EXPRESSION AND FUNCTION OF APELA: A POTENTIAL REGULATOR OF CELL GROWTH IN HUMAN CANCERS

by

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ABSTRACT

Apela, a novel gene identified by our laboratory, is expressed in mouse definitive endoderm, neural tube, and mouse embryonic stem cells (mESCs). In humans, *APELA* is expressed in embryonic stem cells, induced adult pluripotent stem cells (iPSCs) as well as adult kidney and prostate. APELA peptide signals through the G-protein coupled receptor, the APJ receptor, to regulate zebrafish definitive endoderm migration and cardiac development. Interestingly, the mRNA of *Apela* can mediate p53-dependent mESCs cell apoptosis. These findings suggest that Apela can functions as a peptide or as a lncRNA. Signaling pathways that are critical during embryogenesis are also important in cancer development and progression. However, thus far, whether APELA exerts any biological functions that regulate cancer progression is completely unknown.

In this study, analysis of the cancer genome atlas (TCGA) RNA sequencing datasets reveals that *APELA* mRNA is expressed in different human cancer including in ovarian cancer. Real-time quantitative PCR analyses of clinical human ovarian cancer samples show that *APELA* mRNA levels are higher in ovarian clear cell carcinoma (OCCC), than other subtypes. Using a CRISPR/Cas9-mediated knockout approach, I have demonstrated that *APELA* knockout suppresses cell growth in the ovarian clear cell carcinoma cell line, OVISE. Decreased cell growth induced by *APELA* knockout can be partially attenuated by treating cells with recombinant human APELA protein. In addition, flow cytometry analyses show that *APELA* knockout induces G2/M phase arrest in OVISE cells. Western blot results show that the phosphorylation levels of ERK1/2, AKT, and cyclin B1 expression levels are significantly down-regulated in the APELA deficient OVISE cells. Moreover, our results indicate that in the *APELA* knockout cells, decreased cell growth is dependent on the expression of wildtype p53.

Unexpectedly, knockout *APELA* does not affect cell growth in Ewing sarcoma cell line A673, which has high expression of APELA at mRNA level. Interestingly, the APJ receptor is expressed in A673 cells but not in OVISE cells, which strongly suggests that APELA can exert its function through APJ-independent pathway in OVISE cells. In summary, our study demonstrates that APLEA may be an important factor that mediates the progression of OCCC.

PREFACE

The research in this thesis was conducted at Terry Fox Laboratory at BC Cancer Research Centre by Yuyin Yi under the supervision of Dr. Pamela Hoodless. RNA samples from ovarian cancer patients were obtained from Dr. David Huntsman in collaboration with his laboratory. The bioinformatic analysis of TCGA RNA sequencing dataset for APELA expression (Figure 3.1) was performed by Dr. Ewan Gibb from Canada's Michael Smith Genome Sciences Centre. The remaining experiments and data analyses were carried out by Yuyin Yi.

This study was approved by the University of British Columbia - British Columbia Cancer Agency Research Ethics Board under the 'Epigenetic Modifications Regulating Hepatocellular Carcinoma and Hepatocyte Differentiation' (H13-01273). The use of any biohazardous chemicals and material was approved by the University of British Columbia Biosafety Committee under 'Characterization of early mammalian development' (B14-0054).

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LIST OF ABBREVIATIONS

APELA-apelin early endogenous ligand

AA-amino acid

AC-adenylate cyclase

AKT-protein kinase B

APJ-Apelin receptor

cAMP-cyclic adenosine monophosphate

CDK-cyclin-dependent kinase

CHO-Chinese hamster ovarian

CKI-CDK inhibitors

CRISPR/Cas9-clustered regularly interspaced short palindromic repeats; CRISPR associated protein 9

DAG-diacylglycerol

DE-definitive endoderm

DIA-DNA damage-induced apoptosis

E (8.0)-embryonic day 8

ERK-extracellular signal-regulated kinase

ESC-embryonic stem cell

EST- expression sequence tag

GPCR-G protein–coupled receptor

GTP-guanosine triphosphate

GDP-guanosine diphosphate

HnRNPL-heterogeneous nuclear ribonucleoprotein L

HUVEC-umbilical vascular endothelial cells

ICM-inner cell mass

IP3- inositol 1, 4, 5-triphosphate

KO-knockout

lncRNA- long non-coding RNA

MAPK-mitogen-activated protein kinase

mTOR-mammalian target of rapamycin

NOS-nitric oxide synthase

ORF-open reading frame

PI3K-phosphoinositide-3-kinase

PIP2-phosphatidylinositol 4, 5-biphosphate

PKA-protein kinase A

PKC-protein kinase C

PLC-β-phospholipase C-β

qPCR- quantitative PCR

RT-PCR- reverse transcriptase PCR

RTK-receptor tyrosine kinase

SAGE- serial analysis of gene expression

TGF-β-transforming growth factor-beta

UTR-untranslated region

WT-wildtype

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1 CHAPTER 1: INTRODUCTION

1.1 APELA (Apelin Receptor Early Endogenous Ligand)

1.1.1 Discovery of Apela in the definitive endoderm

Early in mouse embryogenesis, during cleavage stages, the embryo generates inside and outside cell populations, and then gives rise to the inner cell mass (ICM) and trophectoderm of the blastocyst by embryonic day 3.5 (E3.5) (Figure 1.1A). During implantation into the maternal uterus (at around E4.5), the ICM gives rise to both the pluripotent epiblast and primitive endoderm (PrE). The epiblast later gives rise to three primary germ layers (ectoderm, mesoderm and definitive endoderm) through a process known as gastrulation. Cells descended from the definitive endoderm (DE) will develop into organs of the respiratory and gastrointestinal tracts including the lungs, liver, pancreas, and intestine (1, 2) (Figure 1.1B).

Previous studies in our laboratory have established serial analysis of gene expression (SAGE) libraries to systematically screen genes specifically expressed in the mouse definitive endoderm (3). At embryonic stage of Somite 0–6, the newly formed endoderm has not yet been patterned. By the stage of Somite 8–12, endoderm patterning has initiated (4) and is divided through the midgut into foregut and hindgut regions (3). Therefore, three longSAGE libraries from early DE of murine embryos were generated and analyzed: 0-6 somite whole endoderm, somite 8-12 foregut and somite 8-12 hindgut. A list of candidate genes, including *Nephrocan* (*Nepn*) and *Peptide YY* (*Pyy*) (3), enriched in endoderm were identified through comparisons within these three endoderm libraries and against mouse longSAGE libraries generated by the Mouse Atlas of Gene Expression Project covering a wide variety of tissues and stages from the fertilized egg to the adult mouse. To identify potential novel genes expressed in the definitive

endoderm, this SAGE study was extended and tag sequences representing non-annotated transcripts were analyzed (5). The analysis results identified that the expression sequence tag (EST), *AK014119* was enriched in the definitive endoderm. *AK014119* lies on chromosome 8 and consists of 4 exons (5). *AK014119* was renamed **End**oderm **e**nriched or *Ende* (5) for simplicity and now its official symbol is *Apela* (also called *Gm10664*).

Spatially, mouse *Apela* is specifically observed in the embryonic regions of the conceptus and the early definitive endoderm in the SAGE analysis (5). In libraries of the DE tissue in the 0-6 somite stage embryos and in the 6-12 somite foregut and hindgut, *Apela* shows highest levels of gene expression. From embryonic day 10.5 (E10.5) onwards, the *Apela* transcript is detected at much lower levels in the developing spleen, heart, kidney, and endodermal derived organs such as liver, intestine and pancreas. Post embryogenesis, *Apela* transcript is observed in the kidney, pituitary gland, brain, urogenital sinus, and testis. Interestingly, *Apela* is also detected in mouse embryonic stem (ES) cells. The expression pattern of *Apela* has also been described by whole mount *in situ* hybridization (5). At E7.0, *Apela* mRNA is specifically detected in a small population of epiblast cells in the distal region of the embryo. The expression of *Apela* in the distal epiblast remains excluded from the extraembryonic region until the early head fold stages (E7.75). Then the cells in the anterior-most region of the embryo also begin to express *Apela*. As the embryo develops into early somite stages (E8.5), *Apela* is highly expressed in both the developing foregut and hindgut pockets, as shown in Figure 1.1 C. By E9.25, cells in the posterior half of the ventral neural tube begin to express *Apela.* At this stage, *Apela* is restricted to a region of the foregut overlying the heart and the posterior-most hindgut. The expression of *Apela* in foregut, hindgut and neural tube can be observed as late as E10.25, at reduced levels.

Thus, *Apela* is expressed dynamically in specific populations of the definitive endoderm during gastrulation in the mouse embryo (5).

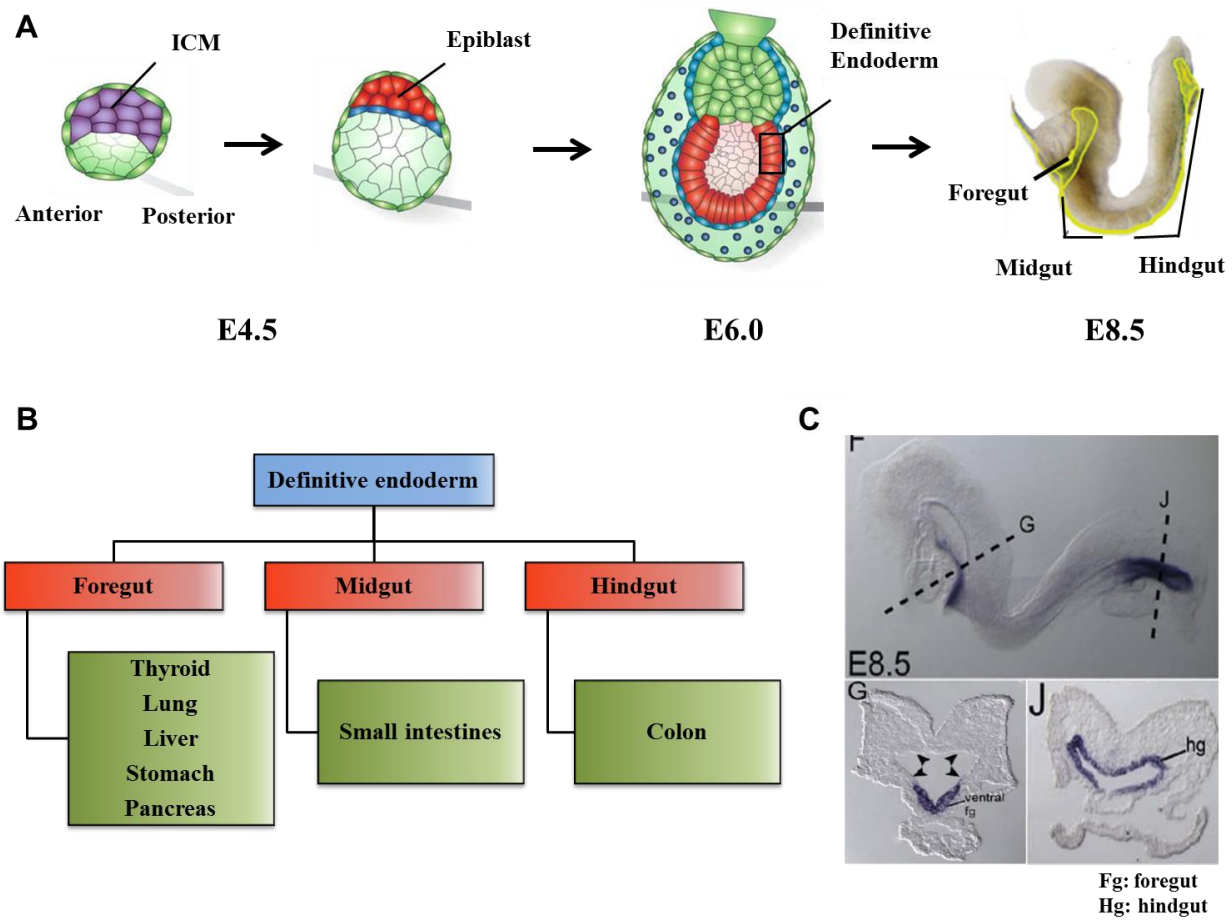

Figure 1.1 *Apela* transcript expression is enriched in mouse definitive endoderm

(A) Formation and development of the mouse definitive endoderm. By embryonic day 4.5 (E4.5), the inner cell mass (ICM) gives rise to both the pluripotent epiblast (red) and primitive endoderm (PrE; blue). The epiblast gives rise to the three embryonic lineages, definitive endoderm (highlighted in yellow), mesoderm and ectoderm. Following gastrulation, the endoderm forms the gut tube divided into foregut, midgut, and hindgut in a precise anterior to posterior order. Organs derived from each segment of the gut tube are shown (B). Adapted from Viotti and Foley, 2014 (6). (C) Whole mount *in situ* hybridization illustrated the restricted expression of *Apela* in both the foregut and hindgut pockets at E8.5. Adapted from Hassan, 2010 (5).

1.1.2 The function of Apela in animal development

Apela was initially annotated as a long non-coding RNA (lncRNA) in zebrafish (*ENSDARG00000094729*), mouse (*Gm10664*) and human (*LOC100506013*, also called *AK092578*). However, ribosome profiling and phylogenetic analysis in mouse (7) and zebrafish (8, 9) suggest that *Apela* mRNA contains an open reading frame (ORF) encoding a small secretory 54 amino acid peptide (aa, 58 aa in zebrafish) called Elabela (10) or Toddler (11). This short peptide includes a signal sequence of 22 aa, and the 11 aa C-terminus is highly evolutionarily conserved in vertebrates, including humans. Using antibodies specific to the predicted human APELA peptide, western blot analysis of *Xenopus laevis* embryos microinjected with human *APELA* ORF mRNA has detected full-length APELA peptide in the embryo extract and processed APELA peptide in the supernatant, suggesting that *APELA* is translated and encodes a secreted peptide (10).

In zebrafish, *apela* gene consists of three exons located on chromosome 1 (10). The *apela* transcript is expressed ubiquitously in zebrafish embryos during late blastula and early gastrula stages and becomes restricted to the lateral mesoderm, endoderm, and anterior and posterior notochord after gastrulation (10, 11). Two groups have shown that disruption of *apela* causes early embryonic lethality due to endoderm and cardiovascular developmental defects (10, 11). It also has been shown that *apela* mutant embryos show delayed internalization of the mesendodermal cells, and impaired movement of ventrolateral endoderm and mesoderm toward the animal pole and during gastrulation (11). Chng et al. reported a crucial role for *apela* in differentiation of the endodermal lineage, which is required for directing proper cardiac ontogeny (10). Moreover, overexpression of *apela* mRNA in wildtype zebrafish embryos at levels greater than required for rescue phenocopied *apela* mutants (11), suggesting that the

precise levels of Apela peptide are critical for early zebrafish development. Both groups have suggested that Apela peptide can bind to the G protein–coupled receptor (GPCR) for Apelin, the APJ receptor, activation of which is required for proper cell movement during zebrafish gastrulation (10, 11). Injection of *apela* mRNA during late blastula and early gastrula stages, but not at later times, rescues *apela* mutants which are able to survive to adulthood and are fertile (11). Therefore, Apela has been considered to be only required during early zebrafish embryogenesis. Of note, it is Apela, not Apelin, which functions in regulating cell migration during early zebrafish embryogenesis (10, 11). Thus, the nomenclature committees for mouse, human, zebrafish and *Xenopus* has named the gene **ap**elin receptor **e**arly endogenous **l**ig**a**nd, and gene symbol is *APELA* in human, *Apela* in mouse and rat, and *apela* in zebrafish and *Xenopus*. In addition, during zebrafish embryo vasculogenesis, Apela peptide and Apelin expressed in the midline has been shown to guide angioblasts migration and regulate vascular patterning via APJ receptor signaling pathway, suggesting a role of Apela in zebrafish angiogenesis (12).

The functions of APELA in mammals remain largely unexplored and only a few studies have been published to date. The role of *Apela* in mouse embryogenesis is currently unknown. However, the *Apela* mRNA has been reported to be present in mouse adult heart non-cardiomyocytes, and increases cardiac contractility through the APJ receptor and activates ERK1/2 signaling (13). In addition, *Apela* mRNA is co-expressed with the APJ receptor in adult kidney in mouse, rat, and humans, and injection of a synthetic APELA peptide in the bloodstream of rats can induce diuresis and water intake increases, suggesting that APELA may have a role in regulating fluid homoeostasis (14). Moreover, recent studies in the central nervous system has demonstrated that the APELA peptide functions as an anorexigenic hormone in adult

mouse brain to regulate food intake, although the expression of *Apela* in the normal brain has not been shown (15).

Remarkably, studies have suggested that *Apela* can not only encode a secreted peptide, which functions via the APJ receptor, but also a lncRNA. *Apela* mRNA is highly expressed in mouse embryonic stem cells (mESCs) and the 3' UTR of *Apela* spliced mRNA has been proposed to act as a regulatory RNA to interact with heterogeneous nuclear ribonucleoprotein L (hnRNPL), an inhibitory regulator of p53 (16). Consequently, *Apela* mRNA inhibits hnRNPL-mediated p53 degradation and increases the amount of mitochondrial p53 to regulate DNA damage–induced apoptosis (DIA). In contrast, *Apela* mRNA expression is repressed by p53, suggesting that *Apela*, hnRNPL, and p53 form a tri-element negative feedback loop in the regulation of DIA in mESCs. Supporting its role as a lncRNA, the coding ability of *Apela* is dispensable for its function in p53-mediated DIA (16).

1.1.3 The expression and function of APELA in human

In humans, the *APELA* gene is located on chromosome 4 and consists of 3 exons (10). The conserved ORF encoding the APELA peptide spans from exon1 to exon2, and the third exon consists entirely of the 3’ UTR. The 3’ UTR of human *APELA* includes two anti-parallel 300 bp Alu repeats, which are not found in rodent (17). The encoded APELA peptide contains a pair of conserved cysteines residues that are predicted form an intermolecular disulfide bridge.

APELA mRNA is most highly expressed in undifferentiated human embryonic stem cells (hESCs) (10, 17, 18). During hESCs embryoid body formation, endodermal, and neuronal differentiation, *APELA* expression is rapidly silenced (17). APELA protein is co-localized with

hESCs Golgi apparatus and detected in the supernatant of cultured *Xenopus laevis* embryos overexpressing the *APELA* ORF mRNA (10), suggesting that APELA is processed through the secretory pathway.

APELA has been shown to promote self-renewal and activate PI3K/AKT signaling pathway in hESCs (17). Knockout of *APELA* by the CRISPR/Cas9 system or knockdown by shRNA in hESCs results in a significant growth disadvantage compared with control hESCs. The growth of sh*APELA* hESCs is entirely rescued by the addition of recombinant APELA. Moreover, an antibody targeting APELA, which has potent APELA-neutralizing activity, is able to inhibit hESCs growth. Therefore, endogenous APELA can promote cell growth of hESCs in an autocrine and paracrine manner (17). Proteomic analysis has identified that APELA could activate the phosphorylation of PRAS40, which is the downstream substrate of AKT. The increased phosphorylation of AKT and p70S6K has been documented in response to addition of recombinant APELA, suggesting that APELA can activate the PI3K/AKT pathway and subsequently activate the mTORC1 pathway. In addition, cell cycle analysis indicates that APELA peptide treatment induces a promotion of G1/S transition in hESCs and this effect can be reversed by a PI3K inhibitor, suggesting that APELA promotes cell cycle progression in a PI3K/AKT pathway dependent manner. Furthermore, genes in the mTORC1 signaling pathway which are associated with growth factor translation are upregulated by APELA gain-of-function, and are downregulated in *APELA* knockdown hESCs. These data suggest APELA promotes the growth of hESCs through PI3K/mTOR-dependent activation of protein translation. APELA is also required to protect against apoptosis induced by a variety of cellular stresses in hESCs (17), which is in contrast with its apoptosis-promoting effect in mESCs (16), suggesting that APELA may function distinctly in different species.

In APELA-treated hESCs, several genes related to the NODAL/TGFβ pathway are upregulated and these genes are enriched for definitive endoderm lineage commitment (17). The increased levels and nuclear localization of phospho-SMAD3 in hESCs treated with APELA indicates that APELA is able to modulate the activation of the NODAL/TGFβ pathway. In addition, many markers of the mesendoderm lineage, including *BRA*, *EOMES*, and *GATA4/6* are positively correlated with APELA expression. However, APELA alone is not sufficient to commit hESCs toward the DE lineage, since cells treated with APELA peptide maintain hESC morphology and remain pluripotent to give rise to all three germ lineages during embryoid body differentiation. Overall, similar to the function in zebrafish embryogenesis, APELA is required in a transcriptional profile that primes hESCs toward the mesendoderm lineage and endodermal differentiation.

Interestingly, the APJ receptor is barely detected in undifferentiated hESCs but is highly upregulated upon mesendoderm differentiation (19, 20). The APELA cell surface binding assay has shown the presence of an endogenous cell-surface receptor that recognizes APELA in undifferentiated hESCs, but the binding is not affected by knockdown of the APJ receptor (17). In addition, APELA-mediated activation of AKT in undifferentiated hESCs is independent of the APJ receptor. Thus, instead of the APJ receptor, an alternate receptor has been postulated to be recognized by APELA peptide and mediate this peptide activity. However, the identity of the postulated receptor is unknown and whether this receptor is responsible for maintaining hESCs self-renewal remains unclear. The APJ receptor is rapidly upregulated upon mesendoderm differentiation, indicating that the APJ receptor may be required for APELA function in poising hESCs toward the mesendoderm lineage (17).

In adult, the human *APELA* transcript is found in kidney and prostate at relatively low levels (10). The regulation of fluid homeostasis by APELA via the APJ receptor pathway in rat kidney (14) implies APELA could have a similar role in humans. In Chinese hamster ovarian (CHO) cells that stably express human APJ, APELA signals through the APJ receptor to suppress cAMP production, stimulates ERK1/2 phosphorylation and slightly induces intracellular calcium mobilization (14, 21). APELA also acts as a hormone regulating the cardiovascular system in adulthood as well as in embryonic development. *In vitro* study in human umbilical vascular endothelial cells (HUVEC) has showed that APELA induces angiogenesis via APJ receptor signaling. APELA also can relax mouse aortic blood vessel via APJ receptor but through a mechanism different from Apelin (21).

1.2 Apelin and the APJ receptor

1.2.1 Discovery of Apelin and APJ receptor

The APJ receptor gene (*APNLR*) (22) was discovered in 1993 based on its sequence similarity with the angiotensin II type 1 receptor (AT-1) and is located on chromosome 11 in humans (22). The human APJ receptor is comprised of 380 amino acids and is a class A (rhodopsin-like) GPCR (22). The APJ receptor is conserved in many species, including mouse (23), rat (24), cow (25), Xenopus (26), and zebrafish (27).

The APJ receptor is not activated by angiotensin II and was therefore designated an orphan receptor until 1998 when a 36-amino acid peptide was identified and named Apelin, for **AP**J **e**ndogenous **li**ga**n**d (28). The human Apelin gene, termed *APLN*, is located on chromosome X and encodes a 77 amino acid preproprotein containing a secretory sequence with a hydrophobic

rich N-terminal region. There are several mature isoforms of the Apelin peptide. A cascade of proteolytic cleavage events of the preproprotein produces several predicted C-terminal Apelin peptides: Apelin-36, Apelin-17, Apelin-13, and a pyroglutamylated form of Apelin-13 [(Pyr^{1}) Apelin-13] (29). These Apelin peptide isoforms are all agonists for the APJ receptor. Full conservation of the Apelin peptide has been shown between the different species in the 23 C-terminal amino acid, suggesting an essential physiological role.

1.2.2 Tissue Distribution of Apelin and APJ receptor in humans

Both the APJ receptor and Apelin are present in the central nervous system and are widely expressed in multiple peripheral tissues (30). The APJ receptor has the highest expression in the spinal cord, corpus callosum, and medulla, with lower levels in the hypothalamus and hippocampus (31-33). In the periphery, human APJ receptor expression is present throughout the organs including heart, lung, kidney, stomach, and small intestine, but is strongest in the spleen and placenta (32, 33). In addition, a number of studies have reported cardiovascular localization of the APJ receptor in vascular endothelial, smooth muscle cells, as well as cardiomyocytes (34). The expression of Apelin mRNA follows that reported for the APJ receptor (33), suggesting a functional dependence between the receptor and ligand. Although different isoforms of Apelin display similar functions, it has been demonstrated that Apelin-13 and [(Pyr^{1}) Apelin-13] represents the predominant form in the heart, while Apelin-36 is predominant it lung, testis, and uterus (35, 36).

1.2.3 The biological actions of the apelinergic system

Since the discovery of APJ receptor signaling, the physiological significance of the apelinergic system has been clarified within many regulatory processes including in the cardiovascular and central nervous systems. In addition, activated by Apelin, the APJ receptor plays a vital role in angiogenesis, fluid homeostasis, and energy metabolism, and acts as a human immunodeficiency virus (HIV-1) co-receptor.

The important insights of APJ receptor signaling into cardiovascular roles have been supported by phenotypes in both the Apelin and APJ receptor knockout mice. Apelin knockout mice have normal heart morphology and blood pressure (37, 38) but develop impaired cardiac contractility with age, severe heart failure in response to overload pressure (37), and pulmonary hypertension under hypoxia (39). Mice lacking the gene encoding for the APJ receptor are born at sub-Mendelian ratios and most die in utero due to cardiovascular developmental defects including poorly looped hearts, defective atrioventricular cushion formation, and deformed vasculature of the yolk sac in the embryo (40). The few surviving mice have normal blood pressure but a modest decrease in basal cardiac contractility, marked decrease in exercise capacity, and heart failure under pressure overload, which phenocopies Apelin knockout mice (37, 41). These data indicate that the apelinergic system is important not only for normal cardiovascular development and function, but also for appropriate cardioprotective effects under stress (42).

Peripheral Apelin functions as an antihypertensive factor *in vivo* (41, 43) and in hypertensive disease, sensitivity to the peripheral administration of Apelin might be altered (44). Mediated primarily by nitric oxide (45), Apelin peptide administration has been reported to modulate the vasomotor tone, which in turn results in blood pressure reduction, heart rate and

myocardial contractility increase (46, 47). Thus, Apelin released from endothelial cells cause vasodilation or vasoconstriction via activation of APJ receptors on the endothelium or underlying smooth muscle cells (48). Additionally, Apelin is also reported to modulate cardiac function by activating APJ receptors present on cardiomyocytes (34).

Apelin acts as an angiogenic factor through the APJ receptor both in normal physiology and in the pathology of cancer. The presence of both receptor and ligand is required for the normal development of frog vasculature (49) and murine blood vessels formation (50). In addition, Apelin is involved in hypoxia-induced retinal angiogenesis (51) and non-neovascular remodeling of retina (52). It has been reported that Apelin is broadly expressed in endothelial cells in tumors of different origins and the expression level of the APJ receptor is also increased in tumor endothelium, implying the potential of apelinergic system as an anticancer therapeutic target.

The expression of Apelin and the APJ receptor in areas of the brain critical for fluid homeostasis regulation implies involvement of Apelin in controlling body fluid balance (53). A number of studies have discussed the controversial role of Apelin and APJ receptor in maintaining fluid homeostasis. Some studies have observed the diuretic effect for Apelin that counteracts arginine vasopressin, the neurohypophyseal hormone for body water retention, to increase diuresis (54). In contrast, APJ-mediated antidiuresis has been reported in APJ receptor knockout mice, which are unable to reduce urine volume in response to water deprivation (55). The regulatory role of Apelin in drinking behaviors is also complicated since central administration of Apelin has been shown to increase water intake in water-replete animals (56), while other studies have reported that Apelin reduces water intake (57) or has no effect (58, 59).

Thus, Apelin and APJ receptor are important for physiological regulation of fluid homeostasis; however, the exact involvement of the apelinergic system in fluid balance is not clear.

Apelin and APJ receptor exert a beneficial regulatory role in energy metabolism and both are upregulated in metabolic disorders (60). Apelin is considered to maintain insulin sensitivity (61) to regulate glucose metabolism. Additionally, accumulating evidence suggests that Apelin is also involved in lipid metabolism by reducing fat mass and promoting fuel consumption (62). Those findings of Apelin are very encouraging in the context of a therapeutic approach in obesity-associated diseases such as type 2 diabetic.

Collectively, APJ receptor mediates a wide range of normal physiological processes including cardiovascular regulation, angiogenesis, energy metabolism, fluid homeostasis, and the neuroendocrine stress response. Moreover, APJ receptor signaling is also implicated in several pathologies, including heart disease, diabetes, obesity, and cancer.

1.2.4 Signal transduction pathways coupled to the APJ receptor

The APJ receptor interacts with multiple G-proteins and can trigger numerous intracellular signaling cascades in different cell types. An overview of the signaling pathways potentially mediated by APJ receptor is shown in Figure 1.2.

The APJ receptor can couple to both $G\alpha_{i/o}$ and $G\alpha_{q/11}$. The classical $G\alpha_{i/o}$-mediated downstream effects such as ERK and AKT activation and decreased cAMP production have been shown in CHO cells (63). Furthermore, mouse APJ receptor couples preferentially to $G\alpha_{i1}$ and $G\alpha_{i2}$, but not to $G\alpha_{i3}$, and subsequently induces inhibition of adenylate cyclase and phosphorylation of ERK1/2 (64). Through coupling to $G\alpha_{q/11,}$ APJ receptor can activate ERK

signaling either via PLCβ–PKC pathway or stimulates RAS directly (65). Activated by Apelin, APJ-mediated signaling cascades usually target transcription factors. It is not yet clear the exact transcription factors or other cellular effectors that transduce via APJ receptor signaling.

It has recently been shown that APJ receptor prompts myocardial hypertrophy in response to mechanical stretch in association with a β-arrestin-dependent mechanism. Interestingly, this response of the APJ receptor is G-protein-independent. Additionally, stretch diminishes Apelin signaling by decreasing G protein activation and recruiting β-arrestin (66).

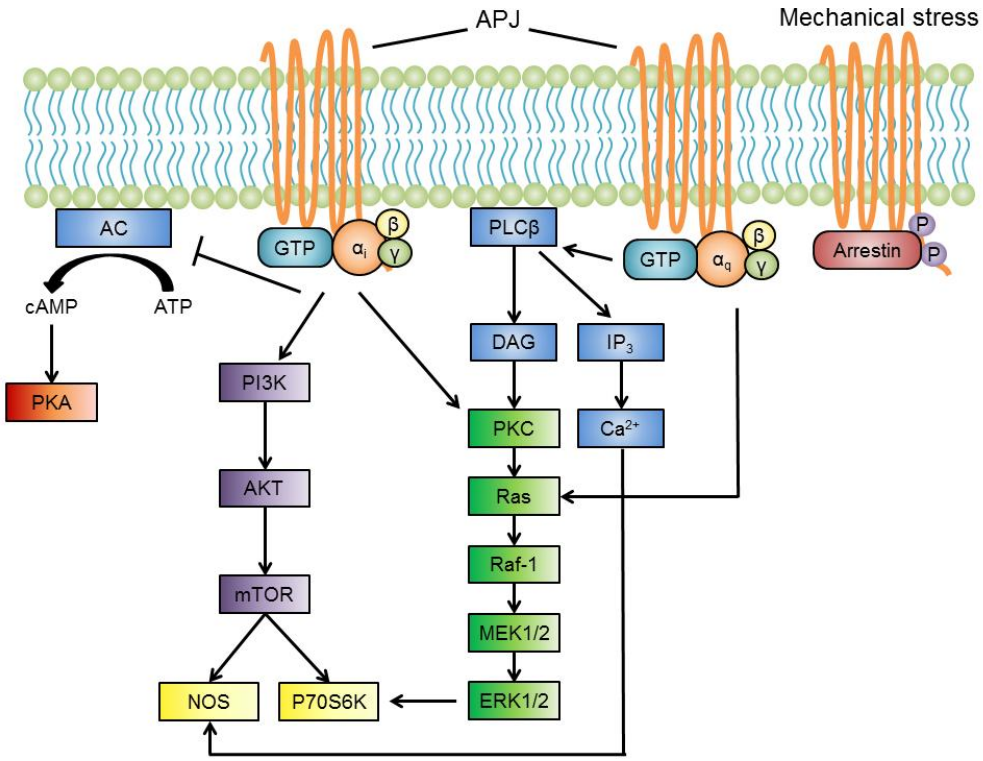

Figure 1.2 Overview of APJ receptor-mediated signaling pathways

Activated APJ receptor couples to $G\alpha_i$ and $G\alpha_q$. $G\alpha_i$ stimulates the MAPK cascade via PKC, and it can also activate phosphoinositide 3-kinase (PI3K) and subsequently activates AKT and mammalian target of rapamycin (mTOR), causing the activation of both p70S6K and endothelial nitric oxide synthase (eNOS). $G\alpha_i$ signaling can also inhibit adenylate cyclase (AC) activity, reducing cAMP production and thereby inhibiting protein kinase A (PKA) activation. $G\alpha_q$ stimulates phospholipase C-β (PLC-β) signaling, inducing diacylglycerol (DAG) and 1, 4, 5-triphosphate (IP3). IP3 stimulates the release of Ca^{2+}, and then activates nitric oxide synthase (NOS). DAG activates protein kinase C (PKC) cascade, the activator of RAS. RAS then activates the MAPK cascade of Raf-1, MAPK-/ERK kinase (MEK1/2) and ERK1/2. $G\alpha_q$ also signals directly via RAS and activates the MAPK cascade bypassing PKC. APJ receptor also mediates a mechanosensory response via a β-arrestin-dependent pathway independent of G-protein. Arrows indicate activation and blunted arrow indicates inhibition. Adapted from Chapman (60) and O'Carroll (53).

1.3 Growth factors and cancer progression

1.3.1 The stepwise progression of tumorigenesis

Cancer is a multifactorial disease involving abnormal, uncontrolled growth of tissue that arises from dysregulation of normal cellular behaviour, resulting in alterations in many physiological processes. Tumor initiation is the result of acquisition of oncogenic attributes, which lead to activation of proliferative signalling, loss of cell growth control and resistance to cell death. Thereby, the initial tumor cells are able to sustain unlimited growth and escape the mechanisms of normal cellular homeostasis. Subsequently, the mutation-bearing clones expand, leading to the progression of the tumor, such as *in situ* carcinoma surrounded by the basal membrane. Cancer cells then migrate and penetrate into neighboring tissues and disseminate through lymphatic and blood vessels. New blood vessels generated by angiogenesis induction support tumor growth and establish large-size tumors. Consequently, tumor cells are able to interact with the cellular microenvironment, avoid immune destruction and invade the tissue architecture to metastasize to distant organs (67). While different types of cancers show significantly different features, there are fundamental changes, referred to as hallmarks of cancer, shared by almost all of them. These properties include sustained proliferative signaling, evasion of growth suppressors, escape from apoptosis, ability to invade and metastasize to other tissue, limitless replicative potential, sustained angiogenesis, evasion of immune surveillance, and deregulated metabolism. In addition, two main enabling characteristics: genome instability, which generates random mutations, and tumor-promoting inflammation, can contribute to expediting the acquisition of multiple hallmarks and fostering their capabilities (68, 69).

In normal tissues, the production and release of growth-promoting signals are under precise control to regulate the cell growth-and-division cycle, thereby ensuring homeostasis of cell

number and thus maintaining normal tissue architecture and functions. Normal cells mostly stay in a quiescent state and require external growth signals that are transmitted through receptors to direct cell grow and divide. In contrast, cancer cells can grow and divide without external growth signals since they can generate their own growth factor ligands and express cognate receptors that respond to these growth signals, resulting in autocrine proliferative stimulation. Alternatively, external growth signals can be facilitated in cancer cells by elevating the receptors protein level or altering their molecular structure, rendering such cells hyper-responsive to limited amounts of growth factor ligand (68). Moreover, cancer cells may send signals to stimulate normal cells within the supporting tumor-associated microenvironment to provide the cancer cells with various growth factors (70). Consequently, tumor cells are able to proliferate in an abnormal manner.

The unscheduled cell growth in tumor cells can also result from mutations of the regulatory proteins in cell cycle control and dysfunction of checkpoint machineries, which leads to uncontrolled cell growth and cell division. Mammalian cell growth is mediated via cell cycle progression. The cell cycle is divided into a series of four distinct phases: mitosis phase (M), gap 1 phase (G1), synthesis phase (S), and gap 2 phase (G2). In the G1 phase, the cell is growing in size and preparing for chromosomal replication. In the subsequent S phase, DNA synthesis occurs and then the cell continues into the G2 phase for cell division preparation. After the G2 phase, replicated chromosomes are segregated, and the cell divides into two genetically identical daughter cells during M (mitosis) phase. Cells will re-enter the G1 phase, or enter a quiescent state known as G0 phase.

Proper progression through the cell cycle is modulated by accurate transitions to ensure that specific events take place in an orderly manner. The regulatory proteins involved in phase

transitions are called cyclin-dependent kinases (CDK), whose activation is driven by cyclin and CDK inhibitors (CKIs). CDK4 and CDK6, complexed with cyclin D1, 2 or 3 are responsible for promoting G1 phase progression, and the CDK2/cyclin E complex functions in driving the cells from late G1 to the early S-phase. Following nuclear envelope breakdown, the formation of the CDK1-cyclin B complexes facilitates cells through mitosis (71). Cell cycle progression is negatively regulated by CKIs, preventing replication of abnormal DNA and allowing cells to maintain in a quiescent state. Cell cycle is also regulated by checkpoints that assure the previous phase has been successfully accomplished before entering the next phase. For instance, once DNA damage has occurred, DNA damage checkpoints are activated and block cell cycle progression, allowing the DNA to be repaired. Therefore, the cell cycle checkpoints also serve for suppressing tumorigenesis.

In addition to inducing and sustaining positive growth-stimulatory signals, cancer cells are generally insensitive to anti-growth signals that negatively regulate cell proliferation. The growth of normal cells is controlled by growth inhibitors to maintain cellular and tissue homeostasis. These growth inhibitors are frequently inactivated in cancer cells, resulting in evasion of growth suppression. For instance, the RB (retinoblastoma-associated) protein receives diverse extracellular and intracellular signals and decides whether to allow cell to proceed through its growth-and-division cycle (72). Cancer cells with a disrupted RB pathway lack a critical gatekeeper of cell cycle progression, which allows persistent cell proliferation. Another mechanism to control cell growth is that normal cells will stop replicating when cells come in contact with each other, known as contact inhibition. Cancer cells show no contact inhibition, and thus can grow in an uncontrolled manner, regardless of their neighbouring cells (73).

Irreversible cell damages can render a cell useless or even harmful to an organism. Programmed cell death (PCD) plays an important role in elimination of damaged cells and thus preserves tissue homoeostasis. Apoptosis, autophagy and programmed necrosis are the three main forms of PCD (74). However, cancer cells are characteristically able to bypass these cell death mechanisms even though cells become abnormal. Alteration of key regulators and many signalling pathways involved in PCD has been shown to be associated with cancer initiation and progression (74). For example, the tumor suppressor gene *TP53* encoding p53 protein is frequently mutated in human cancer (75) and has been linked to apoptosis (76). p53 can be activated by stress signals, such as DNA damage and oxidative stress, and can halt further cell cycle progression until these conditions have been normalized. In addition, p53 also can activate DNA repair proteins to conserve genome stability. Alternatively, in the face of irreparable damage, p53 can initiate apoptosis or senescence. In tumor cells, loss-of-function mutations in *TP53* eliminate the critical damage sensor from the anti-proliferation programs as well as apoptosis-inducing circuitry, result in growth suppressor evasion and apoptosis resistance of tumor cells.

1.3.2 Growth factors and their receptors in regulation of cancer progression

Emerging evidence has indicated that signaling molecules, such as the growth factors and their receptors, previously implicated in embryonic development, are important regulators of multistep tumor initiation and progression. Growth factors are proteins or steroidal hormones that maintain cell homeostasis via regulating a variety of cellular processes, including stimulating cellular growth, healing, and differentiation (77). Furthermore, they act as the key mediator in signaling between cells to establish intracellular or extracellular communications

with their specific receptors present in cellular membranes (78). The overexpression of growth factor and receptor contributes to constitutive signaling in human cancer (79). Although there is considerable overlap and crosstalk, two of the most important signaling pathways activated by various growth factors are RAS/MEK/ERK cascade and PI3K/AKT signaling pathways (80).

In the ERK1/2 kinase pathway, binding of ligands to their receptors causes activation of the Shc/Grb2/SOS coupling complex, which in turn activates RAS. Stimulated RAS exchanges guanosine diphosphate (GDP) to guanosine triphosphate (GTP) and undergoes a conformational change to its active state. Active GTP-loaded RAS molecules stimulate RAF kinases, which activates MEK1/2 and then catalyzes activation of ERK1/2. Active ERK molecules translocate to the nucleus to stimulate multiple transcription processes that are essential for cellular behaviors such as cell growth, cell cycle progression, cell survival, cell migration, and angiogenesis (81).

The PI3K/AKT is an intracellular pathway that regulates cell cycle, cellular quiescence, and growth, and is shown to be involved in cancer. In humans, PI3K exists as a heterodimer of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. Activation of PI3K is achieved by binding of the regulatory p85 subunit to an activated RTK or through the stimulation of the small GTP-binding protein RAS. On activation, the PI3K substrate phosphatidylinositol (3, 4, 5)-phosphate (PIP3) serves as a docking site for proteins containing phospholipid-binding domains, including protein kinase B and AKT. AKT has a large number of substrates that regulate mainly survival and metabolism. Thus, PI3K/AKT can regulate the activation of tumor cells by direct downstream effects. In addition, this pathway can crosslink with the action of ERKs through activation of AKT (77).

1.4 Thesis objectives

Most recent studies on APELA are now focused on its role in embryonic development (10-12) or embryonic stem cells (16, 17). Many of the cellular processes involved in embryonic development are mediated by growth factors and their receptors. In many cases, the abnormal expression or even mutation of these same growth factors and receptors are also of relevance to the origins and development of a wide variety of cancers. Until now, whether APELA has a role in human cancers has not yet been investigated. Therefore, the overall goal of this study is to determine if APELA plays a role in human cancers and identify signaling pathways that are mediated by APELA. Our hypothesis is that *APELA* is a previously unidentified gene that encodes a growth factor and a lncRNA that function to promote cell growth in cancers.

To achieve this, I have three objectives.

Objective 1: To explore the expression of APELA in human cancers. Using in-house RNA-seq data from different types of tumor tissues, I am able to analyze *APELA* mRNA expression in a variety of cancers

Objective 2: To investigate the potential functional roles of APELA in human cancers *in vitro*. Generation of APELA deficient cell lines by CRISPR/Cas9 system enables us to evaluate the effects of APELA expression on basic cellular functions such as cell growth, apoptosis, and survival. siRNA knockdown and over-expression have also been used for APELA functional studies.

Objective 3: To elucidate the signaling mechanisms mediated by APELA to regulate cell behaviors in cancer cell lines. Using biochemical methods, I have examined the activation of internal signaling pathways stimulated by APELA in cancer cells.

2 CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines and culture

Ewing sarcoma cell lines A673, TC71 and TC32 are generously provided by Dr. Poul Sorensen (Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, BC). Ovarian cancer cell lines CAOV3, KURAMOCHI, JHOC-5, JHOC-7, JHOC-9, OVCAR-4, OVISE, OVMANA, OVTOKO, RMG-2, TOV21G, and VOA295 are generous gift from Dr. David Huntsman (Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, BC). Cells were maintained in a humidified incubator at 37˚C with 5% CO_2. See Table 2.1 for a list of cell lines, histology and culture conditions. Growth medium for the cells was changed every two to three days. Cells were passaged using 0.25% Trypsin EDTA (Stemcell).

2.2 Molecular cloning, plasmid preparation, and transfection

To create *APELA*-expressing construct, Human *APELA* open reading frame (ORF) was cloned in pLs lentiviral vector with an IRES-Tomato fluorescent marker downstream of the *APELA* gene. The high fidelity enzyme Phusion (Invitrogen) was used to amplify respective DNA fragments by PCR to make these constructs. DNA was prepared by EndoFree Plasmid Maxi Kit (Invitrogen) and diluted to 1 μg/μl in endotoxin-free TE buffer. Cells were transfected at 60-70% confluency using polyethylenimine (PEI, Polysciences Inc) following a published protocol (82). For p53 protein overexpression, the pCMV-Neo-Bam-p53 vector (Addgene plasmid #16434) was used.

Table 2.1 Ovarian cancer and Ewing sarcoma cell lines

Cell line	Histology	Media
A673	Ewing sarcoma	DMEM/10%FBS (Stemcell)
TC71	Ewing sarcoma	DMEM/10%FBS (Stemcell)
TC32	Ewing sarcoma	DMEM/10%FBS (Stemcell)
CAOV3	Ovarian adenocarcinoma	199/105 (Sigma-Aldrich) /5%FBS
KURAMOCHI	Ovarian undifferentiated carcinoma	RPMI/5%FBS (Stemcell)
JHOC-5	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
JHOC-7	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
JHOC-9	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
OVCAR-4	Ovarian adenocarcinoma	199/105 (Sigma-Aldrich) /5%FBS
OVISE	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
OVMANA	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
OVTOKO	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
RMG-2	Ovarian clear cell carcinoma	RPMI/5%FBS (Stemcell)
TOV21G	Ovarian clear cell carcinoma	199/105 (Sigma-Aldrich) /5%FBS
VOA295	Ovarian clear cell carcinoma	199/105 (Sigma-Aldrich) /5%FBS

2.3 RNA extraction, reverse transcription and cDNA amplification

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Chloroform (EMD Millipore) was then added at 1/5 the volume of Trizol and vigorously shaken. Samples were spun at high speed for 10 minutes at 4˚C. RNA was in the clear upper, aqueous phase and then precipitated with an equal volume of isopropanol (EMD Millipore) at room temperature for 10 min. Samples were spun down at high speed at 4˚C for 10 minutes. Isopropanol was removed and pellets were washed with 70% ethanol (Commercial Alcohols). Pellets were re-suspended in RNase and DNAase free water (Invitrogen). The quality of the RNA was assessed using the Nanodrop 1000 (Pall Corporation) and RNA was stored at -80˚C. RNA was treated with DNAase I (Invitrogen) for 15min at room temperature. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). cDNA was stored at -20 °C.

2.4 Reverse transcription quantitative real-time PCR (qRT-PCR)

Quantitative PCR was performed using the FastStart Universal SYBR Green Master (Rox, Roche) or TaqMan Fast Advanced Master Mix (Invitrogen) according to the manufacturer's protocol on the ABI 7900HT Fast Real-Time PCR System. Cycling conditions were [40x 95°C for 15s, 60°C for 1min, 72°C for 1 min]. All samples and controls were assayed in triplicate. Relative quantification was used for qRT-PCR. A housekeeping gene GAPDH was used as an endogenous control. Obtained values were normalized to GAPDH Ct values and the change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method for gene expression. All primers used in qRT-PCR as well as cloning are found in Table 2.2.

Table 2.2 Primers used in this research

Primers for expression vector cloning		
Name	**Forward Primer sequence 5'to 3'**	**Reverse Primer Sequence 5'to 3'**
APELA	TATGGATCCATGAGATTTCAGCAATTCCT	TATAGATCTTCAGGGAAAGGGTACTCG
Primers for qRT-PCR		
Target	**Forward Primer sequence 5'to 3'**	**Reverse Primer Sequence 5'to 3'**
APELA	TTCAGCAATTCCTTTTTGCAT	CAATTGTGTTTGCGCAGTTT
Primers for Taqman qRT-PCR		
Target	**Company, Catalog #**	
APLN	IDT, Hs.PT.58.1969360	
APLNR	IDT, Hs.PT.58.21382524.g	
GAPDH	IDT, Hs.PT.39a.22214836	
Primers for guide Sequence		
Target	**Forward Primer sequence 5'to 3'**	**Reverse Primer Sequence 5'to 3'**
gRNA outside *APELA* Exon1	CACCGTTTGGAGAGTTCGTGTAAC	AAACGTTTACACGAACTCTCCAAAC
gRNA within *APELA* Exon2	CACCGAGGGAAAGGGTACTCGTGAA	AAACTTCACGAGTACCCTTTCCCTC
Primers for *APELA* knockout analysis		
Target	**Forward Primer sequence 5'to 3'**	**Reverse Primer Sequence 5'to 3'**
Analysis of APELA gene knockout	ATGAGTTTGTGGCTTGCCTG	AATCGGCTCGTTCTGCATAA

2.5 Dicer-substrate RNA-mediated gene silencing and siRNA transfection

To knock down endogenous *APELA*, Dicer-substrate RNAs (DsiRNAs) with 27mer duplex RNAs were designed to specifically target *APELA* (IDT) (83, 84). Cells were transfected with 10nM DsiRNA using Lipofectamine RNAiMAX (Invitrogen). NC1 control (IDT) non-targeting any sequence in human transcriptomes was used as a transfection control. For the inactivation of p53, the cells were transfected with ON-TARGETplus SMARTpool p53 (50 nM) siRNA (Dharmacon). Three independent experiments of knockdowns and non-targeting controls were performed in triplicate. The knockdown efficiency was examined by qRT-PCR.

2.6 Establishment of APELA knockout cell lines using CRISPR/Cas9 system

The targeted gRNA expression oligos were introduced into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector which was a gift from Feng Zhang (Addgene plasmid #42230) (85). gRNA design was based on CRISPR design (http://crispr.mit.edu/). The sequences of these oligos are shown in Table 2.2. 1 μg of pX330 plasmid DNA containing each target gRNA sequence was transfected into A673 and OVISE (1×10^5 cells), respectively. Lipofectamine Lipo2000 (Invitrogen) was used to transfect the plasmids, and transfected cells were trypsinized and seed at low density for single cell colony formation. Single colonies were selected, and each colony was passaged, expanded and genotyped. DNA was isolated using DNAZol reagent (Invitrogen). The genomic region surrounding the CRISPR/Cas9 target site for *APELA* was PCR amplified, and PCR products were purified using a Gel Extraction Kit (Roche) according to the manufacturer's protocol. The amplicons were cloned into the PCR2.1 vector (Invitrogen) and then sequenced.

Table 2.2 shows the primer sequences. *APELA* RNA expression levels in each cell line were examined using qRT-PCR.

2.7 Recombinant peptide and treatment

APELA peptide was generated according the whole mature peptide of 32 amino acids (QRPVNLTMRRKLRKHNCLQRRCMPLHSRVPFP, Biomatik, Wilmington, Delaware, USA). For cell growth analysis, 10uM peptide was added to cells for consecutive days before fixation. For phospho-AKT and phospho-ERK1/2 activation analysis, cells were treated with 10uM peptide for 15 min and then collected for protein extraction.

2.8 Cell viability assay

Crystal violet staining was performed to assess cell viability. The cells were incubated in 24-well plates at 1.0×10^5 cells per well and cell viability was measured at the indicated time points. The culture medium was removed. The cells were washed once with PBS (Stemcell) and then were fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 10 min or at 4˚C overnight. Afterwards, the cells were stained with 0.1% w/v crystal violet (Sigma-Aldrich) in 10% ethanol (Commercial Alcohols) for 20 min. Plates were washed four times in tap water and dried overnight. After the plates were photographed, blue dye was dissolved in 500 μl of 10% acetic acid (Fisher Chemical) and emission spectra were measured at an excitation wavelength of 570 nm using a Multimode Microplate Reader (Thermo Fisher Scientific). Three independent experiments were performed in triplicate.

2.9 Cell cycle analysis with flow cytometry

Cell cycle analysis was performed using Propidium Iodide (PI, Sigma-Aldrich) staining. The cells were seeded in 6-well plates (1×10^6 cells/well). After incubation for 48h or transfected with DsiRNA for 48h, the cells were collected and fixed with in ice cold 70% ethanol at -20 ˚C overnight. Samples were then re-suspended in cell cycle buffer (0.5 mg/ml RNase A and 5 ug/ml PI in PBS) at a concentration of 5×10^5 cells/ml. Samples were analyzed for DNA content (PI staining) by flow cytometry (Becton Dickinson FACSCalibur) measuring emission with a 580 nm filter. . The data were analyzed using FlowJo to generate percentages of cells in G1, S and G2/M phases.

2.10 Immunofluorescence

Cells were cultured in plates with coverslips for two days and then fixed with 4% PFA for 10-20 minutes at room temperature or overnight at 4˚C. After washed with PBS three times for 5 minutes each, cells were blocked with blocking solution containing 5% Bovine Serum Albumin (BSA, Roche) with 0.1% Triton X-100 (Sigma) in PBS for 1 hour at room temperature. Primary antibodies phosphorylated Histone H3 (pHH3) and E-cadherin (Table 2.3) were diluted in blocking solution at 1:100 ratio overnight at 4˚C. The following day, the cells were washed with PBS three times for 5 minutes at room temperature. Alexa 594-labeled goat anti-mouse or Alexa 488-labeled goat anti-rabbit IgG (Thermo Fisher Scientific) were used as a secondary antibody. Cells were counterstained with 4’, 6’-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, 1 µg/mL, Sigma) for 10 minutes at room temperature to label nuclei. Cells were mounted with 50-100 µL of 20 mg/mL DABCO (1, 4-diazabicyclo [2.2.2] octane, Sigma). Coverslips were then turned over on slides and sealed with nail polish for visualization. Images were captured on

Nikon Eclipse A1+ confocal microscopy System with the NIS-Elements Advanced Research software. For immunofluorescence images, cell counts for pHH3 positive nuclei were performed on three independent experiments and averaged using ImageJ software.

2.11 Protein extraction and western blot analysis

Cell lysates were prepared using lysis buffer (50mM Tris-HCl, PH7.4; 150mM NaCl; 1mM EDTA, PH8.0; 0.5% Triton) supplemented with one tablet of protease inhibitor cocktail (Roche). For phosphorylated proteins detection, 10mM NaF, 10mM sodium pyrophosphate and 2mM sodium orthovanadate were added to lysis buffer. The protein concentrations were determined using a protein assay kit with bovine serum albumin (BSA) standards according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with TBST containing 2% BSA for 1h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies (Table 2.3). Membranes were then washed, probed with a secondary horseradish peroxidase (HRP)-conjugated antibody (Table 2.3) for 1h at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL, Pierce) kit. Antibodies were listed in Table 2.3.

2.12 Bioinformatic analysis of RNA-seq data and microarray data

RNA-seq data from normal tissue samples of 122 human individuals representing 32 different tissues are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2836. RNA-seq data from TCGA cancer dataset was analyzed

by Dr. Ewan Gibb (Canada's Michael Smith Genome Sciences Centre, University of British Columbia). The microarray data of ovarian clear cell carcinoma mouse model is available through the Gene Expression Omnibus (GEO: https://www.ncbi.nlm.nih.gov/geo/) under the accession number: GSE57380.

2.13 Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments. The statistical analyses were conducted using a standard Student's *t* test for paired data. Multiple comparisons were first analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. *p* values of <0.05 were considered statistically significant.

Table 2.3 Antibodies for Western blot and immunofluorescence analysis

Primary antibodies				
Antibody against	**Host species**	**Company**	**Catalog #**	**Dilution**
AKT	Mouse	Invitrogen	AH0112	1:1000
Phospho-AKT	Rabbit	Invitrogen	44621G	1:1000
CDK4	Rabbit	Santa Cruz	sc-260	1:200
Cyclin D1	Mouse	Santa Cruz	sc-246	1:200
Cyclin B1	Rabbit	Cell Signaling Technology	4138	1:1000
ERK1/2	Rabbit	Cell Signaling Technology	9102	1:1000
Phospho-ERK1/2	Mouse	Cell Signaling Technology	9106	1:1000
p53	Rabbit	Santa Cruz	sc-6243	1:500
pHH3	Rabbit	EMD Millipore	06-570	1:100
E-cadherin	Mouse	BD Biosciences	610181	1:100
β-Actin	Mouse	Abcam	Ab6276	1:1000
Secondary antibodies				
HRP-conjugated	Mouse	Jackson ImmunoReseach	115-035-003	1:5000
HRP-conjugated	Rabbit	Jackson ImmunoReseach	111-035-003	1:5000
ALEXA FLUOR 488	Rabbit	Thermo Fisher Scientific	A11008	1:500
ALEXA FLUOR 594	Mouse	Thermo Fisher Scientific	A31623	1:500

3 CHAPTER 3: RESULTS

3.1 Examination of APELA expression in human cancers

To determine the tissue distribution of APELA, APJ receptor and Apelin in human normal tissues, published RNA sequencing (RNA-seq) data generated from samples representing all major tissues and organs (n = 32) in the human body (86) were used for analysis. As shown in Figure 3.1A, wide expression of Apelin and APJ receptor mRNA was found in tissues including heart, kidney, lung, placenta, adipose, and the central nervous system. In contrast, mRNA expression of *APELA* was only detected in the adult kidney (FPKM = 0.9) and prostate (FPKM = 2) at very low levels, which is consistent with previous studies on *APELA* tissue distribution (14, 21).

To determine if APELA is expressed in human cancer, the cancer genome atlas (TCGA) RNA-seq datasets (APPENDIX I, June 2015) generated from a broad panel of human cancers (Figure 3.1B and Figure 3.1C) were analysed with the help from Dr. Ewan Gibb (Canada's Michael Smith Genome Sciences Centre, University of British Columbia). In all available tumor (n = 6401) and normal (n = 561) RNA-seq libraries, *APELA* mRNA was detected in many types of tumors including kidney renal clear cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, ovarian serous cystadenocarcinoma (OV) and thyroid carcinoma at low level (mean RPKMS < 5). Thus, the broad expression of *APELA* mRNA in human cancers prompted us to explore the idea that the APELA is potentially critical for cancer initiation and development. In TCGA RNA-seq data, 13.1% of OV samples had detectable *APELA* mRNA expression, although the average *APELA* level was relatively low (RPKMS = 1.23 ± 0.23). Based on RNA-seq analysis of *APELA* expression in normal human tissues (Figure 3.1A), *APELA* is not present in adult human ovary as well as other tissues including fallopian tube, endometrium, from which

the majority of ovarian cancer originated from. Therefore, the high percentage of OV patients with detectable *APELA* levels suggested that APELA may exert biological functions in ovarian cancers.

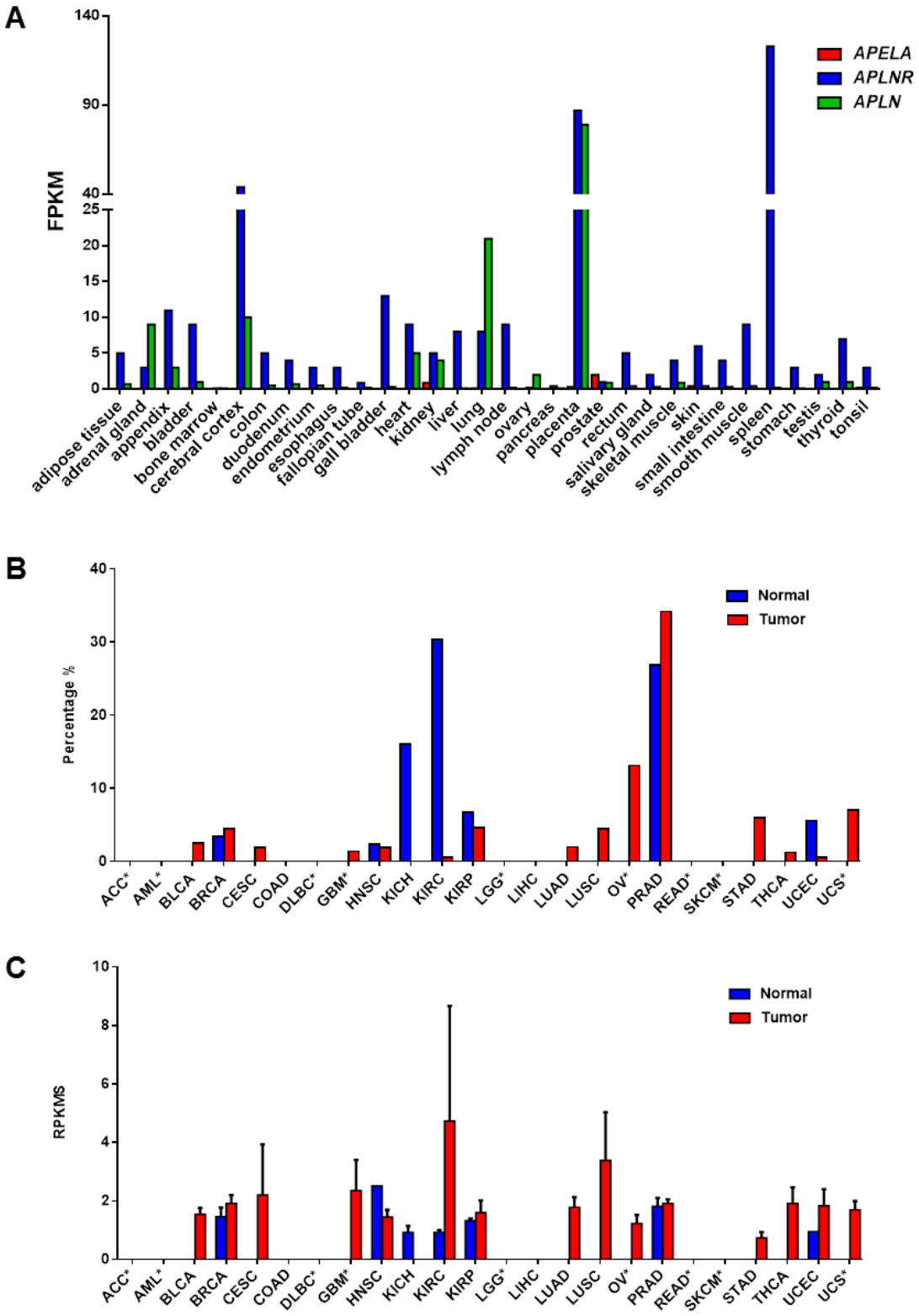
A
FPKM
APELA
APLNR
APLN
adipose tissue
adrenal gland
appendix
bladder
bone marrow
cerebral cortex
colon
duodenum
endometrium
esophagus
fallopian tube
gall bladder
heart
kidney
liver
lung
lymph node
ovary
pancreas
placenta
prostate
rectum
salivary gland
skeletal muscle
skin
small intestine
smooth muscle
spleen
stomach
testis
thyroid
tonsil
B
Percentage %
Normal
Tumor
ACC*
AML*
BLCA
BRCA
CESC
COAD
DLBC*
GBM*
HNSC
KICH
KIRC
KIRP
LGG*
LIHC
LUAD
LUSC
OV*
PRAD
READ*
SKCM*
STAD
THCA
UCEC
UCS*
C
RPKMS
Normal
Tumor

Figure 3.1 APELA expression levels in human normal and tumor tissues

(A) *APELA*, Apelin (*APLN*) and APJ receptor (*APLNR*) expression level in RNA-seq analysis from tissue samples of 122 human individuals representing 32 different tissues. The raw sequencing data are available at ArrayExpress under the accession number E-MTAB-2836 (www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-2836/). (B) The percentage of *APELA* detected samples and C) *APELA* expression in 24 TCGA cancer types and corresponding normal tissues. Red bar indicates the tumor samples and blue bar indicates normal samples analyzed. The star (*) indicates that corresponding normal samples were not available. ACC, adrenocortical carcinoma; AML, Acute Myeloid Leukemia; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma; UCEC, uterine corpus endometrial carcinoma and UCS, uterine carcinosarcoma. For patient sample numbers, see APPENDIX I.

3.2 Characterization of APELA function in ovarian cancer cells

3.2.1 APELA mRNA level is elevated in ovarian clear cell carcinoma

Ovarian cancer is a leading cause of death resulting from gynecological malignancies and is a heterogeneous set of diseases which can arise from different ovarian cell types. The main histotypes of ovarian cancer are epithelial in origin and can be subdivided into 5 specific epithelial subtypes, high grade serous carcinoma (HGSC), clear cell carcinoma (CCC), endometrioid carcinoma (EC), mucinous carcinoma (MC) and low grade serous carcinoma (LGSC). There are several rare types of non-epithelial ovarian cancers, such as granulosa cell tumor (GCT). As mentioned in section 3.1, APELA is not present in normal tissue of ovarian cancer origin, such as fallopian tube and endometrium. To investigate the expression of APELA in ovarian cancer tissues, the mRNA expression level of *APELA* in 20 patients with EC, 16 with CCC, 20 with HGSC, and 14 with GCT was compared by quantitative real-time PCR (qRT-PCR). As shown in Figure 3.2A, *APELA* was detected in some of the CCC samples and is significantly higher compared to other subtypes of ovarian cancer tissues. These results are consistent with the *APELA* levels in different ovarian cancer subtypes from microarray analysis (GSE65986, data not shown). Interestingly, clear cell carcinoma could be divided into two distinct groups: one with high expression of *APELA* mRNA and the other group with low *APELA* levels.

Genetically engineered mouse models facilitate us to investigate the molecular mechanisms of human disease pathogenesis, including tumor initiation and progression. To further examine the expression of APELA in ovarian clear cell carcinoma and normal ovarian tissue, microarray data from a newly reported ovarian clear cell carcinoma (OCCC) mouse model (87) was used. This mouse model genetically and histologically resembles the human

disease by inducing a localized deletion of ARID1A and the expression of the PIK3CA^{H1047R} substitution mutation (87). Compared with control ovarian tissue, *Apela* mRNA was upregulated in primary tumors, consistent with the elevated expression level of *APELA* in human OCCC (Figure 3.2B). Overall, the enhanced expression of APELA in human OCCC patient samples and tumor tissues from mouse OCCC model suggests a potential physiologic role of APELA specifically in ovarian clear cell carcinoma.

3.2.2 APELA and APJ receptor expression pattern in ovarian cancer cell lines

To analyze *APELA* mRNA expression in ovarian cancer cell lines, qRT-PCR was performed on 3 high-grade serous carcinoma and 9 clear cell carcinoma cell lines. As shown in Figure 3.3, *APELA* mRNA was detected in several clear cell carcinoma cell lines (88) but not in high-grade serous carcinoma cell lines. Unexpectedly, both APJ receptor and Apelin mRNA were undetectable in the most cell lines expressing *APELA* mRNA (Figure 3.3), suggesting that APELA may have functions that are independent of Apelin/APJ receptor signaling pathway. To better understand the basic functions of APELA, the ovarian clear cell carcinoma cell line OVISE, which expresses *APELA* mRNA, was used.

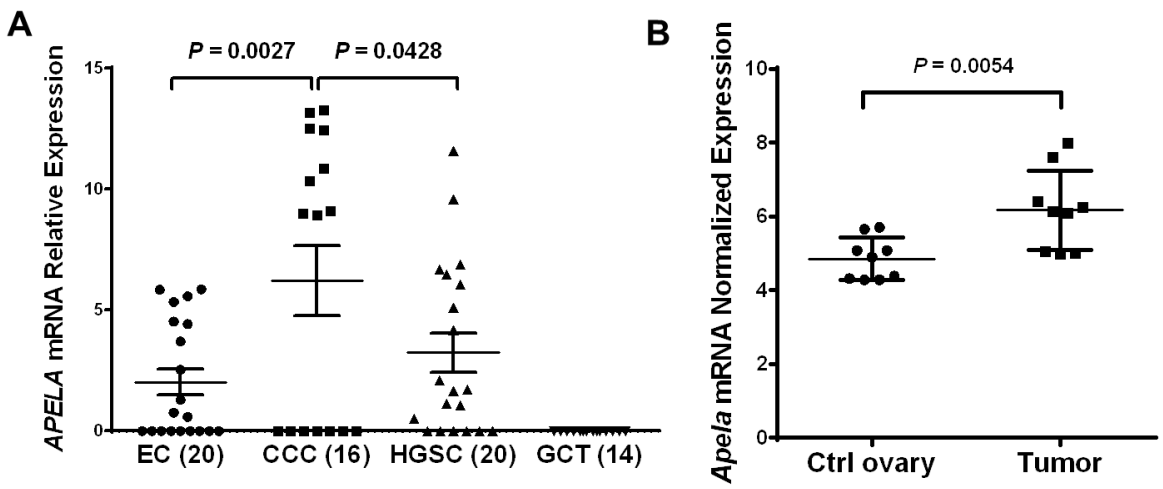

Figure 3.2 APELA mRNA level is upregulated in ovarian clear cell carcinoma

(A) *APELA* mRNA expression from 70 ovarian cancer samples. qRT-PCR showed highest levels of *APELA* in clear cell carcinoma (CCC), which was significantly higher than all other groups. The relative expression of APELA was normalized to GAPDH. Significant differences in *APELA* expression level between endometrioid carcinoma (EC, n=20), high-grade serous carcinoma (HGSC, n=14) or granulosa cell tumor (GCT, n=20) and clear cell carcinoma (CCC, n=16) patient samples were expressed as mean ± SEM and statistical analysis was conducted by one-way ANOVA. (B) Microarray analysis of *Apela* expression in mouse ovary clear cell carcinoma model. Significant differences based on average expression ± SEM between primary tumors and matched control ovaries were calculated using a two-tailed Student's *t* test, n=9.

3.2.3 CRISPR/Cas9-mediated knockout of APELA decreases OVISE cell growth

To assess the function of APELA in ovarian cancer cells, OVISE clones with homozygous genetic deletion of APELA were generated using CRISPR/Cas9 technology (89) (Figure 3.4A). qRT-PCR showed that *APELA* mRNA expression was completely disrupted in *APELA* knockout OVISE cells compared with the parental OVISE cells. Since APELA was reported to promote growth and pluripotency in hESCs (17), I investigated the effect of APELA on cell growth of ovarian clear cell carcinoma cells. Two knockout clones were selected for the crystal violet staining assay and results demonstrated that depletion of APELA expression significantly decreased OVISE cell growth by 60% in one of the *APELA* knockout OVISE clone ($p < 0.001$), and 30% in another knockout OVISE clone ($p < 0.01$) when compared with the parental OVISE cells (Figure 3.4 C). These findings indicate that APELA may play a role in cell growth of ovarian clear cell carcinoma cells.

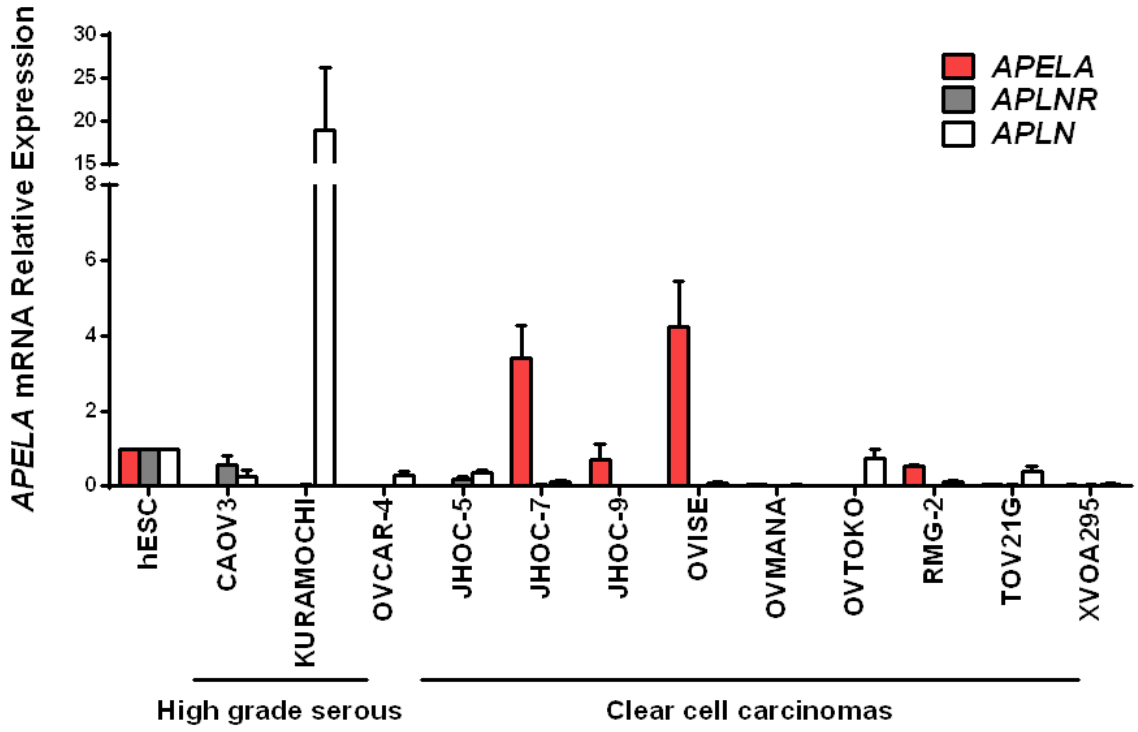

Figure 3.3 APELA, APJ receptor and Apelin mRNA levels in ovarian cancer cell line

The 12 ovarian cancer cells lines were analyzed by qRT-PCR for APELA, APJ receptor (*APLNR*) and Apelin (*APLN*) expression. The relative expression of APELA was normalized to GAPDH, and hESCs were used as a reference cell line. The results of the qRT-PCR are expressed as the mean ± SEM of three independent experiments.

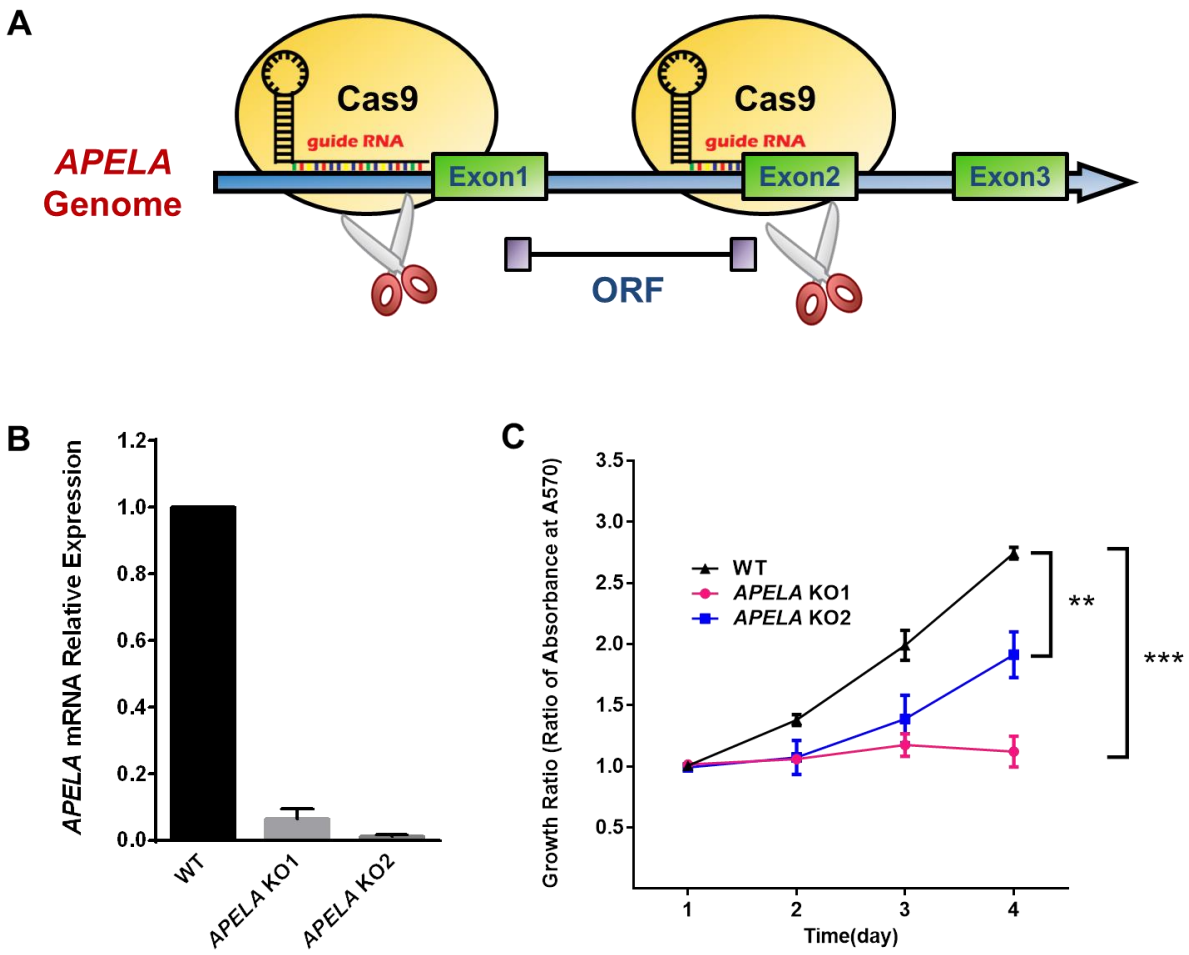

Figure 3.4 APELA knockout decreases cell growth in ovarian clear cell carcinoma cell line OVISE

(A) Schematic diagram of targeted deletion of human *APELA* with CRISPR/Cas9. Guide RNAs (gRNAs) targeting the human *APELA* locus were designed to delete the genomic DNA containing the APELA open reading frame (ORF). (B) The efficiency of targeted deletion with CRISPR/Cas9 was determined by qRT-PCR in two clones and parental OVISE cells. (C) Cell growth of OVISE cells and *APELA* knockout cells (KO) assessed by crystal violet assay at indicated time points. The results are expressed as the mean ± SEM of three independent experiments performed in duplicate. **$p<0.01$, ***$p<0.001$ compared with parental OVISE cells (WT) by two-way ANOVA.

3.2.4 Loss of APELA induces G2/M phase arrest in OVISE cells

To further elucidate the inhibitory effect of APELA deficiency on cell growth, we examined the cell cycle distribution in OVISE cells by flow cytometry. Quantitation of cells in G1, S, and G2/M phase of the cell cycle showed that cells accumulated in G2/M phase in *APELA* knockout OVISE cells, whereas the cell population in G1 was decreased (Figure 3.5A). Consistent with the change of cell cycle distribution, Western blot analysis showed significant decreased levels of Cyclin B1, the G2/M transition regulator, in *APELA* knockout cells compared with parental OVISE cells (Figure 3.5B). However, the level of CDK4 and Cyclin D1 displayed no obvious difference (Figure 3.5B), revealing that knockout of *APELA* mainly affects G2/M transition rather than G1/S transition in OVISE cells. In order to confirm the effect of CRISPR/Cas9-mediated knockout of *APELA*, Dicer-substrate short interfering RNAs (DsiRNAs) were chosen to knock down *APELA*. DsiRNAs are synthesized 27mer duplex RNAs optimized for Dicer processing and show increased potency in RNA interference compared to traditional 21mer siRNAs. DsiRNAs against *APELA* achieved approximately 70% depletion of *APELA* mRNA relative to the control in which the cells were transfected with a non-targeting human transcriptome control DsiRNA (Figure 3.6A). Consistently, cell cycle analysis demonstrated that OVISE cells transfected with Dsi*APELA* also displayed an accumulation of cells in the G2/M phase compared to control cells (Figure 3.6B), which also ruled out the effect of clonal variation by artificial selective pressure of culture adaptation in the CRISPR/Cas9 system.

Furthermore, phospho-histone H3 (pHH3, a marker of mitosis) protein levels was investigated using immunofluorescence staining. pHH3 positive cells were reduced by more than two-fold in the *APELA* knockout cells compared with parental OVISE cells (Figure 3.5C), confirming the growth defect induced by knocking out *APELA*. Moreover, the results

demonstrated a large proportion of knockout cells were not actively dividing, which suggested that loss of *APELA* delays entry into mitosis in OVISE cells. Collectively, these observations suggested that APELA regulates cell growth by promoting G2/M transition in OVISE cells.

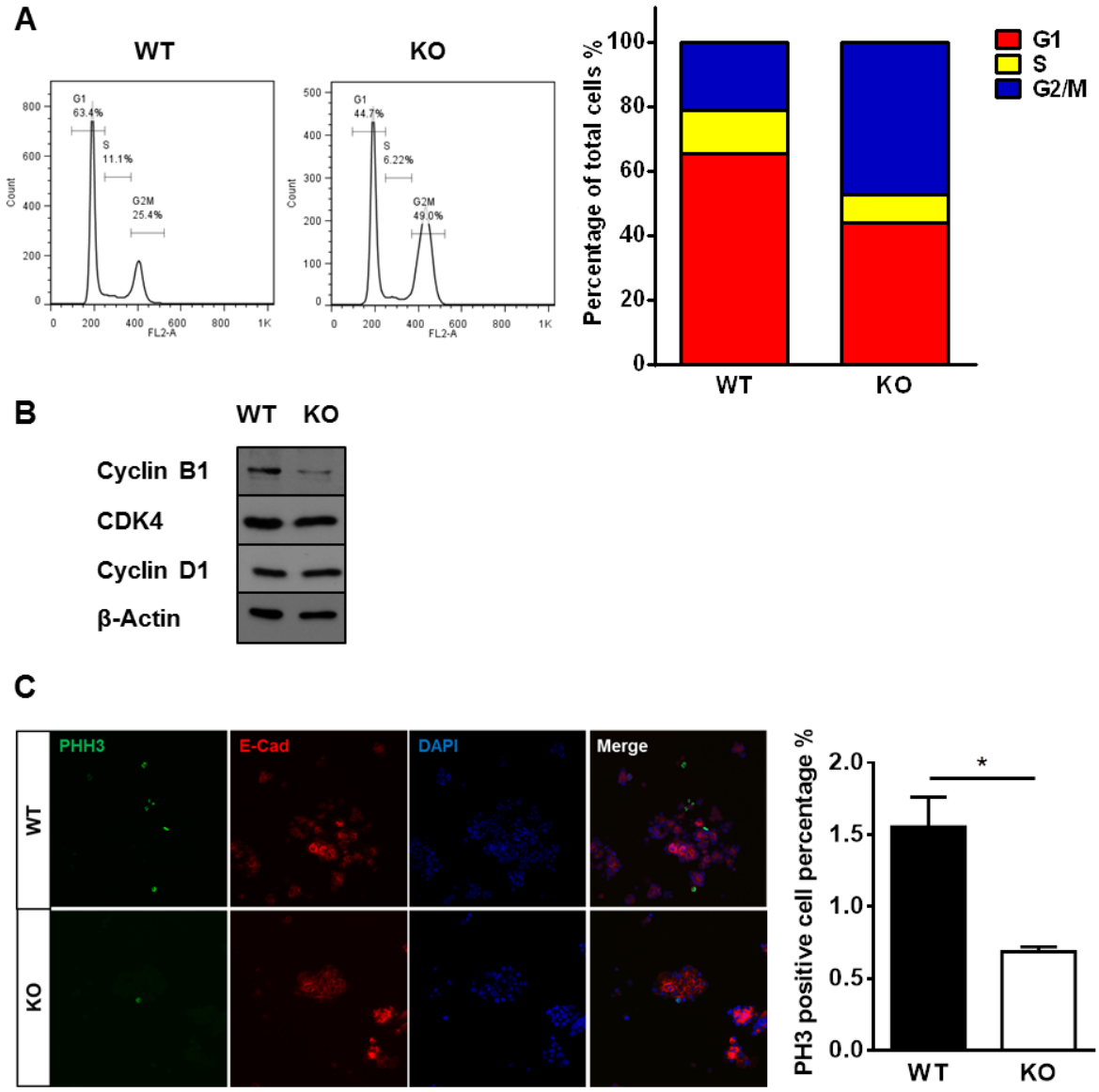

Figure 3.5 APELA knockout induces G2/M phase arrest in OVISE cells

(A) Cell cycle analysis of parental (WT) and *APELA* knockout (KO) OVISE cells. Cells were stained with propidium iodide (PI) and analyzed using flow cytometry. PI-stained cells were gated on the basis of their DNA content and the percentage of cells in different phases of the cell cycle was indicated. (B) Western blot of cyclin B1, CDK4, cyclin D1 in OVISE parental and knockout cells. β-Actin was used as loading control. (C) Immunofluorescent staining and quantification of mitosis marker phospho-Histone 3 in WT and KO OVISE cells. Each experiment was repeated at least three times and performed in triplicate. *p<0.05 compared with parental OVISE cells (WT) by two tailed Student's t test.

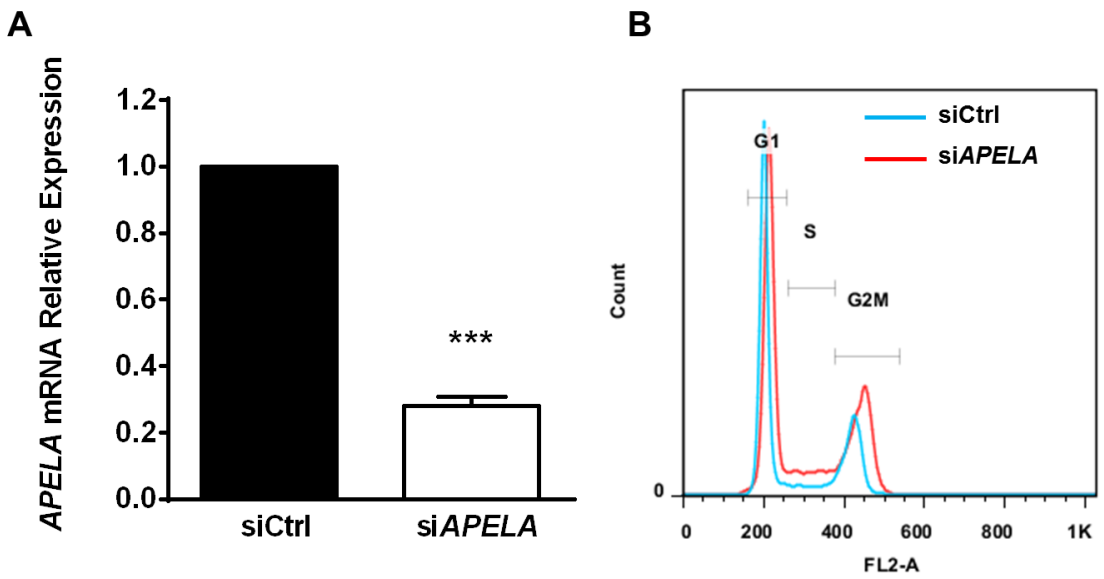

Figure 3.6 APELA knockdown induces G2/M arrest in OVISE cells

(A) qRT-PCR determination of *APELA* mRNA. The *APELA* mRNA levels of cells transfected with control DsiRNA were set as 1. Mean ± SEM are shown for three independent experiments. ***$p < 0.001$ compared with cells transfected with control DsiRNA by two tailed Student's *t* test. (B) Cell cycle analysis by PI-staining on siCtrl and si*APELA* in OVISE cells. Each experiment was repeated three times and performed in triplicate.

3.2.5 APELA peptide partially rescues cell growth defect in *APELA* knockout OVISE cells

To assess the bioactivity of the APELA peptide, a 32-amino acid recombinant APELA peptide was synthesized at 98% purity. OVISE cells treated with APELA peptide at 10μM showed enhanced growth relative to untreated cells (Figure 3.7A), suggesting that APELA peptide can promote OVISE cell growth. Since expression of the APJ receptor is undetectable in OVISE cells (see Figure 3.3), the response to exogenous APELA suggests the existence of an unknown receptor for APELA in OVISE cells. These data provide new evidence for the hypothesis that instead of the APJ receptor, an unidentified receptor can be activated by APELA peptide. Notably, the growth of *APELA* knockout OVISE cells was partially rescued by the addition of recombinant APELA (Figure 3.7B).

3.2.6 APELA is required for activation of AKT and ERK1/2 signaling pathways in OVISE cells

It has been shown that APELA is able to activate ERK1/2 pathway in CHO cells through the APJ receptor (14, 21). In hESCs, APELA is required for cell survival by activating PI3K/AKT/mTORC1 signaling (17). However, it is unknown whether these signaling pathways are also involved in APELA-regulated cancer cell biology. To determine whether the ERK1/2 and PI3K/AKT pathway contribute to the increase of APELA-induced cell growth, the levels of ERK1/2 and AKT and their activated form in OVISE cells were examined. As shown in Figure 3.8, disruption of *APELA* reduced the basal level of phospho-ERK1/2 and phospho-AKT in OVISE cells, suggesting that APELA expression is required for ERK1/2 and AKT signaling pathways activation in OVISE cells. Interestingly, treatment with 10μM APELA peptide only increased the ERK1/2 phosphorylation level but not AKT phosphorylation level in both WT and

KO OVISE cells. Since OVISE cells do not express the APJ receptor, the activation of ERK1/2 signaling again suggests the existence of an unidentified receptor in OVISE cells. The different responses of these two signaling pathways to exogenous APELA peptide in OVISE cells suggests that extracellular APELA activates ERK1/2 signaling pathway through an unknown receptor, while AKT signaling pathway activation is mediated by APELA internally in the cell. These results may explain the partially rescued cell growth by APELA peptide treatment in APELA deficient OVISE cells.

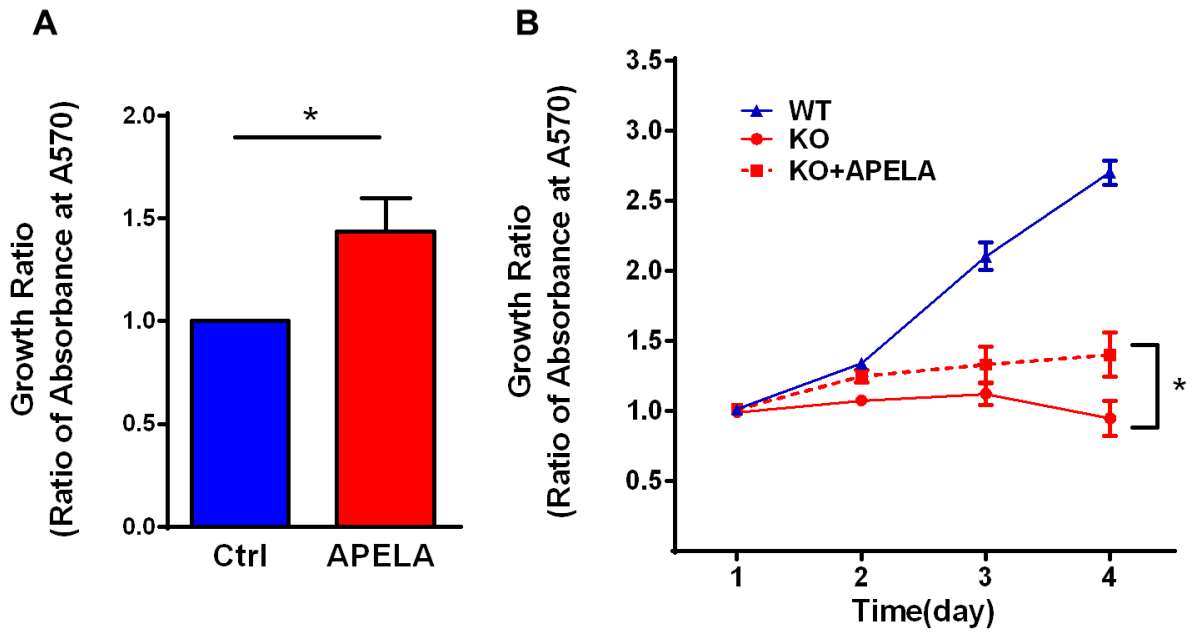

Figure 3.7 Treatment with APELA partially attenuates APELA-knockout-decreases cell growth in OVISE cells

(A) Treatment of APELA synthetic peptide promoted OVISE cell growth. The results are expressed as the mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with parental OVISE cells (WT) by two tailed Student's t test. (B) Cell growth of APELA knockout cells was partially rescued with synthetic APELA. KO: *APELA* knockout OVISE cells. The results are expressed as the mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with parental OVISE cells (WT) two-way ANOVA.

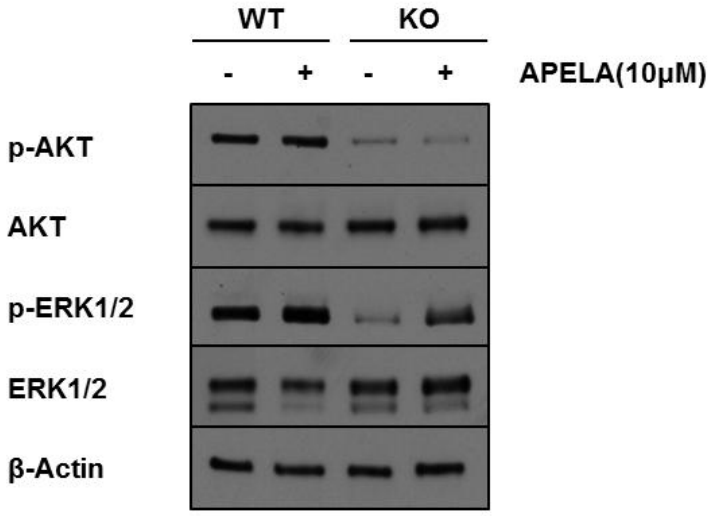

Figure 3.8 APELA regulates AKT and ERK1/2 signaling pathways in OVISE cells

WT cells and *APELA* KO OVISE cells were treated with 10 μM APELA for 15 min. Phosphorylation of ERK1/2, and AKT were determined by western blot using antibodies specific for phosphorylated, activated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT). Membranes were stripped and reprobed with antibodies to total ERK1/2 and AKT. Each experiment was repeated three times.

3.3 Characterization of APELA function in Ewing sarcoma cells

3.3.1 APELA, APJ receptor, and Apelin expression pattern in Ewing sarcoma cell lines

The mRNA expression data from the Cancer Cell Line Encyclopedia (CCLE: http://www.broadinstitute.org/ccle/home) showed that *APELA* is most consistently highly expressed in sarcoma, especially in Ewing sarcoma (Figure 3.9). Ewing sarcoma (ES) is the second most common pediatric malignant sarcoma of bone and soft tissue with a wide spectrum of clinical presentations. Thus, I also tested whether APELA has a role in Ewing sarcoma.

To examine APELA expression in Ewing sarcoma cells, qRT-PCR validation was performed in A673, TC71, and TC32 cell lines (Figure 3.10). Consistent with CCLE microarray data, *APELA* was expressed in A673 and TC71 but not in TC32. In addition, the expression of the APJ receptor and Apelin were also relatively high in A673 and TC71 cells but were hardly detected in TC32 cells. A673 cell line was used to investigate whether APELA also functions in Ewing sarcoma and whether APELA signals via the APJ receptor in these cells.

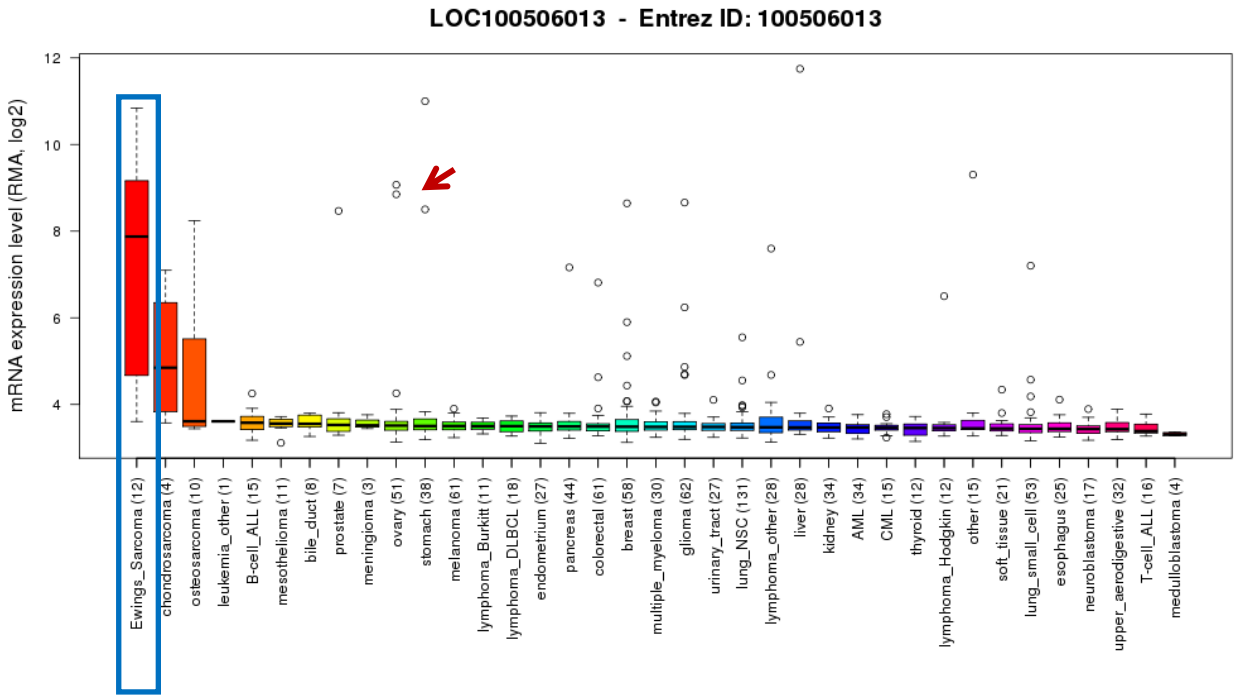

Figure 3.9 *APELA* mRNA is highly expressed in Ewing sarcoma cell lines

APELA mRNA is most highly expressed in Ewing sarcoma cell lines (highlighted with blue rectangle) based on the Cancer Cell Line Encyclopedia (CCLE) dataset. The expression of *APELA* in ovarian clear cell carcinoma cell line OVISE is relatively high (highlighted with red arrow). The CCLE is a compilation of gene expression, chromosomal copy number, and massively parallel sequencing data of a large panel of human cancer cell lines collected by the Broad Institute. The expression data was generated by using Affymetrix U133+2 arrays.

3.3.2 Knockout of APELA does not affect A673 Ewing sarcoma cell growth

Using the same CRISPR/Cas9 system (Figure 3.4A), I generated *APELA* knockout A673 clones with homozygous genetic deletion of APELA. Interestingly, the growth disadvantage as shown in OVISE *APELA* KO cells was not observed in two *APELA* knockout clones of A673 cells compared with parental cells (Figure 3.11B). Moreover, cell cycle distribution remained unchanged upon altering APELA expression by CRISPR/Cas9 knockout or DsiRNA mediated knockdown in A673 cells (Figure 3.11C and Figure 3.11E). Apelin/APJ receptor pathway has been documented to trigger cell growth and regulate cell cycle progression. The relatively high expression of Apelin in A673 is potentially able to compensate for the abolished APELA functionality. Alternatively, the regulatory function of APELA in cancer cell growth or cell cycle may not signal through the APJ receptor.

3.3.3 Knockout of APELA attenuates ERK1/2 but not AKT signaling pathway in A673 cells

I next examined the effect of APELA depletion on ERK1/2 and AKT signaling pathway in A673 cells. Western blot analysis of the relative levels of ERK1/2 phosphorylation displayed a slight reduction in *APELA* KO A673 cells (Figure 3.12). Surprisingly, unlike the situation in OVISE cells, the level of AKT phosphorylation was not affected in the *APELA* KO A673 cells. These inconsistent responses to the knockout of *APELA* imply the regulation of APELA in cancer cells could be cell type-specific.

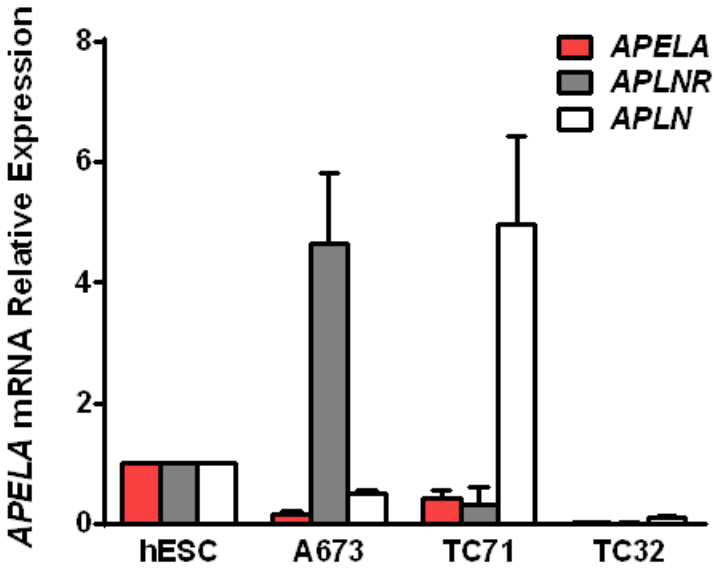

Figure 3.10 APELA, Apelin, and APJ receptor mRNA expression in Ewing sarcoma cell lines

qRT-PCR validation of *APELA* mRNA level in Ewing sarcoma cell line is consistent with CCLE mRNA expression data. APJ receptor (*APLNR*) and Apelin (*APLN*) expression is also shown. The relative expression of APELA was normalized to GAPDH, and hESCs were used as a reference cell line. The results of the qRT-PCR are expressed as the mean ± SEM of three independent experiments.

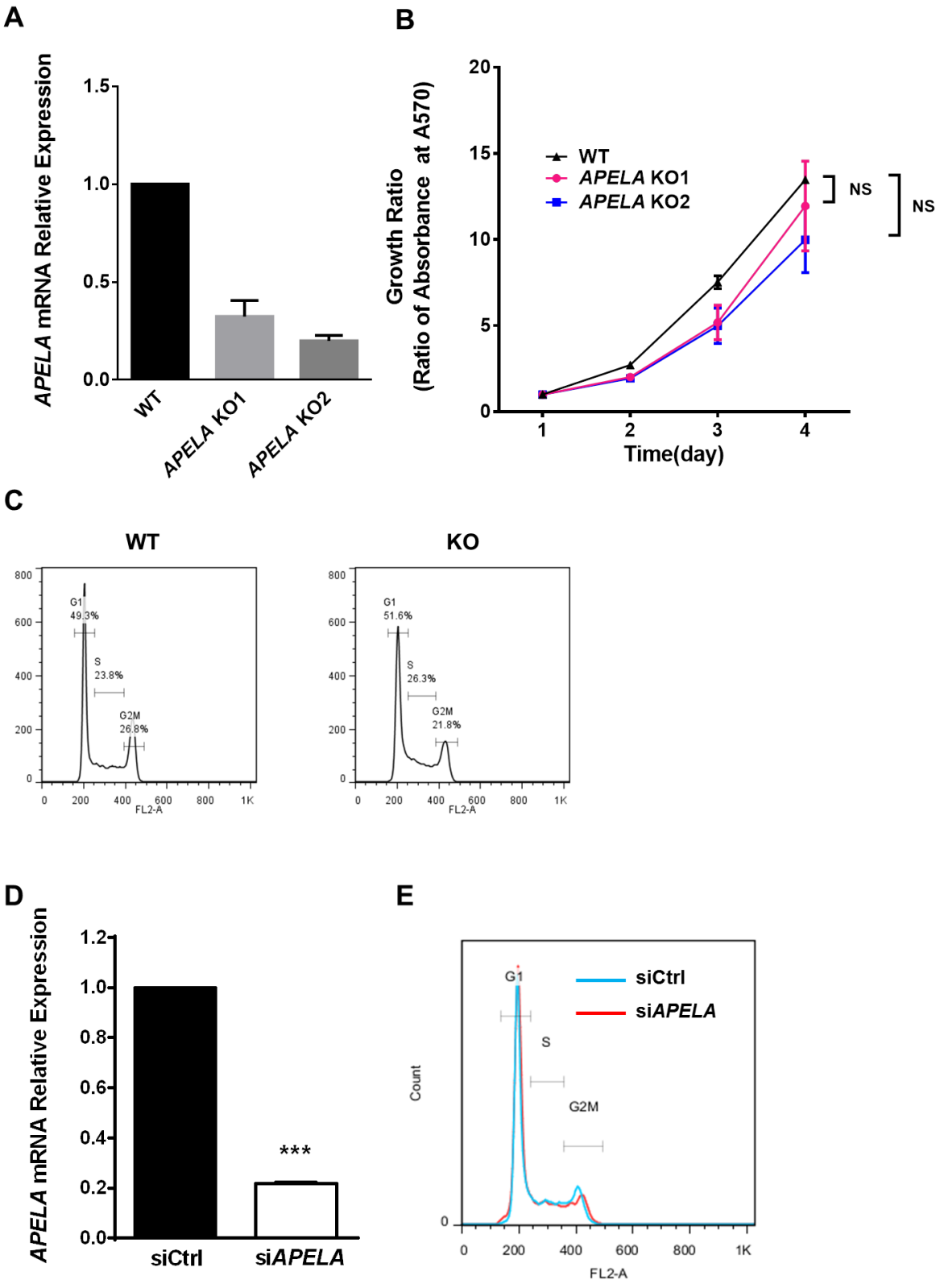
A
APELA mRNA Relative Expression
1.5
1.0
0.5
0.0
WT
APELA KO1
APELA KO2
B
Growth Ratio
(Ratio of Absorbance at A570)
20
15
10
5
0
WT
APELA KO1
APELA KO2
NS
NS
1
2
3
4
Time(day)
C
WT
KO
G1
49.3%
S
23.8%
G2M
26.8%
G1
51.6%
S
26.3%
G2M
21.8%
800
600
400
200
0
0
200
400
600
800
1K
FL2-A
D
APELA mRNA Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
siCtrl
siAPELA
E
G1
S
G2M
siCtrl
siAPELA
Count
0
0
200
400
600
800
1K
FL2-A

Figure 3.11 Knockout of APELA does not affect cell growth in Ewing sarcoma cell line A673

(A) The efficiency of targeted deletion with CRISPR/Cas9 was determined by qRT-PCR in two clones and parental A673 cells. (B) Cell growth of A673 cells and two clones of *APELA* knockout cells (KO) assessed by crystal violet assay at indicated time points. The results are expressed as the mean ± SEM of at least three independent experiments performed in duplicate. (C) Cell cycle analysis of parental (WT) and *APELA* knockout (KO) A673 cells. Cells were stained with PI and analyzed using flow cytometry. (D) The knockdown efficiency of APELA mRNA determined by qRT-PCR. The transcript levels of cells transfected with control DsiRNA (siCtrl) were set as 1. Mean ± SEM are shown for three independent experiments. ***$p < 0.001$ compared with cells transfected with control DsiRNA by two tailed Student's t test. (E) Cell cycle analysis by PI-staining on siCtrl and si*APELA* in A673cells. Each experiment was repeated three times and performed in triplicate.

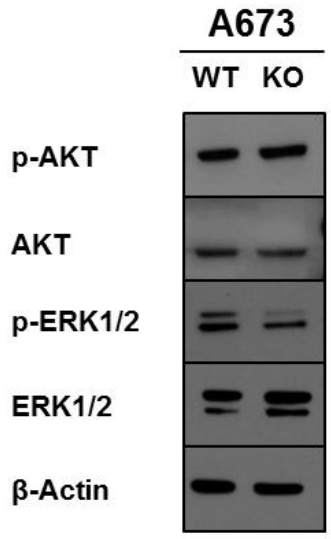

Figure 3.12 Knockout of APELA inhibits ERK1/2 activation in Ewing sarcoma cell line A673

WT cells and *APELA* KO A673 cells were lysed for Western blots. Phosphorylation of ERK1/2, and AKT were determined by western blot using antibodies specific for phosphorylated, activated forms of ERK1/2 (p-ERK1/2) and AKT (p-AKT). Membranes were stripped and re-probed with antibodies to total ERK1/2 and AKT. The experiment was repeated three times.

3.4 APELA regulates cancer cell growth through the p53 signaling pathway

Recently, a study reported that APELA regulates p53-mediated DIA as a regulatory RNA in mouse ESCs, and it also described a tri-element negative feedback loop composed of Apela, p53, and its inhibitory binding partner hnRNPL (16). This theory may explain why OVISE and A673 showed distinct phenotypes upon knockout of APELA, since expression of wildtype (functional) p53 has been documented in OVISE (88) and the A673 cell line is characterized as a p53-null cell line (90).

To better investigate whether the role of APELA in cancer cells is dependent on p53 levels, three additional APELA-positive cells: two ovarian cancer cells (JHOC-7, JHOC-9) and one Ewing sarcoma cell (TC71) were used for further analysis. As shown in Figure 3.13 and Table 3.1, western blot analysis showed that OVISE and JHOC-7 express p53 at modest levels and p53 level in JHOC-9 is relatively high. However, p53 was barely detected in TC71 and A673 at the protein level.

Table 3.1 APELA, APJ receptor and p53 expression in ovarian clear cell carcinoma and Ewing sarcoma cell lines

Cell line	OVISE	JHOC-7	JHOC-9	TC71	A673
	Ovarian Clear cell carcinoma			Ewing sarcoma	
APELA	+	+	+	+	+
APJ	-	-	-	+	+
p53	+	+	++	-	-

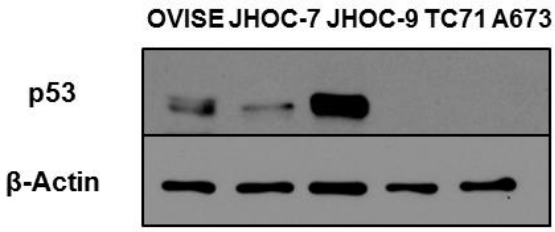

Figure 3.13 p53 expression levels in ovarian clear cell carcinoma and Ewing sarcoma cell lines

The protein level of p53 was analyzed by western blot in ovarian clear cell carcinoma cell lines OVISE, JHOC-7, JHOC-9, and Ewing's sarcoma cell lines TC71 and A673. The experiment was repeated three times.

3.4.1 APELA knockout-mediated cell cycle disruption is dependent on wildtype p53 expression

To investigate whether p53 expression is correlated with APELA-mediated cell cycle regulation, OVISE, JHOC-7, JHOC-9, TC71, and A673 cells were transfected with DsiRNA against APELA to knockdown its expression. The depletion efficiency was examined by qRT-PCR (Figure 3.14A). Using flow cytometry, cell cycle analysis showed that knockdown of APELA in wildtype p53 positive cells OVISE, JHOC-7, and JHOC-9 altered cell cycle distribution to different degrees (Figure 3.14B). In JHOC-9 cells, the high level of p53 is associated with dramatically disrupted cell cycle, and cell death was observed from one-day post-transfection. On the contrary, in p53 negative cells TC71 and A673, cell cycle distribution remained unchanged upon decreasing *APELA* expression (Figure 3.14C). Of interest, APJ receptor was only detected in TC71 and A673 but not in OVISE, JHOC-7 and JHOC-9 cells (Figure 3.3, Figure 3.10 and Table 3.1). Thus, the effect induced by *APELA* knockdown in APJ receptor negative cells is independent of the APJ receptor.

To further determine whether p53 level is important for APELA to regulate cancer cell growth, loss- and gain-of-function approaches were used in A673 and JHOC-9 cells. In p53-null A673 cells, pCMV-p53 plasmid was used to overexpress p53 for two days, and then knocked down APELA level by Dsi*APELA* for another two days. On the other hand, JHOC-9 cells, which have high level of p53, were transfected with siRNA against p53 for two days to reduce p53 protein level, and then transfected with Dsi*APELA* for another two days to knockdown *APELA* expression. As shown in Figure 3.15A, the cell number of p53-expressing A673 cells was reduced significantly after knockdown of *APELA*, while overexpressing p53 or knockdown of *APELA* alone did not significantly affect cell growth in A673 cells. Conversely, a decrease of

JHOC-9 cell number induced by knockdown of *APELA* was not observed in p53-knockdown JHOC-9 cells. These results suggest that p53 is required for APELA-regulated cancer cell growth and cell cycle regulation. In addition, our observations that APELA mediates cell cycle regulation in cells without the APJ receptor but not in cells expressing the APJ receptor strongly support our hypothesis that the regulatory role of APELA in cancer cells is independent of the APJ receptor signaling pathway.

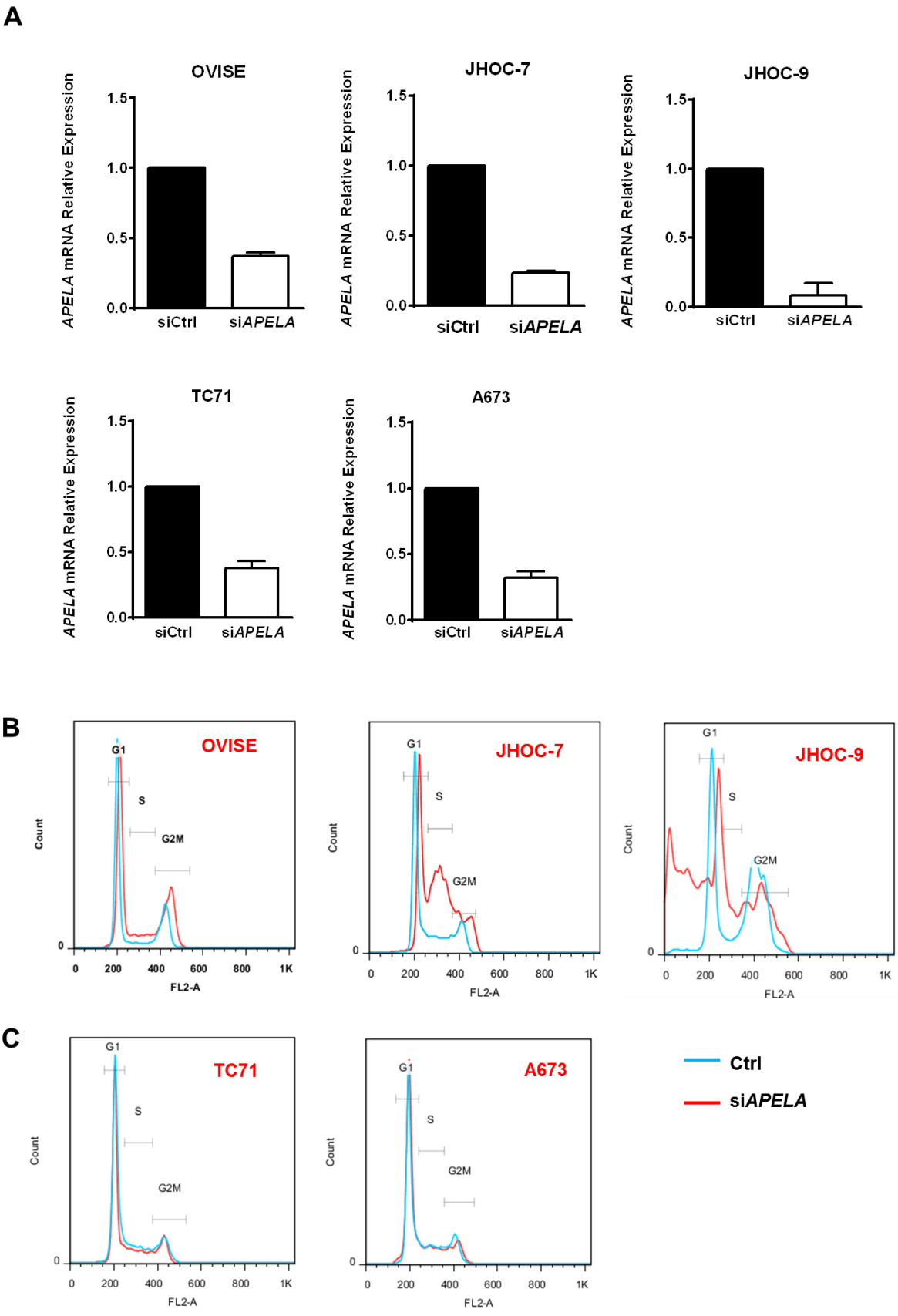
A
OVISE
JHOC-7
JHOC-9
TC71
A673
APELA mRNA Relative Expression
1.5
1.0
0.5
0.0
siCtrl
siAPELA
B
C
G1
S
G2M
Count
FL2-A
0
200
400
600
800
1K
Ctrl
siAPELA

Figure 3.14 The regulatory effect of APELA on cell cycle depends on the expression level of wildtype p53

(A) The knockdown efficiency of APELA mRNA determined by qRT-PCR. The transcript levels of cells transfected with control DsiRNA (siCtrl) were set as 1. Mean ± SEM are shown for three independent experiments. (B) Cell cycle analysis by PI-staining on siCtrl and si*APELA* in ovarian clear carcinoma cell lines JHOC-7, JHOC-9, OVISE, and Ewing sarcoma cells TC71 and A673. Each experiment was repeated three times and performed in triplicate.

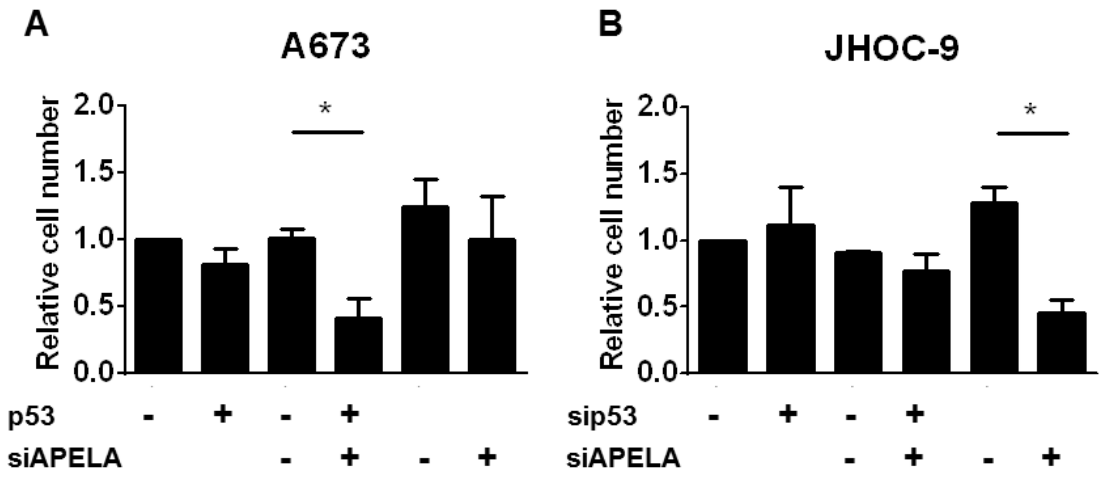

Figure 3.15 p53 is required for APELA-induced decrease of cell growth in A673 and JHOC9 cells

(A) A673 cells were transfected for 48 hours with control (pCMV) or p53-encoding (pCMV-p53) vectors pCMV-p53 to overexpress p53, and then transfected with DsiRNA against APELA for another 48 hours to knock down APELA. The numbers of cells were counted by trypan blue. (B) JHOC-9 cells were transfected for 48 hours with siRNA against p53 and then transfected with DsiRNA against APELA for another 48 hours. The numbers of cells were counted by trypan blue. Each experiment was repeated three times and performed in triplicate. Mean ± SEM are shown for three independent experiments. Differences between two groups were analyzed by One-way ANOVA. *$p<0.05$ compared with the control samples.

3.4.2 APELA regulates p53 expression in cancer cells

In mESCs, it was confirmed that *Apela* positively regulates p53 activity, while *Apela* expression level is suppressed by p53 (16). To determine whether APELA could activate p53, I have evaluated whether APELA increased p53 protein levels. p53-null cells A673 were transfected with pCMV-p53, and JHOC-9 cells expressing wildtype p53 were transfected with siRNA against p53. The p53 levels of the cell extracts were assessed by immunoblotting. Western blot analysis showed increased p53 level induced by knockdown of *APELA* in JHOC-9 cells compared with cells transfected with siCtrl (Figure 3.16B). The level of p53 increased significantly in p53-overexpressing A673 cells in comparison with control cells. Interestingly, knockdown of *APELA* in p53–overexpressing A673 cells stimulated exogenous p53 overexpression by approximately 2-fold (Figure 3.16A). These results suggest that APELA expression may inhibit p53 level in cancer cells.

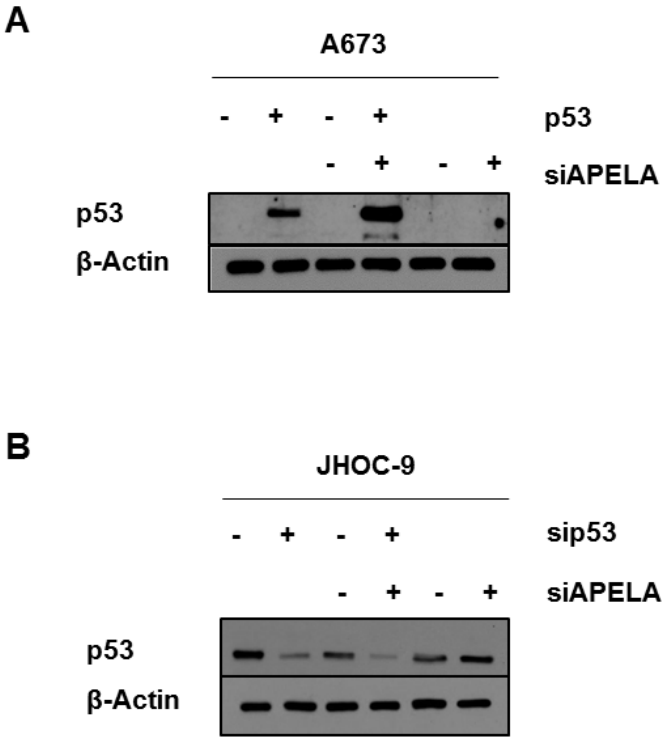

Figure 3.16 Knockdown of APELA enhances the overexpressed and endogenous p53 protein levels in A673 and JHOC9 cells

(A) A673 cells were transfected for 48 hours with control (pCMV) or p53-encoding (pCMV-p53) vectors pCMV-p53 to overexpress p53 and then transfected with DsiRNA against APELA for another 48 hours to knock down APELA. p53 protein levels were analyzed by western blot. (B) JHOC-9 cells were transfected for 48 hours with siRNA against and then transfected with DsiRNA against APELA for another 48 hours. p53 protein levels were analyzed by Western blot. Each experiment was repeated three times.

4 CHAPTER 4: DISCUSSION

Although originally annotated as a lncRNA, studies in zebrafish have suggested that *Apela* encodes a small secreted peptide that is evolutionarily conserved among vertebrate species including humans. This small peptide functions through the G-protein coupled receptor, APJ, to regulate definitive endoderm migration and cardiac development (10, 11). In addition, *Apela* mRNA can potentially mediate p53-dependent cell apoptosis in mESCs (16). In humans, APELA is expressed in embryonic stem cells and induced pluripotent stem cells, as well as adult kidney and prostate. It has been shown that APELA peptide promotes hESCs self-renewal via cell cycle progression and protein translation (17). This suggests that *Apela* can encode a peptide growth factor and function as a lncRNA. However, thus far, whether APELA exerts any biological functions that regulate cancer progression is completely unknown. My work is the first study that demonstrates the tumor growth regulatory role of APELA in ovarian clear cell carcinoma.

4.1 APELA stimulates ovarian clear cell carcinoma growth mainly by regulating the cell cycle

Generation of *APELA* knockout cancer cell lines using CRISPR/Cas9 enables us to study the function of APELA in *in vitro*. Analysis of *APELA* knockout cancer cell lines showed that APELA deficiency resulted in a cell growth defect by disrupting cell cycle progression. The cell growth disadvantage in APELA deficient cell lines could be partially rescued by treating cells with an APELA peptide, suggesting that APELA can promotes ovarian clear cell carcinoma cell growth *in vitro*. Cell growth defects induced by APELA siRNA knockdown was also detected in the other OCCC cell lines JHOC-7 and JHOC-9 that express *APELA* mRNA. Our study also indicated that APELA is required for ERK1/2 and AKT phosphorylation in OVISE cells, which

implicates the involvement of the ERK and PI3K/AKT signaling pathways in APELA-regulated cancer cell growth. To explore whether *APELA* expression can affect tumor growth *in vivo*, the APELA deficient OVISE cells and the parental cells will be subcutaneously injected into NOD/SCID mice for evaluation of tumor formation and metastasis potential. Overall, this work has shown the biological effects of APELA expression in OCCC cell lines and these results will provide insight into the contribution of APELA to cancer progression.

4.2 APELA regulates cell growth via an APJ receptor independent pathway

Interestingly, all of the OCCC cell lines that displayed cell cycle disruption and a cell growth defect in response to APELA depletion do not express the APJ receptor, revealing that the regulatory role of APELA in ovarian clear cell carcinoma cells is through an APJ-independent pathway. Recent studies by another group have suggested that APELA peptide promotes self-renewal and activates the PI3K/AKT pathway in undifferentiated hESCs, in which the APJ receptor mRNA level is relatively low (17). Consequently, they put forward the hypothesis that APELA can recognize another unidentified receptor and function via this alternative receptor in hESCs (17). Our observation of APELA peptide induced cell growth and ERK1/2 signaling pathway activation in cell lines without APJ receptor expression provides further evidence for the existence of an alternative receptor for APELA which mediates APELA function in ovarian clear cell carcinoma cells. Future studies will be directed at identifying the unknown receptor by proteomic analysis and investigating how the signaling pathways activated by APELA regulate cancer cell functions.

4.3 The function of APELA in cancer cells depends on p53 status

A striking finding in this study is that knockout of *APELA* had no effect on cell growth or cell cycle distribution in Ewing sarcoma cell line A673 even though it expresses the APJ receptor. Several reasons could explain this observation. First of all, A673 cells express APJ receptor and Apelin mRNA at high levels as shown in Figure 3.1. Apelin has been shown to promote cell growth in cancer cells and tumor growth *in vivo* by activating the APJ receptor signaling pathway (91). Thus, Apelin and APELA may have functional redundancy in A673 cells, and Apelin may compensate for the APELA deficiency to maintain normal cell growth and cell cycle distribution. This compensation was not observed in OVISE cells possibly because of lack of expression of APJ receptor and Apelin in OVISE cells. This hypothesis has not been assessed yet in this thesis. Using siRNA knockdown of Apelin and the APJ receptor combined with Apelin and APELA peptide treatment in A673 cells, future work will explore whether Apelin and APELA function redundantly and whether they can activate APJ receptor signaling in A673 cells. Also, it is not clear whether A673 cells have the hypothesized alternative receptor that could be activated by APELA in OVISE cells. Identification of the alternative receptor for APELA will help to address this hypothesis.

My present work has indicated that the expression of wildtype p53 is required for APELA function in cancer cells. APELA expression has been shown to be regulated by p53 activation and APELA is involved in p53-dependent DIA in mESCs (16). I therefore asked whether p53 could account for different effects of *APELA* knockout in OVISE and A673 cells. Analysis of several cancer cell lines with different p53 levels revealed that APELA-regulated cell cycle distribution depended on p53 status. APELA deficiency resulted in cell cycle disruption and cell growth reduction in cells expressing p53 at modest levels. In high p53-expressing cells, loss of

APELA led to a cell number decrease due to cell death, which may result from cell apoptosis, necrosis or autophagy (92, 93). In the future, using the autophagy marker (LC3), apoptosis marker (Annexin V, Caspase 3), and DNA content staining (PI staining), I will clarify the exact effects of APELA deficiency on cell death. In p53-null cells APELA knockdown had no effect on cell growth and cell cycle progression. Interestingly, the role of APELA in regulating cell growth was elucidated by overexpressing p53 protein in p53-null cells, and *vice versa*. These results suggest that APELA functions in a p53-dependent manner in cancer cells.

p53 plays an essential role in controlling multiple intra- and extracellular processes, such as regulation of cell cycle, cell repair, as well as cell death (94). Normally, p53 is inactive and is expressed at low levels. In response to various genotoxic or cytotoxic stress, including DNA damage, p53 becomes activated and results in cell growth arrest or apoptosis by regulating target genes transcription, allowing damaged cells to self-repair or be eliminated (95). In this manner, p53 protects against oncogenesis of normal cells and thus the gene *TP53* is called the tumor suppressor gene (96). p53 has been shown to suppress oncogenesis by inhibiting cancer cell growth (97) and inducing cancer cell apoptosis (98). In this study, it has been shown that wildtype p53 cancer cell lines (OVISE, JHOC-7 and JHOC-9) were more sensitive to APELA depletion than p53-null mutant cancer cell lines (A673 and TC71), suggesting that the status of p53 is essential for APELA functions.

The ERK1/2 and PI3K/AKT pathways have been shown to interact with the p53 pathway (73). Both ERK1/2 and PI3K/AKT signaling pathways can be activated by APELA in OVISE cells that express wildtype p53; while in p53-null A673 cells, knockout of APELA only altered ERK1/2 signaling activity but not AKT pathway. The differential activation of PI3K/AKT

signaling pathways by APELA in cells with different p53 status implies potential crosstalk between the p53 pathway and PI3K/AKT pathway in APELA-regulated cellular functions.

4.4 APELA modulates p53 protein stabilization in cancer cells

In this study, I have demonstrated that APELA can regulate p53 protein expression in cancer cells. First, APELA silencing not only increases endogenous p53 level in the JHOC-9 cells expressing wildtype p53, but also induces exogenous p53 accumulation in p53-null cells A673. Second, p53 mRNA levels are not altered after modulating APELA expression, further supporting the hypothesis that APELA regulates p53 expression at the protein level and not at the transcriptional level. However, instead of functioning as an activator of p53 in mESCs, my study suggested that human APELA inhibits p53 protein level and functional activity in cancer cells. The regulation of p53 expression and function are extensively stress-, species-, and cell type-specific (95). It was shown that the p53 signaling in mESCs is regulated by an ESC-specific transcriptome, and the tri-element loop between APELA, p53, and hnRNPL is mESCs-specific (16). In line with our observations, it points to a possible alternative role of APELA in mouse ESC maintenance compared to APELA in human cancer progression.

To our knowledge, this is the first time that APELA has been reported as a potential p53 protein inhibitor, although it is not clear whether this function of APELA depends on its coding ability. It is thought that the control of p53 activation or accumulation is mainly at the translational and post-translational levels (95). Recently, the regulation of p53 protein synthesis by alternative translation initiation machineries (99) and RNA-binding proteins (100, 101) has been discussed for p53-mediated tumor suppression. Both proteins and microRNAs have been shown to be involved in such regulation (102). For instance, ribosomal protein L26 (RPL26)

binds to p53 mRNA to increase p53 translation, resulting in an increase in p53-mediated apoptosis (103). Also, the stabilization and activation of p53 can be regulated by a series of post-translational modifications, including phosphorylation, acetylation, methylation, glycosylation, ubiquitination, sumoylation, and neddylation (104). The most prevalent mechanism of p53 regulation mainly operates within a negative-feedback loop with the downstream target of p53, MDM2 (murine double minute 2), an E3 ubiquitin ligase which facilitates the ubiquitylation and proteasomal degradation of p53 (105). Thus, the inhibitory effect of APELA on p53 can be at both protein translation and stability levels. Our data demonstrates that AKT phosphorylation is affected by APELA knockout in the wildtype p53 cell line OVISE. But this effect was not observed in the p53-null cell line A673. These results suggest that APELA potentially regulates PI3K/AKT pathway through altering p53 protein levels. Further elucidation of the mechanisms will help us fully understand the complexity and functions of APELA-mediated p53 inactivation and degradation.

4.5 APELA expression: a signature for the clear cell carcinoma subtype of ovarian cancers

In this thesis, I have examined the expression level of *APELA* mRNA in human normal tissues and different cancer tissues. Analysis of TCGA datasets showed that *APELA* mRNA is expressed in several types of human cancer at very low levels, including in ovarian cancers. I have further demonstrated that *APELA* mRNA is specifically elevated in ovarian clear cell carcinomas (OCCC) compared with other ovarian cancers by analyzing *APELA* mRNA expression in different subtypes of ovarian cancer samples. However, in the normal ovary tissues and other organs that most ovarian cancers originate from, *APELA* mRNA is hardly detected. In

addition, using gene expression profile from a mouse model of OCCC (87), I have confirmed that *Apela* mRNA is significantly elevated in ovarian clear cell carcinomas but not expressed in normal mouse ovaries.

OCCC accounts for 10% of epithelial ovarian cancer and is a distinct entity among epithelial ovarian carcinomas (106). It is associated with endometriosis that arises from endometriotic cyst, a benign ovarian tumor. OCCC has unique clinical, histological, and biological characteristics. While a majority of the epithelial cancers are relatively sensitive to chemotherapy, OCCC mostly shows strong resistance to platinum-based chemotherapy, leading to poorer prognosis (107, 108).

Recently, numerous genetic alterations involved in OCCC have been documented. OCCCs are usually p53 wildtype and have a lower frequency of breast cancer 1 (*BRCA1*) and *BRCA2* mutations compared to high-grade serous carcinomas (109, 110). The most frequent alterations are *ARID1A* mutations, which were seen in 46%–57% of ovarian clear cell carcinomas, 30% of endometrioid carcinomas, and none of the high-grade serous ovarian carcinomas. The subsequent loss of expression of BAF250a, encoded by *ARID1A*, is also frequently observed in OCCC (111). It has been shown that ARID1A collaborates with p53 to regulate *CDKN1A* and *SMAD3* transcription and tumor growth in OCCC and other endometriosis-related ovarian cancers (112). It is worthwhile to explore whether loss of ARID1A is associated with the p53-dependent function of APELA.

Another event in OCCC is that the P13K/AKT/mTOR pathway is frequently abnormal. The *PIK3CA* gene has been found to be specifically mutated in OCCC, with a 33% frequency, and results in over-activation of PI3K kinase activity (113). Mutations of *PTEN* and loss of PTEN protein in OCCC has been reported to activate PI3K (114). In addition, mTOR is

overexpressed in 80 % of CCC patients (115). All of these alterations lead to an aberrant functioning PI3K/AKT/mTOR pathway. The PI3K/AKT/mTOR pathway plays an essential role in both normal cell physiology and in cancer cell proliferation, tumorigenesis, and metastasis. I have shown that loss of APELA in OCCC cells decreases AKT activity, indicating that APELA is required for PI3K/AKT/mTOR signaling pathway activation in OCCC. Further work is required to better understand the mechanism of how APELA is involved in this pathway to regulate OCCC tumor development.

Another important molecular feature of OCCC is the strong expression of vascular endothelial growth factor (VEGF) in both early stage and advanced stage OCCC (116). It has been shown that APELA peptide promotes angiogenesis *in vitro* (21). Whether APELA expression in OCCC correlates with VEGF-mediated angiogenesis is still unknown.

The overexpression of hepatocyte nuclear factor-1β (HNF1β) is now regarded as an OCCC hallmark, as it is expressed in almost all OCCC patients (117). Loss of HNF-1β expression has been shown to cause apoptotic cell death in OCCC cell lines, indicating that HNF-1β expression could be essential for its survival (118).

In my analysis, the higher level of APELA distinguishes OCCC from other ovarian cancer subtypes. However, whether *APELA* mRNA expression in OCCC is associated with the other molecular features or whether APELA is involved in regulation of the altered signaling pathways is still unclear. In future work, I will test for *APELA* expression and localization using *in situ* hybridization on frozen tumor samples from human OCCC patients. To obtain a better understanding of the correlations between *APELA* mRNA expression and OCCC features, I will combine analysis of tumor histological features, such as tumor grade and vascularization, with OCCC molecular characteristics including *PIK3CA* mutations, ARID1A, HNF1β and p53

expression. One caveat of my APELA expression analysis is that I am unable to show protein APELA levels in human tissues due to lack of an appropriate antibody specific to the APELA peptide. Thus, I will attempt to generate our own antibody and use immunohistochemistry to analyze the correlation between APELA peptide expression and ovarian cancer subtype and patient outcomes. Together with the well-known molecular characteristics, APELA expression may serve as another important diagnostic marker for OCCC.

4.6 Does APELA function as a peptide or non-coding RNA in human cancer cells?

The other possibility for an APJ-independent role of APELA is that the *APELA* RNA acts as a regulatory RNA to regulate cancer cell growth. A recent study described the non-coding role of Apela in mESCs to promote p53-mediated DIA (16). The 3'UTR of Apela mRNA forms a putative secondary structure that interacts with the p53 inhibitor heterogeneous nuclear ribonucleoprotein L (hnRNPL) protein and thus increased the mitochondrial localization of p53 and inhibited p53 degradation to promote p53-mediated apoptosis. The coding ability is dispensable for this regulatory function of Apela because Apela peptide treatment had no effect on DIA in mESCs (17). Therefore, Apela can function both as a peptide growth factor and as a non-coding RNA. Studies in hESCs have shown that *APELA* mRNA functions to prevent DIA, which is contrary to the function of *Apela* in mESCs (17). They argued that the secondary structure and protein-binding ability of *Apela* mRNAs are species specific because of the different length and low homology of the 3'UTR between species. Moreover, they proposed APELA acts as an endogenous secreted growth factor to promote hESCs growth and pluripotency. These studies lead us to question whether human APELA functions as a peptide or as a non-coding RNA in cancer cell biology.

In our studies, although exogenous APELA peptide could slightly promote cell growth, treatment of APELA peptide only partially attenuated the cell growth defect in *APELA* knockout cells. Interestingly, another group who did the cell growth rescue experiment in hESCs self-renewal used a synthetic peptide with a disulfide bond modification between the conserved C39 and C44 residues. This modified APELA peptide displayed a dose-dependent activity for enhanced hESCs growth (17). It is known that disulfide bonds are crucial post-translational modifications that are required for the stability and function of a large number of proteins. For example, changes in the disulfide arrangement are associated with altered activity of hormones, such as insulin (119). To explore whether an internal disulfide bond is required for APELA peptide function, APELA ligands with and without the disulfide bond will be generated and tested for cell growth by cell counting assay. This analysis will help us understand whether the weak rescue with the APELA peptide is due to low activity of the unmodified peptide.

Another possible reason for the partial rescue of cell growth by APELA peptide in knockout cells could be that it is the portion of APELA inside of cells that plays the major role in promoting cell growth. Knockout *APELA* in OVISE cells reduced ERK1/2 and AKT activation, while treatment of APELA peptide in *APELA* knockout OVISE cells only increased ERK1/2 phosphorylation but not AKT phosphorylation. These results suggest that AKT signaling is activated by internal APELA rather than external APELA in OVISE cells. Therefore, internal APELA may regulate cell growth through AKT signaling pathway either as a peptide or non-coding RNA. Many studies have investigated how non-coding RNAs regulate various cellular processes, such as apoptosis (120), cell cycle, and cell growth (121). One caveat in this thesis is whether APELA also has non-coding RNA functions in human cancers has not been well elucidated. In future studies, lentiviral vectors will be generated for ectopic expression of

wildtype *APELA* RNA, or without the ATG codon of the ORF, or without the ORF sequence. Using this method, I will be able to compare the effects of the APELA peptide and lncRNA on cancer cells and further explore how different forms of APELA regulate the cellular function through different signaling pathways.

4.7 Long non-coding RNAs as a source of new peptides and function in human cancers

Long non-coding RNAs (lncRNAs) are a group of transcribed RNA molecules more than 200 nucleotides in length that do not encode proteins. lncRNAs are emerging as key players participating in various processes, such as imprinting and development (122). Various studies have elucidated different mechanisms of lncRNA actions (123). Nuclear lncRNAs can regulate gene transcription by acting as enhancer RNA (eRNA) or by recruiting chromatin modifying complexes like Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2) (124), or as decoys to bind to transcription factors (125), or modulating the splicing of pre-mRNA. Cytoplasmic lncRNAs can modulate mRNA stability, mRNA translation, and act as miRNA sponges to compete for microRNA binding (123).

In the last several years, many studies have investigated how lncRNAs are functionally associated with human cancers, including ovarian carcinoma (126, 127). Dysregulation of lncRNAs have been shown to affect cellular functions such as cell proliferation, apoptosis, angiogenesis, metastasis, and evasion of tumor (128). For instance, a lncRNA called *HOST2* is specifically highly expressed in ovarian cancer, and it acts as a miRNA sponge to maintain the expression of oncogenes, and subsequently to promote proliferation, migration, and invasion in cancer cells as well as tumor growth in xenografted mice (129). In contrast to the above example,

the mechanisms of action have not been fully explored for many lncRNAs which exhibit altered expression in ovarian cancers and which have been shown to regulate cellular functions, (127).

The emerging studies have discussed the idea that lncRNAs also contain small open reading frames (ORFs) that can be translated in biological active small peptides (7, 130), and APELA is among this lncRNA category. Recent studies have shown that in addition to function as peptide, Apela mRNA can functions as lncRNA in mESCs (17). In this study, we discovered that previously annotated lncRNA, APELA has peptide functions in cancer cells, providing an important evidence for the ability of long non-coding RNAs to produce functional small peptide. In addition, APELA peptide alone is not sufficient to rescue *APELA* knockout induced cell growth disadvantage, suggesting *APELA* potentially also can bear noncoding function in cancer cells. My work raises the possibility that lncRNA that previously showed importance in human cancers may also produce small peptide and exert biology functions in cancer cells.

4.8 Research impact and future work

Taken together, in this work I have analyzed APELA expression in different human organs and tumor tissues and shown that APELA is elevated in a broad range of human cancers, especially in ovarian clear cell carcinoma. As summarized in Figure 4.1, our data highlights the important role of APELA in promoting human ovarian clear cell tumor growth in a p53-dependent manner. I propose in cancer cells, APELA can regulate cell growth through both internal and external pathways, and the regulatory role of APELA depends on p53 status. By negatively regulating p53 protein activity, internal APELA potentially modulates AKT phosphorylation and subsequently controls cell growth. On the other hand, external APELA can signal through an unknown receptor, which can regulate cell growth. This effect possibly is

through the ERK1/2 signaling pathway. To date, limited studies have examined the biological functions of APELA in humans, and these studies were focused on kidney physiology or hESCs renewal (14, 17). We are the first group to demonstrate the biological effects of APELA in cancer cell behaviors and this study provides insight into the contribution of APELA to cancer progression.

As discussed previously, it is the internal APELA that mainly regulates ovarian clear cell carcinoma growth. Future analysis will focus on understanding whether internal APELA functions as a long non-coding RNA or peptide by overexpressing or silencing APELA, as well as disrupting the translation of *APELA* RNA. In addition, the secreted form of APELA modestly promotes cell growth in ovarian clear cell lines, suggesting the existence of another receptor of the APELA peptide. To discover the novel receptor for APELA, LC-MS/MS analysis will be performed for identification of proteins bound to the APELA peptide. In this way, I will also be able to identify the upstream or downstream components that interact with the APELA peptide. As consequence, I will further explore the APELA signaling pathway and understand how this pathway functions in ovarian clear cell carcinoma.

Our study describes a p53-dependent role of APELA and its ability to inhibit p53 in cancer cells. This exciting discovery demonstrates the remarkable complexity of cell context-dependent regulatory processes that affect APELA functions. At present, the function of p53 protein has been extensively studied, making p53 a well-known tumor suppressor. To gain a better understanding of the role of p53 in APELA-mediated function, I will look into the underlying mechanism, such as translational machinery or protein degradation pathway, for the regulation of p53 level that is modulated by APELA. Additionally, in cancer cells, whether APELA interacts with hRNPL as illustrated in mESCs to positively activate p53 could also be an important study

to follow up. It is of interest that instead of binding to the promoter of *Apela*, p53 binds to three sites within the enhancer region of the *Apela* gene and potentially interferes with the interaction between transcriptional factors and the enhancer to repress *Apela* transcription in mESCs (16). The involvement of p53 in APELA expression regulation has not yet been illustrated in human cells. Therefore, it is valuable to investigate whether and how p53 can also regulates APELA expression in cancer cells.

To further understand how APELA functions in cancer development and progression, I will continue to evaluate the effects of APELA expression on basic cellular functions such as cell migration, apoptosis, and survival in cancer cells. Moreover, future study is necessary to elucidate whether APELA function is cancer type specific.

OCCC is one histologic subtype of epithelial ovarian carcinoma. Compared with other subtypes, it is associated with significantly poorer prognosis and relatively resistant to conventional platinum-based chemotherapy (131). Currently, available treatments for OCCC are challenging and new therapeutic strategies are needed. Thus, elucidation of the molecular mechanisms underlying OCCC tumorigenesis and progression is critical to the treatment of OCCC. In the future, our laboratory will collaborate with Dr. David Huntsman (Department of Pathology and Laboratory Medicine, University of British Columbia) to analyze the correlation between APELA expression and prognosis and outcome information from OCCC patients. Furthermore, our laboratory will cooperate with Dr. Aly Karsan (Department of Pathology and Laboratory Medicine, University of British Columbia) to generate a xenograft mouse model for OVISE and APELA knockout OVISE cells. This model will allow us to evaluate the tumor formation and metastasis potential to further understand the function of APELA in human cancers. In the future, I can also use these models to evaluate association between APELA

expression and chemotherapy drugs and radiation effects. In summary, our investigations identified the APELA signaling pathway as a novel pathway that is involved in OCCC tumor growth. In addition, I have elucidated a potential molecular mechanism for OCCC progression, which may provide a promising target to facilitate the development of novel therapeutic approaches in the management of this disease.

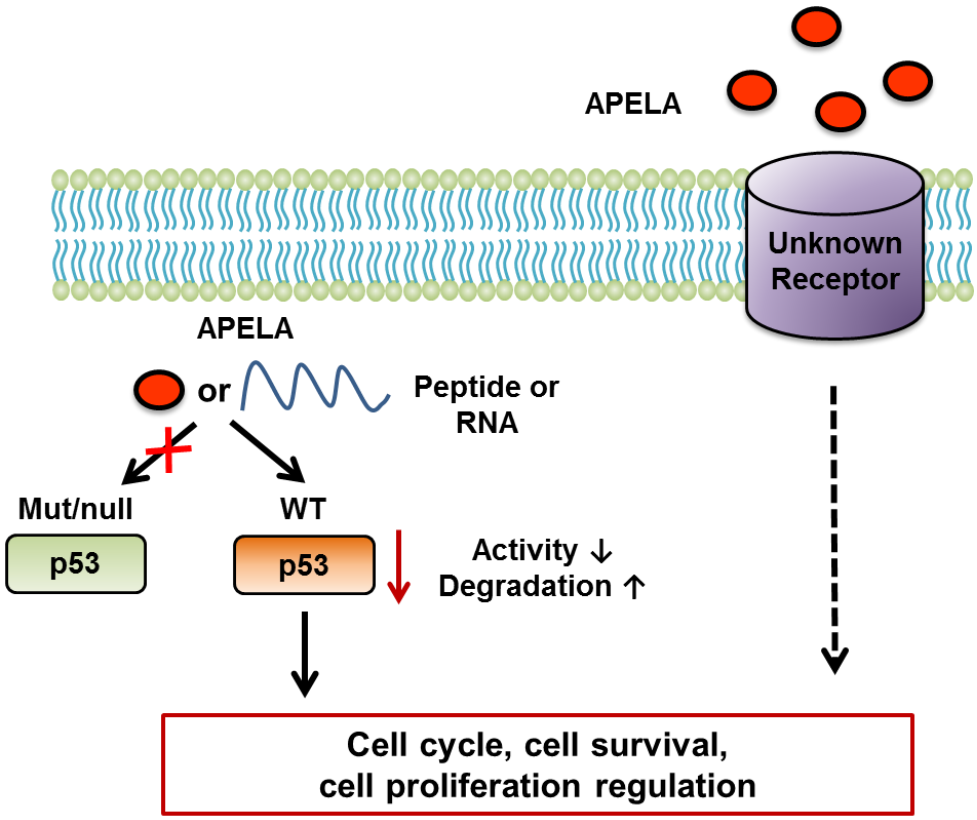

Figure 4.1 The hypothesized model for the role of APELA in cancer cells

The role of APELA in cancer cell growth regulation mainly relies on internal signaling independent of its receptors. The RNA or non-cleaved peptide of APELA functions in a p53-dependent manner. In cancer cells expressing wildtype p53, APELA maintains p53 levels by regulating p53 activity or protein degradation to control key cellular processes such as cell cycle progression, cell survival, and cell growth. In addition, the secreted form of APELA peptide signals through an unknown receptor and contributes to the regulation in cancer. Solid black arrows indicate interactions demonstrated by our data while dashed black arrows specify hypothesized regulations.

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APPENDIX I. Information of TCGA cancer samples

Disease	Abbreviation	#Processed normal	#Detected normal	#Processed tumor	#Detected tumor*
Adrenocortical carcinoma	LAML	0	0	79	0
Acute Myeloid Leukemia	ACC	0	0	91	0
Bladder Urothelial Carcinoma	BLCA	19	0	244	6
Breast invasive carcinoma	BRCA	87	3	696	31
Cervical squamous cell carcinoma and endocervical adenocarcinoma	CESC	2	0	104	2
Colon adenocarcinoma	CHOL	17	0	350	0
Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	LCML	0	0	9	0
Glioblastoma multiforme	GBM	0	0	217	3
Head and Neck squamous cell carcinoma	HNSC	42	1	424	8
Kidney Chromophobe	KICH	25	0	66	0
Kidney renal clear cell carcinoma	KIRC	33	10	348	2
Kidney renal papillary cell carcinoma	KIRP	30	2	172	8
Brain Lower Grade Glioma	LGG	0	0	329	0
Liver hepatocellular carcinoma	LIHC	50	0	165	0
Lung adenocarcinoma	LUAD	58	0	503	10
Lung squamous cell carcinoma	LUSC	50	0	490	22
Ovarian serous cystadenocarcinoma	OV	0	0	420	55
Prostate adenocarcinoma	PRAD	41	11	167	57
Rectum adenocarcinoma	READ	0	0	71	0
Skin Cutaneous Melanoma	SKCM	0	0	266	0
Stomach adenocarcinoma	STAD	32	0	283	17
Thyroid carcinoma	THCA	57	0	485	6
Uterine Corpus Endometrial Carcinoma	UCEC	18	1	365	2
Uterine Carcinosarcoma	UCS	0	0	57	4

*Detected represents the samples that APELA can be detected in RNA-seq data.