The Role of RUNX3 in Chemoresistance in Epithelial Ovarian Cancer

by

Nubia Iracema Zepeda

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Experimental Oncology

Department of Oncology
University of Alberta

Abstract:

Chemoresistance is one of the major hurdles to induce a cure in patients with epithelial ovarian cancer (EOC). RUNX3, belonging to the family of RUNX transcription factors, has been shown to act in an oncogenic manner by promoting proliferation and colony formation when overexpressed, in EOC. However, to date the role of RUNX3 in chemoresistance in EOC has not been studied. Based on its oncogenic role in EOC, we want to determine whether RUNX3 contributes to chemoresistance in EOC.

In our study we found that RUNX3 expression is elevated in human EOC samples when compared to non-cancerous ovarian cells. RUNX3 expression is variable in EOC cell lines. Interestingly A2780s cells, a cisplatin-sensitive ovarian adenocarcinoma cell line, show little expression of RUNX3 at the protein level. A2780cp cells, which were derived from A2780s cells by exposure to step-wise increasing doses of cisplatin, show high expression of RUNX3. In A2780s and A2780cp cells, treatment with carboplatin increases the expression of RUNX3 at the protein level. When RUNX3 is overexpressed in EOC cells, cells become slightly more resistant to carboplatin treatment. When we knock down the expression of RUNX3 in A2780cp cells, no effect on cell sensitivity to carboplatin is observed. However, dominant-negative (dn) expression of RUNX3 in A2780cp cells made cells more sensitive to the effects of carboplatin. The dn form may be binding to and occupying the sites that RUNX family members recognize, or binding to the common co-factor CBFβ. Based on these results, we conclude that RUNX3 plays a minor role in chemoresistance when overexpressed

in EOC and that other factors, such as other RUNX family members, may be compensating for the loss of RUNX3 when it is knocked down. In the future, it would be interesting to block all the RUNX family members, and observe whether chemoresistant cells become more sensitive to carboplatin treatment.

Next we took a systematic approach to understanding chemoresistance by examining the paired human EOC cell lines, A2780s and its derivative A2780cp, using a microarray analysis. To identify networks and pathways that may be key during acquired chemoresistance we conducted an extensive literature search and put the data into Ingenuity Pathway Analysis (IPA) software. Our microarray and IPA suggest that Wnt signaling may be upregulated in A2780cp cells, although further experiments are needed to confirm this. In the future we plan to modulate the canonical Wnt pathway in A2780cp and A2780s cells, and determine whether activation of the canonical Wnt pathway is playing a role in chemoresistance in EOC.

Table of Contents:

Section 1: Introduction **1**

1.1 Ovarian Cancer 1

1.1.1 Classification 1

1.1.2 Diagnosis and treatment 2

1.2 Platinum Agents 3

1.2.1 Cisplatin and carboplatin 3

1.2.2 Mechanism of action 4

1.2.3 Molecular basis of resistance in ovarian cancer 7

1.3 The RUNX family of transcription factors 10

1.3.1 Overview of the RUNX family 10

1.3.2 Diversification of roles 16

1.3.3 Regulation of gene expression of the RUNX family 17

1.3.4 Biological processes regulated by the RUNX family 17

1.4 RUNX3 and Cancer 20

1.4.1 RUNX3‟s tumor suppressive potential 20

1.4.2 RUNX3‟s oncogenic potential 26

1.4.3 RUNX3 and ovarian cancer 28

1.4.4 RUNX3 and cell sensitivity to chemotherapeutic agents 31

1.5 Hypothesis 33

Section 2: Methods and Materials **34**

2.1 Cell culture and tumor specimens 34

2.2 Generating RUNX3 overexpression cells 34

2.3 Generating RUNX3 knockdown cells 35

2.4 Generating RUNX3 dominant-negative cells 37

2.5 Neutral red uptake assay 37

2.6 Collection of cell lysates 38

2.7 Western blotting 38

2.8 RNA isolation and real-time PCR analysis 39

2.9 Statistics 40

Section 3: Results 42

3.1 Expression of RUNX3 in normal OSE, IOSE, primary EOC cells and EOC cell lines 42

3.2 RUNX3 is localized to the nucleus of EOC cells 46

3.3 RUNX3 protein level is increased after carboplatin treatment in EOC cells 46

3.4 RUNX3 overexpression in EOC cell lines confers resistance to carboplatin treatment 49

3.5 Silencing RUNX3 expression in A2780cp cells has no effect on cell viability 52

3.6 Dominant-negative mutant of RUNX3 in A2780cp cells sensitizes cells to carboplatin treatment 56

3.7 Expression of the RUNX transcription factors in EOC cell lines 59

Section 4: Discussion and Future Directions 61

4.1 RUNX3 and chemoresistance in ovarian cancer 61

4.2 Systematic approach to identify gene expression and signaling pathways affected during acquired chemoresistance 67

4.3 The Wnt signaling pathway 70

Section 5: Conclusion **78**

Section 6: References **80**

List of tables:

Table 1: List of PCR primer sequences 40

Table 2: Clinical information on primary serous EOC tissues 43

Table 3: Clinical information on primary EOC cells derived from patient ascites 44

Table 4: Genes involved in Wnt signaling that are differentially expressed between A2780s and A2780cp cells 73

Table 5: Validation of microarray by qRT-PCR 74

List of Figures:

Figure 1: Activation and mode of action of cisplatin 6

Figure 2: Mechanisms of acquired chemoresistance in ovarian cancer 11

Figure 3: The RUNX family of transcription factors 13

Figure 4: Interaction between RUNX3 and the TGFβ pathway 22

Figure 5: RUNX3 expression is elevated in human EOC 42

Figure 6: RUNX3 expression is variable in human EOC cell lines 46

Figure 7: RUNX3 is localized to the nucleus in EOC cells 47

Figure 8: A2780s cells are more sensitive to carboplatin treatment than A2780cp cells 49

Figure 9: Carboplatin increases RUNX3 expression in EOC cell lines 50

Figure 10: Overexpression of RUNX3 makes SKOV3 cells slightly more resistant to carboplatin treatment 52

Figure 11: Overexpression of RUNX3 makes A2780s cells slightly more resistant to carboplatin treatment 53

Figure 12: Knockdown of RUNX3 by shRNA in A2780cp cells has no effect on cell viability following carboplatin treatment 54

Figure 13: Knockdown of RUNX3 by siRNA in A2780cp cells has no effect on cell viability following carboplatin treatment 56

Figure 14: Dominant-negative mutant of RUNX3 sensitizes A2780cp cells to carboplatin treatment 57

Figure 15: Expression of RUNX genes in EOC cell lines 59

Figure 16: Microarray analysis setup 68

Figure 17: The canonical Wnt/β-catenin signaling pathway 71

Figure 18: Wnt target genes are upregulated in A2780cp cells 75

Abbreviations:	Full name:
AML:	Acute myeloid leukemia
APC:	Adenomatous polyposis coli
APS:	Ammonium persulfate
ATM:	Ataxia telangiectasia mutated protein
ATR:	ATM- and Rad3-related protein
BCC:	Basal cell carcinoma
BMP:	Bone-morphogenetic proteins
CBF:	Core-binding factor
CCD:	Cleidocranial dysplasia
CDK:	Cyclin-dependent kinase
CHEK:	Checkpoint kinase
CK1α/ε:	Casein kinase 1α/ε
CMV:	Cytomegalovirus
CTR1:	Copper transporter 1
DKK:	Dickkopf
DN:	Dominant-negative
dNTP:	Dinucleotide triphosphate
DOC:	Sodium deoxycholate
DSB:	Double strand breaks
DSH:	Dishevelled
DTT:	Dithiothreitol
EOC:	Epithelial ovarian cancer

EMT:	Epithelial to mesenchymal transition
ERCC:	Excision repair cross-complementing rodent repair deficiency, complementation group
FACS:	Fluorescence-activated cell sorting
FBS:	Fetal bovine serum
GFP:	Green fluorescent protein
GSH:	Glutathione
GSK3β:	Glycogen synthase kinase 3β
HBS:	Hepes-buffered saline
HOSE:	Human ovarian surface epithelial
HNSCC:	Head and neck squamous cell carcinoma
HR:	Homologous recombination
IOSE:	Immortalized ovarian surface epithelial
LEF/TCF:	Lymphoid enhancer factor/T-cell factor
LOH:	Loss of heterozygosity
LRP:	LDL-receptor-related protein
MDR:	Multidrug resistant
miRNA:	MicroRNA
MLH:	MutL homolog
MLV:	Moloney murine leukemia virus
MMR:	Mismatch repair
NER:	Nucleotide excision repair
NHEJ:	Non-homologous DNA end joining

OSE:	Ovarian surface epithelial
P/S:	Penicillin-streptomycin
PBS:	Phosphate buffered saline
PEBP2:	Polyomavirus enhancer binding protein 2
PIC:	Protease inhibitor cocktail
qRT-PCR:	Quantitative reverse-transcription polymerase chain reaction
R-Smads:	Receptor-regulated Smads
RIPA:	Radioimmuno-precipitation assay buffer
RUNX:	Runt-related
SDS:	Sodium dodecyl sulfate
SEM:	Standard error of mean
SFRP:	Secreted Frizzled-related protein
TEMED:	Tetramethylethylenediamine
TGFβ:	Transforming growth factor-β
UTR:	Untranslated-region
VEGF:	Vascular endothelial growth factor
WHO:	World Health Organization
WIF:	Wnt inhibitory factor
WT:	Wild-type
β-TrCP:	β-transducin repeat-containing protein

Section1: Introduction

1.1: Ovarian cancer

1.1.1: Classification

The World Health Organization (WHO) classifies ovarian tumors into three major categories according to their derivation: epithelial ovarian tumors, germ cell tumors and sex cord stromal tumors[1]. The majority of ovarian cancers are of epithelial origin (approximately 90%)[2]. Epithelial ovarian cancer (EOC) is a heterogeneous group of diseases made up of different tumor types, with distinct clinical outcomes and defining features[3]. Traditionally, classification was largely based on histological characteristics, although a better understanding of the changes occurring at the molecular level has led to an integrated classification system based on histological and molecular features. Histological categories are classically divided into 4 subtypes: serous, which is the most common type of EOC, endometrioid, clear cell and mucinous subtypes[4]. Other subtypes such as Brenner (transitional cell), mixed epithelial, undifferentiated and unclassified ovarian cancers are now also recognized as distinct histological subtypes[2]. EOC can also be classified as type I (low-grade) and type II (high-grade) cancers based on histological and molecular changes[3]. Histological tumors classified as type I include low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas[3]. Genetic changes common in type I tumors include mutations in KRAS, BRAF, ARID1A, PTEN and PIK3CA[2,5]. Type II tumors include high-grade serous, high-grade endometrioid and undifferentiated carcinomas[3]. Changes in type II tumors include aberrations in p53 and BRCA1 and BRCA2 genes[2].

BRCA1 and BRCA2 mutations can arise from somatic mutations or, in the case of hereditary disease, be inherited through germline mutations[2]. Type I tumors generally respond poorly to platinum-based therapy whereas type II tumors are initially responsive to platinum therapy[5,6].

1.1.2: Diagnosis and treatment

EOC is the leading cause of death from gynecologic malignancies[7]. Diagnosis at advanced stages of the cancer, combined with ineffective current therapies contribute to the recurrence and chemoresistance displayed by the disease[8]. When diagnosed and treated at stage 1 (tumor is limited to the ovaries), the majority of patients can be cured using current therapies[9]. However, most patients with early-stage disease do not present symptoms and when symptoms are present they are often nonspecific, making early diagnosis challenging[4]. Thus, the majority of cases are detected after the disease has metastasized to the pelvic region (stage 2), the abdomen or lymph nodes (stage 3) or to distant organs (stage 4), making the cure rate for EOC approximately 30%[9].

Currently cytoreductive surgery followed by a combination of platinum-paclitaxel agents is used as the first-line treatment for ovarian cancer[10-12]. Despite an initial positive response to first-line chemotherapy, most women eventually relapse[6,8]. If patients relapse, they can be re-treated with the same drugs given during first-line chemotherapy (paclitaxel and carboplatin)[13]. The decision to use platinum therapy or to pursue a regimen with non-platinum cytotoxic agents for relapsed patients depends on the length of the remission[4]. Generally, patients that

relapse after more than six months are considered to have "platinum-sensitive" disease and can be re-treated with carboplatin alone or carboplatin in combination with other agents. Patients that relapse after less than 6 months are considered to have "platinum-resistant" disease, whereas patients who do not respond to platinum chemotherapy from the beginning are considered to have "platinum-refractory" disease[4,8]. Patients with platinum-resistant or platinum-refractory disease can be treated with other agents in place of platinum chemotherapy which include anthracyclines, topoisomerase inhibitors, nucleoside analogues, taxanes, vinca alkaloids or hormonal therapies[14]. However, the rate at which patients respond to these other agents is fairly low and relatively short in duration, and falls each time a patient relapses[14]. Moreover, it is not well established which treatment or combination of treatment options are the most effective in relapsed disease[4].

As a consequence of the chemoresistance and recurrence, the 5-year survival rate is only 30% in patients with advanced EOC[9]. A better understanding of the changes occurring at the molecular level during acquired drug resistance is necessary to better manage this disease[14].

1.2: Platinum agents

1.2.1: Cisplatin and carboplatin

Cisplatin has been used for the treatment of numerous cancers[15,16]. In specific tumor types, treatment with cisplatin can initially lead to tumor regression or stabilization[15,16]. Nonetheless, many patients are intrinsically resistant to

platinum therapies, or develop acquired resistance after the initial positive response[15,16], as seen in ovarian cancer (reviewed by Agarwal *et al.*[14]). Cisplatin has numerous toxic side effects including nephrotoxicity and neurotoxicity. The intrinsic and acquired resistance displayed by cancer cells to cisplatin, as well as the severe side effects, led to the development of platinum derivatives. In the mid-1980"s carboplatin was discovered and shown to cause almost no nephrotoxicity, and less gastrointestinal tract toxicity and neurotoxicity. The main side-effects noted involve the bone marrow, and more specifically reversible thrombocytopenia (as reviewed in Kellan *et al.*[17]). The survival rates for carboplatin and cisplatin were the same in ovarian cancer patients, leading to the approval of carboplatin for ovarian cancer treatment[18].

1.2.2: Mechanism of action

Cisplatin is a square planar complex that interacts with DNA to exert its cytotoxic effects[19]. Cisplatin is activated when it enters into the cell, due to the low amount of chloride atoms found in the cytoplasm compared to the extracellular space[20]. Upon entry into the cell, one or both chlorine ligands are removed and replaced with water molecules, leading to the creation of a highly reactive electrophilic species[20,21]. Reactive cisplatin preferentially interacts with purine bases in DNA, and forms inter- and intrastrand crosslinks[22,23], although it can also interact with intracellular stores of nucleophiles, such as reduced glutathione (GSH) or methionine[15,24] (as reviewed in Galluzzi *et al.*[15] and Siddik *et al.*[25]). Carboplatin, a derivative of cisplatin, undergoes similar processes in the

cell as cisplatin and forms the same adducts in DNA, although carboplatin reacts with slower kinetics[26].

DNA repair pathways can recognize adducts formed by cisplatin in DNA, and consequentially signal for repair or cell death. Nucleotide excision repair (NER) is the main pathway responsible for the removal of cisplatin adducts[27,28], while the mismatch repair (MMR) system is key in triggering apoptosis[29]. If the damage caused by cisplatin is limited, cell cycle arrest will occur and allow repair mechanisms to attempt to repair the damaged DNA. However, if there is extensive damage and it cannot be repaired, cells will undergo apoptotic death[15]. The key signaling cascade involved in triggering apoptosis following cisplatin-induced DNA lesions includes the activation of ATM (ataxia telangiectasia mutated protein), ATR (ATM- and Rad3-related protein) and Chek1 (checkpoint kinase 1). This in turn leads to the phosphorylation and activation of p53. Activated p53 can act through intrinsic and extrinsic apoptosis, leading to cellular death (as reviewed by Galluzzi *et al*.[15], and Siddik *et al*.[25]). (Mechanism of action summarized in Figure 1).

1.2.3: Molecular basis of resistance in ovarian cancer:

Uptake of cisplatin into cells was believed to solely occur through passive diffusion. However, using yeast and mouse cells it was shown that copper transporter 1 (CTR1), a protein involved in the uptake of copper, also participates in cisplatin uptake[30]. Increased expression of CTR1 leads to greater accumulation

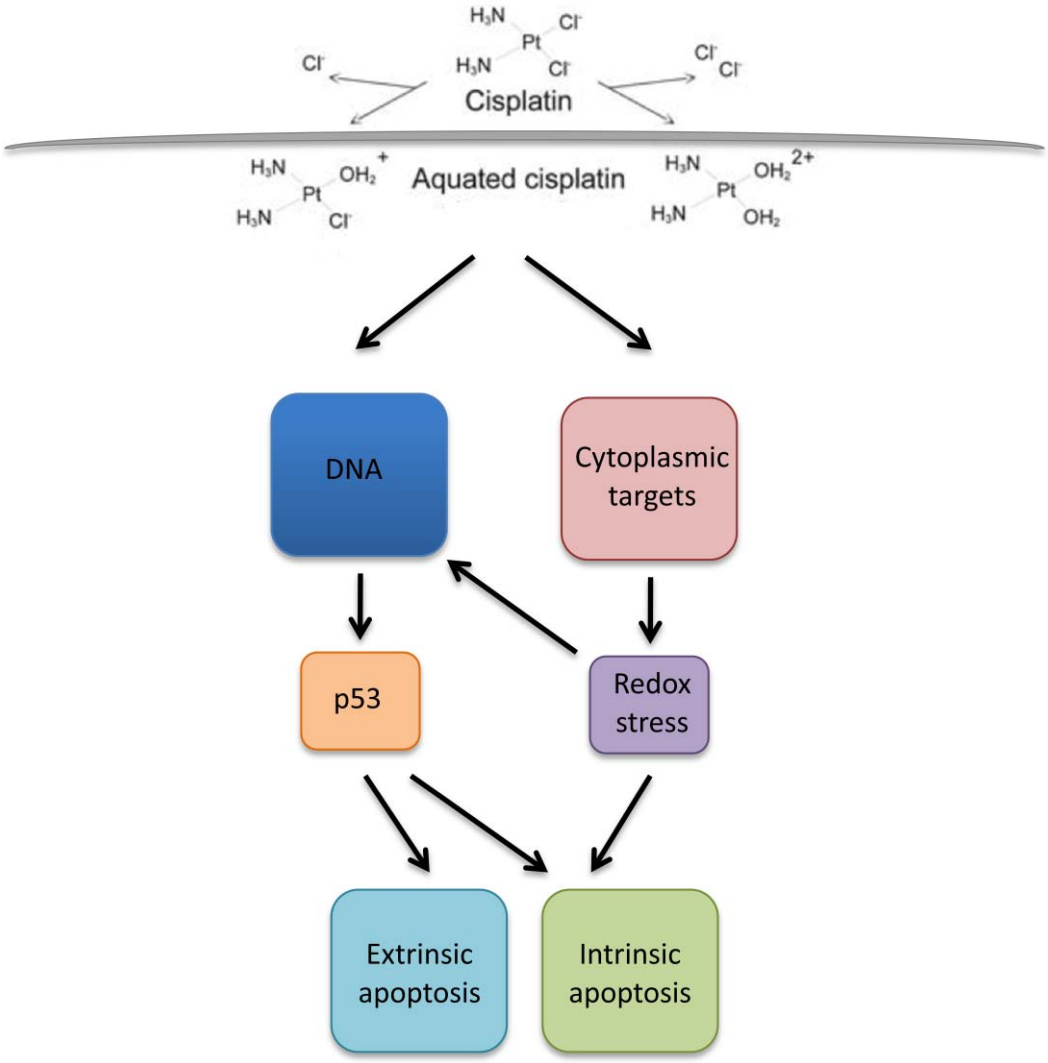

Figure 1. Activation and mode of action of cisplatin. Cisplatin is activated upon entry into the cytoplasm, where one or both of the chloride groups spontaneously become aquated, due to the relatively low concentration of chloride ions in the cytoplasm, compared to the extracellular space. Activated cisplatin can interact with and deplete cytoplasmic substrates including endogenous nucleophiles (eg. reduced glutathione (GSH), methionine, metallothioneins), causing oxidative stress. The oxidative stress can amplify cisplatin-induced DNA damage or trigger intrinsic apoptosis. The main mechanism by which cisplatin induces cell death is through the formation of DNA inter- and intrastrand adducts. These adduct cause distortions in the DNA that are recognized by DNA repair pathways that try and repair the DNA. If the DNA damage is beyond repair, apoptotic death is triggered through a signaling cascade involving ATM, ATR and Chek1 that phosphorylates and stabilizes the tumor suppressor p53. p53 exerts cell death via intrinsic and extrinsic apoptosis. Figure and text adapted from [15].

of cisplatin in ovarian cancer cells[31]. Moreover, ovarian cancer cells downregulate the expression of the CTR1 in a time-dependent manner when exposed to cisplatin[32,33], implicating CTR1 in cisplatin resistance. Other proteins involved in mediating copper homeostasis were also investigated and found to be altered in cisplatin resistant cells[34]. Specifically, the copper-extruding P-type ATPases, ATP7A and ATP7B, mediate resistance to platinum agents[35,36]. High expression of ATP7A has been correlated with poor survival in patients[37], while high expression of ATP7B has been correlated with a poor response to chemotherapy[38].

Cisplatin can interact with and deplete the cellular stores of nucleophiles[24], and thus cause oxidative stress[15]. The oxidative stress can promote the DNA damage created by cisplatin adducts. However, these endogenous stores of nucleophiles can also lead to the inactivation of cisplatin[15]. GSH is synthesized by consecutive reactions involving two key enzymes. First, ɣ-glutamylcysteine is synthesized from L-glutamate and cysteine by the enzyme ɣ-glutamylcysteine synthetase. Next lysine is added by the enzyme glutathione synthetase, forming GSH. Glutathione transferase catalyzes the interaction between GSH and electrophilic species, making GSH necessary for antioxidation[39]. Studies have shown increased levels of GSH and enzymes involved in GSH synthesis in cell lines obtained after the acquisition of drug resistance in ovarian cancer, implicating high levels of GSH and GSH synthesis in chemoresistance in ovarian cancer[40,41].

The ability of ovarian cancer cells to repair damaged DNA can also be altered and promote acquired drug resistance. In particular, the NER system is responsible for repairing the majority of cisplatin adducts from DNA[28]. Cisplatin adducts are recognized by the binding and assembly of NER proteins, followed by an incision on both sides of the lesion. Damaged nucleotides are excised and DNA synthesis occurs to re-establish the integrity of the previously damaged DNA[42]. ERCC1 (excision repair cross-complementing rodent repair deficiency, complementation group 1) is an endonuclease that participates in NER and is responsible for incising the 5‟ side of DNA adducts[42]. Clinical studies in ovarian cancer have demonstrated a role for enhanced NER, and in particular ERCC1 expression, in drug resistance[43,44]. One study analyzed the RNA levels of ERCC1 in tumor tissue derived from ovarian cancer patients, and found that patients who were clinically resistant to platinum-based therapy had a higher expression level of ERCC1, compared to patients who responded to therapy[43]. The MMR system is key in repairing misincorporations of DNA bases that can arise during normal cellular processes such as replication or recombination, or due to DNA damage[45]. The MMR system can also detect cisplatin-adducts, but it cannot repair them, and will thus signal for apoptosis. The mechanism by which MMR proteins transmit a pro-apoptotic signal is unknown, although it is speculated that they may physically interact with ATM and ATR to activate pro-apoptotic signaling[45]. In line with this, a study found lower expression of hMLH1 (human MutL homolog), a protein involved in the MMR system, in samples collected from ovarian cancer patients after treatment with cisplatin, compared with samples from untreated

patients[46]. The group suggested that loss of hMLH1 may be a step in the development of chemoresistance in ovarian cancer[46]. Cisplatin adducts can also lead to double-strand breaks (DSBs), which can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ)[42]. Two components of HR are encoded by BRCA1 and BRCA2[42], which as previously mentioned, are genes frequently mutated in a subset of familial ovarian tumors[2]. In general, BRCA1/2-deficient ovarian cancers have a high rate of metastasis beyond the peritoneal cavity, while sporadic EOCs are usually confined to the peritoneal cavity[47]. Nonetheless, patients with BRCA1/2 mutations have a higher rate of survival[48,49] and better response rates to first and subsequent lines of platinum-based therapy[50,51].

Another mechanism of drug-resistance in ovarian cancer involves the decreased ability of cells to undergo apoptosis following exposure to cisplatin. p53 is a key mediator in the process. In ovarian cancer, patients that have p53 mutations generally respond poorly to platinum-based regimens, when compared to patients without p53 mutations[52]. The study emphasized the need to determine the status of p53 when choosing a therapeutic regimen to treat ovarian cancer patients[52]. Expression of factors important in blocking apoptosis, including Bcl-2 and Bcl-xl, have also been implicated in poor-response to cisplatin-based chemotherapy[53,54], although another study demonstrated an association between high expression of Bcl-2 and improved survival[55]. The latter group proposed that the relative levels of anti-apoptotic and pro-apoptotic proteins may be more important in determining a cells ability to transmit apoptotic signals, rather than

the absolute levels of Bcl-2[55]. Other factors involved in blocking apoptosis, such as survivin[56], have also been shown to be important in mediating drug resistance in ovarian cancer (Mechanism of cisplatin resistance summarized in Figure 2).

1.3 The RUNX family of transcription factors

1.3.1. Overview of the RUNX family

The RUNX (Runt-related) family are a group of transcription factors that are important in regulating cell proliferation and differentiation (reviewed in Coffman *et al*.[57]). The RUNX family is made up of three members RUNX1/AML1 (acute myeloid leukemia 1 protein), RUNX2 and RUNX3. All members share a conserved runt domain, which is located at the N-terminus of the protein[57]. The runt domain is responsible for binding with the common cofactor of the RUNX family (described in the following paragraph), and for recognizing and binding DNA[58] at the common consensus sequence PyGPyGGT[57,59]. The specificity of the RUNX family in gene regulation is determined by binding to this consensus sequence in the promoter or enhancer region of target genes[60]. Genes targeted by the RUNX family are reviewed in Otto *et al.*[60] and will be discussed in more detail below. The C-terminus is important in the regulation of DNA binding, and activation and repression of transcription, through the activation and inhibition domains[57] (Figure 3). The expression of RUNX genes starts from two promoters, the P1 and the P2 promoter, which gives rise to two isoforms of proteins[61] (reviewed in Levanon *et al*.[62]). Each isoform has a distinct N-terminal sequence[62]. Moreover, the RUNX family can also undergo alternative

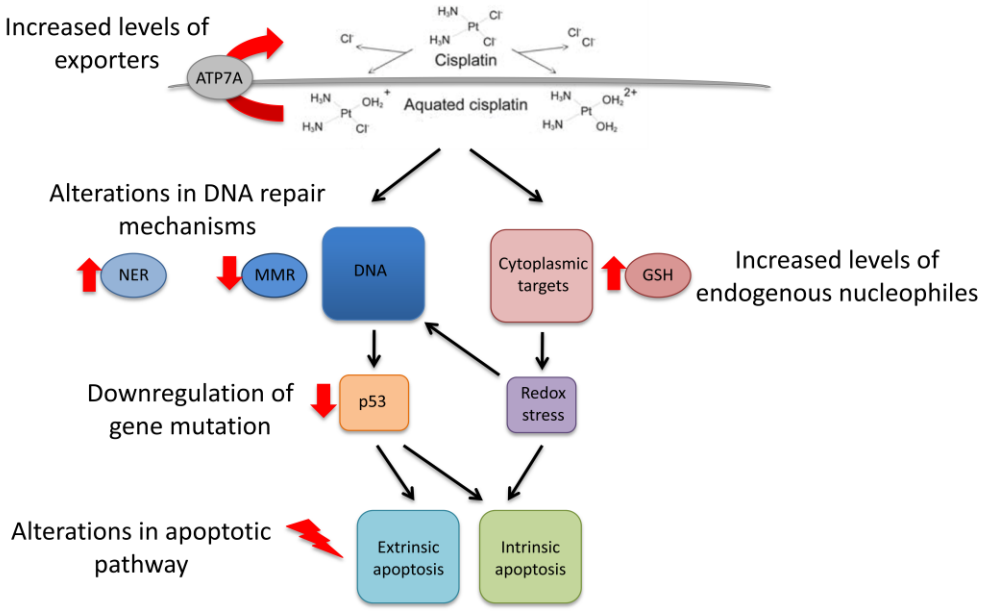

Figure 2. Mechanisms of acquired chemoresistance in ovarian cancer. Chemoresistance occurs at multiple levels in ovarian cancer. Decreased levels of copper importers (which can also import cisplatin), and increased levels of copper exporters such as ATP7A and ATP7B have been observed in resistant cells. Alterations in DNA repair mechanisms, such as increases in nucleotide excision repair (NER) proteins, and decreases in mismatch repair proteins (MMR) are present in resistant cells. Increases in the endogenous levels of nucleophiles such as reduced glutathione (GSH), or enzymes that aid in the production of GSH exist in resistant cells. Mutations in the status of p53 and alterations in the apoptotic pathway are also evident in platinum resistant ovarian cancer cells. Figure adapted from[15].

splicing, which generates multiple splice variants [57,63-65].

The RUNX family share a common co-factor named CBFβ (core-binding factor β) or PEBP2β (polyomavirus enhancer binding protein 2β), which binds to the runt domain of the RUNX transcription factors and enhances DNA binding[66]. The free form of CBFβ normally interacts with the actin cytoskeleton and is thus located in the cytoplasm. In order for CBFβ to translocate to the nucleus, it must interact with the runt domain present on the RUNX transcription factors[67,68]. The RUNX family binds to the promoter or enhancer region of target genes and can act as transcriptional activators or repressors on their own, or by interacting with transcriptional co-activators and co-repressors[69]. Durst *et al.* provides examples of the transcriptional co-activators and co-repressors that are known to interact with the RUNX family[69]. In general, the RUNX family members act as weak transcriptional activators and potent transcriptional repressors, on their own[69,70].

RUNX transcription factors play important roles in cell proliferation and differentiation. For instance, in the sea urchin the Runx gene, spRunt, has different functions depending on the cell type under examination and the time in development. Early in development it is needed for cell division whereas later, it is necessary for the expression of genes involved in differentiation[71-73]. In myeloid progenitor cells RUNX1 has been shown to promote proliferation, and accelerates the entry of cells from G1 into S phase[74].

As mentioned above, the RUNX genes can undergo alternative splicing leading to the generation of multiple splice variants. These splice variants can

a)

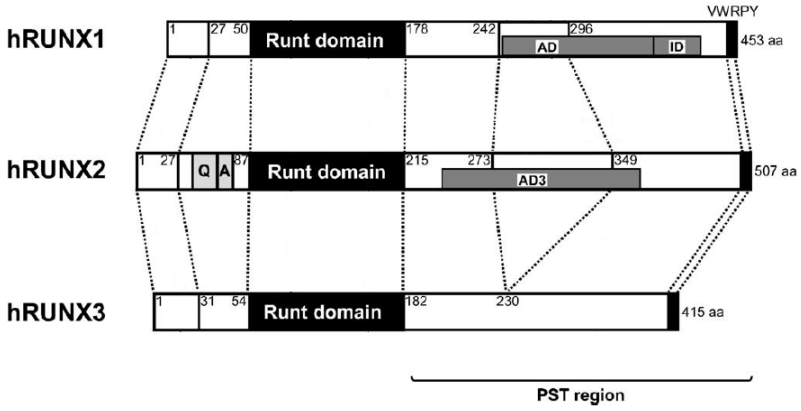

Figure 3. The RUNX family of transcription factors. The RUNX family is made up of three members RUNX1, RUNX2 and RUNX3. All members share a conserved runt domain, located near the N-terminus of the protein. The domain is important for recognizing and binding to specific DNA sequences at the promoter or enhancer region of target genes, and for binding to the common co-factor CBFβ (core-binding factor β). The c-terminus is important for the regulation of DNA binding and activation or repression of transcription. Figure adapted from [61]. Text adapted from [57,61,62].

have antagonistic functions in terms of proliferation and differentiation, depending on the levels of each splice variant[57]. One example involves RUNX1 (previously referred to as AML1). When alternatively spliced it can give rise to two forms of proteins referred to as AML1a and AML1b. Both forms contain the runt domain that is responsible for binding DNA. However, AML1a lacks the transcriptional activation domain present in AML1b, due to alternative splicing. When AML1a is overexpressed in a murine myeloid cell line, proliferation is stimulated while differentiation of cells is suppressed. However, the effects of AML1a can be canceled by overexpression of AML1b[65].

The RUNX family can undergo post-translational modifications, which affect the levels of RUNX proteins and the activity of the RUNX family as summarized in Bae *et al.*[61]. For instance, all RUNX family members undergo phosphorylation at various sites, which can disrupt or promote the interaction between RUNX proteins and transcriptional activators or repressors, or affect the levels of RUNX protein degradation, and thus modulate the activity of RUNX proteins[61]. A ubiquitin ligase-mediated pathway degrades RUNX proteins[75]. Bae *et al.*[61] proposed a model where the cellular levels of RUNX proteins are regulated by the amount of acetyl transferase and deacetylase activity. Since acetylation and ubiquitination of RUNX proteins both recognize the same lysine residues, when there are high levels of deactylase activity, the lysine residues on the RUNX protein can be ubiquitinated thus leading to protein degradation. This would occur when the cell requires low amounts of RUNX activity. However, if high amounts of RUNX activity are necessary, lysine residues undergo

acetylation due to relatively high amounts of acetyl transferase, protecting the RUNX protein from degradation[61].

1.3.2: Diversification of roles

Gene knockout mice have been used to determine the specific function of each RUNX family member. Runx1 is essential for hematopoiesis[76]. Runx2 is necessary for bone formation[77,78]. Runx3 is needed for the development of dorsal root ganglia neurons[79,80], T-cell differentiation[81] and growth of the gastric epithelium[82], although growth of the gastric epithelium was not confirmed in a study conducted by Levanon *et al*.[80]. The RUNX family has also been implicated in a variety of human diseases with the most well studied being the association between mutations in RUNX1 and human leukemia[83]. Frequently, mutations consist of chromosomal translocation between the N-terminal half of RUNX1, where the runt domain is located, and the C-terminus of another gene[84]. Cleidocranial dysplasia (CCD) is a disease associated with dysregulation of RUNX2. The disease is characterized by bone malformation which can lead to skeletal abnormalities[85]. RUNX3 has also been implicated in human diseases with the main one being cancer[86]. This topic is the focus of the thesis and will be discussed in more detail below. The importance that this family of genes has during development is evident by the variety of diseases arising from the dysregulation of the RUNX family. In broad terms, the diseases are associated with an imbalance between cell proliferation and differentiation.

1.3.3: Regulation of gene expression of the RUNX family

As previously mentioned, transcription of all three family members is initiated from either the P1 or P2 promoter[61,62]. The sequences of the P1 and P2 promoter regions contain binding sites for the RUNX proteins, indicating that auto regulation of RUNX proteins by either themselves or other family members is possible[60,64,87,88]. There is convergence between the vitamin D and retinoic acid pathway at a common hormone response element that is present in the promoter region of RUNX genes, and controls their expression. Expression of RUNX1 and RUNX3 is positively regulated by retinoic acids[89,90], while the expression of RUNX2 is downregulated by vitamin D[60,91]. Distinct signaling pathways and transcription factors can also modulate the expression of each RUNX gene, as summarized by Otto *et al.*[60] and Levanon *et al.*[62].

1.3.4: Biological processes regulated by the RUNX family

The TGFβ (transforming growth factor β) pathway regulates cellular growth, differentiation and migration. In cancer, the TGFβ pathway has been shown to play a tumor suppressive role, although it can also promote tumor growth and metastasis, when pathological forms of the pathway are active (reviewed in Massague *et al.*[92]). Two main branches of the TGFβ super family exist, consisting of the TGFβ and the BMP (bone-morphogenetic proteins) pathways. When bound by the appropriate ligands, TGFβ receptors are activated and act as serine/threonine kinases to phosphorylate downstream signal transducers called R-Smads (receptor regulated Smads). R-Smads include Smad2

and Smad3 (act downstream of the TGFβ branch) and Smad1, Smad5 and Smad8 (act downstream of the BMP branch). R-Smads bind the common Smad, Smad4, and together form complexes that control the transcription of downstream target genes[92]. The RUNX family interacts with the TGFβ superfamily at multiple points and can modulate the activity of the pathway[93]. In a study conducted by Hanai *et al*.[94], they determined that all three RUNX family members can interact with Smad1, Smad2, Smad3 and Smad5[94]. RUNX3 has been shown to play a tumor suppressive role in gastric cancer by interacting with and modulating the activity of the TGFβ pathway[82]. Conversely, in a recent study, RUNX2 was shown to induce EMT (epithelial to mesenchymal transition) in a breast cancer cell line, in part by stimulating SNAI2 (a family member in a group of transcription factors shown to promote EMT and tumor dissemination), in a TGFβ dependent manner[95]. These findings implicate RUNX2, SNAI2 and TGFβ in EMT and invasiveness in breast cancer[95].

The canonical Wnt signaling pathway is important in numerous biological processes. Activation of the canonical Wnt pathway requires β-catenin to accumulate in the cytoplasm and translocate into nucleus, where it associates with lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors, and activates the transcription of Wnt-target genes[96]. One study found that the runt domain was necessary for interaction between LEF1 and RUNX1 or RUNX2[97]. Moreover, LEF1 disrupted the activity of RUNX2 on the osteocalcin promoter[97]. Since all RUNX family members contain the conserved runt-domain, it is hypothesized that LEF1 may interact with all the RUNX transcription factors, and

possibly modulate their activity[98]. The interaction between RUNX3 and Wnt signaling has been studied in more detail and will be discussed below.

The RUNX family can interact with and influence the progression of the cell cycle. For example, RUNX1 accelerates the progression of myeloid cells from G1 to S phase[74], and the protein level of RUNX1 increases at the G1 to S phase transition[99]. In osteoblastic MC3T3 cells, the expression of RUNX2 increases upon induction of quiescence, while low levels are found during late G1/S phase and mitosis, supporting an antiproliferative role for RUNX2 in these cells[100]. In addition, RUNX3 can induce the expression of the key cell cycle regulator p21[101], in cooperation with Smads, in gastric epithelial cells[102]. CDKs (cyclin-dependant kinases) and cyclins have also been shown to affect the function of RUNX transcription factors by phosphorylation of RUNX proteins and by kinase independent mechanisms as summarized in Chuang *et al.*[98].

The RUNX family is also important during stress response in cells. The NHEJ pathway is one of the main pathways for repairing DSBs. When DNA DSBs occur, the two broken DNA ends are brought into close proximity so that they can be repaired. The Ku protein, which is a heterodimer consisting of Ku70 and Ku80, binds to the ends of the DSB and is important in maintaining the proximity of the DNA ends and recruiting other factors in the NHEJ pathway[103]. RUNX3 has been shown to bind with Ku70[101]. Interestingly, binding of Ku70 with RUNX3 negatively regulates the transactivation of p21 by RUNX3[101]. The group suggested that Ku70 might interrupt the activity of RUNX3 as a transcription factor, since it was shown to bind to the transcriptional activation

domain of RUNX3[101]. In line with this, RUNX2 and Ku70 have also been shown to interact[104]. However, this group found that RUNX2 and Ku70 synergistically activate the transcription of osteocalcin gene expression[104], demonstrating the multifaceted role of RUNX transcription factors. Neither of the two studies summarized above[101,104] indicated that the RUNX-Ku70 complex plays a role in the response to DSB. Nonetheless, the fact that the RUNX family members can interact with the Ku70 protein suggests that the family could be involved in the DNA damage response. RUNX transcription factors also induce cell cycle inhibitors[102], and proapoptotic genes such as Bax[105] and Bim[106, 107], demonstrating their importance in detecting and responding to cellular stress.

1.4: RUNX3 and Cancer

1.4.1: RUNX3s tumor suppressive potential

Like the other RUNX family members, RUNX3 has been implicated in human diseases, and has been shown to play a key role in cancer development. The first study to examine RUNX3 in the context of cancer demonstrated that RUNX3 is an important regulator of gastric epithelial cell growth, and that approximately half of human gastric cancer cells do not significantly express RUNX3[82]. Since then several groups have shown that RUNX3 is inactivated in various human cancers, and the idea of RUNX3 as a tumor suppressor has emerged. RUNX3 inactivation occurs due to numerous mechanisms[86]. One of the most common mechanisms of inactivation involves silencing via hypermethylation of the CpG island at the RUNX3 promoter, which has been

reported in numerous types of tissues as summarized by Subramaniam *et al*.[86]. RUNX3 function is also dysregulated by protein mislocalization from the nucleus to the cytoplasm and hemizygous deletion[86], and in one study a mutation in the runt domain was found[82].

Much work supporting a tumor suppressive role for RUNX3 comes from studies in gastric cancer. $Runx3^{-/-}$ mice were generated by Li *et al*.[82], and died soon after birth [82]. They observed that the stomach wall of the knockout mice was thicker than the stomach wall of the wild-type (WT) animals, and found that gastric epithelial proliferation was stimulated while apoptosis was reduced in the knockout mice. Moreover, $Runx3^{-/-}$epithelial cells were less responsive to the growth-inhibitory effects of TGFβ treatment. Since $Runx3^{-/-}$ mice died soon after birth, no tumor growth was observed[82]. In order to determine whether RUNX3 acts as a tumor suppressor in gastric cancer, Li *et al.*[82] generated cell lines from epithelial cells of the stomachs of $RUNX3^{-/-}p53^{-/-}$ mice and $RUNX3^{+/+}p53^{-/-}$ mice. $RUNX3^{-/-}p53^{-/-}$ cells led to tumor formation in nude mice, while $RUNX3^{+/+}p53^{-/-}$ cells did not[82].

In gastric cancer, RUNX3 acts in a tumor suppressive manner by interacting with the TGFβ pathway to activate downstream genes (Figure 4). Specifically, RUNX3 can interact with the Smad2 and Smad3, and the transcriptional co-activator p300[93]. When gastric cancer cells were treated with TGFβ1, cells expressing RUNX3 demonstrated higher levels of p21 as well as reduced growth. RUNX3 binding sites were found on the p21 promoter. RUNX3 was found to regulate p21 expression in cooperation with Smad3[102]. Another

study found that in a gastric cancer cell line (SNU16), TGFβ-induced apoptosis was RUNX3-dependent. Increased levels of Bim were found in cells treated with TGFβ, and were further induced by exogenous expression of RUNX3. Putative RUNX3 and Smad binding sites were located on the Bim promoter[106]. In support of this, a previous study also found that RUNX3 binds to the Bim promoter via two RUNX-binding elements, and activates the transcription of Bim[107]. RUNX3 can also downregulate the expression of vascular endothelial growth factor (VEGF), by binding to RUNX3 response elements on the VEGF promoter in gastric cancer cells, and suppresses the angiogenic potential of endothelial cells[108].

The TGFβ pathway can interact with RUNX3 in other cancers as well. For example, the SEG1 cell line is an esophageal cell line that does not respond to the growth-inhibitory effects of TGFβ. TGFβ receptors are present in SEG1 cells as well as normal levels of Smad3 and Smad4[109]. In a study conducted by Torquati *et al.*[110], they demonstrated that SEG1 cells do not express RUNX3[110]. Interestingly, exogenous expression of RUNX3 restored an antiproliferative and pro-apoptotic effect, when cells were treated with TGFβ. This effect was not noted in cells transfected with RUNX3 in the absence of TGFβ[110]. In a study by Sakakura *et al.*[111], the expression of RUNX3 was downregulated by hypermethylation of the promoter in radioresistant esophageal cancer cell lines. Exogenous expression of RUNX3 induced cell sensitivity to radiation and increased Bim expression, as well as enhanced the apoptotic effects of TGFβ treatment. The study concluded that when RUNX3 is inactivated, the cells

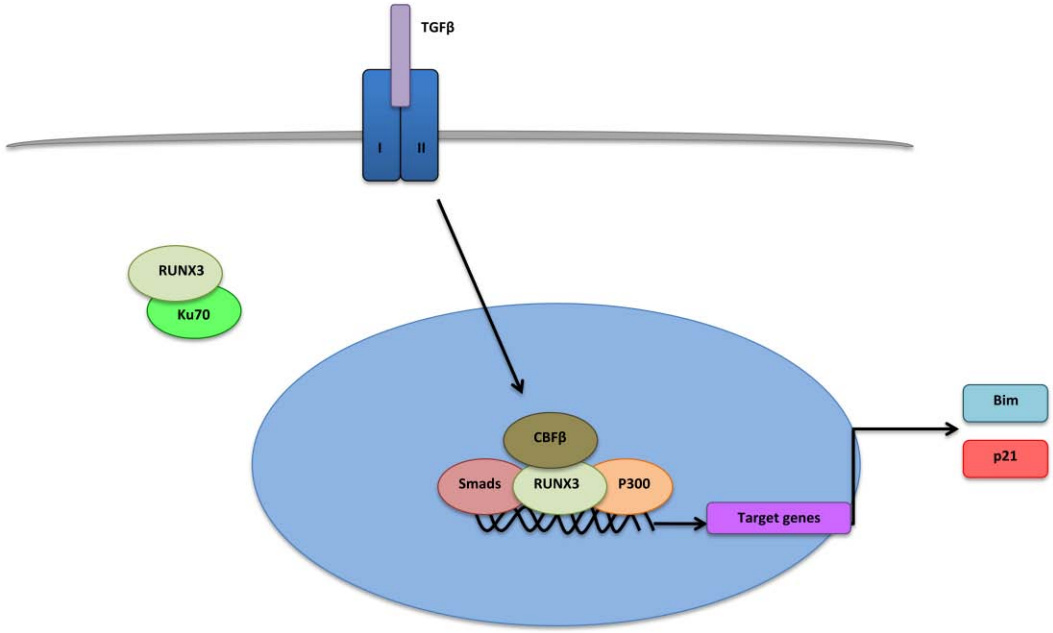

Figure 4. Interaction between RUNX3 and the TGFβ pathway. RUNX3 activates the transcription of target genes by interacting with R-Smads (Smad2 and Smad3) and p300, downstream of the TGFβ (transforming growth factor β) pathway. RUNX3 activates the expression of Bim and p21, thereby exerting an effect on apoptosis and cell growth, respectively. RUNX3 also interacts with Ku70, a protein involved in non-homologous end joining (NHEJ) repair. CBFβ (core-binding factor β). Figure and text adapted from [86].

become resistant to radiotherapy, whereas induction of RUNX3 promotes radiosensivity in esophageal cancer[111].

In colorectal cancer, the tumor suppressive capability of RUNX3 seems to negatively regulate the oncogenic Wnt pathway. Specifically Ito *et al.*[112] demonstrated that TCF4, β-catenin and RUNX3 interact with one another, by co-immuoprecipitation. Moreover, in $Runx3^{-/-}$ intestinal epithelia, increased Wnt signaling was demonstrated by an upregulation of Wnt target genes[112]. Inactivation of the adenomatous polyposis coli (APC) gene, a key regulator of the canonical Wnt pathway that binds to and destabilizes β-catenin, is a known step in the progression of colon carcer[96]. In the study conducted by Ito *et al.*[112], they examined $Runx3^{+/-}$ and $Apc^{Min/+}$ mice (a mouse model used for colon cancer studies that mimics the inactivation of one APC gene in humans and leads to the development of adenomatous polyps[113]). They found that adenomas developed in the small intestine at similar rates between the two mice. In $Apc^{Min/+}$ mice, accumulation of nuclear β-catenin was observed, whereas in $Runx3^{+/-}$ mice, β-catenin was not localized to the nucleus in significant amounts. Either inactivation of RUNX3 or Apc can lead to intestinal adenomas independently. Next, $Runx3^{+/-}$ $Apc^{Min/+}$ mice were investigated and invasive adenocarcinomas and adenomatous polyps were observed in the small and large intestine. The study concluded that RUNX3 affects that "strength" of activated Wnt signaling. When the Wnt pathway is active, either by binding of Wnt ligands or through pathological activation (eg. inactivation of Apc), RUNX3 can interact with TCF-4/β-catenin, and affect the strength of Wnt signaling. However, when RUNX3 is absent and

the Wnt pathway is active, the TCF-4/β-catenin complex may have enhanced or prolonged activity. The study suggests that both RUNX3 inactivation and β-catenin activation are necessary for the progression of intestinal tumors, and that RUNX3 can modulate the strength of Wnt signaling and act as a "brake" to oncogenic Wnt signaling in colorectal cancer[86].

RUNX3 is located on chromosome 1p36, which is a region that commonly undergoes changes in various types of cancers[114,115]. Moreover, in colon cancer cells, introduction of a normal human chromosome 1p36 reduced the tumorigenicity of these cells, suggesting that this region carries a tumor suppressor gene for colon cancer[116]. Such observations have led researchers to believe that the 1p36 region may contain several tumor suppressors, and that RUNX3 may be one of those tumor suppressors.

To summarize, several groups have found inactivation of RUNX3"s function by hypermethylation, hemizygous deletion, functional mutations or protein mislocalization to the cytoplasm[86]. Researchers have also found strong evidence for a tumor suppressive role, with key studies being conducted in gastric[82] and colon cancer[112] (Summarized by Subramaniam *et al*.[86]). Furthermore, RUNX3 is located on chromosome 1p36, which is a region believed to contain numerous tumor suppressors[117]. Such observations led to the widely accepted paradigm of RUNX3 as a tumor suppressor in human cancers. However, evidence demonstrating the oncogenic potential of RUNX3 also exists.

1.4.2: RUNX3's oncogenic potential

Moloney murine leukemia virus (MLV) commonly integrates at the Dsi1 locus in rat thymic lymphomas. The Dsi1 locus is 30kb upstream of the P1 promoter of the Runx3 gene on mouse chromosome 4, which is homologous with human chromosome 1, where RUNX3 is located. When cells are infected with the MLV virus and integration occurs at the Dsi1 locus, high levels of Runx3 transcripts arise from the P1 promoter[118]. Moreover, by examining common retroviral integration sites in murine tumors, Runx3 was also identified as a "candidate cancer gene", amongst other transcription factors[119]. Runx3 was also identified as playing a role in disease persistence in a retroviral insertional mutagenesis assay performed with the goal of "identifying genes that contribute to disease persistence in cells treated with imatinib". Imatinib is a kinase inhibitor that targets the oncoprotein Bcr-Abl in chronic myeloid leukemia[120]. In this study, bone marrow cells were transduced with the Bcr-Abl oncogene and transplanted into mice. Next, animals were treated with imatinib to select for cells where integration of Bcr-Abl had affected genes that could affect the response to imatinib. Integration near the Runx3 promoter was observed as well as increased Runx3 expression. The study concluded that expression of Runx3 in Bcr-Abl positive cell lines protected cells from imatinib-induced apoptosis[120]. Such insertional mutagenesis screens implicate RUNX3 in tumor development and suggest that RUNX3 can play an oncogenic role in a context dependent manner.

In support of the retroviral insertional work[118-120] RUNX3 can also function as an oncogene when overexpressed in specific human tissues. The first

observation suggesting an oncogenic role for RUNX3 occurred in basal cell carcinoma (BCC) tissues[121]. A tissue microarray comparing BCC and normal skin samples was performed to examine the expression of β-catenin and RUNX3. Approximately half of samples analyzed expressed nuclear β-catenin, whereas all of the samples analyzed showed nuclear localization of RUNX3. The study found that the overexpressed RUNX3 protein was normal and full length[121]. This was the first study to find overexpression of RUNX3 localized to the nucleus of cells. Next, RUNX3 overexpression was observed in head and neck squamous cell carcinoma (HNSCC) samples, when compared to normal tissues[122]. A separate study examined the levels of RUNX3 in HNSCC cell lines, and found high expression of RUNX3 at the mRNA and protein level, with little or no methylation detected at the promoter region[123]. Moreover, RUNX3 was localized to the nucleus, where it can act as a transcription factor. When a HNSCC cell line was transfected with a RUNX3 expression vector, increased proliferation and tumorsphere formation were observed, as well as an inhibition of apoptosis[123]. Interestingly, overexpression of RUNX3 made cells resistant to the apoptotic effects of adriamycin. Results were confirmed by knocking down RUNX3 expression in a HNSCC cell line that endogenously expresses high levels of RUNX3[123].

Previous studies had found RUNX3 overexpression[124-126] in cancerous cells, but in those studies they determined that RUNX3 was mislocalized from the nucleus into the cytoplasm[86]. However, in BCC and HNSCC, RUNX3 was

overexpressed and localized to the nucleus and in HNSCC, is implicated in the promotion of cancer as an oncogene.

1.4.3: RUNX3 and ovarian cancer

The understanding of RUNX3 as either an oncogene or tumor suppressor in EOC has evolved. Initial observations on the methylation status of RUNX3 suggested that it was silenced by hypermethylation of the promoter region in EOC[127]. Specifically, "RUNX3 methylation was detected in 53.1% of primary EOC tumors, 16.7% of benign epithelial ovarian tumors and 28% of nonmalignant tissues surrounding ovarian cancers". Non-cancerous ovarian tissues displayed no methylation. The data supported the notion of RUNX3 as a tumor suppressor and the group concluded that inactivation of RUNX3 may lead to impairment of the TGFβ pathway and other tumor suppressive functions[127]. It is important to note that this group performed no functional studies supporting RUNX3‟s role as a tumor suppressor in ovarian cancer[127].

Next, RUNX3 expression and localization were examined by immunohistochemical and immunofluorescent staining in ovarian cancer cell lines and tissues, and cytoplasmic localization was determined[128]. The group found high expression of RUNX3 in primary ovarian cancer samples, when compared to non-cancerous samples. A subgroup of patients with serous ovarian cancer was examined, and a "difference in the five-year survival of 75% versus 32% in weakly and strongly expressing groups", was observed. When examined at the mRNA and protein level, RUNX3 expression was upregulated in approximately

50% of the ovarian cancer cell lines, when compared to human ovarian surface epithelial (HOSE) cell lines. Next, to evaluate the potential oncogenic role of RUNX3 in ovarian cancer, an ovarian cancer cell line that did not express detectable levels of endogenous RUNX3, OVCAR429, was transduced with lentivirus containing RUNX3 or an empty vector, as a control. OVCAR429 cells transduced with RUNX3 showed a significant increase in cell proliferation when compared to the control cells. When RUNX3 was silenced in ovarian cancer cells expressing high levels of RUNX3, SKOV3 cells, a slight decrease in proliferation was noticed, when compared to control cells. The group concluded that RUNX3 is overexpressed and plays a growth stimulatory role in EOC although the mechanism by which it promotes growth is unknown. The group also determined that RUNX3 is mislocalized to the cytoplasm[128].

Soon after, another group investigated the expression and functional role of RUNX3[129]. Endogenous levels of RUNX3 were examined in five ovarian cancer cell lines, and the levels of expression were variable. By cellular fractionation and Western blotting, the group observed strong nuclear localization of RUNX3 with no cytoplasmic RUNX3 detected. These results were confirmed by immunofluorescence. Ovarian cancer cells, which express high levels of endogenous RUNX3 (TOV112D and SKOV3 cells), were stably infected with lentivirus delivering control shRNA or an shRNA specifically targeting RUNX3. A major reduction in the growth rate was observed in cells infected with a vector encoding an shRNA targeting RUNX3, when compared to control cells. RUNX3-overexpressing A2780 cells were established using a lentivirus construct. A

modest increase in cell proliferation was observed in A2780 cells expressing RUNX3 when compared to control cells. Reduction of RUNX3 expression decreased the ability of ovarian cancer cells to form soft agar colonies, while stable overexpression led to a modest increase in colony formation. Lee *et al.*[129] concluded that RUNX3 is overexpressed and functions as an oncogene in ovarian cancer. In reference to the discrepancy between RUNX3 localization in this study[129] and the study by Nevadunsky *et al*.[128], (referred to in the previous paragraph), the group stated that it was likely due to differences in the procedure of immunofluorescent staining used to detect endogenous RUNX3[129].

RUNX1 and RUNX2 have also been found to act in an oncogenic manner in serous EOC by contributing to cell proliferation, migration and invasion[130,131]. Specifically, a study compared the methylation status of early and late stage ovarian tumors, and found that advanced ovarian tumors generally display DNA hypomethylation in several genes that have been implicated in tumor progression and chemoresistance[132]. Specifically, they found that RUNX1 and RUNX2 are among the genes that are hypomethylated in serous EOC samples obtained post-chemotherapy treatment, when compared to samples derived from the same patients pre-chemotherapy[132]. The group went on to examine the role of RUNX1 and RUNX2 in serous EOC[130,131]. They found significantly higher expression in EOC tissue samples, when compared to normal ovarian tissue samples. Knockdown of RUNX1 or RUNX2 in EOC cells caused a decrease in cell proliferation, colony formation, cell migration and invasion. Next, gene expression profiling and pathway analyses were performed to compare RUNX

expressing and knockdown cells, and distinct molecular profiles confirmed the role of RUNX1 and RUNX2 in cell proliferation, migration and invasion in EOC[130,131].

The RUNX family of transcription factors have been shown to act in an oncogenic manner in EOC[128,129]. More specifically, RUNX3 is an oncogene that promotes proliferation and colony formation in ovarian cancer[128,129]. However, the role that RUNX3 plays in chemoresistance of EOC remains unknown.

1.4.4: RUNX3 and cell sensitivity to chemotherapeutic agents

To date, no group has studied the role of RUNX3 in chemoresistance in EOC. However, RUNX3 has been shown to play a role in promoting cell sensitivity to chemotherapeutic agents in other tumor types, when it acts as a tumor suppressor[133-135]. In hepatocellular carcinoma patients, the levels of miR-130a were significantly increased in tumors following treatment with cisplatin chemotherapy. miR-130a levels were higher in cisplatin-resistant Huh7-R cells, a cisplatin-resistant hepatocellular carcinoma cell line, than in parental Huh7 cells. Overexpression of miR-130a in Huh7 made the cells cisplatin-resistant, while knockdown of miR-130a in Huh7-R cells made the cells more sensitive to cisplatin treatment. Upregulation of miR-130a directly inhibited expression of RUNX3, when examined at the transcriptional level and at the protein level. Wnt/β-catenin signaling was also activated in Huh7-R cells, compared to Huh7 cells. The group speculated that upregulation of miR-130a, through its inhibition

of RUNX3 expression, can activate Wnt/β-catenin signaling and lead to increased cisplatin resistance[133].

In gastric cancer, the levels of miR-106a are frequently upregulated[136], and it has been suggested as a potential biomarker to diagnose gastric cancer[137]. Recently, elevated levels of mir-106a have been observed in two MDR (multidrug resistant) human gastric cancer cell lines (SGC7901/ADR and SGC7901/VCR), when compared to the parental gastric cancer cell line (SGC7901)[134]. SGC7901 cells transfected with miR-106a mimic were more resistant to the cytotoxic effects of adriamycin, cisplatin and 5-fluourouracil. In contrast, suppression of miR-106a in SGC7901/VCR cells resulted in enhanced sensitivity to the same set of drugs. When SGC7901 cells were transfected with miR-106a mimic, the efflux of adriamycin was enhanced along with an increase in the mRNA levels of MDR1 and the protein P-glycoprotein, which is encoded by the MDR1 gene. Conversely, when SGC7901/ADR cells were transfected with a miR-106a inhibitor, the efflux of adriamycin decreased. Using TargetScan, RUNX3 was predicted as a target of miR-106a. SCG7901 cells were cotransfected with miR-106a mimic or control mimic, and WT or mutated RUNX3 sensitive promoter luciferase construct. SGC7901 cells cotransfected with miR-106a mimic and WT RUNX3 luciferase promoter showed decreased luciferase activity compared with control mimic. The protein level of RUNX3 was significantly decreased in SGC7901/ADR and SGC7901/VCR cells, when compared to SGC7901 cells. Overexpression of RUNX3 in SGC7901 cells transfected with miR-106a mimic reversed the effect of miR-106a, and made the cells more sensitive to anticancer drugs, suggesting

that miR-106a might induce MDR in gastric cancer cells by inhibiting RUNX3 expression[134].

Taken together, these studies suggest that RUNX3 plays an important role in promoting cell sensitivity to chemotherapeutic agents when it acts as a tumor suppressor[133,134]. However, the role that RUNX3 plays in chemoresistance in ovarian cancer, where it acts as an oncogene, has yet to be studied.

1.5: Hypothesis

The main focus of our research is to identify the molecular mechanisms underlying chemoresistance in ovarian cancer. RUNX3 has been shown to play an oncogenic role in ovarian cancer, but its role in chemoresistance in ovarian cancer remains unknown. Based on its oncogenic role in ovarian cancer, we hypothesize that RUNX3 contributes to chemoresistance in ovarian cancer. By targeting RUNX3 expression, we expect ovarian cancer cells to become more sensitive to platinum agents.

Section 2: Materials and methods

2.1: Cell culture and tumor specimens

Human ovarian cancer cell lines SKOV3 (human adenocarcinoma), A2780s (human adenocarcinoma), and A2780cp (human adenocarcinoma) cells were cultured in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco®) and 100U/ml penicillin-streptomycin (P/S) (Gibco®). OVCAR3 (human adenocarcinoma) cells were cultured in RPMI 1640 with the same supplements. 293T (human embryonic kidney) and Phoenix-Ampho (human kidney) cells were cultured in DMEM with the same supplements. 13 primary cultures of normal human ovarian surface epithelial (OSE) cells were collected and prepared by scraping the surface of the ovary from patients with benign gynecologic diseases as described in[138]. Two immortalized ovarian surface epithelial (IOSE) cell lines were used. Cells were immortalized by SV40 large T-antigen[139]. 11 primary EOC cells isolated from ovarian cancer patients‟ ascites were collected. Cells were cultured in M199/MCDB105 with the same supplements listed above. Cells were maintained in a humidified 37˚C incubator with 5% CO_2. 22 primary serous EOC tissues were kindly donated by the CBCF Tumor Bank.

2.2: Generating RUNX3 overexpression cells

All vectors were obtained from the BC Cancer Research Institute. Phoenix-Ampho cells were transfected with an empty retroviral vector (MSCVpac) or a retroviral vector containing the human RUNX3 gene

(MSCVpac-hRUNX3) by the calcium phosphate method as follows. At least 1 hour prior to transfecting Phoenix-Ampho cells, the medium was changed. 10 μg of the expression plasmid (MSCVpac or MSCVpac-hRUNX3) was added to dH_2O to a final volume of 450 μL. Tubes were vortexed 4-5 times and then briefly spun. 50 μL of 2.5 M $CaCl_2$ was added to each tube dropwise. Next, 500 μL of 2 X HBS (hepes-buffered saline) was added dropwise to the solution, and mixed by pipetting. The solution was incubated at room temperature for 5 minutes. The solution was added dropwise to the Phoenix-Ampho cells, and cells were incubated overnight at 37˚C. The next day, the medium was removed and fresh medium was added. 48 and 72 hrs post-transfection, the medium from the Phoenix-Ampho cells was filtered (Millex ® Syringe-driven Filter Unit 0.33 μm) and added to target cells (SKOV3 or A2780s cells). Polybrene (Sigma) (8 μg/mL) was added to the target cells (1/1000 dilution). SKOV3 and A2780s cells were infected with retrovirus containing either MSCVpac or MSCVpac-hRUNX3. Infected cells were selected by treatment with puromycin (Sigma) to generate pooled SKOV3/MSCVpac, SKOV3/hRUNX3, A2780s/MSCVpac and A2780s/hRUNX3 cells.

2.3: Generating RUNX3 knockdown cells

All vectors were obtained from the BC Cancer Research Institute. 293T cells were transfected with a lentivirus vector (pLentiLox) containing an shRNA targeted against a random sequence (shRandom: 5‟-GTT GCT TGC CAC GTC CTA GAT-3‟) or an shRNA targeted against the RUNX3 gene (shRUNX3D: 5‟-GGA CCC TAA CAA CCT TCA AGA-3‟ or shRUNX3E: 5‟-GCC GTC TCA

TCC CAT ACT TCT-3‟) by the calcium phosphate method as follows. At least 1 hour prior to transfecting 293T cells, the medium was changed. 10 μg of the expression plasmid (shRandom or shRUNX3D or shRUNX3E), 5 μg of RRE, 5 μg of REV and 5 μg of VSVG were added to dH_2O to a final volume of 450 μL. Tubes were vortexed 4-5 times and then briefly spun. 50 μL of 2.5 M $CaCl_2$ was added to each tube dropwise. Next, 500 μL of 2 X HBS was added dropwise to the solution, and mixed by pipetting. The solution was incubated at room temperature for 5 minutes. The solution was added dropwise to the 293T cells, and cells were incubated overnight at 37˚C. The next day, the medium was removed and fresh medium was added. 48 and 72 hrs post-transfection, the medium from the 293T cells was filtered (Millex ® Syringe-driven Filter Unit 0.33 μm) and added to target cells (A2780cp cells). Polybrene (Sigma) (8 μg/mL) was added to the target cells (1/1000 dilution). A2780cp cells were infected with either shRandom or shRUNX3D or shRUNX3E. Infected cells were purified by fluorescence-activated cell sorting (FACS) for green fluorescent protein (GFP) positive cells; the lentiviral vector contains a cytomegalovirus (CMV)-driven GFP. Pooled A2780cp/shRandom, A2780cp/shRUNX3D and A2780cp/shRUNX3E cells were generated.

A mixture of 4-pooled siRNAs targeted against RUNX3 (On-TARGETplus human RUNX3) was used to transiently knock down the expression of RUNX3 in A2780cp cells. An siRNA targeted against a scrambled sequence was used as the control (On-TARGETplus non-targeting pool). Cells were transfected using Lipofectamine® RNAiMAX reagent. The procedure was

carried out according to the Lipofectamine ® RNAiMAX Reagent Protocol 2013 (96-well plate for cytotoxicity assays and 6-well plate for collection of cell lysate).

2.4: Generating RUNX3 dominant-negative cells

A2780cp cells were transfected with pcDNA3.1 vector or pcDNA-Flag-RUNX3 (1-187) (kindly provided by Dr. Yoshiaki Ito, Cancer Science Institute of Singapore) using the Novagen GeneJuice ® Transfection Reagent according to Novagen User Protocol TB289 (10 cm dish format). Transduced cells were selected by treatment with multiple rounds of G418 (Invitrogen). 6 colonies of A2780cp-pcDNA and 6 colonies of A2780cp-Flag-RUNX3 (1-187) were picked using cloning rings and expanded.

2.5: Neutral red uptake assay

Cell viability assays determining the cytotoxic effects of carboplatin were performed using the neutral red uptake assay. Cells were seeded into 96-well plates at a density of 3000 cells per well (unless otherwise indicated). The next day, cells were left untreated or treated with increasing concentrations of carboplatin (Sigma). 48 or 72 hrs post-treatment the medium was replaced with new medium containing 33 μg/ml of neutral red dye (Sigma) and incubated at 37˚C for 3 hrs. The cells were washed once with phosphate buffered saline (PBS) and lysed with 100 μl of lysis buffer (98% ethanol and 2% acetic acid). Absorbance was read at 540 nm using the FLUOstar Omega microplate reader.

2.6: Collection of cell lysates

Whole cell lysates were prepared using modified radioimmuno-precipitation buffer (RIPA) as described previously[140]. RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS (sodium dodecyl sulfate), 1.0% DOC (sodium deoxycholate), 1.0% Triton X-100, 10 mM NaP_2O_7, 10 mM NaF , 1 mM Na_3OV_2 , 1 X PIC (protease inhibitor cocktail)) was added to cells, and cell lysate was scraped and collected. Cell lysates were briefly sonicated and centrifuged at 13000 rpm for 15 minutes at 4˚C. Supernatant containing the whole cell lysate was collected. Cytoplasmic and nuclear fractions were extracted using Buffer A (10 mM Hepes-KOH pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl) and Buffer C (50 mM Hepes-KOH pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol), respectively. Briefly, cells were rinsed with PBS and Buffer A was added. Cell lysates were scraped and collected. Cell lysates were incubated on ice for 10 minutes, and then centrifuged for 10 minutes at 12000 rpm at 4˚C. The supernatant containing the cytosolic fraction was collected. The remaining pellet was resuspended in Buffer C and rotated for 20 minutes at 4˚C. The cell lysate was centrifuged for 10 minutes at 12000 rpm at 4˚C, and the supernatant containing the nuclear fraction was collected. Protein concentration was quantified using the DC protein assay (Bio-Rad).

2.7: Western Blotting

50 μg of protein were loaded into each lane of a 10% SDS polyacylamide gel (Stacking gel: 4X Upper Buffer, 30% Bis-Acrylamide, 10% APS (ammonium

persulfate), TEMED (tetramethlethylenediamine), dH_2O; Separating gel: 4X Lower Buffer, 30% Bis-Acrylamide, 10% APS, TEMED, dH_2O). Gels were run at a constant 100 V for 30 minutes and then at 150 V for approximately 60 minutes. Proteins were transferred onto nitrocellulose paper at a constant 100 V for 90 minutes. Immunoblotting was performed using an anti-RUNX3 monoclonal antibody (Abcam R3-5G4, 1:1000 dilution). Protein loading was normalized by immunoblotting with an anti-β-actin (Abcam, 1:1000), anti-tubulin (Abcam, 1:1000), anti-PARP (Cell Signaling, 1:1000) or anti-GAPDH (Cell Signaling, 1:1000) antibody. Membranes were scanned and analyzed using an Odyssey® IR scanner and Odyssey® imaging software 3.0.

2.8: RNA isolation and real-time PCR analysis

Using the RNeasy Mini Kit (Qiagen), total RNA was isolated. To remove DNA contamination, 2.5 μg of RNA was incubated with 2 μ of DNase I Amplification Grade (Invitrogen), 2.5 μl of 10 X DNase I Reaction Buffer (Invitrogen) and RNA free dH_2O (final volume 25 μl) and incubated at room temperature. 2.5 μl of 25 mM EDTA was added to RNA and heated to 70˚C from 10 minutes in the Verti® 96 well thermal cycler (Life Technologies). 5 μl of 10 X random primers (Invitrogen) were added and the samples were heated to 70˚C for 10 minutes in the Verti® 96 well thermal cycler (Life Technologies). First strand cDNA was reverse transcribed using 1 μl of SuperScript ® II Reverse Transcriptase (Invitrogen) in the presence of 1 μl of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 5 μl of 0.1 M DTT (dithiothreitol) (Invitrogen), 1 μl of 25 mM dNTP (dinucleotide triphosphate) (Invitrogen) and 10

µl of 5 X First Strand Buffer (Invitrogen). Reverse transcriptase PCR was done in the Verti® 96 well thermal cycler (Life Technologies). The PCR reaction conditions for Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) were 10 µl of 2 X SYBER green buffer, 1 µl of 10 µM forward/reverse primers, 1 µl of 50 ng/µl cDNA and 8.5 µl of dH_2O. qRT-PCR was carried out using the Mastercycler® ep Realplex real-time PCR system (Eppendorf). PCR primers are listed in Table 2.

2.9: Statistics

Where applicable, data are expressed as mean ± SEM (standard error of mean). Unpaired two-tailed t tests were used for the comparison of mean values. Statistical significance was determined at P value <0.05. Experiments were repeated a minimum of 3 times. Statistical analyses were performed using Prism Software.

Table 1. List of PCR primer sequences.

Gene	Sequence (Forward)	Sequence (Reverse)
RUNX1	5‟-ACT ATC CAG GCG CCT TCA CCT ACT-3‟	5‟-TAG TAC AGG TGG TAG GAG GGC GAG-3‟
RUNX2	5‟-ACG AAT GCA CTA TCC AGC CAC CTT-3‟	5‟-ATA TGG AGT GCT GCT GGT CTG GAA-3‟
RUNX3	5‟-TGG CAG GCA ATG ACG AGA ACT ACT-3‟	5‟-TGA ACA CAG TGA TGG TCA GGG TGA-3‟
APC2	5‟-GCT GTT ATG AAG CTG TCC TTT G-3‟	5‟-GGG TCA TCT TGT GCA TCT CAT A-3‟
DKK1	5‟-AGC GTT GTT ACT GTG GAG AAG-3‟	5‟-CTG ACA AGT GTG AAG CCT AGA A-3‟
DKK3	5‟-GAA GAG ATG GAG GCA GAA GAA G-3‟	5‟-CGT GTC TGT GTT GGT CTC AT-3‟
FRZB (SFRP3)	5‟-CCC ATC TGC ACC ATT GAC TT-3‟	5‟-GTG GCG GTA CTT GAT GAG TAT G-3‟
SFRP1	5‟-GCT TGT GCT GTA CCT GAA GA-3‟	5‟-TCT TGT CCC ACT TGT GGA TG-3‟
WNT3	5‟-TGA CTT CGG CGT GTT AGT G-3‟	5‟-GTG CAT GTG GTC CAG GAT AG-3‟
WNT3a	5‟-GAC TTC CTC AAG GAC AAG TAC G-3‟	5‟-GGC ACC TTG AAG TAG GTG TAG-3‟
WNT11	5‟- AAC AGG ATC CCA AGC CAA TAA-3‟	5‟-CCA TGG CAC TTA CAC TTC ATT TC-3‟
c-MYC	5‟-AGC GAC TCT GAG GAG GAA-3‟	5‟-TGT GAG GAG GTT TGC TGT G-3‟
CCND1 (Cyclin D)	5‟-CTC GGT GTC CTA CTT CAA ATG T-3‟	5‟-TTC CTC GCA GAC CTC CA-3‟
c-JUN	5‟-AAC GAC CTT CTA TGA CGA TGC CCT-3‟	5‟-TCA GGG TCA TGC TCT GTT TCA GGA-3‟
GAPDH	5‟-GGA CCT GAC CTG CCG TCT AGA A-3‟	5‟-GGT GTC GCT GTT GAA GTC AGA G-3‟
18S	5‟- GTA ACC CGT TGA ACC CCA TT-3‟	5‟-CCA TCC AAT CGG TAG TAG CG-3‟

Section 3: Results

3.1: Expression of RUNX3 in normal OSE, IOSE, primary EOC cells and EOC cell lines

To confirm the elevated expression of RUNX3 in EOC reported in the two previous studies[128,129], we examined the protein expression of RUNX3 in 22 serous EOC tissues and compared them to 2 IOSE cell cultures, which were used as non-cancerous controls. As shown in Figure 5a, RUNX3 was expressed in the majority of cancerous samples, but in none of the IOSE cells. All of the samples were serous EOC, and the majority was stage III (Table 2). Next we evaluated RUNX3 protein expression in primary EOC cells isolated from patient‟s ascites. 6 out of 10 of the samples expressed high levels of RUNX3, when compared to non-cancerous primary OSE cells (Figure 5b). All cancer samples were serous EOC and stage III or IV tumors, with the exception of samples 12 and 16. Sample 12 was found to be benign and sample 16 was found to be an adenocarcinoma that metastasized from the colon (Table 3). Similar to IOSE cells, the ten OSE cell cultures did not express RUNX3 (Figure 5b). A panel of 4 EOC cell lines was evaluated for RUNX3 expression. One normal OSE cell culture and two IOSE cell lines were used as non-cancerous controls. Low RUNX3 expression was detected in OVCAR3, SKOV3 and A2780s cells (Figure 6). Interestingly A2780cp cells, which were derived from A2780s cells by exposing A2780s cells to increasing-stepwise concentrations of cisplatin[141-144], express a high level of

Figure 5a)

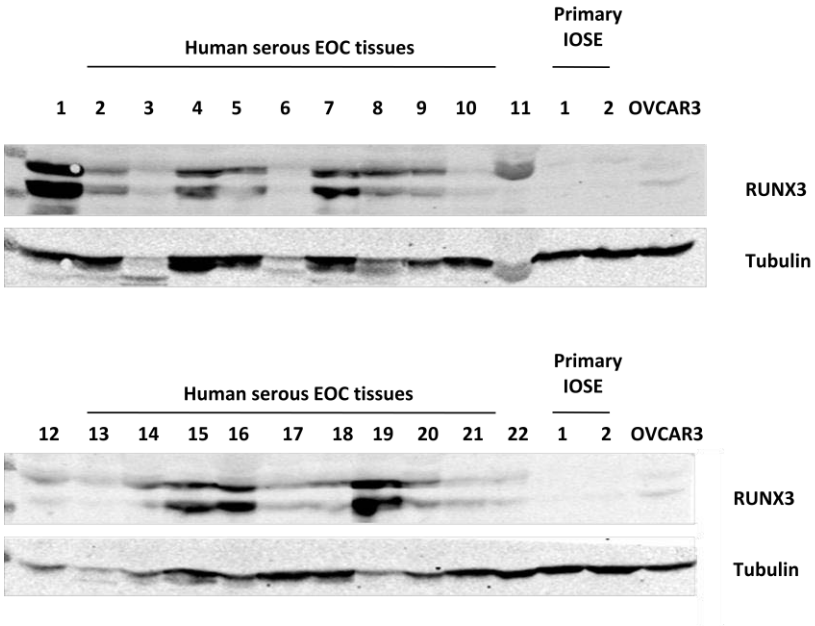

Figure 5b)

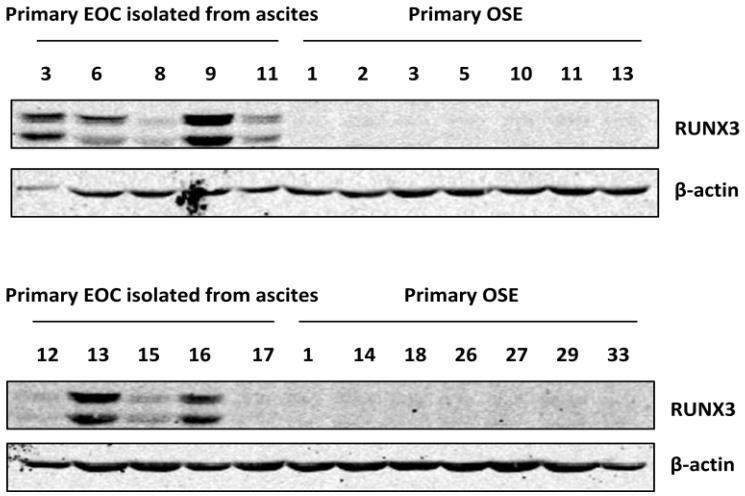

Figure 5. RUNX3 expression is elevated in human EOC. **a)** RUNX3 expression in 22 primary serous EOC tissue. Immortalized ovarian surface epithelial (IOSE: non-cancerous) cells were used as a non-canerous control. Tubulin was used as a loading control. **b)** RUNX3 expression in primary EOC cells collected from patients ascites. Ovarian surface epithelial cells (OSE: non-cancerous) were used as a non-cancerous control. β-actin was used as a loading control.

Table 2. Clinical information on primary serous EOC tissues. Samples donated by CBCF Tumor Bank. Unknown whether samples were obtained before or after treatment.

Sample ID	EOC:	Stage	Grade	Recurrence
1	Serous	IIB	-	No
2	Serous	IIIC	-	Yes
3	Serous	IIIC	-	No
4	Serous	IIIC	-	Yes
5	Serous	IIIC	-	Yes
6	Serous	IIA	-	Yes
7	Serous	IIIC	High	No
8	Serous	IIIC	High	Yes
9	Serous	IV	High	Yes
10	Serous	IV	High	Yes
11	Serous	IIIC	High	Yes
12	Serous	IIIC	High	Yes
13	Serous	IIIC	High	Yes
14	Serous	IIIC	High	Yes
15	Serous	IIIC	High	Yes
16	Serous	IIIC	High	No
17	Serous	IIIC	High	Yes
18	Serous	IIIC	High	Yes
19	Serous	IIIA	High	No
20	Serous	IC	High	Yes
21	Serous	IC	High	No
22	Serous	IIIC	High	Yes

Table 3. Clinical information on primary EOC cells derived from patient ascites. Samples were obtained with the help of Dr. Helen Steed.

Sample ID	EOC	Stage	Grade	Sample obtained before or after treatment	Treatment	Response to treatment
3	-	-	-	-	-	-
6	Serous	IV	High	Before	Upfront diagnosis	-
8	Serous	IV	High	After	Carboplatin, taxol and gemcitabine	Platinum Resistant
9	Serous	IIIC	High	Before	Upfront diagnosis	Platinum Sensitive
11	Serous	IIIC	High	After	Carboplatin and taxol	Platinum Sensitive
12	Benign	-	-	-	-	-
13	Serous	IIIC	High	Before	Upfront diagnosis	Platinum Resistant
15	Serous	IIIC	High	After	Carboplatin and taxol	Platinum Resistant
16	Metastatic adenocarcinoma from colon	-	-	-	-	-
17	Serous	IIIC	High	After	Carboplatin, taxol and liposomal doxorubicin	Platinum Sensitive

RUNX3 (Figure 6). Two RUNX3-related bands were detected in primary EOC samples and EOC cell lines, which likely represent two different RUNX3 isoforms[61,129]. Together, our results confirmed that RUNX3 is expressed in EOC cells, but not in normal OSE and IOSE cells.

3.2: RUNX3 is localized to the nucleus of EOC cells

Results regarding the subcellular localization of RUNX3 in EOC cells have been conflicting[128,129]. We examined the subcellular localization of RUNX3 in our study to ensure that it is localized to the nucleus in EOC cell lines and primary EOC cells, where it can functionally act as a transcription factor. Consistent with the results reported by Lee *et al.*[129], we confirmed that RUNX3 is localized to the nucleus of the 4 EOC cell lines used throughout the study, with minimal amounts localized to the cytoplasm (Figure 7a). Importantly, our data showed clear nuclear localization of RUNX3 with no appreciable cytoplasmic localization detected in three primary EOC cells, which express high levels of RUNX3 (Figure 7b). PARP and tubulin or GAPDH were used a nuclear and cytoplasmic loading controls, respectively.

3.3: RUNX3 protein level is increased after carboplatin treatment in EOC cells

A2780cp cells, which were derived from A2780s cells by exposing A2780s cells to increasing stepwise concentrations of cisplatin, are cisplatin resistant[141-144]. Currently carboplatin is a first-line therapeutic agent in the treatment of ovarian cancer because of its low toxicity profile when compared to

Figure 6)

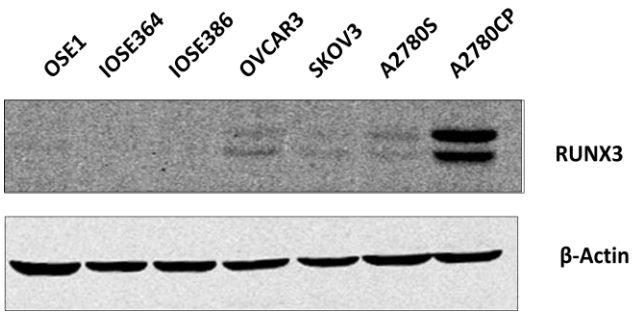

Figure 6. RUNX3 expression is variable in human EOC cell lines. RUNX3 expression in a panel of EOC cell lines. Ovarian surface epithelial and immortalized ovarian surface epithelial (OSE and IOSE: non-cancerous) cells were used as non-cancerous controls. β-actin was used as the loading control.

Figure 7a)

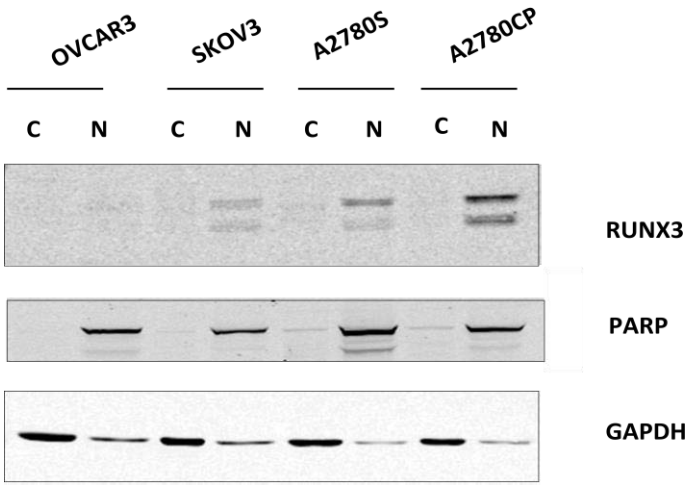

Figure 7b)

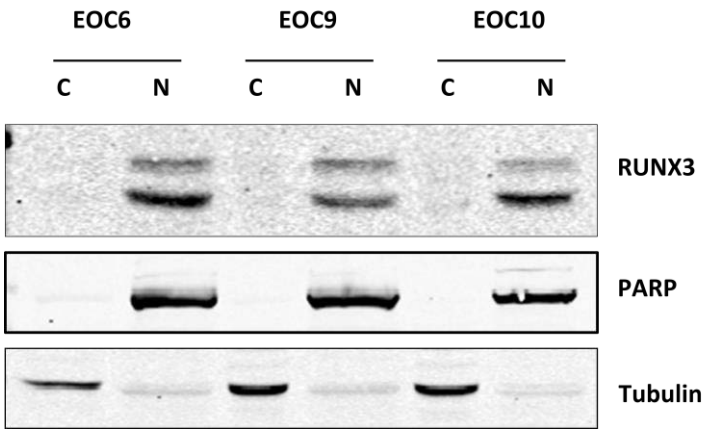

Figure 7. RUNX3 is localized to the nucleus in EOC cells. a) EOC cell lines underwent cellular fractionation. PARP and GAPDH were used as nuclear and cytoplasmic loading controls, respectively. **b)** Primary EOC cells isolated from the ascites of EOC patients underwent cellular fractionation. PARP and tubulin were used as nuclear and cytoplasmic loading controls, respectively. C: cytoplasm, N: Nucleus.

cisplatin[10-12]. We thus used carboplatin in this study. To confirm that A2780cp cells are also resistant to carboplatin we performed a dose response experiment and found that A2780cp cells are indeed more resistant to carboplatin than A2780s cells (IC50 for A2780s cells: 13.62 μM and A2780cp cells: 163.2 μM) (Figure 8).

The higher expression of RUNX3 in A2780cp cells compared to A2780s cells (Figure 8) suggests that RUNX3 expression increases after prolonged exposure to cisplatin. To determine whether RUNX3 expression increases upon short-term exposure to carboplatin, we examined the expression of RUNX3 in A2780s and A2780cp cells after treatment with carboplatin at various time points. By Western blotting we determined that RUNX3 expression indeed increased by carboplatin in both A2780s and A2780cp cells, 24, 48 and 72 hrs after treatment (Figure 9).

3.4: RUNX3 overexpression in EOC cell lines confers resistance to carboplatin treatment

Previous studies have demonstrated that RUNX3 acts as an oncogene in ovarian cancer[128,129]. However, the role RUNX3 plays during chemoresistance in EOC has yet to be examined. The higher expression of RUNX3 in A2780cp cells and increase in expression of RUNX3 by carboplatin suggests that RUNX3 might contribute to carboplatin resistance of EOC cells. To assess the role of RUNX3 in chemoresistance of EOC we overexpressed RUNX3 in SKOV3 and A2780s cells, which express low levels of endogenous RUNX3 (Figure 6). SKOV3 and

Figure 8a) **Figure 8b)**

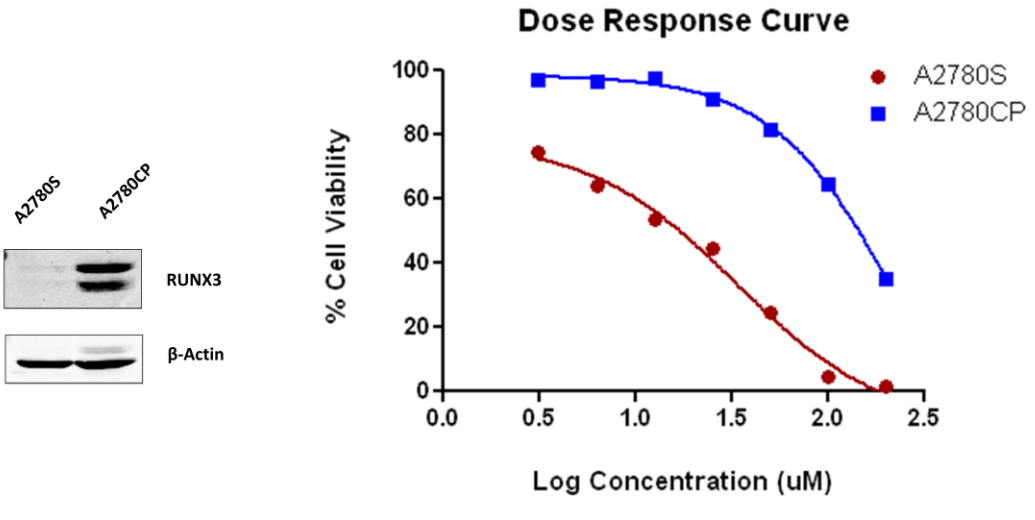

Figure 8. A2780s cells are more sensitive to carboplatin treatment than A2780cp cells. a) RUNX3 expression in A2780s and A2780cp. β-actin was used as a loading control. **b)** A2780s and A2780cp cells were treated with increasing concentrations of carboplatin for 72 hrs. Cell viability was measured by the neutral red assay. A2780s IC50: 13.62 μM. A2780cp IC50: 163.2 μM. Experiments were repeated 3 times.

Figure 9a)

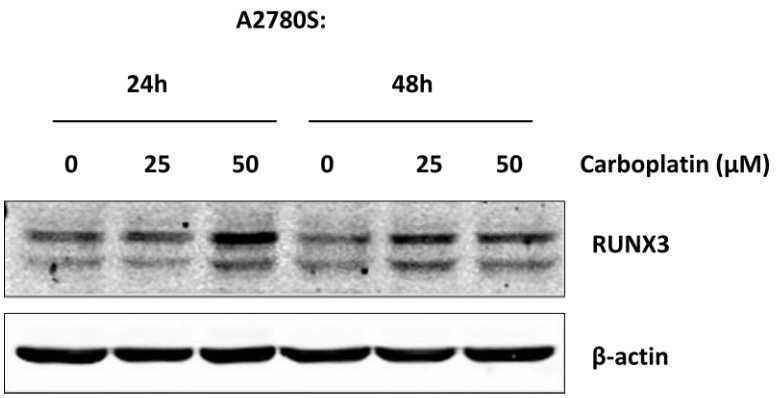

Figure 9b)

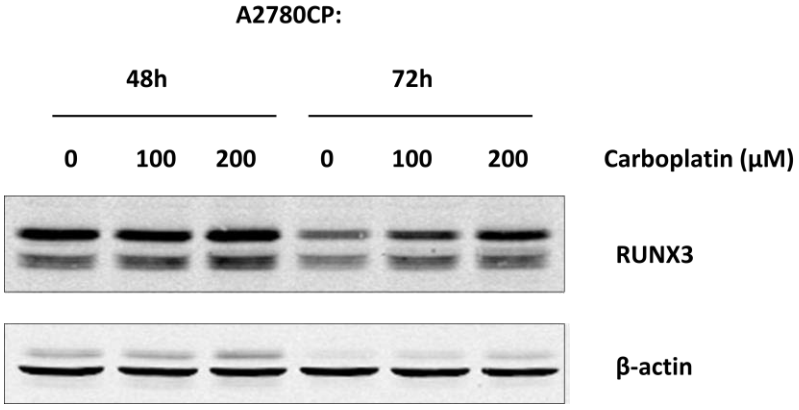

Figure 9. Carboplatin increases RUNX3 expression in EOC cell lines. a) A2780s cells were treated with increasing concentrations of carboplatin (0, 25 and 50 μM). Cell lysate was collected 24 and 48 hrs post-treatment. RUNX3 expression was examined by Western blotting. β-actin was used as a loading control. **b)** A2780cp cells were treated with increasing concentrations of carboplatin (0, 100 and 200μM). Cell lysate was collected 48 and 72 hrs post-treatment. RUNX3 expression was examined by Western blotting. β-actin was used as a loading control.

A2780s cells were infected by a retrovirus delivering either a control MSCVpac vector or an MSCVpac vector expressing RUNX3. RUNX3 overexpression in SKOV3 and A2780s cells was verified by Western blotting (Figure 10a and Figure 11a). Next, SKOV3 and A2780s cells expressing MSCVpac or RUNX3 were treated with increasing concentrations of carboplatin and cell viability was determined using the neutral red assay. 48 and 72 hrs post-treatment, we observed that SKOV3/RUNX3 and A2780s/RUNX3 cells are slightly more resistant to carboplatin treatment than SKOV3/MSCVpac and A2780s/MSCVpac cells (Figure 10b and Figure 11b). Together, our results show that overexpression of RUNX3 renders SKOV3 and A2780s cells modestly resistant to carboplatin.

3.5: Silencing RUNX3 expression in A2780cp cells has no effect on cell viability

As shown in Figure 6 and Figure 8a, cisplatin-resistant A2780cp cells express a higher level of endogenous RUNX3 protein than cisplatin-sensitive A2780s cells. To determine whether RUNX3 contributes to carboplatin resistance in A2780cp cells, we stably knocked down RUNX3 expression in A2780cp cells using two lentivirus delivered shRNA constructs (shRUNX3D and shRUNX3E). The partial knockdown of RUNX3 in A2780cp by the two different constructs was confirmed by Western blotting (Figure 12a). Cell viability assays demonstrate that knockdown of RUNX3 by shRNA has no significant effect on cell viability in A2780cp cells treated with carboplatin at 48 and 72 hrs (Figure 12b). With the shRNA we were only able to achieve partial knockdown of RUNX3 protein expression (Figure 12a). Next we used a mixture of 4 pooled

Figure 10a)

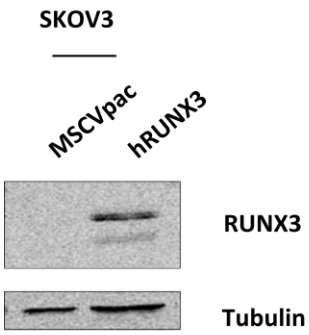

Figure 10b)

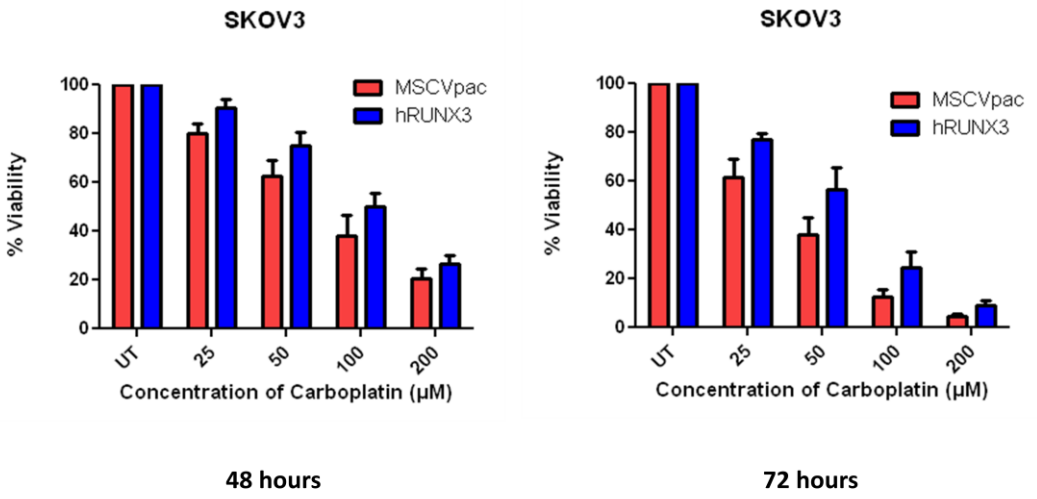

Figure 10. Overexpression of RUNX3 makes SKOV3 cells slightly more resistant to carboplatin treatment. a) Overexpression of RUNX3 in SKOV3 cells was confirmed by Western blotting. Tubulin was used as a loading control. **b)** SKOV3/MSCVpac and SKOV3/hRUNX3 cells were treated with increasing concentrations of carboplatin for 48 and 72 hrs. Cell viability was measured by the neutral red assay. Statistics: Mean + SEM. Unpaired two-tailed t test. Not statistically significant. Experiments repeated 5 times.

Figure 11a)

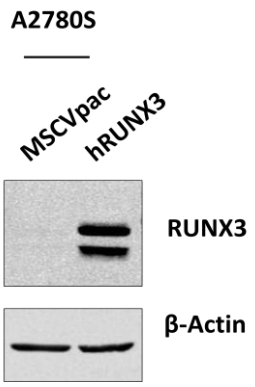

Figure 11b)

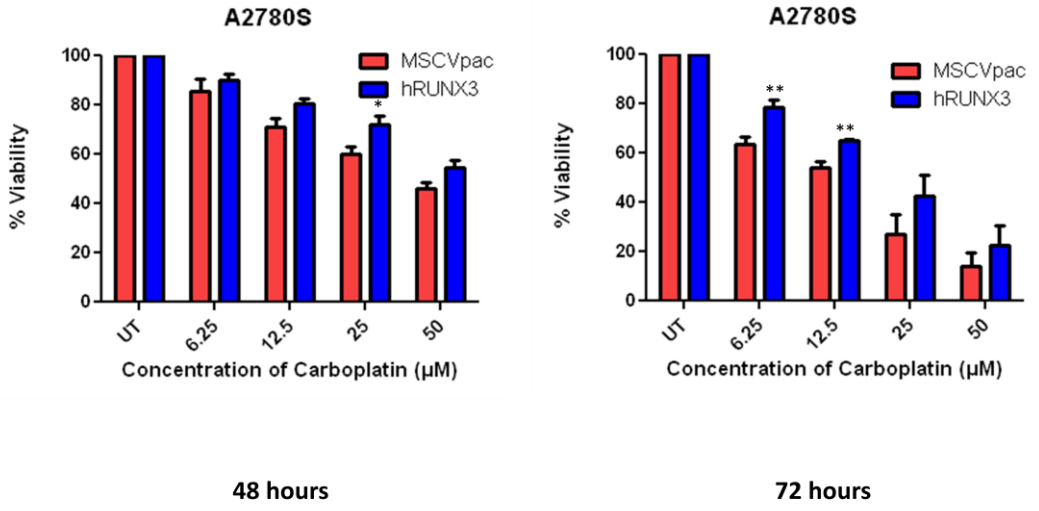

Figure 11. Overexpression of RUNX3 makes A2780s cells slightly more resistant to carboplatin treatment. a) Overexpression of RUNX3 in A2780s cells was confirmed by Western blotting. β-actin was used a loading control. **b)** A2780s/MSCVpac and A2780s/hRUNX3 cells were treated with increasing concentrations of carboplatin for 48 and 72 hrs. Cell viability was measured by the neutral red assay. Statistics: Mean + SEM. Unpaired two-tailed t test, * P value <0.05, ** P value < 0.005. Experiments repeated 4 times.

Figure 12a)

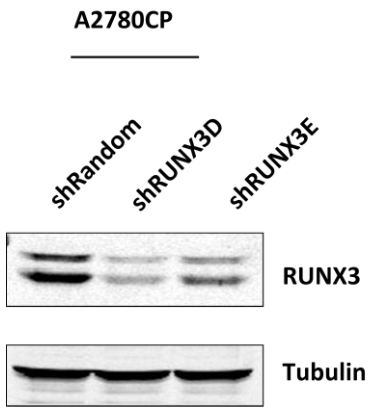

Figure 12b)

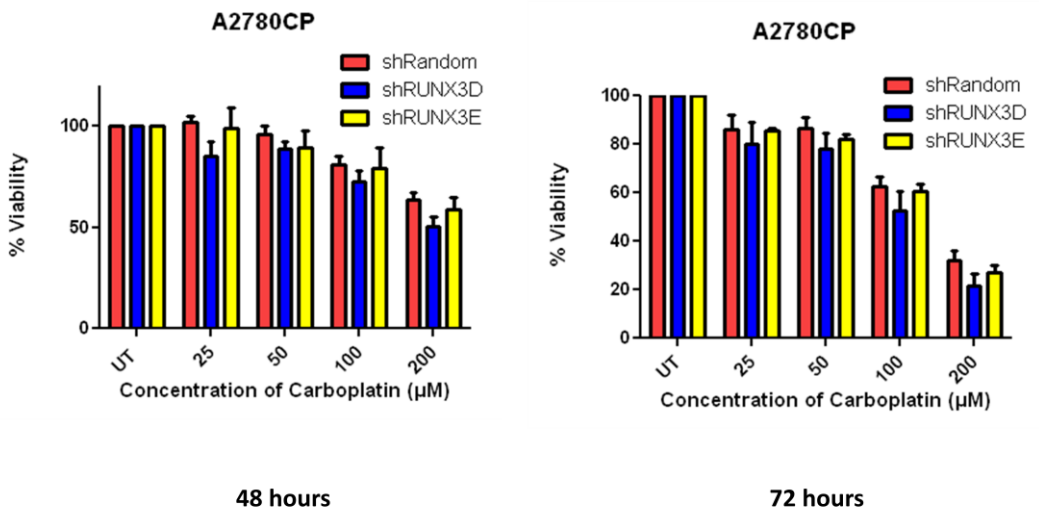

Figure 12. Knockdown of RUNX3 by shRNA in A2780cp cells has no effect on cell viability following carboplatin treatment. a) Knockdown of RUNX3 in A2780cp cells by two different shRNA constructs (shRUNX3D and shRUNX3E) was confirmed by Western blotting. Tubulin was used as a loading control. **b)** A2780cp/shRandom, A2780cp/shRUNX3D and A2780cp/shRUNX3E cells were treated with increasing concentrations of carboplatin for 48 and 72 hrs. Cell viability was measured by the neutral red assay. Statistics: Mean + SEM. Unpaired two-tailed t test. Not statistically significant. Experiments repeated 3 times.

siRNAs targeted against RUNX3 to transiently silence RUNX3 expression in A2780cp cells. A2780cp cells were also transfected with an siRNA targeted to a scrambled sequence (siControl) as a control. By Western blotting we observed almost complete knockdown of RUNX3 protein expression by siRUNX3 (Figure 13a). Similar to the results obtained in the shRNA experiments, knockdown of RUNX3 by siRNA has no effect on cell viability in A2780cp cells treated with carboplatin, when compared to siControl cells at 48 and 72 hrs (Figure 13b). Our data suggests that RUNX3 knockdown alone is not adequate to sensitize A2780cp cells to carboplatin treatment.

3.6: Dominant-negative mutant of RUNX3 in A2780cp cells sensitizes cells to carboplatin treatment

To further examine the role of RUNX3 in carboplatin resistance of EOC, we stably transfected A2780cp cells with a FLAG-tagged dominant negative (dn) form of RUNX3 (1-187) (dnRUNX3), which has an intact DNA-binding domain (Runt domain) but lacks the transactivation domain (Figure 14a). The empty vector pcDNA was used as a control. FLAG expression in the dn clones was confirmed by Western blotting (Figure 14b). Clones 2, 3 and 6 expressed FLAG. However, only clones 2 and 6 were used for cell viability assays as clone 3 had a different morphology from the parental cell line (A2780cp cells). A2780cp/pcDNA clones 1 and 2 and A2780cp/dnRUNX3 clones 2 and 6 were treated with carboplatin for 48 and 72 hrs, and cell viability was determined using the neutral red viability assay. A2780cp/dnRUNX3 clones 2 and 6 were more sensitive to carboplatin treatment than A2780cp/pcDNA clones 1 and 2, at both

Figure 13a)

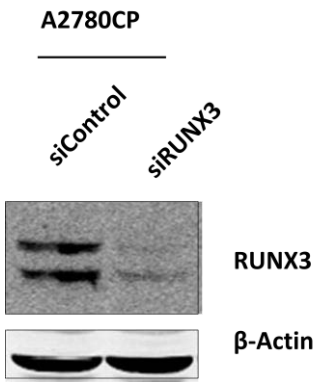

Figure 13b)

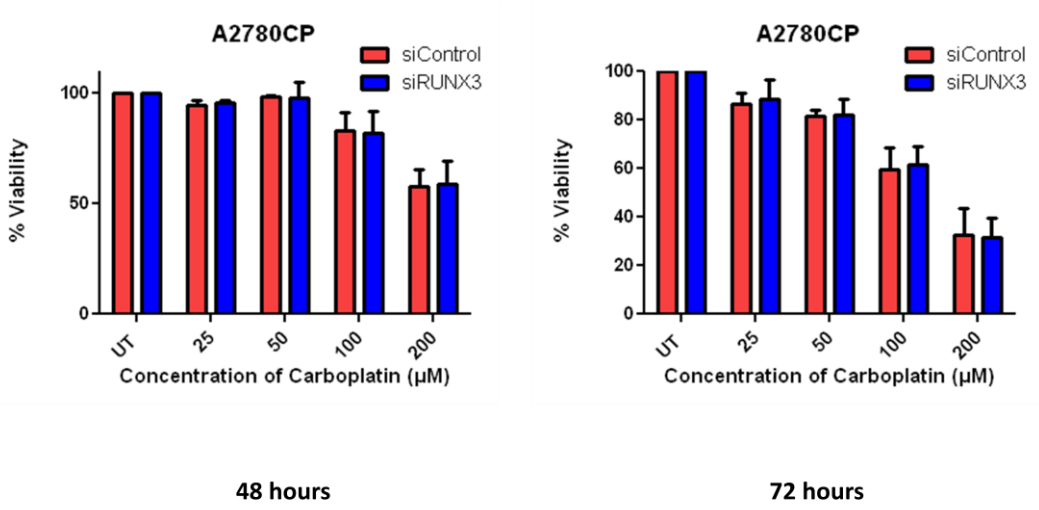

Figure 13. Knockdown of RUNX3 by siRNA in A2780cp cells has no effect on cell viability following carboplatin treatment. a) Knockdown of RUNX3 in A2780cp cells by pooled siRNA targeted to RUNX3 was confirmed by Western blotting, 48 hrs post-siRNA transfection. β-actin was used a loading control. **b)** A2780cp/siControl and A2780cp/siRUNX3 cells were treated with increasing concentrations of carboplatin for 48 and 72 hrs. Cell viability was measured by the neutral red assay. Statistics: Mean + SEM. Unpaired two-tailed t test. Not statistically significant. Experiments repeated 3 times.

Figure 14a)

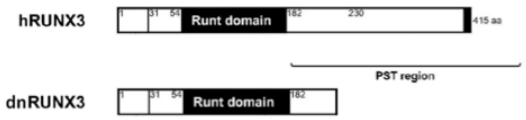

Figure 14b)

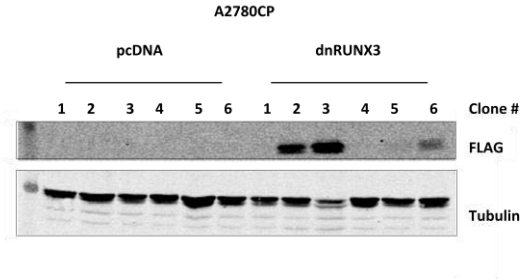

Figure 14c)

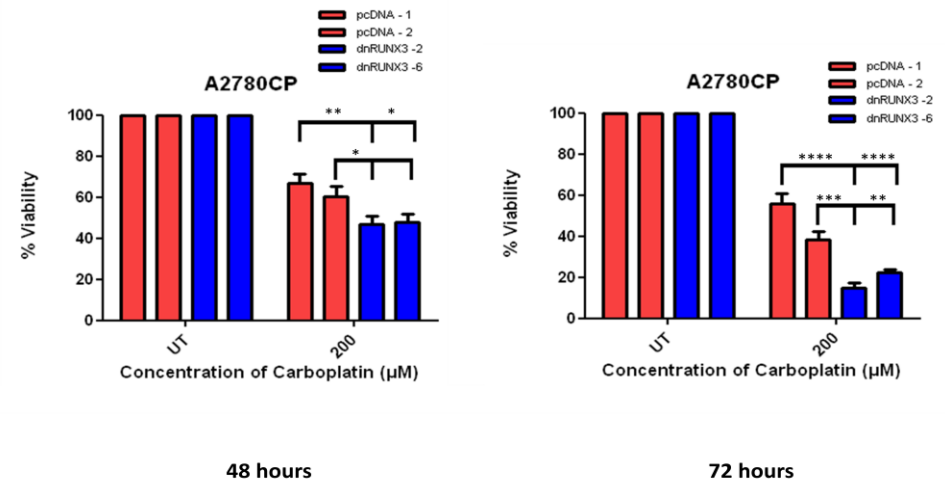

Figure 14. Dominant-negative mutant of RUNX3 sensitizes A2780cp cells to carboplatin treatment. a) Diagram depicting dominant-negative (dn) form of RUNX3. Diagram modified from Bae et al.[61] **b)** Expression of FLAG-tagged dnRUNX3 in A2780cp cells was confirmed by Western blotting. Tubulin was used as a loading control. **c)** A2780cp/pcDNA clone 1 and clone 2, and A2780cp/dnRUNX3 clone 2 and clone 6 were treated with 200 μM carboplatin for 48 and 72 hrs. Cell viability was measured by the neutral red assay. Statistics: Mean + SEM. Unpaired two-tailed t test, * P value <0.05, ** P value < 0.005, *** P value < 0.0005, **** P value < 0.0001 Experiments repeated 5 times.

48 and 72 hrs (Figure 14c). Expression of dnRUNX3 in A2780cp cells renders cells more sensitive to carboplatin treatment.

3.7: Expression of the RUNX transcription factors in EOC cell lines

We wanted to determine if all three RUNX family members are expressed in the EOC cell lines used throughout our study. To do so, RNA was collected from A2780s, A2780cp and SKOV3 cells and qRT-PCR was performed to look at the levels of RUNX gene expression, relative to the control gene 18S. All three RUNX genes were expressed in the three cell lines (Figure 15).

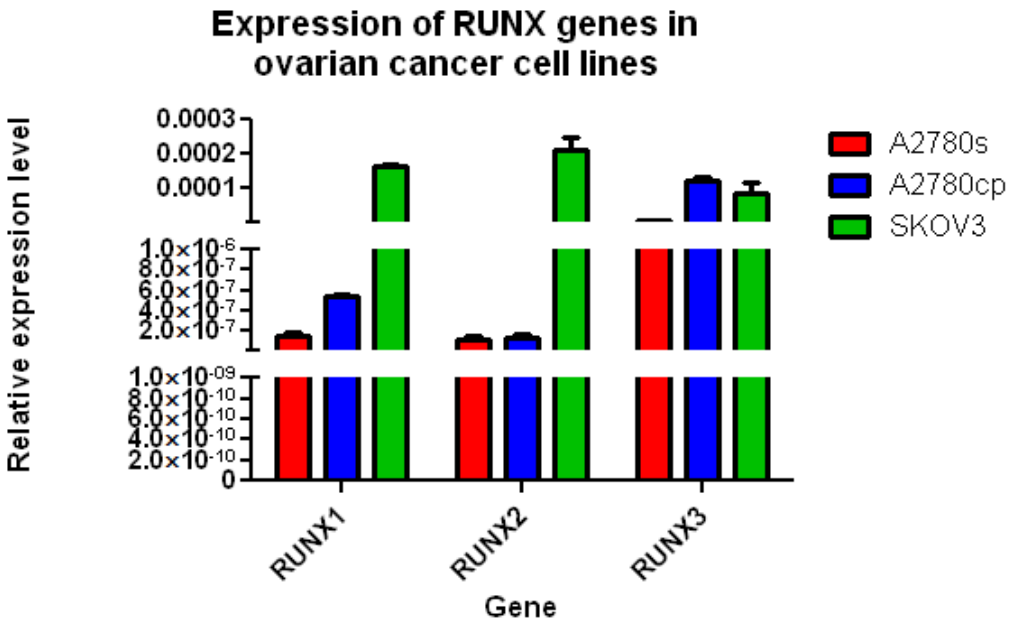

Figure 15. Expression of RUNX genes in EOC cell lines. RNA from A2780s, A2780cp and SKOV3 cells was collected, reverse transcribed into cDNA and the levels of RUNX genes were quantified by qRT-PCR. Expression levels are relative to the expression of the control gene 18S. Primer sequences are included in Table 1. Experiments were repeated 3 times.

Section 4: Discussion and Future Directions

4.1: RUNX3 and chemoresistance in ovarian cancer

The RUNX transcription factors can function as tumor suppressors or oncogenes in a context dependent manner[145]. The majority of the early literature supported a tumor suppressor role for RUNX3, following the discovery that RUNX3 acts as a tumor suppressor in gastric carcinoma[82]. In ovarian cancer the first study to examine the methylation status of RUNX3 found hypermethylation and gene silencing in primary EOC cells and EOC cell lines, and they concluded that inactivation of its function may result in impairment of the TGFβ pathway and other tumor suppressive functions carried out by RUNX3[127]. However, no functional studies were performed to support the suggested tumor suppressive role. Later, two studies demonstrated that RUNX3 expression is increased in EOC samples and cell lines, when compared to non-cancerous controls[128,129]. Overexpression of RUNX3 in EOC cell lines that express low levels of endogenous RUNX3 led to increased proliferation of cells, while knockdown of RUNX3 in EOC cell lines that express endogenous levels of RUNX3 had the opposite effect[128,129]. Both groups concluded that RUNX3 plays an oncogenic role in EOC by promoting proliferation. RUNX3 also has been shown to modulate cell sensitivity to chemotherapeutic agents in other tumors[133-135]. However, whether RUNX3 expression is associated with chemoresistance of EOC has not been studied. In this study we demonstrate that RUNX3 expression is increased by carboplatin treatment and overexpression of RUNX3 renders EOC cell lines slightly more resistant to carboplatin treatment.

We found that RUNX3 is expressed in EOC tissues (Figure 5a) and primary EOC cells derived from ascites (Figure 5b). Variable levels of expression were found in EOC cell lines (Figure 6). In various cancers, RUNX3 overexpression has been associated with protein inactivation by mislocalization to the cytoplasm[86,124,125,146]. Inconsistent results have been reported regarding the cellular localization of RUNX3 in EOC cells. Nevadunsky *et al.*[128] reported that RUNX3 is localized to the cytoplasm in EOC cell lines[128], whereas Lee *et al.*[129] observed nuclear localization of RUNX3 in EOC cells[129]. In this study we confirm that RUNX3 is localized to the nucleus in human EOC cell lines and primary EOC samples (Figure 7), which is consistent with the localization reported by Lee *et al.*[129] and supports the functional role of RUNX3 as a transcription factor.

As mentioned previously, A2780cp is a cisplatin-resistant EOC cell line derived from cisplatin-sensitive (A2780s) cells by exposure to increasing stepwise concentrations of cisplatin[141-144]. These paired cell lines have previously been used as a model for development of resistance to platinum agents[34,142]. When we compared the levels of RUNX3 in these two cell lines, we found that RUNX3 expression was significantly higher in the cisplatin-resistant A2780cp cells (Figure 8a). Interestingly, RUNX3 expression increased in A2780s and A2780cp cells after short-term exposure to carboplatin treatment (Figure 9). However, how carboplatin treatment increases RUNX3 expression in EOC cells remains to be determined. Taken together, these data suggest that RUNX3 may be one of several genes modified during acquired chemoresistance in patients treated with

platinum agents. Moreover, the levels of RUNX3 may increase soon after treatment with platinum agents begins.

Members of the RUNX family contain a homologous runt-domain that controls binding to specific DNA sequences and binds with the common co-factor, CBFβ. The RUNX-CBFβ complex regulates the expression of target genes by binding to their promoters or enhancers[57]. All RUNX proteins bind the same DNA motif and can activate or repress the transcription of target genes by recruiting transcriptional co-activators or co-repressors[57]. Recently, RUNX1 and RUNX2 have been found to act in an oncogenic manner in serous EOC by contributing to cell proliferation, migration and invasion[130,131]. Also, as previously mentioned, RUNX3 has also been shown to act in an oncogenic manner in EOC by promoting cell proliferation[128,129]. Even though the RUNX family members seem to act in an oncogenic manner in EOC, their role in chemoresistance in EOC has yet to be studied. To find out whether RUNX3 has an effect on cell sensitivity to carboplatin treatment, we overexpressed RUNX3 in EOC cell lines, which express low levels of endogenous RUNX3. Using SKOV3 and A2780s cells, we demonstrated that RUNX3 makes cells modestly resistant to carboplatin treatment at both 48 and 72 hrs post-treatment, with the greatest effect observed at 72 hrs (Figure 10 and Figure 11). However, when RUNX3 expression was knocked down in A2780cp cells, which endogenously expresses high levels of RUNX3, using an shRNA or siRNA specifically targeted against RUNX3, no significant effect on cell sensitivity to carboplatin treatment was observed (Figure 12 and Figure 13).

When examining the oncogenic potential of the RUNX family it is important to decipher whether the distinct roles they play arise because the members have diverged in function, or whether their distinct roles arise from tissue-specific expression of each gene[145]. The family members have been shown to have overlapping functions using a transcriptional reporter assay, supporting the notion of tissue-specific expression[147]. All RUNX proteins are known to recognize the same DNA-binding motifs[57] and in EOC act in an oncogenic manner[128-131], suggesting that RUNX1 and/or RUNX2 may be able to compensate for the loss of RUNX3 when it is knocked down. In the three cell lines used in our study we found that all the RUNX family members are expressed (Figure 15), raising the possibility that RUNX1 and/or RUNX2 may be compensating for the loss of RUNX3. To better examine the effect of RUNX3 on chemoresistance, it may be necessary to also inhibit the other RUNX family members and examine whether either of them together or alone have an effect on chemoresistance.

No significant difference in cell viability was observed when RUNX3 expression was knocked down by an shRNA or siRNA specifically targeted to RUNX3, and cells were treated with carboplatin (Figure 12 and Figure 13). Next, we wanted to see whether a dn form of RUNX3 would have an effect on cell sensitivity to carboplatin. A2780cp cells were transfected with a truncated form of RUNX3 that contains amino acids 1-187. The runt domain is present and functional in this dn form, while the c-terminus, which is responsible for the activation and repression of transcription[57], is missing[125]. Cells stably transfected with dnRUNX3 were significantly more sensitive to carboplatin treatment than

control cells (Figure 14). The effect observed may be due to the binding of dnRUNX3 to the DNA-motif recognized by endogenous RUNX family members or to the binding and occupancy of CBFβ by dnRUNX3. In either case, dnRUNX3 may be disrupting the function of the entire RUNX family and not simply RUNX3. In line with the results observed in the knockdown experiments, results in the dnRUNX3 experiments support the notion that the greatest effect on cell sensitivity to carboplatin would be observed if all three RUNX members or their common co-factor CBFβ were silenced. In a study by Davis *et al*.[148], CBFβ expression was silenced in SKOV3 cells and was found to significantly repress the ability of the cells to form colonies in soft agar compared to control cells[148], which is in line with the study examining RUNX3 knockdown in ovarian cancer and colony formation[129] and the oncogenic role associated with the RUNX transcription factors in EOC[130,131,128,129]. It would be interesting to knockdown the expression of CBFβ and examine the effect it has on chemoresistance of EOC.

Each of the three RUNX genes is transcriptionally regulated by two promoters, the P1 promoter and the P2 promoter, which gives rise to two protein isoforms[61]. The family can also undergo alternative splicing, which generates multiple splice variants[63-65]. Moreover, the RUNX family can also undergo various post-translational modifications such as phosphorylation or ubiquitination, further increasing the variations that exist between RUNX proteins[61]. In the study examining RUNX3 overexpression in BCC, the group observed two RUNX3-related bands, which they stated could represent different RUNX3 isoforms, although the doublet could also be caused by phosphorylation or proteolytic

degradation[121]. The group found that the RUNX3 proteins observed in BCC are full-length and intact, and likely represent the two RUNX3 isoforms[121]. In the study conducted by Lee *et al.*[129], these two bands were also observed in ovarian cancer samples. In our study we observed two RUNX3 bands at approximately 46kDa and 44kDa, by Western blotting, which are in line with the molecular weight observed by Salto-Tellez *et al*.[121] in BCC and Lee *et al.*[129] in EOC. Based on this information we assumed that the two bands present in our samples are the two RUNX3 isoforms originating from the P1 and P2 promoter.

To date both isoforms originating from P1 and P2 promoters have been assumed to perform synonymous functions in cells[61], even though different 5‟-untranslated regions (5‟UTRs) and N-terminal coding regions are generated from these two promoters[62]. It would be interesting to specifically overexpress or knockdown one isoform in EOC cells and observe the effects on cell proliferation and differentiation, and chemoresistance. Although the differences between the two isoforms are minimal, and do not affect the runt domain[62], they may perform distinct functions.

EOC is a heterogeneous disease with numerous genetic and epigenetic aberrations that drive oncogenic signaling pathways that lead to chemoresistance. In this study we show that overexpression of RUNX3 makes A2780s and SKOV3 cells slightly more resistant to carboplatin. In gastric cancer, RUNX3 has been shown to interact with and regulate proteins involved in the cell cycle (p21), apoptosis (Bim), and DNA repair (Ku70)[86]. Perhaps RUNX3 is modulating the activity of these systems when overexpressed in SKOV3 or A2780s cells.

Knockdown of RUNX3 did not have an effect on cell sensitivity to carboplatin treatment in A2780cp cells. However, dn expression of RUNX3 made cells sensitive to the effects of carboplatin, supporting the idea that the effect that RUNX3 has in chemoresistance may be masked when RUNX3 alone is knocked down, as the other RUNX family members are still present and exerting an effect that can compensate for the loss of RUNX3.

4.2: Systematic approach to identify gene expression and signaling pathways affected during acquired chemoresistance

Chemoresistance is one of the major challenges in treating advanced EOC. Numerous molecular changes are known to occur during acquired chemoresistance in EOC including alteration in the expression of molecules involved in critical biological processes such as platinum importers and exporters, DNA repair proteins, and apoptotic molecules (reviewed in Introduction: Molecular mechanisms of acquired chemoresistance). Although several molecules and pathways have already been identified as being altered in chemoresistant EOC cells, no major improvements in treating recurrent and chemoresistant EOCs have been made since the establishment of carboplatin and paclitaxel as first-line therapeutic agents[8]. Thus we want to take a systematic approach to find which molecules are altered during acquired chemoresistance and see if there is a key pathway or molecule that can be targeted to overcome this chemoresistance or be used as a biomarker of chemoresistant disease.

To identify genes altered during acquired chemoresistance in EOC cells, we examined the paired human EOC cell lines A2780s (cisplatin-sensitive) and it derivative A2780cp (cisplatin-resistant) using a microarray approach. Briefly, RNA from A2780s and A2780cp cells was extracted using the Qiagen RNAeasy mini kit, and labeled using the Low InputQuick Amp Labeling Kit, one-color (Agilent Technologies). cRNA was hybridized onto Agilent Whole Human Genome Microarray slides (kindly donated by Dr. Helen Steed) using the Gene Expression Hybridization Kit (Agilent Technologies). Microarray results were analyzed using Agilent Feature Extraction and Gene Spring Software (Figure 16).

Thousands of genes were differentially expressed between A2780s and A2780cp cells. To identify networks and pathways that may be key during acquired resistance in EOC we conducted an extensive literature search and put the data into Ingenuity Pathway Analysis software (IPA). The software can identify associations and common pathways between differentially expressed genes. IPA shows that among the top canonical pathways differentially expressed between A2780s and A2780cp cells, the Wnt pathway was one of the most affected. After a literature search, we found that the Wnt pathway is important in chemoresistance in EOC[149]. We thus decided to take a closer look at the Wnt pathway in our study.

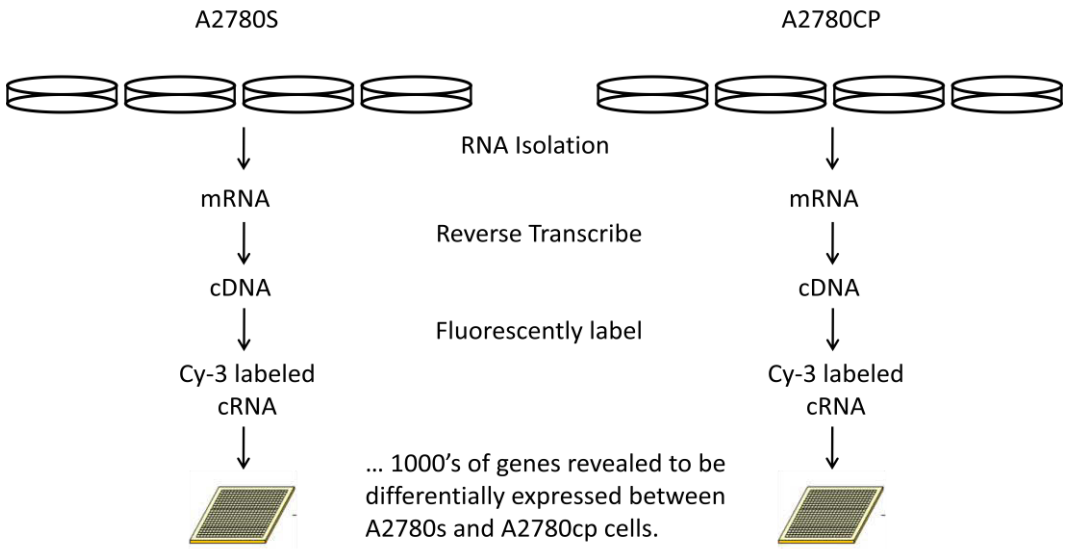

Figure 16: Microarray analysis setup. A2780s and A2780cp cells were compared for differential expression of genes by One-Color Microarray-Based Gene Expression (Low Input Quick Amp Labeling). Briefly, A2780s and A2780cp RNA was isolated. RNA was reverse-transcribed into cDNA. cDNA was amplified and labeled with -Cy3 to form labeled cRNA. Labeled cRNA was purified and hybridized onto Agilent Whole Genome Microarray chips. Results between A2780s and A2780cp cells were compared using GeneSpring software, and thousands of genes were found to be differentially expressed between A2780s and A2780cp cells. Statistics: T test unpaired. P-value cut-off: 0.02. Fold-change cut-off: 2.0. Multiple testing correction: no correction.

4.3: The Wnt signaling pathway

The Wnt signaling pathway is important in regulating cell growth and differentiation during development[150]. Wnt signaling can be divided into two categories referred to as the canonical and non-canonical Wnt pathway. The main difference between these two categories is the presence of β-catenin in the canonical pathway and its absence in the non-canonical pathway. Wnt signaling is further broken down into three branches which includes the canonical Wnt/β-catenin pathway, the non-canonical planar cell polarity pathway and the non-canonical Wnt/Ca^{2+} pathway[150]. The main focus of our research in Wnt signaling thus far has surrounded the canonical pathway (Figure 17) (reviews on canonical Wnt signaling by Clevers *et al*.[96] and Lustig *et al*.[150]).

Wnts bind to members of two families of cell-surface receptors, the Frizzled gene family, and the LDL-receptor-related protein (LRP) family, which act as co-receptors. Inhibitors can modulate Wnt signaling. Wnt inhibitory factor (WIF) and secreted Frizzled-related proteins (SRFPs) (also referred to as Frzb) inhibit Wnt signaling by binding to Wnt ligands, whereas Dickkopf (DKK) binds to and causes endocytosis of the LRP co-receptors. Other factors such as Kremen and Cerberus are also known to inhibit Wnt signaling[150,96].

Canonical Wnt signaling depends upon the accumulation and localization of β-catenin. The levels of cytoplasmic β-catenin are regulated by the destruction complex, which is a "multiprotein complex" consisting of Axin, which acts as a scaffold protein, APC and two kinases (glycogen synthase kinase 3β (GSK3β)

and casein kinase 1 (CK1)). When the pathway is inactive, β-catenin is held in the destruction complex, and phosphorylated by CK1 and GSK3β. Phosphorylated β-catenin is recognized and ubiquitinated by β-TrCP (β-transducin repeat-containing protein), and degraded by the proteasome. In the absence of Wnt ligands, LEF/TCF transcription factors, which are present in the nucleus, are held in an inactive state. This prevents the transcription of downstream Wnt target genes[150,96].

In the presence of Wnt ligands, Wnt-Frizzled/LRP complexes activate the canonical pathway. Dishevelled (Dsh) is recruited to the plasma membrane where it interacts with the activated the Frizzled/LRP complex. It remains unclear precisely how Dsh functions in Wnt signal transduction. CK1 and GSK3β phosphorylate the LRP co-receptors, which causes the recruitment of Axin to the plasma membrane and the inactivation of the destruction complex. The inactivation of the destruction complex allows for the accumulation of cytoplasmic β-catenin. Once β-catenin has accumulated in the cytoplasm it can translocate into the nucleus and associate with LEF/TCF transcription factors, causing the transcription of Wnt downstream target genes (Figure 17) [96,150].

Numerous Wnt/β-catenin target genes have been identified. As summarized by Klaus *et al*.[151], well known targets include genes that function in cell differentiation, signaling (VEGF), proliferation (cyclin D and MYC) and adhesion (E-cadherin)[151]. However, it is difficult to state whether there are any

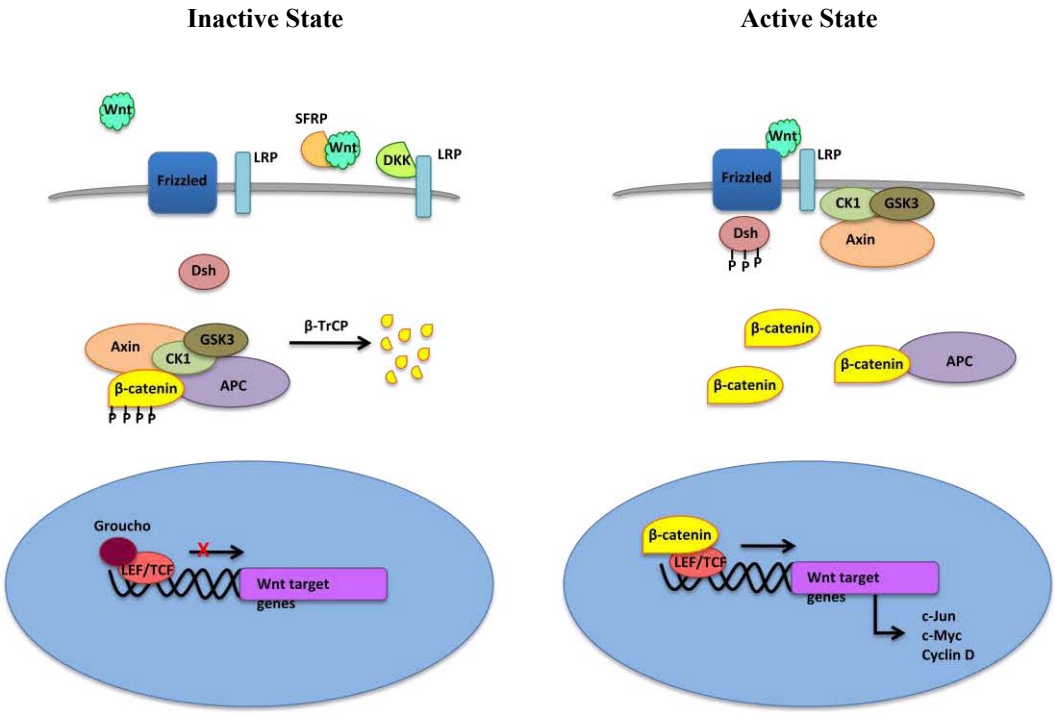

Figure 17. The canonical Wnt/β-catenin signaling pathway. Inactive State: In the absence of Wnt ligands, β-catenin is held in the destruction complex with APC (adenomatous polyposis coli) and the axins. Here it is phosphorylated by the kinases CK1 (casein kinase) and GSK3β (glycogen synthase kinase 3β) and subsequently ubiquitination by β-TrCp (β-transducin repeat-containing protein) and degraded by the proteasome. LEF/TCF (lymphoid enhancer factor/T-cell factor), a transcription factor that β-catenin can bind with and activate, is held in a repressive state by the corepressor Groucho. Active State: In the presence of Wnt ligands, LRP (LDL-receptor-related protein) is phosphorylated by CK1 and GSK3β and Dishevelled (DSH) is recruited to the plasma membrane. Phosphorylated LRP leads to the recruitment of Axin to the plasma membrane and the inactivation of the destruction complex. β-catenin stabilization and accumulation leads to its translocation into the nucleus, where is can form a transcriptionally active complex with LEF/TCF by displacing Groucho. DKKs (dickkopfs) can inhibit Wnt signaling by binding to and causing internalization of LRP. SFRPs (secreted frizzled-related protein) inhibit Wnt signaling by binding to Wnt ligands. Figure adapted from [96]. Text adapted from [151].

"universal" Wnt/β-catenin targets, as most genes seem to be cell type specific, although as described by Clevers *et al.*[96], Axin2[152] and SP5[153] seem to be good candidates[150,96,151].

Using the IPA software to analyse the data, we found that the Wnt/β-catenin pathway seems to be activated in A2780cp (cisplatin-resistant) cells, when compared to A2780s (cisplatin-sensitive) cells. Specifically, Wnt inhibitors such as DKK1, DKK3, SFRP1 and SFRP3 (FRZB) were found to be downregulated in A2780cp cells, when compared to A2780s cells. APC2, which is part of the destruction complex and important in degrading β-catenin, was also downregulated in A2780cp cells. Moreover, Wnt glycoproteins such as Wnt3 and Wnt3a, which are known to activate the Wnt/β-catenin pathway, are upregulated in A2780cp cells, when compared to A2780s cells (Table 4).

However, other proteins such as WIF1, an inhibitor of the Wnt pathway, is upregulated in A2780cp cells and TCF4, a trancription factor that β-catenin binds with to activate downstream targets, is downregulated in A2780cp cells, which is not in line with activation of the Wnt/β-catenin pathway in A2780cp cells. Thus it is necessary to confirm that the Wnt/β-catenin pathway is indeed more active in A2780cp cells than A2780s cells. The results obtained by microarray were confirmed by qRT-PCR (Table 5). In order to confirm that the Wnt/β-catenin pathway is activated in A2780cp cells, we wanted to see whether downstream genes are activated. We specifically looked at the expression of c-Jun, c-Myc and cyclin D[151]. The expression of c-Jun and cyclin D are upregulated in A2780cp cells compared to A2780s cells (Figure 18). c-Myc

Table 4. Genes involved in Wnt signaling that are differentially expressed between A2780s and A2780cp cells.

Symbol	Gene name	Fold change (A2780cp/A2780s)	Location
DKK1	Dickkopf 1 homolog	-47.643	Extracellular space
FZD7	Frizzled family receptor 7	-15.761	Plasma membrane
SFRP1	Secreted-frizzled related protein 1	-14.484	Plasma membrane
SFRP3 (FRZB)	Frizzled-related protein	-6.568	Extracellular space
APC2	Adenomatosis polyposis coli 2	-4.961	Cytoplasm
TCF4	Transcription factor 4	-4.359	Nucleus
DKK3	Dickkopf 3 homolog	-2.771	Extracellular space
FZD2	Frizzled family receptor 2	-2.125	Plasma membrane
FZD4	Frizzled family receptor 4	-2.109	Plasma membrane
WIF1	WNT inhibitory factor 1	2.312	Extracellular space
WNT3	Wingless-type MMTV integration site family 3	2.737	Extracellular space
WNT11	Wingless-type MMTV integration site family, member 11	3.651	Extracellular space
FZD8	Frizzled family receptor 8	4.225	Plasma membrane
WNT3A	Wingless-type MMTV integration site family, member 3A	4.370	Extracellular space

Table 5. Validation of microarray by qRT-PCR.

Gene	Fold change: Microarray (A2780cp/A2780s)	Fold change: qRT-PCR (A2780cp/A2780s)
DKK1	-47.643	-6.46
SFRP1	-14.484	-7.4975
SFRP3 (FRZB)	-6.568	-4.4125
APC2	-4.961	-2.53
DKK3	-2.771	-2.335
WNT3	2.737	1.2125
WNT11	3.651	2.4925
WNT3A	4.370	3.1591

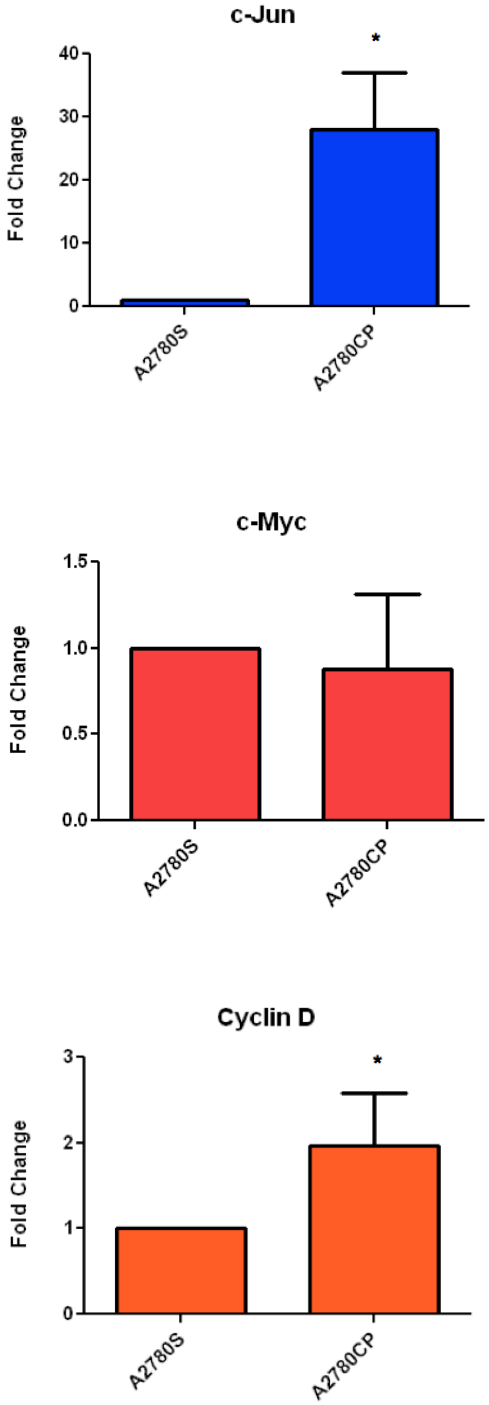

Figure 18. Wnt target genes are upregulated in A2780cp cells. RNA was isolated from A2780s and A2780cp cells using the Qiagen RNeasy mini kit. cDNA was generated by reverse-transcription PCR. The expression of Wnt target genes (c-Myc, c-Jun and cyclinD) in A2780s and A2780cp cells were examined by qRT-PCR. Statistics: Mean + SEM. Unpaired two-tailed t test, * P value <0.05. Experiments repeated 3 times.

expression is similar between A2780s and A2780cp cells. Even though the levels of c-Myc are similar between A2780s and A2780cp cells, these results support the idea that the Wnt/β-catenin pathway is upregulated in A2780cp cells, compared to A2780s cells. Other experiments need to be carried out to further confirm the upregulation of the Wnt/β-catenin pathway. Specifically, we plan to perform a luciferase assay to measure the activity of β-catenin. We also want to look at the localization of β-catenin in the nucleas of A2780s and A2780cp cells. If the Wnt pathway is more active in A2780cp cells, we expect to see greater nuclear expression of β-catenin in A2780cp cells, compared to A2780s cells.

If the pathway is in fact upregulated in A2780cp cells we want to target specific components of the pathway and modulate the activity of the Wnt/β-catenin pathway and see whether this has an effect on chemoresistance in the paired cell lines. Of particular interest to us are the Wnt inhibitors DKK1, SFRP1 and FRZB. We want to overexpress these genes in A2780cp cells to downregulate the activity of the Wnt/β-catenin pathway and treat cells with carboplatin to determine whether modulating the Wnt/β-catenin pathway has an effect on cell sensitivity to carboplatin treatment.

Section 5: Conclusions

Ovarian cancer is a disease with numerous genetic and epigenetic aberrations that drive oncogenic signaling pathways, and lead to acquired chemoresistance. In our first study we showed that RUNX3 expression is elevated in human EOC tissues, primary EOC cell cultures and EOC cell lines compared to primary OSE cells and IOSE cells. Overexpression of RUNX3 in two ovarian cancer cell lines (A2780s and SKOV3) made the cells slightly more resistant to carboplatin treatment, suggesting that RUNX3 may contribute to carboplatin resistance in EOC. Nonetheless, knockdown of RUNX3 alone is not adequate to re-sensitize the cisplatin-resistant A2780cp cells, to carboplatin. Expression of dn RUNX3 made the cells more sensitive to carboplatin treatment, which is in line with our idea that other factors, such as the other RUNX family members may be compensating for the loss of RUNX3. It will be interesting to test whether blocking all the RUNX family members will re-sensitize the chemoresistant cells to carboplatin treatment.

Our microarray study shows that the expression of many genes are altered during acquired chemoresistance in A2780cp cells. Therefore, it will be critical to identify the changes that are causing or playing a prominent role in the development of chemoresistance. By IPA analysis and an extensive literature review, we determined that the Wnt signaling pathway seems to be upregulated in cisplatin-resistant A2780cp cells and may play a role in chemoresistance of EOC. We plan to confirm whether the pathway is in fact upregulated in A2780cp cells

and then modulate the pathway to determine whether the canonical Wnt signaling pathway plays a role in chemoresistance in EOC.

Section 6: References

1. RE, S. *Histological typing of ovarian tumors*. *World Health Organization International Histological Classification of Tumours* (Springer Verlag, 1999).
2. Ledermann, J. A. *et al.* Newly diagnosed and relapsed epithelial ovarian carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **24 Suppl 6,** vi24–32 (2013).
3. Kurman, R. J. & Shih Ie, M. The Origin and Pathogenesis of Epithelial Ovarian Cancer: A Proposed Unifying Theory. *The American journal of surgical pathology* **34,** 433–443 (2010).
4. Cannistra, S. A. Cancer of the Ovary. *New England Journal of Medicine* **351,** 2519–2529 (2004).
5. Shih, I.-M. & Kurman, R. J. Ovarian Tumorigenesis: A Proposed Model Based on Morphological and Molecular Genetic Analysis. *The American journal of pathology* **164,** 1511–1518 (2004).
6. Colombo, P.-E. *et al.* Sensitivity and resistance to treatment in the primary management of epithelial ovarian cancer. *Crit. Rev. Oncol. Hematol.* **89,** 207–216 (2014).
7. Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2013. *Ca-Cancer J Clin* **63,** 11–30 (2013).
8. Yap, T. A., Carden, C. P. & Kaye, S. B. Beyond chemotherapy: targeted therapies in ovarian cancer. *Nature reviews. Cancer* **9,** 167–181 (2009).
9. Bast, R. C., Hennessy, B. & Mills, G. B. The biology of ovarian cancer: new opportunities for translation. *Nature reviews. Cancer* **9,** 415–428 (2009).
10. Ozols, R. F. *et al.* Phase III Trial of Carboplatin and Paclitaxel Compared With Cisplatin and Paclitaxel in Patients With Optimally Resected Stage III Ovarian Cancer: A Gynecologic Oncology Group Study. *Journal of Clinical Oncology* **21,** 3194–3200 (2003).
11. Bois, du, A. *et al.* A Randomized Clinical Trial of Cisplatin/Paclitaxel Versus Carboplatin/Paclitaxel as First-Line Treatment of Ovarian Cancer. *J. Natl. Cancer Inst.* **95,** 1320–1329 (2003).
12. Neijt, J. P. *et al.* Exploratory Phase III Study of Paclitaxel and Cisplatin Versus Paclitaxel and Carboplatin in Advanced Ovarian Cancer. *Journal of Clinical Oncology* **18,** 3084–3092 (2000).
13. Gore, M. E., Fryatt, I., Wiltshaw, E. & Dawson, T. Treatment of relapsed carcinoma of the ovary with cisplatin or carboplatin following initial treatment with these compounds. *Gynecologic oncology* **36,** 207–211 (1990).
14. Agarwal, R. & Kaye, S. B. Ovarian cancer: strategies for overcoming resistance to chemotherapy. *Nature reviews. Cancer* **3,** 502–516 (2003).
15. Galluzzi, L. *et al.* Molecular mechanisms of cisplatin resistance. *Oncogene* **31,** 1869–1883 (2012).
16. Giaccone, G. Clinical Perspectives on Platinum Resistance. *Drugs* **59,** 9–17 (2000).

17. Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nature reviews. Cancer* **7,** 573–584 (2007).
18. Aabo, K. *et al.* Chemotherapy in advanced ovarian cancer: four systematic meta-analyses of individual patient data from 37 randomized trials. Advanced Ovarian Cancer Trialists' Group. *British journal of cancer* **78,** 1479–1487 (1998).
19. Alderden, R. A., Hall, M. D. & Hambley, T. W. The discovery and development of cisplatin. *Journal of chemical …* (2006).
20. Kelland, L. Preclinical Perspectives on Platinum Resistance. *Drugs* **59,** 1–8 (2000).
21. El-Khateeb, M. *et al.* Reactions of cisplatin hydrolytes with methionine, cysteine, and plasma ultrafiltrate studied by a combination of HPLC and NMR techniques. *Journal of Inorganic Biochemistry* **77,** 13–21 (1999).
22. Fichtinger-Schepman, A. M. J., Van der Veer, J. L., Hartog, Den, J. H. J., Lohman, P. H. M. & Reedijk, J. Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry* **24,** 707–713 (1985).
23. Eastman, A. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacology & Therapeutics* **34,** 155–166 (1987).
24. Timerbaev, A. R., Hartinger, C. G., Aleksenko, S. S. & Keppler, B. K. Interactions of antitumor metallodrugs with serum proteins: advances in characterization using modern analytical methodology. *Chem. Rev.* **106,** 2224–2248 (2006).
25. Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22,** 7265–7279 (2003).
26. Knox, R. J., Friedlos, F., Lydall, D. A. & Roberts, J. J. Mechanism of Cytotoxicity of Anticancer Platinum Drugs: Evidence That cis-Diamminedichloroplatinum(II) and cis-Diammine-(1,1-cyclobutanedicarboxylato)platinum(II) Differ Only in the Kinetics of Their Interaction with DNA. *Cancer Res* **46,** 1972–1979 (1986).
27. Chaney, S. G. & Sancar, A. DNA Repair: Enzymatic Mechanisms and Relevance to Drug Response. *J. Natl. Cancer Inst.* **88,** 1346–1360 (1996).
28. Furuta, T. *et al.* Transcription-coupled Nucleotide Excision Repair as a Determinant of Cisplatin Sensitivity of Human Cells. *Cancer Res* **62,** 4899–4902 (2002).
29. Perez, R. P. Cellular and molecular determinants of cisplatin resistance. *Eur. J. Cancer* **34,** 1535–1542 (1998).
30. Ishida, S., Lee, J., Thiele, D. J. & Herskowitz, I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *P Natl Acad Sci USA* **99,** 14298–14302 (2002).
31. Holzer, A. K. *et al.* The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. *Mol. Pharmacol.* **66,** 817–823 (2004).
32. Holzer, A. K., Katano, K., Klomp, L. W. J. & Howell, S. B. Cisplatin rapidly down-regulates its own influx transporter hCTR1 in cultured

human ovarian carcinoma cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10,** 6744–6749 (2004).
33. Holzer, A. K. & Howell, S. B. The Internalization and Degradation of Human Copper Transporter 1 following Cisplatin Exposure. *Cancer Res* **66,** 10944–10952 (2006).
34. Katano, K. *et al.* Acquisition of resistance to cisplatin is accompanied by changes in the cellular pharmacology of copper. *Cancer Res* **62,** 6559–6565 (2002).
35. Samimi, G. *et al.* Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10,** 4661–4669 (2004).
36. Kalayda, G. V., Wagner, C. H., Buss, I., Reedijk, J. & Jaehde, U. Altered localisation of the copper efflux transporters ATP7A and ATP7B associated with cisplatin resistance in human ovarian carcinoma cells. *BMC Cancer* **8,** 175 (2008).
37. Samimi, G. *et al.* Increase in expression of the copper transporter ATP7A during platinum drug-based treatment is associated with poor survival in ovarian cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9,** 5853–5859 (2003).
38. Nakayama, K. Prognostic Value of the Cu-Transporting ATPase in Ovarian Carcinoma Patients Receiving Cisplatin-Based Chemotherapy. *Clinical Cancer Research* **10,** 2804–2811 (2004).
39. Balendiran, G. K., Dabur, R. & Fraser, D. The role of glutathione in cancer. *Cell Biochem. Funct.* **22,** 343–352 (2004).
40. Lewis, A. D. A., Hayes, J. D. J. & Wolf, C. R. C. Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: intrinsic differences and cell cycle effects. *Carcinogenesis* **9,** 1283–1287 (1988).
41. Godwin, A. K. *et al.* High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *P Natl Acad Sci USA* **89,** 3070–3074 (1992).
42. Sancar, A., Lindsey-Boltz, L. A., Ünsal-Kaçmaz, K. & Linn, S. M OLECULARM ECHANISMS OFM AMMALIANDNA R EPAIR AND THEDNA D AMAGEC HECKPOINTS. *Annu. Rev. Biochem.* **73,** 39–85 (2004).
43. Dabholkar, M. *et al.* ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients. *J. Natl. Cancer Inst.* **84,** 1512–1517 (1992).
44. Dabholkar, M., Vionnet, J., Bostick-Bruton, F., Yu, J. J. & Reed, E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J. Clin. Invest.* **94,** 703–708 (1994).
45. Li, G.-M. Mechanisms and functions of DNA mismatch repair. *Cell Res* **18,** 85–98 (2008).

46. Brown, R. *et al.* hMLH1 expression and cellular responses of ovarian tumour cells to treatment with cytotoxic anticancer agents. *Oncogene* **15,** (1997).
47. Gourley, C. *et al.* Increased Incidence of Visceral Metastases in Scottish Patients With BRCA1/2-Defective Ovarian Cancer: An Extension of the Ovarian BRCAness Phenotype. *Journal of Clinical Oncology* **28,** 2505–2511 (2010).
48. Ben David, Y. *et al.* Effect of BRCA mutations on the length of survival in epithelial ovarian tumors. *J. Clin. Oncol.* **20,** 463–466 (2002).
49. Chetrit, A. *et al.* Effect of BRCA1/2 Mutations on Long-Term Survival of Patients With Invasive Ovarian Cancer: The National Israeli Study of Ovarian Cancer. *Journal of Clinical Oncology* **26,** 20–25 (2008).
50. Tan, D. S. P. *et al.* „BRCAness"Syndrome in Ovarian Cancer: A Case-Control Study Describing the Clinical Features and Outcome of Patients With Epithelial Ovarian Cancer Associated With BRCA1 and BRCA2 Mutations. *Journal of Clinical …* (2008).
51. Cass, I. *et al.* Improved survival in women with BRCA-associated ovarian carcinoma. *Cancer* **97,** 2187–2195 (2003).
52. Gadducci, A., Cosio, S., Muraca, S. & Genazzani, A. R. Molecular mechanisms of apoptosis and chemosensitivity to platinum and paclitaxel in ovarian cancer: biological data and clinical implications. *Eur. J. Gynaecol. Oncol.* **23,** 390–396 (2002).
53. Mano, Y. *et al.* Bcl-2 as a predictor of chemosensitivity and prognosis in primary epithelial ovarian cancer. *Eur. J. Cancer* **35,** 1214–1219 (1999).
54. Williams, J. *et al.* Expression of Bcl-xL in ovarian carcinoma is associated with chemoresistance and recurrent disease. *Gynecologic oncology* **96,** 287–295 (2005).
55. Herod, J. J. O. *et al.* The Prognostic Significance of Bcl-2 and p53 Expression in Ovarian Carcinoma. *Cancer Res* (1996).
56. Sui, L. *et al.* Survivin expression and its correlation with cell proliferation and prognosis in epithelial ovarian tumors. *Int. J. Oncol.* **21,** 315–320 (2002).
57. Coffman, J. A. Runx transcription factors and the developmental balance between cell proliferation and differentiation. *Cell Biol. Int.* **27,** 315–324 (2003).
58. Kagoshima, H. *et al.* The Runt domain identifies a new family of heteromeric transcriptional regulators. *Trends Genet.* **9,** 338–341 (1993).
59. Speck, N. A. & Stacy, T. A New Transcription Factor Family Associated with Human Leukemias. *Crit Rev Eukar Gene Expr* **5,** 337–364 (1995).
60. Otto, F., Lübbert, M. & Stock, M. Upstream and downstream targets of RUNX proteins. *J. Cell. Biochem.* **89,** 9–18 (2003).
61. Bae, S.-C. & Lee, Y. H. Phosphorylation, acetylation and ubiquitination: The molecular basis of RUNX regulation. *Gene* **366,** 58–66 (2006).
62. Levanon, D. & Groner, Y. Structure and regulated expression of mammalian RUNX genes. *Oncogene* **23,** 4211–4219 (2004).
63. Levanon, D. *et al.* A large variety of alternatively spliced and

differentially expressed mRNAs are encoded by the human acute myeloid leukemia gene AML1. *DNA Cell Biol.* **15,** 175–185 (1996).
64. Bangsow, C. *et al.* The RUNX3 gene--sequence, structure and regulated expression. *Gene* **279,** 221–232 (2001).
65. Tanaka, T. *et al.* An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. *Embo J* **14,** 341–350 (1995).
66. Ogawa, E. *et al.* Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. *Virology* **194,** 314–331 (1993).
67. Tanaka, Y. *et al.* The protooncogene product, PEBP2beta/CBFbeta, is mainly located in the cytoplasm and has an affinity with cytoskeletal structures. *Oncogene* **15,** 677–683 (1997).
68. Lu, J. *et al.* Subcellular localization of the alpha and beta subunits of the acute myeloid leukemia-linked transcription factor PEBP2/CBF. *Mol Cell Biol* **15,** 1651–1661 (1995).
69. Durst, K. L. & Hiebert, S. W. Role of RUNX family members in transcriptional repression and gene silencing. *Oncogene* **23,** 4220–4224 (2004).
70. Perry, C., Eldor, A. & Soreq, H. Runx1/AML1 in leukemia: disrupted association with diverse protein partners. *Leukemia Research* **26,** 221–228 (2002).
71. Coffman, J. A., Kirchhamer, C. V., Harrington, M. G. & Davidson, E. H. SpRunt-1, a new member of the runt domain family of transcription factors, is a positive regulator of the aboral ectoderm-specific CyIIIA gene in sea urchin embryos. *Dev. Biol.* **174,** 43–54 (1996).
72. Robertson, A. J., Dickey, C. E., McCarthy, J. J. & Coffman, J. A. The expression of SpRunt during sea urchin embryogenesis. *Mech. Dev.* **117,** 327–330 (2002).
73. Coffman, J. A., Kirchhamer, C. V., Harrington, M. G. & Davidson, E. H. SpMyb functions as an intramodular repressor to regulate spatial expression of CyIIIa in sea urchin embryos. *Development* **124,** 4717–4727 (1997).
74. Strom, D. K. *et al.* Expression of the AML-1 oncogene shortens the G(1) phase of the cell cycle. *J. Biol. Chem.* **275,** 3438–3445 (2000).
75. Huang, G. *et al.* Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. *Embo J* **20,** 723–733 (2001).
76. Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G. & Downing, J. R. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84,** 321–330 (1996).
77. Komori, T. *et al.* Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89,** 755–764 (1997).

78. Otto, F. *et al.* Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89,** 765–771 (1997).
79. Inoue, K.-I. *et al.* Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. *Nature neuroscience* **5,** 946–954 (2002).
80. Levanon, D. *et al.* The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. *The EMBO Journal* **21,** 3454–3463 (2002).
81. Taniuchi, I. *et al.* Differential Requirements for Runx Proteins in CD4 Repression and Epigenetic Silencing during T Lymphocyte Development. *Cell* **111,** 621–633 (2002).
82. Li, Q.-L. *et al.* Causal Relationship between the Loss of RUNX3 Expression and Gastric Cancer. *Cell* **109,** 113–124 (2002).
83. Speck, N. A. & Gilliland, D. G. Core-binding factors in haematopoiesis and leukaemia. *Nature reviews. Cancer* **2,** 502–513 (2002).
84. Westendorf, J. J. & Hiebert, S. W. Mammalian runt-domain proteins and their roles in hematopoiesis, osteogenesis, and leukemia. *J. Cell. Biochem.* **Suppl 32-33,** 51–58 (1999).
85. Otto, F., Kanegane, H. & Mundlos, S. Mutations in the RUNX2 gene in patients with cleidocranial dysplasia - Otto - 2002 - Human Mutation - Wiley Online Library. *Human mutation* (2002).
86. Subramaniam, M. M. *et al.* Molecular pathology of RUNX3 in human carcinogenesis. *Biochimica et biophysica acta* **1796,** 315–331 (2009).
87. Ghozi, M. C., Bernstein, Y., Negreanu, V., Levanon, D. & Groner, Y. Expression of the human acute myeloid leukemia gene AML1 is regulated by two promoter regions. *P Natl Acad Sci USA* **93,** 1935–1940 (1996).
88. Drissi, H. *et al.* Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. *J. Cell. Physiol.* **184,** 341–350 (2000).
89. Tanaka, K. *et al.* Increased expression of AML1 during retinoic-acid-induced differentiation of U937 cells. *Biochem. Biophys. Res. Commun.* **211,** 1023–1030 (1995).
90. Le, X. F. Regulation of AML2/CBFA3 in Hematopoietic Cells through the Retinoic Acid Receptor alpha -Dependent Signaling Pathway. *Journal of Biological Chemistry* **274,** 21651–21658 (1999).
91. Drissi, H. 1,25-(OH)2-Vitamin D3 Suppresses the Bone-Related Runx2/Cbfa1 Gene Promoter. *Experimental Cell Research* **274,** 323–333 (2002).
92. Massagué, J. TGFβ in Cancer. *Cell* **134,** 215–230 (2008).
93. Ito, Y. & Miyazono, K. RUNX transcription factors as key targets of TGF-beta superfamily signaling. *Curr. Opin. Genet. Dev.* **13,** 43–47 (2003).
94. Hanai, J. *et al.* Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. *J. Biol. Chem.* **274,** 31577–31582 (1999).
95. Chimge, N.-O. *et al.* Regulation of breast cancer metastasis by Runx2 and

estrogen signaling: the role of SNAI2. *Breast Cancer Res.* **13,** R127 (2011).
96. Clevers, H. Wnt/β-Catenin Signaling in Development and Disease. *Cell* **127,** 469–480 (2006).
97. Kahler, R. A. Lymphoid Enhancer Factor-1 and beta -Catenin Inhibit Runx2-dependent Transcriptional Activation of the Osteocalcin Promoter. *Journal of Biological Chemistry* **278,** 11937–11944 (2003).
98. Chuang, L. S. H., Ito, K. & Ito, Y. RUNX family: Regulation and diversification of roles through interacting proteins. *Int J Cancer* **132,** 1260–1271 (2012).
99. Bernardin-Fried, F. AML1/RUNX1 Increases During G1 to S Cell Cycle Progression Independent of Cytokine-dependent Phosphorylation and Induces Cyclin D3 Gene Expression. *Journal of Biological Chemistry* **279,** 15678–15687 (2004).
100. Galindo, M. The Bone-specific Expression of Runx2 Oscillates during the Cell Cycle to Support a G1-related Antiproliferative Function in Osteoblasts. *Journal of Biological Chemistry* **280,** 20274–20285 (2005).
101. Tanaka, Y. *et al.* Runx3 interacts with DNA repair protein Ku70. *Experimental Cell Research* **313,** 3251–3260 (2007).
102. Chi, X. Z. *et al.* RUNX3 Suppresses Gastric Epithelial Cell Growth by Inducing p21WAF1/Cip1 Expression in Cooperation with Transforming Growth Factor -Activated SMAD. *Mol Cell Biol* **25,** 8097–8107 (2005).
103. Lieber, M. R., Ma, Y., Pannicke, U. & Schwarz, K. Mechanism and regulation of human non-homologous DNA end-joining. *Nat. Rev. Mol. Cell Biol.* **4,** 712–720 (2003).
104. Willis, D. M. *et al.* Regulation of osteocalcin gene expression by a novel Ku antigen transcription factor complex. *J. Biol. Chem.* **277,** 37280–37291 (2002).
105. Eliseev, R. A., Dong, Y. F., Sampson, E. & Zuscik, M. J. Runx2-mediated activation of the Bax gene increases osteosarcoma cell sensitivity to apoptosis. *Oncogene* (2008).
106. Yano, T. *et al.* The RUNX3 Tumor Suppressor Upregulates Bim in Gastric Epithelial Cells Undergoing Transforming Growth Factorβ - Induced Apoptosis. *... and cellular biology* (2006).
107. Yamamura, Y., Lee, W. L., Inoue, K.-I., Ida, H. & Ito, Y. RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells. *J. Biol. Chem.* **281,** 5267–5276 (2006).
108. Peng, Z. *et al.* RUNX3 inhibits the expression of vascular endothelial growth factor and reduces the angiogenesis, growth, and metastasis of human gastric cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12,** 6386–6394 (2006).
109. Lebman, D. A., Edmiston, J. S., Chung, T. D. & Snyder, S. R. Heterogeneity in the transforming growth factor beta response of esophageal cancer cells. *Int. J. Oncol.* **20,** 1241–1246 (2002).
110. Torquati, A. *et al.* RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness

in esophageal adenocarcinoma cells. *Surgery* **136,** 310–316 (2004).
111. Sakakura, C. *et al.* Frequent silencing of RUNX3 in esophageal squamous cell carcinomas is associated with radioresistance and poor prognosis. *Oncogene* **26,** 5927–5938 (2007).
112. Ito, K. *et al.* RUNX3 Attenuates β-Catenin/T Cell Factors in Intestinal Tumorigenesis. *Cancer Cell* **14,** 226–237 (2008).
113. Leclerc, D., Deng, L., Trasler, J. & Rozen, R. ApcMin/+ mouse model of colon cancer: gene expression profiling in tumors. *J. Cell. Biochem.* **93,** 1242–1254 (2004).
114. Weith, A. *et al.* Report of the second international workshop on human chromosome 1 mapping 1995. in *Cytogenet. Cell Genet.* **72,** 114–144 (1996).
115. Bae, S.-C. & Choi, J.-K. Tumor suppressor activity of RUNX3. *Oncogene* **23,** 4336–4340 (2004).
116. Tanaka, K. *et al.* Suppression of tumourigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. *Oncogene* **8,** 2253–2258 (1993).
117. Herzog, C. R., Wang, Y. & You, M. Allelic loss of distal chromosome 4 in mouse lung tumors localize a putative tumor suppressor gene to a region homologous with human chromosome 1p36. *Oncogene* **11,** 1811–1815 (1995).
118. Stewart, M., MacKay, N., Cameron, E. R. & Neil, J. C. The Common Retroviral Insertion Locus Dsi1 Maps 30 Kilobases Upstream of the P1 Promoter of the Murine Runx3/Cbfa3/Aml2 Gene. *Journal of Virology* **76,** 4364–4369 (2002).
119. Kim, R. *et al.* Genome-based identification of cancer genes by proviral tagging in mouse retrovirus-induced T-cell lymphomas. *Journal of Virology* **77,** 2056–2062 (2003).
120. Miething, C. *et al.* Retroviral insertional mutagenesis identifies RUNX genes involved in chronic myeloid leukemia disease persistence under imatinib treatment. *P Natl Acad Sci USA* **104,** 4594–4599 (2007).
121. Salto-Tellez, M. *et al.* RUNX3 protein is overexpressed in human basal cell carcinomas. *Oncogene* **25,** 7646–7649 (2006).
122. Ginos, M. A. *et al.* Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. *Cancer Res* **64,** 55–63 (2004).
123. Tsunematsu, T. *et al.* RUNX3 Has an Oncogenic Role in Head and Neck Cancer. *PloS one* **4,** e5892 (2009).
124. Lau, Q. C. *et al.* RUNX3 is frequently inactivated by dual mechanisms of protein mislocalization and promoter hypermethylation in breast cancer. *Cancer Res* **66,** 6512–6520 (2006).
125. Ito, K. *et al.* RUNX3, a novel tumor suppressor, is frequently inactivated in gastric cancer by protein mislocalization. *Cancer Res* **65,** 7743–7750 (2005).
126. Kim, W. J. *et al.* RUNX3 inactivation by point mutations and aberrant DNA methylation in bladder tumors. *Cancer Res* **65,** 9347–9354 (2005).

127. Zhang, S. Q., Wei, L. X., Zhang, A. F., Zhang, L. L. & Yu, H. RUNX3 Gene Methylation in Epithelial Ovarian Cancer Tissues and Ovarian Cancer Cell Lines. *Omics* **13,** 307–311 (2009).
128. Nevadunsky, N. S. *et al.* RUNX3 protein is overexpressed in human epithelial ovarian cancer. *Gynecologic oncology* **112,** 325–330 (2009).
129. Lee, C. W. *et al.* RUNX3 functions as an oncogene in ovarian cancer. *Gynecologic oncology* **122,** 410–417 (2011).
130. Keita, M. *et al.* The RUNX1 transcription factor is expressed in serous epithelial ovarian carcinoma and contributes to cell proliferation, migration and invasion. *Cell Cycle* **12,** 972–986 (2013).
131. Wang, Z.-Q. *et al.* Inhibition of RUNX2 Transcriptional Activity Blocks the Proliferation, Migration and Invasion of Epithelial Ovarian Carcinoma Cells. *PloS one* **8,** e74384 (2013).
132. Keita, M. *et al.* Global methylation profiling in serous ovarian cancer is indicative for distinct aberrant DNA methylation signatures associated with tumor aggressiveness and disease progression. *Gynecologic oncology* **128,** 356–363 (2013).
133. Xu, N. *et al.* Upregulated miR-130a increases drug resistance by regulating RUNX3 and Wnt signaling in cisplatin-treated HCC cell. *Biochem. Biophys. Res. Commun.* **425,** 468–472 (2012).
134. Zhang, Y., Lu, Q. & Cai, X. MicroRNA-106a induces multidrug resistance in gastric cancer by targeting RUNX3. *FEBS Lett.* **587,** 3069–3075 (2013).
135. Zheng, Y. *et al.* Epigenetic downregulation of RUNX3 by DNA methylation induces docetaxel chemoresistance in human lung adenocarcinoma cells by activation of the AKT pathway. *Int. J. Biochem. Cell Biol.* **45,** 2369–2378 (2013).
136. Wang, Z. *et al.* miR-106a is frequently upregulated in gastric cancer and inhibits the extrinsic apoptotic pathway by targeting FAS. *Mol. Carcinog.* **52,** 634–646 (2013).
137. Xiao, B. *et al.* Detection of miR-106a in gastric carcinoma and its clinical significance. *Clin. Chim. Acta* **400,** 97–102 (2009).
138. Shepherd, T. G., Thériault, B. L., Campbell, E. J. & Nachtigal, M. W. Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients. *Nat Protoc* **1,** 2643–2649 (2006).
139. El-Sehemy, A. *et al.* Notch activation augments nitric oxide/soluble guanylyl cyclase signaling in immortalized ovarian surface epithelial cells and ovarian cancer cells. *Cell. Signal.* **25,** 2780–2787 (2013).
140. Fu, Y., Sies, H. & Lei, X. G. Opposite Roles of Selenium-dependent Glutathione Peroxidase-1 in Superoxide Generator Diquat- and Peroxynitrite-induced Apoptosis and Signaling. *Journal of Biological Chemistry* **276,** 43004–43009 (2001).
141. Rixe, O. *et al.* Oxaliplatin, tetraplatin, cisplatin, and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's Anticancer Drug Screen panel. *Biochem. Pharmacol.* **52,** 1855–1865 (1996).

142. Yang, X., Fraser, M., Moll, U. M., Basak, A. & Tsang, B. K. Akt-Mediated Cisplatin Resistance in Ovarian Cancer: Modulation of p53 Action on Caspase-Dependent Mitochondrial Death Pathway. *Cancer Res* **66,** 3126–3136 (2006).
143. Al-Bahlani, S. *et al.* P73 regulates cisplatin-induced apoptosis in ovarian cancer cells via a calcium/calpain-dependent mechanism. *Oncogene* **30,** 4219–4230 (2011).
144. Im-aram, A. *et al.* The mTORC2 component rictor contributes to cisplatin resistance in human ovarian cancer cells. *PloS one* **8,** e75455 (2013).
145. Blyth, K., Cameron, E. R. & Neil, J. C. The runx genes: gain or loss of function in cancer. *Nature reviews. Cancer* **5,** 376–387 (2005).
146. Subramaniam, M. M. *et al.* RUNX3 inactivation by frequent promoter hypermethylation and protein mislocalization constitute an early event in breast cancer progression. *Breast cancer research and treatment* **113,** 113–121 (2009).
147. Javed, A. *et al.* runt Homology Domain Transcription Factors (Runx, Cbfa, and AML) Mediate Repression of the Bone Sialoprotein Promoter: Evidence for Promoter Context-Dependent Activity of Cbfa Proteins. *Mol Cell Biol* **21,** 2891–2905 (2001).
148. Davis, J. N. *et al.* Association of core-binding factor β with the malignant phenotype of prostate and ovarian cancer cells. *J. Cell. Physiol.* **225,** 875–887 (2010).
149. Su, H.-Y. *et al.* Epigenetic silencing of SFRP5 is related to malignant phenotype and chemoresistance of ovarian cancer through Wnt signaling pathway. *Int J Cancer* **127,** 555–567 (2010).
150. Lustig, B. & Behrens, J. The Wnt signaling pathway and its role in tumor development. *J. Cancer Res. Clin. Oncol.* **129,** 199–221 (2003).
151. Klaus, A. & Birchmeier, W. Wnt signalling and its impact on development and cancer. *Nature reviews. Cancer* **8,** 387–398 (2008).
152. Jho, E.-H. *et al.* Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* **22,** 1172–1183 (2002).
153. Weidinger, G., Thorpe, C. J. & Wuennenberg-Stapleton, K. The Sp1-Related Transcription Factors sp5 and sp5-like Act Downstream of Wnt/β-Catenin Signaling in Mesoderm and Neuroectoderm Patterning. *Current biology* (2005).