A** and **Figure 7**. Protein expressions were normalized with the protein quantities in the parental cell lines. The quantification was done with a built-in analysis tool in ImageStudio software (Li-COR).

ROR1. ROR1 was expressed in A2780cis but not in A2780. Quantitative comparison of A2780 and A2780cis (**Figure 6B**) showed that ROR1 expression was more than 6-fold higher in the cisplatin-resistant cell line. ROR1 was not expressed in OVCAR3 and OVCAR3cis. JHOS2, SKOV3, Kuramochi, and Ovsaho all had ROR1 expression. There seems to be a correlation of ROR1 expression with higher cisplatin resistance in ovarian cancer cell lines.

ROR2. ROR2 expression was increased in A2780cis and OVCAR3cis when compared to their cisplatin-sensitive parental cell lines. Quantitative comparison of A2780 and A2780cis showed that ROR2 expression increased 3-fold in the cisplatin-resistant cell line (**Figure 6B**). OVCAR3cis had a 2-fold increase in ROR2 expression in comparison to OVCAR3 (**Figure 6B**). While Ovsaho was the only studied cell line to not express ROR2, SKOV3 had also very low ROR2 expression.

LRP6. Receptor LRP6 was expressed in all of the studied cell lines. A2780cis had higher LRP6 expression than A2780, but conversely, OVCAR3 had higher expression than OVCAR3cis.

PTK7. Receptor PTK7 was expressed strongly in all of the studied cell lines. There was a decrease in the expression in A2780cis in comparison to A2780. By contrast, the expression was higher in OVCAR3cis than in OVCAR3. The pattern was similar to LRP6.

Wnt5a. Expression of ligand Wnt5a was drastically increased in OVCAR3cis in comparison to OVCAR3, which suggests that cisplatin resistance upregulated Wnt5a expression. Wnt5a expression was lower in all other cell lines.

β-catenin. Canonical Wnt pathway signaling molecule β-catenin was increased in OVCAR3cis in comparison to OVCAR3. There was no β-catenin expression in A2780 and A2780cis. Expression in Ovsaho was weak in comparison to other cell lines that expressed β-catenin.

RhoA. There was a drastic increase in RhoA GTPase expression in OVCAR3cis in comparison to OVCAR3. Also, SKOV3, JHOS2, Kuramochi and Ovsaho had constitutively higher levels of RhoA compared to A2780 and A2780cis.

4.2.2 AKT/PI3K pathway

We then wanted to investigate the expression of AKT/PI3K pathway in relation to cisplatin resistance in OC cells. For this purpose, cells were lysed after a 48h in culture and Western blot was done on the lysates with various antibodies as shown in **Figure 7**.

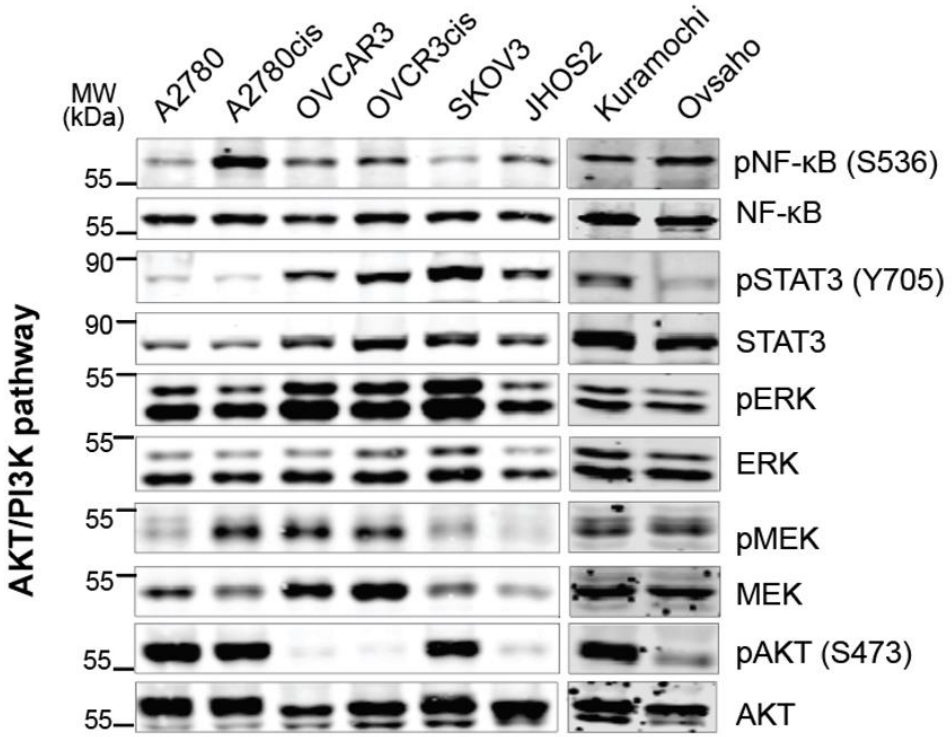

Figure 7. Western blot analysis of proteins associated with the AKT/PI3K pathway.

NF-κB/pNF-κB. Expression of unphosphorylated NF-κB was increased in A2780cis and moderately in OVCAR3cis in comparison to their parental cell lines. Expression of pNF-κB (S536) was prominently higher in A2780cis than in A2780 and was relatively weak in SKOV3.

***STAT3/pSTAT3*.** STAT3 was expressed in all the studied cell lines. It was weakly phosphorylated on Y705 in A2780 and A2780cis. A2780 and A2780cis expressed STAT3 less than the other studied cell lines. Levels of both STAT3 and pSTAT3 Y705 were increased in OVCAR3cis in comparison to OVCAR3. SKOV3 had a high level of STAT3 (Y705) phosphorylation. Comparison of STAT3 and pSTAT3 in Ovsaho showed that the level of STAT3 (Y705) phosphorylation was weak in this particular cell line.

ERK/pERK. ERK expression was slightly higher in OVCAR3cis than OVCAR3 but no major differences were observed in other cell lines. The highest pERK levels were detected in SKOV3.

MEK/pMEK. Phosphorylation of MEK was prominently increased in A2780cis in comparison to A2780, which suggests that the pathway activity was increased in A2780cis. Although cisplatin-resistant OVCAR3cis expressed more MEK than OVCAR3, the level of phosphorylation did not increase the same way in these cell lines. JHOS2 showed weak expression for both MEK and pMEK.

AKT/pAKT. AKT was expressed in all the studied cell lines. While AKT expression was higher in OVCAR3cis than OVCAR3, AKT phosphorylation was very weak in both cell lines. JHOS2 and Ovsaho had weak pAKT (S473) expression.

4.2.3 The most differentially expressed proteins in parental and cis-resistant cell lines

The most differentially expressed proteins of Western blot analyses of Wnt (**Figure 6A**) and AKT/PI3K (**Figure 7**) pathways were further quantified as shown in the column charts in **Figure 6B**. Protein expressions were normalized according to protein levels in parental A2780 and OVCAR3 cell lines.

The most drastic differences between A2780 and A2780cis were expressions of ROR1 and ROR2 and the phosphorylation rate of NF-κB S536, while NF-κB expression remained relatively uniform in both cell lines. These results suggest that ROR1 and ROR2 and NF-κB-mediated signaling could have important roles in chemoresistance.

The most drastic differences between OVCAR3 and OVCAR3cis were the expressions of ROR2, Wnt5a, and RhoA. The analysis suggested that Wnt5a-ROR2 signaling is more increased in the cisplatin-resistant cell line. These results suggest that Wnt5a-ROR2 and RhoA GTPase signaling could have important roles in chemoresistance.

4.2.4 Stem cell markers

Next, we wanted to investigate the association of cisplatin resistance and the expression of stem cell-associated proteins in OC cells. For this purpose, OC cells were lysed after a 48h culture and Western blot was done on the lysates with antibodies for Bmi1, YAP/TAZ, Snai1, and β-tubulin.

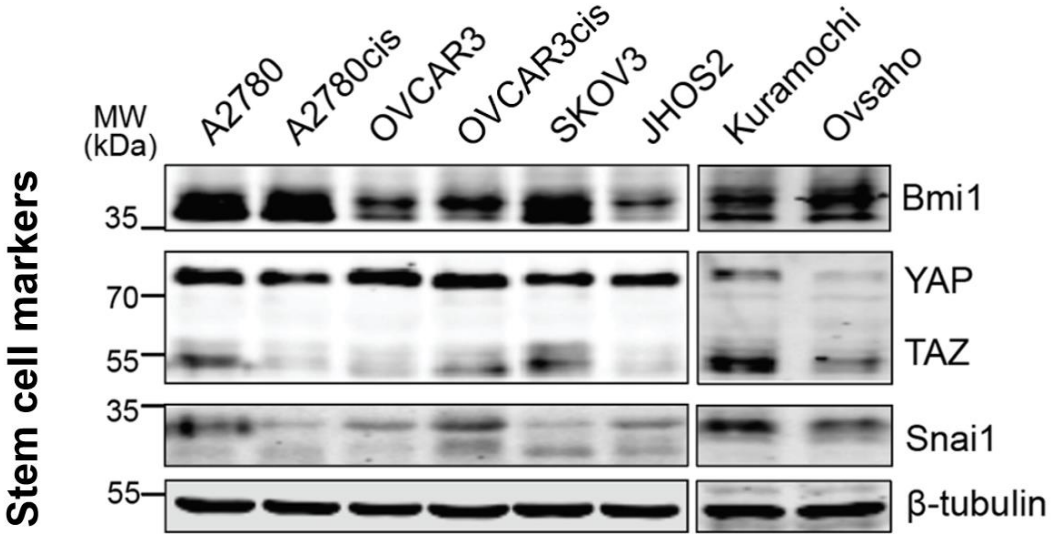

Figure 8. Western blot analysis of expression of stem cell-associated proteins in the studied OC cell lines.

The results of the Western blot analyses are presented in **Figure 8**.

Bmi1. Comparisons between OVCAR3 and OVCAR3cis showed that the cisplatin-resistant OVCAR3cis cell line had higher levels of Bmi1 expression. JHOS2 and OVCAR3 had weak Bmi1 expression when compared to the other cell lines.

YAP/TAZ. YAP was expressed abundantly in all the cell lines while expression in Ovsaho was relatively weak. TAZ was expressed in A2780 but not A2780cis. The difference in OVCAR3 and OVCAR3cis was opposite, as OVCAR3cis had higher TAZ expression than OVCAR3.

***Snai1*.** Snai1 expression patterns were similar to TAZ in all the other cell lines except for SKOV3, where TAZ was expressed abundantly while Snai1 expression was weak.

Apart from YAP, JHOS2 expressed all the studied proteins quite weakly in comparison to the expression levels in the other cell lines while Kuramochi cells expressed all the studied stem cell markers.

4.3 The effect of shRNA targeted expression KO of ROR1 or ROR2 in ovarian cancer cell lines A2780/A2780cis

Next, we wanted to investigate the effect of targeting ROR1 and ROR2 expression in A2780 and A2780cis cell lines on the downstream ROR signaling pathways. For this purpose, we used shRNA lentivirus system. A2780 and A2780cis cells were stable transfected with doxycycline-inducible shRNA plasmid (Ctrl, ROR1 and ROR2) and then subjected to puromycin selection. Stable cells were then treated with doxycycline (Sigma-Aldrich) to induce targeted shRNA expression. The stable shRNA cell lines were created in the lab by another scientist.

A2780 and A2780cis shRNA cell lines were treated with doxycycline for 8 days and cell lysates were prepared. Western blot was performed to analyze the expression of ROR proteins as well as downstream STAT3, NF-κB, and AKT/pAKT.

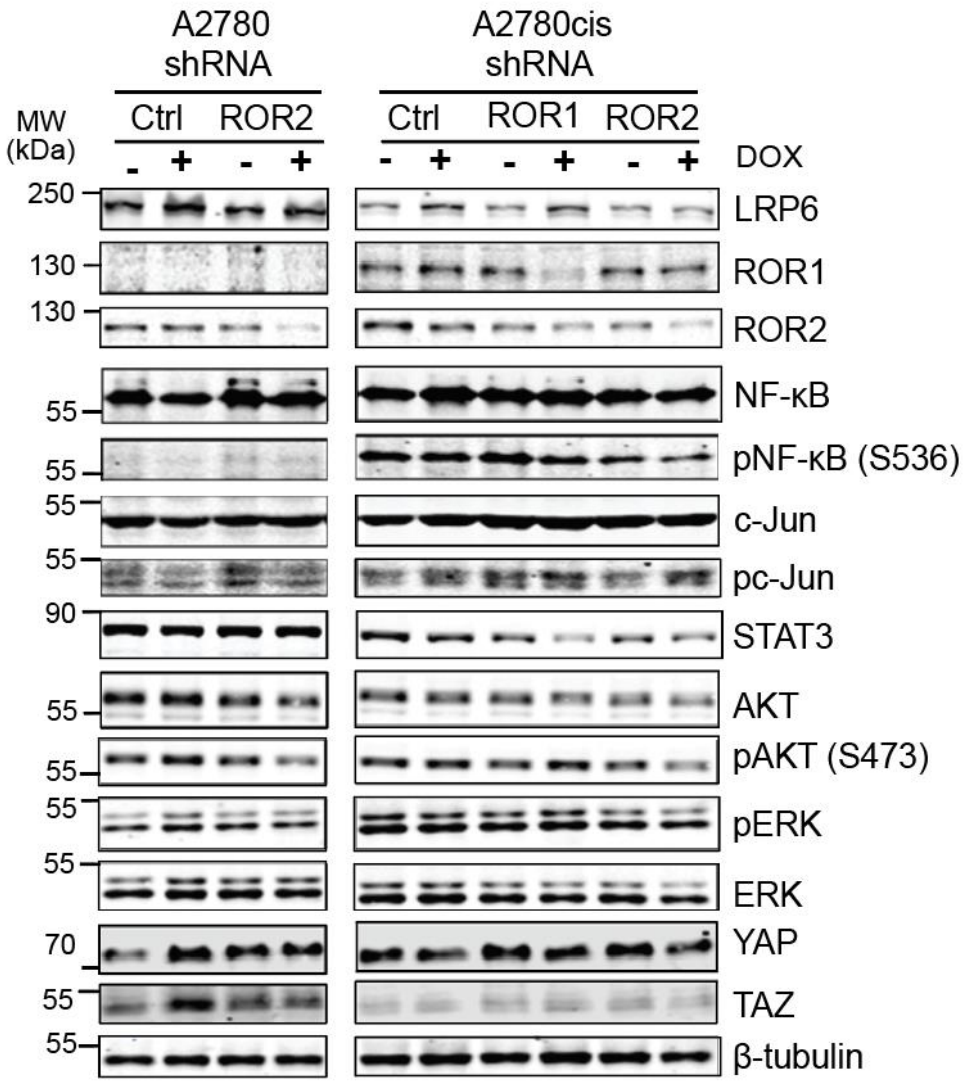

Figure 9. Western blot analysis of shRNA-induced A2780 and A2780cis cell lines where ROR1 and ROR2 expression have been silenced.

The results of the analysis are presented in **Figure 9**.

Non-canonical Wnt receptors. ROR1 was silenced in A2780cis shROR1+ cell line. ROR2 was silenced in A2780 and A2780cis shROR2+ cell lines, and the expression was slightly down-regulated in A2780cis shROR1+. shROR1 was not used for A2780 given there was no endogenous ROR1 expression as shown in **Figure 6A**.

STAT3. The Western blot analysis showed that STAT3 was downregulated in A2780cis shROR1+ and shROR2+. This effect was not seen in A2780. The downregulation was more visible in shROR1+.

NF-κB/pNF-κB. The analysis also showed that expression of phosphorylated **NF-κB** was downregulated in A2780cis shROR2+ cells, which means that targeting ROR2 downregulates NF-κB phosphorylation. A2780 cell lines barely expressed pNF-κB, which was also shown in analysis in **Figure 7**.

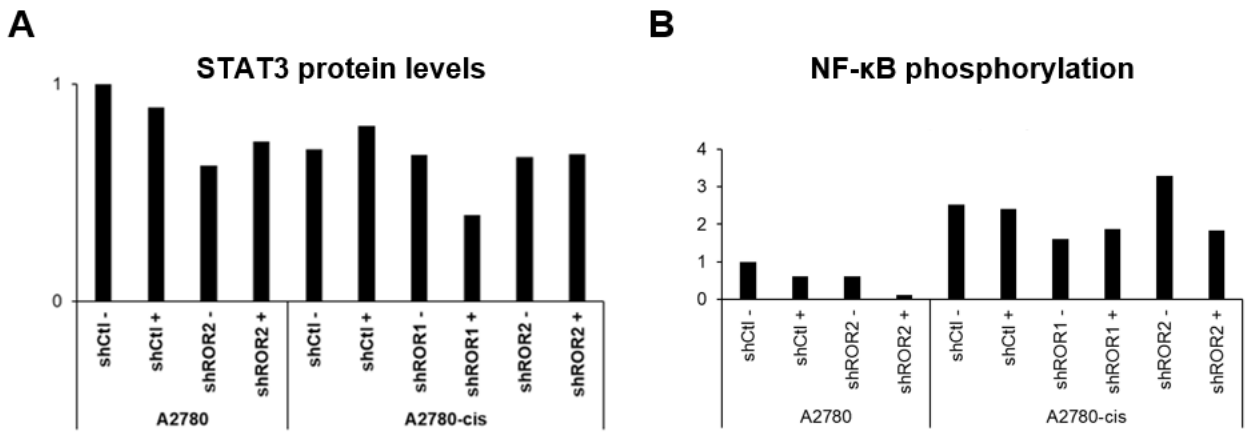

Figure 10. A. Differential expression level of STAT3 in shRNA-induced A2780/A2780cis cell lines. Quantification of Western blot analysis shows that silencing ROR1 in A2780cis down-regulates STAT3 expression. **B.** Differential phosphorylation levels of NF-κB in shRNA-induced A2780/A2780cis cell lines. Decrease of NF-κB phosphorylation can be seen in shROR2+ in both A2780 and A2780cis cell lines.

Stem cell markers

Next, we wanted to investigate how stem cell-related proteins were expressed in A2780 and A2780cis shRNA cell lines. Cell lines were treated with doxycycline for 8 days. Cells were lysed and the lysates were balanced the same way as in **Figure 9**. Western blots were done on cell lysates with antibodies for Bmi1, YAP/TAZ, and Snai1.

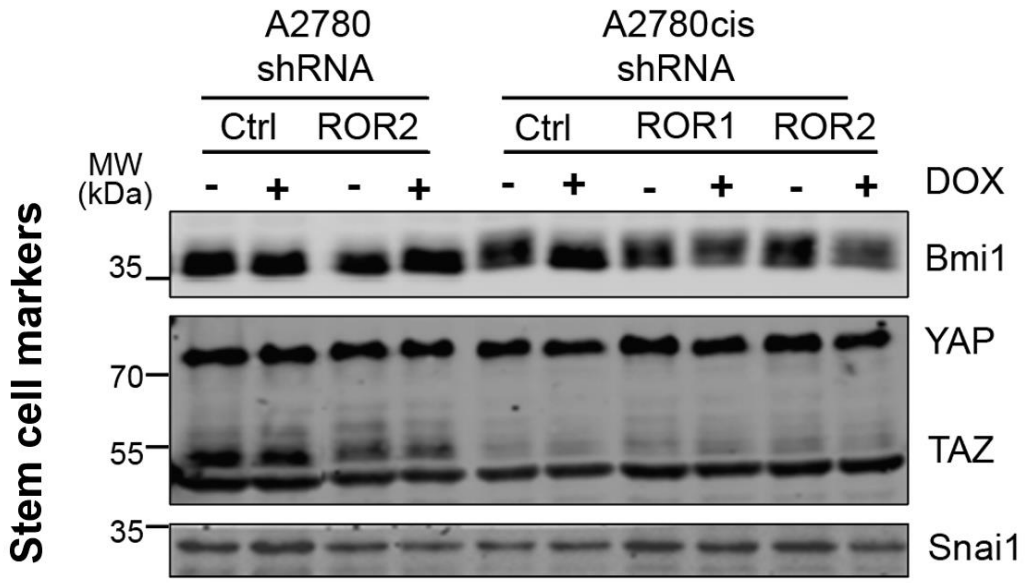

Figure 11. Expression of stem cell pathway-associated proteins in shRNA-induced cell lines.

The results of the analysis are presented in **Figure 11**.

Bmi1. According to Western blot analysis, Bmi1 expression was downregulated in A2780cis shROR1+ and shROR2+. The effect is more prominent in shROR2+. This indicated that ROR1 and ROR2 participate in mediation of the signals that promote Bmi1 expression.

YAP/TAZ. A2780 samples expressed more TAZ than A2780cis. YAP was slightly downregulated in A2780cis shROR1+ and shROR2+. TAZ expression remained uniform.

EMT markers

We then wanted to investigate the expression of EMT markers in A2780 and A2780cis cell lines. Cell lines were treated with doxycycline for 8 days. Cells were lysed and the lysates were balanced the same way as in **Figure 9**. Western blots were done on cell lysates with antibodies for ZO-1, TCF8, N-cadherin, E-cadherin, vimentin, Oct4, and Snai1.

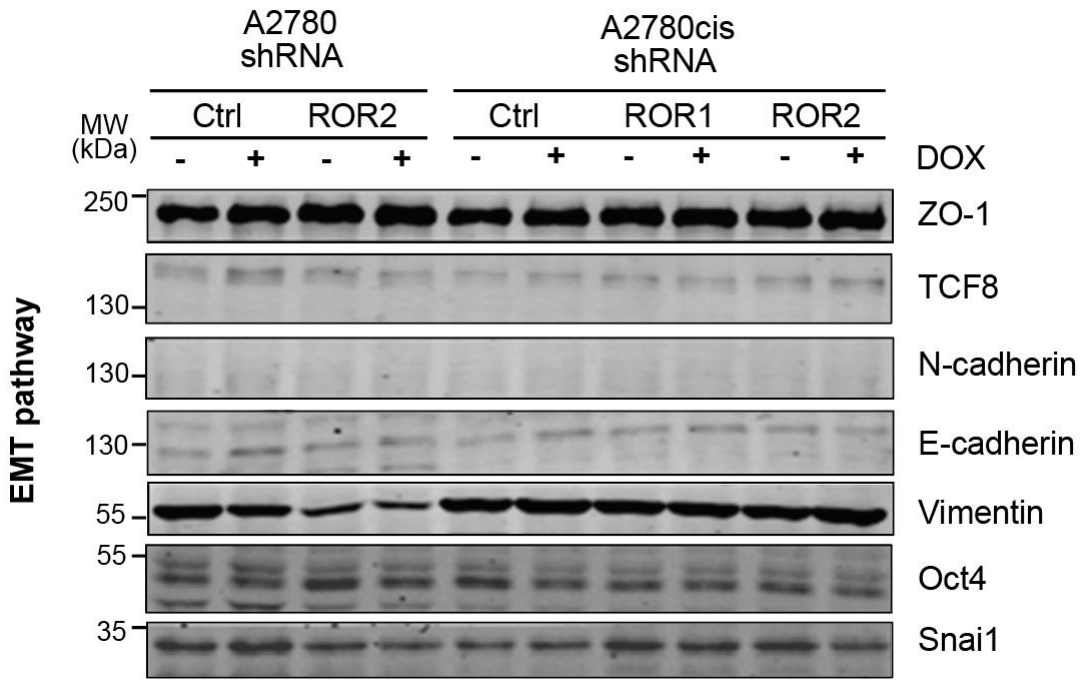

Figure 12. Western blot analysis of EMT-associated protein expressions in shRNA-induced A2780 and A2780cis cell lines.

The Western blots did not show signifiant changes in EMT marker expressions in the studied cell lines, as shown in **Figure 12**.

Wnt5a/Ca^{2+} pathway

We wanted to investigate the expression of Wnt5a/Ca^{2+} pathway-associated proteins in JHOS2, A2780, and A2780cis cell lines. JHOS2 shRNA cell lines were created the same way as A2780 and A2780cis cell lines, as described on page 38. Cell lines were treated with doxycycline for 8 days. Western blots were done on cell lysates with antibodies for ATP2A1, ATP2A2, PKA C-α, pPKA C, Phospholamban, and pPhospholamban.

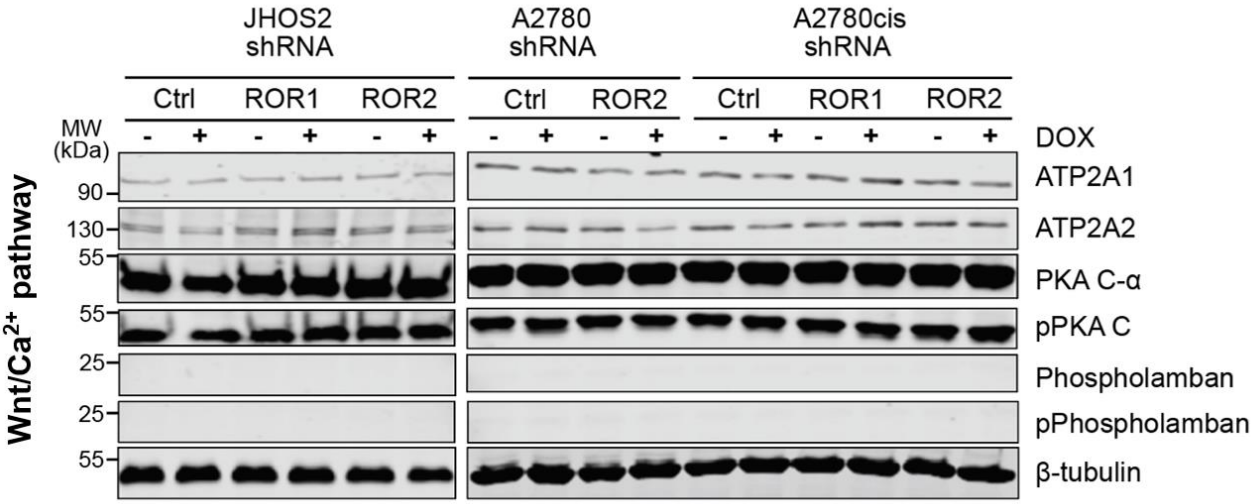

Figure 13. Western blot analysis of expression of Wnt/Ca^{2+} pathway-associated proteins in shRNA induced cell lines.

Significant changes in protein levels were not detected, as shown in **Figure 13**. Expressions of Wnt/Ca^{2+} pathway were not affected by shRNA knockout of ROR1 and ROR2.

4.4. Wnt5a stimulation

Next, we wanted to investigate whether Wnt5a stimulation via ROR1/ROR2 activates intracellular signaling pathways, such as AKT/pAKT and ERK/pERK, downstream of RORs. For this purpose, we used Kuramochi, Ovsaho and SKOV3 cells. Cells were plated in 6-well plates for 24h. The cells were then starved 24h using starvation media with 0% FBS and then stimulated with Wnt5a at a concentration of 50ng/mL for the indicated time points. Cells were then lysed and Western blots were performed to investigate the expression of AKT/pAKT (S473) and ERK/pERK.

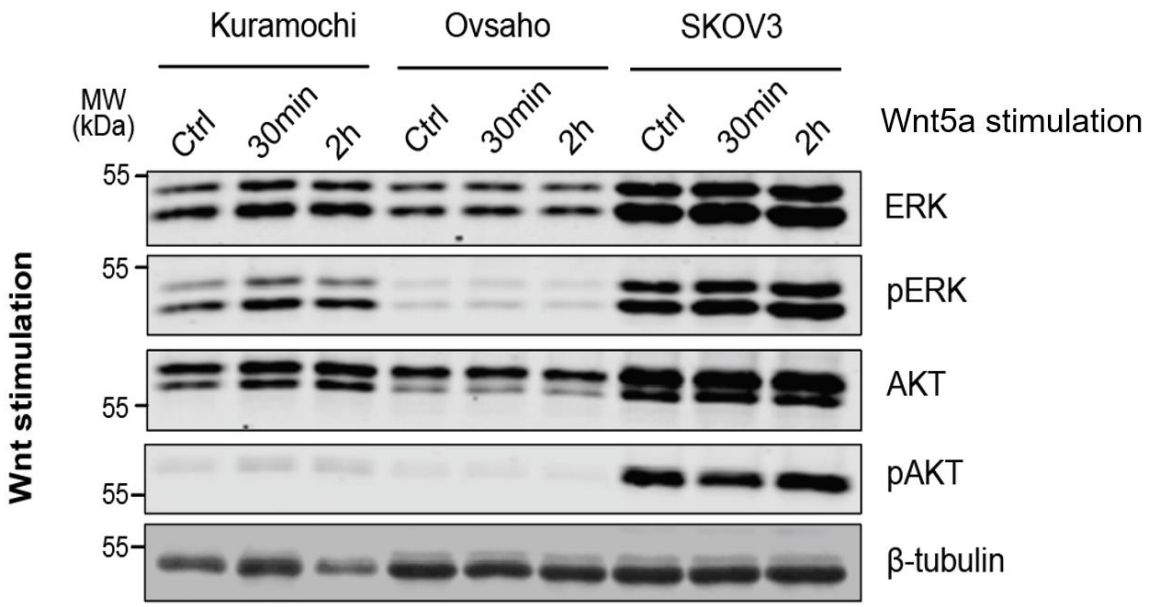

Figure 14. Western blot analysis of protein expression after Wnt5a stimulation of cells at different time points as indicated.

Basal pERK expression in Ovsaho and basal pAKT expression in both Ovsaho and Kuramochi were decreased after starvation, which can be seen by comparing the results in **Figure 14** to the Western blot analysis in **Figure 7.** On the other hand, SKOV3 showed high levels of basal pAKT and pERK even after 24h starvation, suggesting that these pathways are strongly activated in this cell line.

After Wnt5a stimulation, elevated pAKT and pERK levels were observed in Kuramochi but not in Ovsaho and SKOV3. This could be explained by the lack of endogenous Wnt5a expression in Ovsaho and SKOV3 (see **Figure 6A**) and consequently, the Wnt5a pathway is not active in these cells. Another difference is that SKOV3 has high levels of basal phosphorylation of pAKT and pERK, and it therefore is difficult to induce extra activation on these cells using Wnt5a.

Increase of pERK shown in Kuramochi indicates that ERK is phosphorylated and activated and the ERK pathway is upregulated upon Wnt5a stimulation.

5 Discussion

Chemosensitivity assays showed that cell lines with higher expressions of ROR1 and ROR2 were more resistant to cisplatin. A2780cis had higher resistance to cisplatin than the parental A2780. We showed that ROR1 and ROR2 were both more strongly expressed in A2780cis, which supports that ROR1 and ROR2 are connected to cisplatin resistance at least in this cell line. Similarly, OVCAR3cis had higher survival in the cytotoxic assays for cisplatin than OVCAR3cis and, while neither cell line expressed ROR1, OVCAR3cis had higher ROR2 expression. ROR1 and ROR2 expression is known to correlate with poorer prognosis in OC (e.g. Zhang S. et al., 2014; Henry et al., 2016). This was demonstrated in the respective comparisons of A2780 and A2780cis as well as OVCAR3 and OVCAR3cis in regard to their protein expressions and cisplatin resistance assays. However, considering the heterogeneous expression patterns of ROR1/2 in the other studied cell lines – SKOV3, JHOS2, Kuramochi, and Ovsaho – and the results of cisplatin sensitivity assays, showed that ROR1/2 expression alone did not explain cisplatin sensitivity in OC cells; most probably other factors are involved.

We also observed other changes in signaling pathways associated with cisplatin resistance in OC cell lines that we studied. It has been established that NF-κB and STAT3 are important mediators of PI3K/AKT cell survival pathway in OC (e.g. Veskimäe et al., 2018). In this study, the phosphorylation levels of NF-κB (S536) was increased in cisplatin-resistant cell line A2780cis in comparison to A2780 (**Figure 6B**). Previously, it has been found that pSTAT3 (Y705) expression is connected to high expression of ROR1 and ROR2 (Veskimäe et al., 2018). pSTAT3 (Y705) as well as STAT3 levels were upregulated in OVCAR3cis cell line in comparison to OVCAR3 in this study, which suggested that the pathway activity was increased in OVCAR3cis (**Figure 7**). However, there was no such difference in pSTAT3 between A2780 and A2780cis. We also observed that stem cell marker Bmi1 was upregulated in A2780cis and OVCAR3cis in comparison to their parental cell lines.

We also investigated the effect of targeting ROR1 or ROR2 expression in OC cell lines to observe changes in downstream signaling molecules. In this study, targeting ROR2 resulted in decreased pNF-κB (S536) in A2780cis (**Figure 10B**) while targeting ROR1 resulted in decrease of STAT3 expression in A2780cis (**Figure 10A**). This suggests that STAT3 is a downstream effector of ROR1 pathway activation in A2780cis cells. It has been previously shown in our lab that STAT3 is downstream Wnt5a-ROR1 signaling in B-cell leukemia cells (Karvonen et al., 2019), therefore it is very likely that STAT3 is also a mediator of ROR1 in OC cells.

Other changes observed upon ROR1 or ROR2 targeted downregulation was in the levels of Bmi1, which was downregulated in A2780cis cells (**Figure 11**). We did not observe changes in protein expressions in shRNA-induced ROR1 and ROR2 downregulation in A2780 and A2780cis cells regarding EMT, PCP, and Wnt/Ca^{2+} pathways. Baseline expressions of ROR1 and ROR2 in A2780 and A2780cis were relatively low and this may explain why no significant changes were observed in shRNA-induced cells. Cell lines with higher ROR1/2 expression should be used instead to see the effects of targeting these proteins better.

AKT/PI3K and MEK/ERK pathways are known drivers of cancer (Tan F. et al., 2019) and these pathways have been implicated in chemoresistance (Kim S. et al., 2018). Phosphorylation of ERK can promote survival of cancer cells by activating transcription of target genes that contribute to proliferation, survival, and metastasis (Rojas et al., 2016; Pieterse et al., 2019). The AKT/PI3K pathway activity is often altered in tumor cells (LoRusso, 2016).In this study, Wnt5a stimulation increased pERK and pAKT levels in Kuramochi cells which suggests that AKT/PI3K and MEK/ERK pathways were activated. The results show that in the presence of Wnt5a, these pathways could contribute to growth and proliferation of cancer cells. We have also shown that Wnt5a binding to ROR1 could activate pAKT and pERK in BaF3-ROR1 clones but not in parental BaF3 cells that do not express ROR1, clearly suggesting that one way Wnt5a promotes cell survival is by activating pAKT and pERK pathways downstream ROR1 (Sheetz et al., 2020).

6 Conclusions

The purpose of this master's thesis was to characterize the Wnt5a-ROR pathway in cisplatin-resistant OC cell lines and find improvements for the treatment of OC by targeting ROR1 and ROR2 receptors. We wanted to investigate if the expression of ROR1/ROR2 is correlated with cisplatin resistance in OC cell lines and which intracellular pathways are affected.

Increased expression of Wnt5a, ROR1, and ROR2 correlated with cisplatin resistance in cell line pairs A2780/A2780cis and OVCAR3/OVCAR3cis. Targeting ROR1 with shRNA resulted in downregulation of STAT3 and targeting ROR2 resulted in downregulation of pNF-κB levels. Targeting ROR1 and ROR2 decreased the expression of stem cell-associated Bmi1.

Based on the results, targeting ROR1 and ROR2 could provide a good approach for treating chemoresistant OC. The effects of targeting ROR1 and ROR2 on EMT, PCP, and stem cell pathways could be further studied using cell lines with higher basal ROR1 and ROR2 expression, as the changes that the used cell lines showed were modest. In order to further underline the effect on cell stemness, additional stem cell-related proteins could be studied, such as signaling proteins in Hedgehog and Notch pathways. In addition, these experiments should be conducted on patient-derived cell cultures to further validate the results.

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