DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE INVESTIGATION OF PLANT SECONDARY METABOLITES EFFECTS ON METABOLISM OF EUKORYATIC MODELS

by
Zehra TAVŞAN

July, 2017
İZMİR

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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"THE INVESTIGATION OF PLANT SECONDARY METABOLITES EFFECTS ON METABOLISM OF EUKORYATIC MODELS"** completed by **ZEHRA TAVŞAN** under supervision of **PROF. DR. HÜLYA AYAR KAYALI** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

Epithelial ovarian cancer (EOC) is the fourth or fifth most common cause of death from all cancers among women. As the disease is asymptomatic at late detection, the tumor has metastasized at the time of diagnosis and after surgery it recurrences within 16–22 months with the development of a chemoresistant disease. Approximately 90 percent of primary malignant ovarian tumors are epithelial (carcinomas). Recent studies focus on the mechanism of initiation and progression of carcinoma by investigating the role played by cell-cell adhesion during the transformation of epithelial cells.

In general, this thesis focused on two main goals, which mechanism(s) cause to ovarian cancer progression and whether phytochemicals can be used for the treatment. We first investigated whether or which of claudins, tetraspanins, and nontraditional cell adhesion molecule, EpCAM are located in tetraspanin-enriched microdomains and by which PKC isoform regulate these possible interactions. The results showed that EpCAM-claudin-4/7 interactions were responsible for the ovarian cancer progression. These interactions were characterized with the further studies. Also, claudins in these interactions were phosphorylated by PKC delta or eta, showing phosphorylation modulates the complex formation, membrane localization, and stability.

There is an urgent need to find some safer cancer therapeutic and preventive agents, due to unexpected adverse side-effects of using chemotherapeutic drugs. Epidemiological studies have suggested that specific dietary consumption patterns are associated with risk of developing cancer. Targets for chemoprevention of phytochemicals, especially flavonoids may be multiple including induced antioxidant system, apoptosis or autophagy, and cell cycle arrest. We tested the effects of apigenin, myricetin, and luteolin on the ROS production, apoptosis, autophagy and cell cycle as well as the cell adhesion. These flavonoids affected ovarian cancer progression

mechanism depending on the flavonoid type and concentration. The results directed flavonoids may be used to block, retard, or reverse the development and progression of ovarian carcinogenesis.

Keywords: Ovarian cancer, cell adhesion, protein kinase C, flavonoid, cell signalling pathways.

BİTKİ SEKONDER METABOLİTLERİNİN ÖKARYOTİK MODELLERDE METABOLİZMAYA ETKİSİNİN ARAŞTIRILMASI

ÖZ

Epitel ovaryum kanseri (EOK), kadınlar arasındaki görülen kanser nedenli ölümlerde dördüncü veya beşinci sıradadır. Hastalık asemptomatik ve geç tespit edilirken teşhis sırasında tümör metastatik hale gelmiştir ve ameliyattan sonra 16-22 ay içinde nüks eden tümörler kemoreterapötik dirençlidir. Primer habis yumurtalık tümörlerinin yaklaşık yüzde 90'ı epitel kökenlidir (karsinomalar). Son çalışmalar, epitel hücrelerinin dönüşümü sırasında hücre-hücresi adezyonunun oynadığı rolü araştırarak karsinomun başlatılması ve ilerlemesindeki mekanizma üzerine odaklanmaktadır**.**

Bu tez, hangi mekanizma(lar) yumurtalık kanserinin ilerlemesine neden olmaktadır ve tedavide fitokimyasalların kullanımı olarak iki ana hedefe odaklanmıştır. İlk önce, tetraspanin ile zenginleştirilmiş mikro alanlarda claudinler, tetraspaninler ve geleneksel olmayan hücre adezyon molekülü EpCAM'in olup olmadığını ve PKC izoformunun bu olası etkileşimleri nasıl regüle ettiklerini araştırdık. Sonuçlar, yumurtalık kanseri ilerlemesinde EpCAM-claudin-4/7 etkileşimlerinin sorumlu olduğunu gösterdi. Bu etkileşimler daha sonraki çalışmalarla karakterize edildi. Ayrıca, bu etkileşimlerde bulunan claudinler, PKC delta veya eta ile fosforile edilmiştir ve fosforilasyonun kompleks oluşumu, membran lokalizasyonu ve kararlılığını modüle ettiğini göstermektedir.

Kullanılan kemoterapötik ilaçların beklenmeyen olumsuz yan etkileri nedeniyle bazı daha güvenli kanser terapötik ve önleyici ajanlar bulmak çok acil bir ihtiyaçtır. Epidemiyolojik araştırmalar, spesifik diyet tüketimlerinin kanser gelişme riski ile ilişkili olduğunu gösterdi. Fitokimyasal maddelerin, özellikle flavonoidlerin hedefleri antioksidan sistem, apoptoz veya otofajinin indüklenmesi ve hücre döngüsünün durudurlması gibi çoklu olabilir. Apigenin, myricetin ve luteolin'in ROS üretimi, apoptozisi, otofajisi ve hücre döngüsü üzerindeki etkilerinin yanı sıra hücre

adezyonundaki etkisini inceledik. Bu flavonoidler, flavonoid türüne ve konsantrasyonuna bağlı olarak yumurtalık kanseri lerlemesinde rol alan mekanizmaları etkiledi. Flavonoidler, yumurtalık kanseri oluşumu, gelişimini ve ilerlemesini engellemek, geciktirmek veya tersine çevirmek için kullanılabilir.

Anahtar kelimeler: Ovaryum kanseri, hücre adezyonu, protein kinaz C, flavonoid, hücre sinyal yolları.

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER 1
INTRODUCTION

1.1 Epithelial Ovarian Cancer

Cancer is one of the most serious diseases in the world due to its high mortality rate (Zini, Czerninski, & Sgan-Cohen, 2010). The burden of cancer has continuously increased not only in developing countries but also in developed countries. Although current strategies, including surgery, chemotherapy, radiation, and immunosuppressants, make a good progress in reducing cancer incidence and mortality rates and improving survival, cancer still accounts for more deaths than heart disease in persons younger than 85 years (Doyle et al., 2006).

Epithelial ovarian cancer (EOC) is the fourth or fifth most common cause of death from all cancers among women. Frequently more than 75% of EOCs are diagnosed at advanced stage (Auersperg, Wong, Choi, Kang, & Leung, 2001). As the disease is asymptomatic, early detection is difficult so that at the time of diagnosis the tumor has metastasized (FIGO stages III–IV). Even with optimal debulking surgery followed by aggressive front-line chemotherapy, which results in an 80% initial cure rate, advanced-stage disease in the majority of cases is incurable. This is due to the development of a chemoresistant disease which results in recurrence within 16–22 months and a 5-year survival rate of only ~27% (Kipps, Tan, & Kaye, 2013). Although screening tests are available for patient follow-up and for the detection of advanced cases (Herbst, 1994), there are no reliable means for early detection of EOCs except for genetic screening in a small proportion of individuals (Auersperg, Edelson, Mok, Johnson, & Hamilton, 1998). Nonetheless, the gains about diagnosis and treatment are rather modest, there clearly remains a need to better understand the molecular pathogenesis of ovarian cancer so new drug targets and biomarkers that facilitate early detection can be identified.

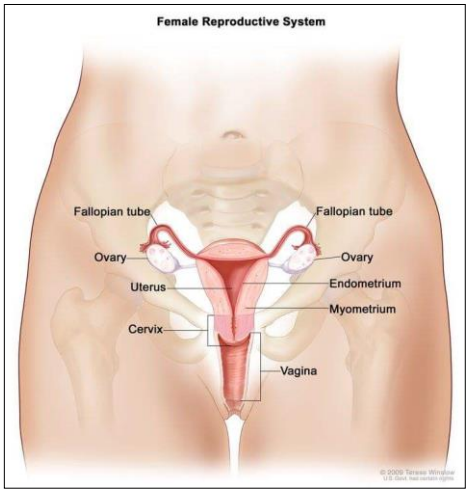


Figure 1.1 The reproductive system of woman (National Cancer Institute).

Approximately 90% of primary malignant ovarian tumors are epithelial (carcinomas), and are thought by most investigators to arise from the ovarian surface epithelium (OSE) or more likely from surface epithelial inclusion cysts (Bell, 2005; Feeley & Wells, 2001). The OSE is the modified pelvic mesothelium that covers the ovary and composed of a single layer of flat-to-cuboidal epithelial cells characterized by low-molecular weight keratins, simple desmosomes, apical microvilli, and a basal lamina (Auersperg et al., 2001). In culture, the OSE produces not only epithelial (laminin and collagen IV) but also mesenchymal (collagen types I and III) components of extracellular matrix (Adams & Auersperg, 1983), and it modulates from an epithelial to a mesenchymal morphology in response to a variety of environmental cues (Auersperg, Siemens, & Myrdal, 1984; Nicosia, Narconis, & Saunders, 1988). The relatively simple phenotype of the adult OSE and its capacity to modulate to a mesenchymal form and to differentiate into a striking variety of EOCs support the concept that adult OSE is relatively uncommitted and pleuripotent compared with other adult coelomic epithelial derivatives.

Histologically, the ovarian tumors form polarized epithelia, papillae, cysts, and glandular structures. Thus, unlike carcinomas in most other organs in which epithelial cells become less differentiated in the course of neoplastic progression than the epithelium from which they arise, the differentiation of EOCs is more complex. Only

in the late stages do these specialized epithelial features diminish although they can persist even when the tumors are metastatic or in the ascites form (Maines-Bandiera & Auersperg, 1997). Tissue culture studies have shown that with neoplastic progression OSE cells not only develop complex epithelial phenotypes, but also become firmly committed to these phenotypes and unresponsive to signals causing mesenchymal conversion of normal OSE. Such unresponsiveness to environmental cues reflects the autonomy from normal control mechanisms that characterizes malignant tumors in general.

1.1.1 The Etiology of EOC

The precise cause of EOC is unknown, but several risk and contributing factors have been identified. Epidemiological studies point to possible racial, geographic, social and hormonal causative factors (Cramer & Welch, 1983; Fraumeni Jr, Grundy, Creagan, & Everson, 1975; Greene, Clark, & Blayney, 1984; Herbst, 1994). Several rist factors were showed in Table 1.1.

Table 1.1 The risk factors for EOC

Patient Characteristics	**Environmental factors**
Increased age	Obesity and high fat diet
Personal history of breast and ovarian cancer	Talc exposure
	Cigarette smoking
Reproductive factors	**Genetic factors**
Nulliparity	Family history of ovarian cancer
Early menarche	BRCA1/2 mutations
Late menopause	Hereditary nonpolyposis colorectral cancer
Infertility	
Polycyctic ovarian syndrome	
Endometriosis	
Ovulation inducing drugs	
Hormone replacement therapy	

Hyperovulation treatment for infertility increases the risk of ovarian cancer, while oral contraceptives and pregnancies are protective. Also, the risk of EOC is increased in women who have not had children and possibly those with early menarche or late menopause. These observations support the hypothesis proposed by Fathalla (1971) and subsequently supported by epidemiological and experimental studies (Auersperg, Siemens, & Myrdal, 1984; Godwin et al., 1992; Testa et al., 1994), that frequent ovulation contributes to increased risk because the repeated rupture and repair of the OSE during ovulation provide an opportunity for genetic aberrations. Another major known risk factor is a strong family history of ovarian cancer, which accounts for 5–10% of cases. The lifetime risk for developing EOS is 1.6% in the general population. This compares with a 4-5% risk when 1 first-degree family member is affected, rising to 7% when 2 relatives are affected.

1.1.2 The Staging of Ovarian Cancers

Ovarian carcinomas can be sub-classified based on degree of differentiation (tumor grade). Historically, the most commonly used grading systems have been those proposed by the International Federation of Gynecology and Obstetrics (FIGO), the World Health Organization (WHO), and the Gynecologic Oncology Group (GOG) (Silverberg, 2000). The FIGO system uses 3 grades based on architectural criteria, i.e., the proportion of glandular or papillary structures relative to areas of solid tumor growth (Table 1.2).

Table 1.2 The stages of ovarian cancer

Stage I	disease confined to one or both ovaries
Stage IA	involves one ovary; capsule intact; no tumor on the ovarian surface; no malignant cell on the peritoneal washings or ascites
Stage IIB	involves both ovaries; capsule intac; no tumor on the ovarian surface; negative washings
Stage IIC	tumor limited to the ovaries with any of the followings: capsule ruptured, tumor on the ovarian surface, positive washings
Stage III	pelvic extensions or implants
Stage IIIA	extension onto the uterus or fallopian tubes, negative washings
Stage IIIB	pelvic extension or implants onto the peritoneal structures; negative washings
Stage IIIC	pelvic extension or implants with positive peritoneal washings
Stage IV	microscopic peritoneal implants ouside of the pelvis, or limited to pelvis with extension to small bowel or omentum

The dualistic model accommodates and confirms the heterogeneous nature of EOC and places the major histological types into 2 groups (types I and II) based on their distinctive clinicopathologic and molecular genetic features. Tumors that are amenable to current strategies for early detection are usually those belonging to the Type I group, which presents as large masses that are confined to one ovary (stage Ia), are indolent. Unfortunately, these account only for a minority (<25%) of malignant ovarian epithelial tumors. The type I tumors are relatively genetically stable and typically display a variety of somatic sequence mutations that include KRAS, BRAF, PTEN, PIK3CA, CTNNB1 (the gene-encoding β-catenin), ARID1A, and PPP2R1A but very rarely TP53 (Jones et al., 2010; Shih & Kurman, 2004; Wiegand et al., 2010). In contrast, the majority of what is considered "ovarian cancer" belongs to the Type II group, which present in advanced stage (stages II-IV) in more than 75% of cases; they grow rapidly and are highly aggressive. Type II tumors are chromosomally highly unstable and harbor TP53 mutations in more than 95% of cases (Ahmed et al., 2010); they rarely display the mutations found in the type I tumors.

1.1.3 Molecular Mechanisms of Ovarian Carcinogenesis

A currently view of ovarian tumorigenesis is that carcinoma begins from inclusion cysts in the ovary, undergoes sequential stages of tumor progression, and then spreads to the pelvic and abdominal cavities, and accumulation of ascites (Lengyel, 2010). Inclusion cysts are defined as cysts in the ovarian cortex that are lined by one layer of cells resembling the surface epithelium (Gusberg & Deligdisch, 1984) and result from entrapment of the surface epithelium during ovulation (Okamura, Katabuchi, Nitta, & Ohtake, 2006; Scully, 1995). Direct extension of cancer to sites proximal to primary tumors, through a series of complex processes which involves cellular proliferation, epithelial-to-mesenchymal transition (EMT) which results in tumor cells migration to distant sites, and mesenchymal-to-epithelial transition (MET) for colonization (Naora & Montell, 2005; Ahmed, Thompson, & Quinn, 2007). But still, expression patterns of epithelial marker, cadherins in the normal and neoplastic ovary are complex and do not fully follow the model which is seen during malignant transformation and tumor progression in the majority of carcinomas (Cavallaro & Christofori, 2004; Pecina-Slaus, 2003). In contrast to other epithelial originated cancer types, normal OSE with flat morphology expresses N-cadherin, but is E-cadherin negative, and the latter is expressed only in clefts and inclusion cysts, postulated to be ovarian carcinoma precursors (Hudson, Zeineldin, & Stack, 2008). Furthermore, while E-cadherin expression is reduced in some primary ovarian carcinomas, this protein is re-expressed in ovarian carcinoma effusions, with significantly higher levels compared to patient-matched primary carcinomas (Davidson et al., 2000).

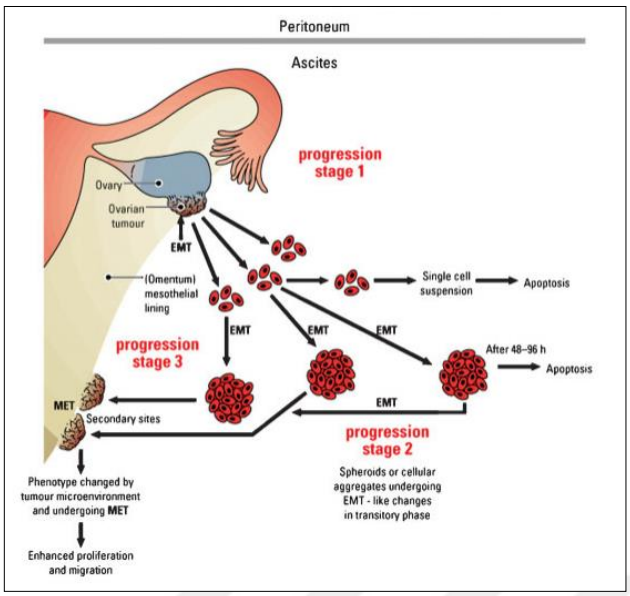


Figure 1.2 A working model of ovarian cancer progression (Ahmed, Thompson, & Quinn, 2007).

In recent years, it has become clear that partial or incomplete EMT can occur in tumors whereby cells retain some epithelial features and migrate or invade while maintaining cell:cell contacts (Brabletz, Jung, Spaderna, Hlubek, & Kirchner, 2005; Christiansen & Rajasekaran, 2006). This intermediate phenotype is observed in normal ovarian epithelium (Ahmed et al., 2006), at the invasive front of colon (Brabletz et al., 2005) and breast (Come et al, 2006) cancer and in normal epidermal tissue during wound repair (Arnoux, Come, Kusewitt, Hudson, & Savagner, 2005). In contrast to the well defined events that characterize EMT in development, tumor-associated EMT in ovarian cancer is currently viewed as a continuum of phenotypic plasticity and gain of mesenchymal characteristics. Another complicated matter is the co-expression of epithelial marker, E-cadherin and mesenchymal marker, N-cadherin in the ovarian carcinoma cells (Sivertsen, Berner, Michael, Bedrossian, & Davidson, 2006). Although there is no definite explanation for the formation of the inclusion cysts, the initiation of EMT in those sites, two hypotheses have been suggested that may promote mesenchymal transformation of OSE within the inclusion cyst in either a paracrine or an autocrine fashion (Auersperg et al., 2001).

In the early steps of EOC, ovarian tumor capsules also disrupt and malignant cells shed from the primary tumors into the peritoneum where they survive as single cells or free-floating multicellular aggregates, commonly known as spheroids, in the ascites. Attachment and disaggregation of cells on mesothelial extracellular matrix (ECM) allows them to anchor as secondary lesions on the walls of the peritoneal cavity with a varying extent of peritoneal invasion and at a later stage, metastasize to distant organs (Burleson, Boente, Pambuccian, & Skubitz, 2006; Shield, Ackland, Ahmed, & Rice, 2009), which has been suggested to occur via transcoelomic, lymphatic, or hematogenous routes (Feki et al., 2009; Tan, Agarwal, & Kaye, 2006). In many cases lymph node metastases and peritoneal spread co-exist. Metastasis through transcoelomic route is frequently associated with the production of ascites (Tan et al., 2006), observed in advanced-stage patients. The ascites may contain tumor cells with rapid proliferative rates indicative of rapid progression of the disease.

1.2 Cell Adhesion and Cancer

Epithelia are sheets of cells that line body cavities and external surfaces in multicellular organisms. Epithelia contain an apical membrane that faces the lumen and a basolateral surface that interacts with the neighboring cells and the basement membrane. This asymmetric organization is a characteristic trait of epithelial cells. Epithelial cells adhere to their neighbors and the extracellular matrix, mediated by different types of junctional complexes, including tight junctions (TJ), adherens junctions (AJ), gap junctions, desmosomes, cell–matrix adhesion complexes, tetraspanin-enriched microdomains, caveolae and lipid rafts, clathrin-coated pits (Figure 1.3). These complexes not only mediate adhesion but are also engaged in the transmission of signals from the plasma membrane to the nucleus to regulate gene expression. The regulation of cell adhesion is an important step to contribute to tissue homeostasis allowing processes such as differentiation and cell migration. Therefore, recent studies focus on to highlight the new insights gained on initiation and progression of carcinoma by investigating the role played by cell-cell adhesion during transformation of epithelial cells.

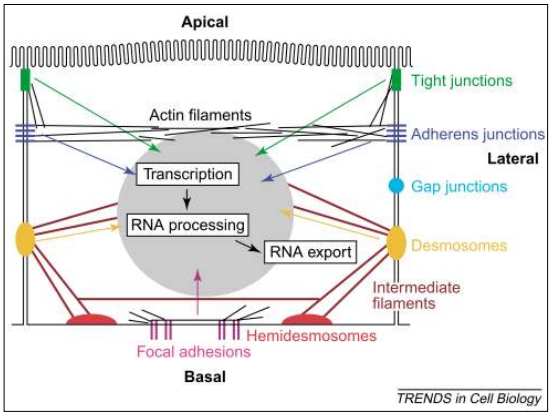


Figure 1.3 Schematic representations of cell adhesions in epithelial cell (Balda & Matter, 2003).

1.2.1 Claudins

Tight junctions (TJs) are composed of transmembrane proteins such as occludins and claudins and intracellular proteins such as Zonula occludens-1 (ZO-1) that coalesce apical to AJ and seal the spaces between neighboring epithelial cells, separate apical and basal membrane domains, and interact with the cytoskeletal network (Shin, Fogg, & Margolis, 2006). The appositions between the exocytoplasmic leaflets of the lateral membranes of adjacent cells extend in a beltlike network that surrounds each cell and attaches it to the neighboring cell to form a continuous seal. In epithelial cells the TJ functions in an adhesive manner and can prevent cell dissociation (Hollande et al., 2001). It has however, become increasingly apparent that the TJ has a vital role in maintaining cell to cell integrity and that the loss of structure integrity can cause to invasion and metastasis of cancer cells.

An important step in the formation of cancer metastases is interaction and penetration of the vascular endothelium by dissociated cancer cells. TJ are therefore the first barrier that cancer cells must overcome in order to metastasize, demonstrated the correlation between the reduction of TJ and tumor differentiation (Hoevel, Macek, Mundigl, Swisshelm, & Kubbies, 2002; Martin, Mansel, & Jiang, 2002; Ren, Hamada, Takeichi, Fujikawa, & Kobayashi, 1990; Satoh et al., 1996). Aberrant expression of claudins is also common in epithelial cancers. Different cancer sites and histological subtypes seem to affect different claudins. In general though, claudin-1 and -7 are

downregulated in esophageal cancer (Lioni et al., 2007), but upregulated in others (Dhawan et al., 2005; Pan et al., 2007). The most common claudins to be affected are claudin-3 and -4 are usually upregulated in cancers. While the mislocalization of claudin-7 in esophageal squamous cell carcinoma leads to the loss of E-cadherin expression, N-glycosylation of E-cadherin has been shown to stabilize tight junctions (Nita-Lazar, Rebustini, Walker, & Kukuruzinska, 2010). In vitro studies suggest that dysregulation of claudin expression may play a pathogenic role in tumorigenesis, but the mechanisms seem to vary between different cancers and claudin isoforms and are mostly poorly understood (Günzel & Yu, 2013).

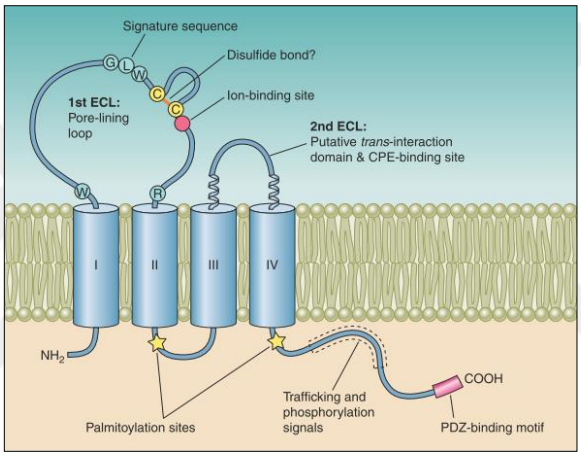


Figure 1.4 A model of claudin structure (Günzel & Yu, 2013).

Claudins which also show Ca^{2+}-independent cell adhesion activity (Kubota et al., 1999), are 18- to 27-kDa tetraspan proteins which have a short cytoplasmic N-terminus, two extracellular loops and a C-terminal cytoplasmic domain (Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998; Tsukita, Furuse, & Itoh, 2001). The C-terminal cytoplasmic tail of claudins is required for their stability and targeting (Arabzadeh, Troy, & Turksen, 2006; Müller, Kausalya, Meij, & Hunziker, 2006; Rüffer & Gerke, 2004). In addition, through a PDZ-binding motif at their C-terminus, claudins directly bind to PDZ (PSD-95/Dlg/ZO-1) domain-containing peripheral membrane (Hamazaki, Itoh, Sasaki, Furuse, & Tsukita, 2002; Itoh et al., 1999; Roh, Liu, Laurinec & Margolis, 2002). Importantly, the position and number of charged amino acids in the first extracellular loop show a wide variation depending on each claudin.

1.2.2 E-cadherin

Adherens junctions (AJs) which localize to the basal side of tight junctions are cell–cell microdomains where calcium-dependent cadherin receptors bind with their extracellular domains to cadherins on opposing cells. The cytoplasmic tails of cadherin receptors connect—via adaptors—to filamentous actin and thereby connect the actin cytoskeleton of neighboring cells through transmembrane cadherin receptors and a network of adaptor proteins (Green, Getsios, Troyanovsky, & Godsel, 2010; Takeichi, 1991). These interactions are tightly controlled to facilitate cell junction assembly or disassembly in response to changes in external or internal forces and/or signaling.

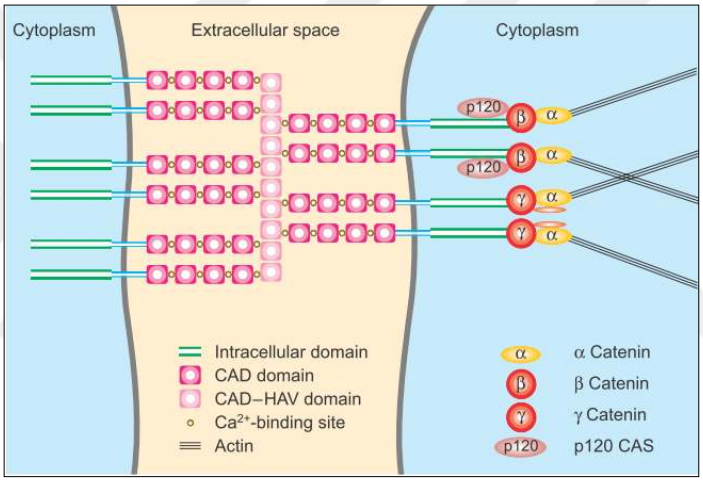


Figure 1.5 E-cadherin-mediated cell–cell adhesions (Christoforiand & Semb, 1999).

The core of the AJ includes interactions among transmembrane glycoproteins of the classical cadherin superfamily, such as E-cadherin, and the catenin family members including p120-catenin, β-catenin, and α-catenin. E-cadherin is a single-pass Type-I transmembrane glycoprotein that is localized to the adherens junction and basolateral membrane in epithelial cells. It consists of a large extracellular domain which contains 5 extracellular cadherin domain repeats that bind calcium ions, a transmembrane segment, and a conserved cytoplasmic domain. Homophilic interactions between cadherin molecules occur first between adjacent cells and then within the same cell by lateral association. These associations form a zipper-like structure that strengthens the

adhesion between the neighboring cells. The cytoplasmic domain interacts with the catenins and a variety of actin-binding proteins to anchor the cadherin–catenin complex to the actin cytoskeleton (Gumbiner, 2005). The adhesive function of E-cadherin plays a vital role in epithelial physiology. The fully formed cadherin–catenin complex, with its associated actin filaments, forms the core of the adherens junction. It brings together 2 apposed plasma membranes with an intercellular gap (only ~25 nm) (Fedor-Chaiken, Hein, Stewart, Brackenbury, & Kinch, 2003), which halts the movement of individual epithelial cells (known as contact inhibition of motility) and permits their organization into tightly bound layers with functionally distinct apical and basolateral plasma membrane domains (David & Rajasekaran, 2012).

A pivotal hallmark of cancer, which is the ability of cancer cells to break free from the primary tumor and migrate to distant sites within the body to form new tumors, is arised through the progressive loss of epithelial characteristics and cell-cell adhesion as cancer cells adopt a more mesenchymal phenotype in a process termed epithelial-to-mesenchymal transition (EMT) (Christiansen & Rajasekaran, 2006; Frixen et al., 1991). A multitude of clinical and experimental studies have revealed that E-cadherin's adhesion function frequently is lost during the development of most, if not all, human epithelial cancers, including carcinomas of the breast, colon, prostate, stomach, liver, esophagus, skin, kidney and lung (Birchmeier & Behrens, 1994; Bracke, van Roy, & Mareel, 1996). Several different mechanisms appear to cause the loss of E-cadherin function, including deletion or mutational inactivation of the E-cadherin gene (Birchmeier & Behrens, 1994; Bracke, van Roy, & Mareel, 1996). Although the prevailing belief is that induction of EMT and loss of E-cadherin is a prerequisite for progression to metastatic disease, tumors are actually remarkably heterogeneous and cancer cells may undergo only a partial transition that enhances invasion while still retaining certain epithelial characteristics, such as E-cadherin (Christiansen & Rajasekaran, 2006).

1.2.3 EpCAM

Many cell adhesion molecules (CAMs) have been characterized in recent decades and function in different processes. Four archetypal CAM families have been identified as the cadherins, the selectins, the integrins, and the immunoglobulin CAM (Ig-CAM) superfamily. In addition to the identified CAMs, several CAMs exist that do not share any of the structural patterns of the four CAM families, i.e. the epithelial cell adhesion molecule (EpCAM).

EpCAM is a type I, transmembrane, 39–42 kDa glycoprotein that functions as a homophilic, epithelial-specific intercellular cell–adhesion molecule (Baeuerle & Gires, 2007; Balzar, Briaire-de, & Rees-Bakker, 2001; Litvinov et al., 1994). The 314 amino acid-long EpCAM is comprised of an extracellular domain (EpEX) with epidermal growth factor (EGF)-and thyroglobulin repeat-like domains, a single transmembrane domain, and a short 26-amino acid intracellular domain (EpICD) (Baeuerle & Gires, 2007; Novinec et al., 2006).

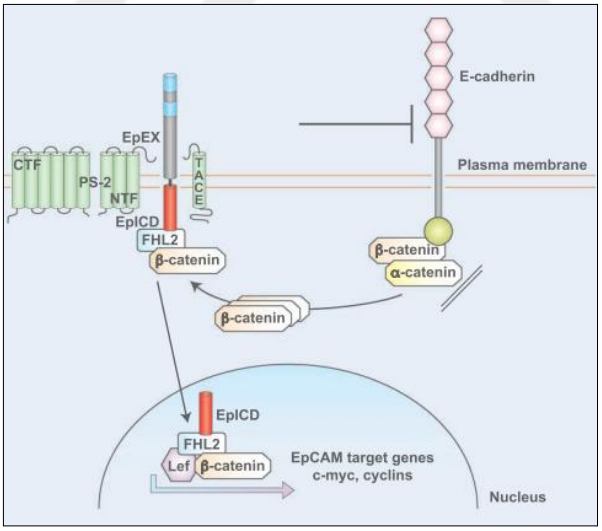


Figure 1.6 Schematic of related signaling pathways of EpCAM (Munz, Baeuerle, & Gires, 2009).

In contrast to the broad expression pattern of most other CAMs in normal tissues, the expression of EpCAM is restricted to normal epithelial cells. Aberrant expression, such as up-regulation, down-regulation, or de novo expression, is characteristic for

EpCAM during and after malignant transformation. EpCAM is typically overexpressed in a variety of epithelial cancers, ie, carcinomas. EpCAM is not expressed on tumors of mesodermal and ectodermal origin, such as neurogenic tumors, sarcomas, melanomas, or lymphomas (Momburg, Moldenhauer, Hammerling, & Moller, 1987). Its overexpression usually correlates with a decrease in survival, for instance in breast and ovarian cancer (Spizzo et al., 2004; Spizzo et al., 2006). An exception is renal cell carcinoma, in which EpCAM expression correlates with an increase in life expectancy (Seligson et al., 2004). EpCAM acts as an oncogenic signal transducer. Several biological functions of EpCAM have been described: i) EpCAM is able to abrogate E-cadherin-mediated cell–cell adhesion by disrupting the link between α-catenin and F-actin thereby loosening cell–cell adhesion (Winter et al., 2003), ii) Association of EpCAM with claudin-7 interferes with EpCAM-mediated homotypic cell–cell adhesion, promoting cell motility, proliferation, survival, carcinogenesis and metastasis formation (Nubel et al., 2009), iii) Upon sequential cleavage of EpCAM by tumor-necrosis-factor alpha converting enzyme (TACE/ADAM17) and a gamma-secretase complex containing presenilin 2 (PS-2) results in release of EpEX into the culture medium, and release of EpICD into the cytoplasm. EpICD functions as part of a transcriptional complex inducing c-myc and cyclin A and E expression with transcriptional regulators β-catenin and Lef, which are both components of the wnt pathway (Maetzel et al., 2009).

In contrast to its promoting role regarding tumour formation, EpCAM is also a tumour suppressive protein. The dual role of EpCAM is reflected by the studies that showed colorectal carcinoma cells transfected with EpCAM complementary DNA (cDNA) increased cell–cell adhesion, attenuated tumor cell invasion in matrigel and decreased tumour incidence and metastasis (Basak et al., 1998).

1.2.4 Tetraspanin-Enriched Microdomains

Structural and compositional gradients of plasma membrane proteins in epithelial cells constitute several epithelial surface domains. The ability of individual cells to differentiate their plasma membrane to form specialized domains with distinct protein and lipid compositions is crucial for many cell biological processes, including cell adhesion, signaling, directed migration, asymmetric cell division and cell fate determination, the development of neuronal networks, and the development of functional epithelial and endothelial barriers (Nelson, 2003; Wodarz & Näthke, 2007). The first ordered membrane domains identified were related to the lipid component of the plasma membrane and were termed lipid rafts (Edidin, 2003; Simons & Toomre, 2000). Independently, protein–protein and protein–lipid interactions were described for various members of the tetraspanin superfamily (Boucheix & Rubinstein, 2001; Tarrant, Robb, van Spriel, & Wright, 2003).

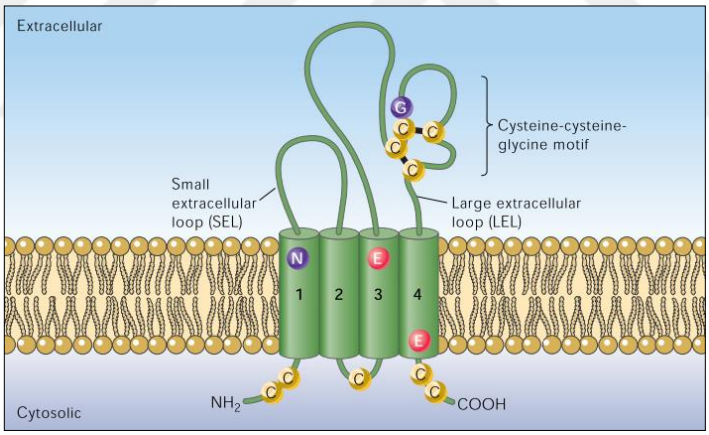


Figure 1.7 Structural features of tetraspanins (Levy & Shoham, 2005).

Tetraspanins contain four transmembrane (TM) domains. The tetraspanin TM domains flank a small and a large extracellular loop (SEL and LEL, respectively) (Figure 1.8). The extracellular loops vary among family members. On the other hand, the LEL contains characteristic motifs, especially the conserved cysteine-cysteine-glycine (CCG) motif, which is the focal point for the formation of two to four disulfide

bridges with additional cysteine residues at fixed positions within the LEL (Seigneuret, Delaguillaumie, Lagaudriere-Gesbert, & Conjeaud, 2001).

Many studies have found correlations between tetraspanins and cancer progression. Most tetraspanins were downregulated in metastatic tumours except CD151, which is the first tetraspanin member to be identified as a promoter of metastasis (Testa, Brooks, Lin, & Quigley, 1999). Similarly, TSPAN8 was also up-regulated at the advanced stages of pancreatic, hepatic, oesophageal, gastric and colorectal carcinomas (Richardson, Jennings, & Zhang, 2011). Although most studies showed CD9 to be down-regulated during cancer progression, the opposite has been found in osteosarcoma, prostate cancer and breast cancer (Kubista, Erovic, Klinger, Sulzbacher, & Trieb, 2004; Zvierev, Wang, & Chevrette, 2005; Kischel et al., 2012).

1.2.5 Regulation of Cell Adhesion

When considering regulatory mechanisms which determine assembly, remodeling/modulation, and dynamics of cell adhesion, regulation occurs on various levels, such as regulation of transcription, post-translational modification, interaction with scaffolding proteins, and homo- or heterodimeric interactions within the same membrane (cis-interaction) as well as between neighboring cells (trans-interaction). After controlling whether proteins are synthesized or not, the cell determines its localization by the balance between its transports by exocytosis and endocytosis. The activity and interactions of a protein at *in situ* location is determined by posttranslational modifications. These modifications include glycosylation, lipidation, ubiquitination, acetylation, proteolysis, and phosphorylation (Clark, Bayir, & Jenkins, 2005).

1.2.5.1 Phosphorylation

Phosphorylation of tyrosine, serine, or threonine residues is a rapid and reversible form of regulation affecting the majority of adhesion molecules, turning “on” or “off” their interactions with other proteins and/or their enzymatic activity. Phosphorylated

tyrosine, serine, or threonine residues can affect a protein in three major ways: i) It can increase the affinity for another protein, ii) It can inhibit a protein-protein interaction, or iii) It can activate enzymatic activity. In proteins with an intramolecular interaction, phosphorylation and dephosphorylation can elicit a conformational change in the protein. The recent studies showed that the balance between kinases and phosphatases leads to the assembly or disassembly of cell adhesions, highlighting the important role of phosphorylation switches in regulating their dynamics.

Phosphorylation has been linked to both increases and decreases in tight junction assembly and function. While Protein Kinase A-mediated phosphorylation has been shown to decrease assembly of claudin-3 into tight junctions (D'Souza, Agarwal, & Morin, 2005), claudin-1 and claudin-4 phosphorylation by Protein Kinase C- theta is required for assembly into intestinal epithelial tight junctions (Banan et al., 2005). A computer-aided search on possible claudin phosphorylation sites for various kinases (PKC, PKA, MAP kinase, WNK, MLCK, c-Src, RhoK, and the Eph receptor family), carried out by González-Mariscal et al. (González-Mariscal, Garay, & Quirós, 2010) showed that there were between 0 and 20 phosphorylation sites on claudins 1 to 22 and some of these sites have already been described to be of physiological importance. Claudin-1 is a target of MAP kinase, aPKC and PKA, and dephosphorylation is achieved by PP2A. MAP kinase as well as aPKC and PKA induced phosphorylation promotes claudin-1 insertion into the tight junction (French et al., 2009; Fujibe et al., 2004; Nunbhakdi-Craig et al., 2002). S208 in claudin-2 was predicted to be a cPKC/nPKC (González-Mariscal, Garay, & Quirós, 2010). Claudin-4 was phosphorylated through PKC or aPKC and the latter process proved to be essential for tight junction formation (D'Souza, Agarwal, & Morin, 2005).

While it is most likely every kinase can recognize at least one docking site within adhesion domains, it is not currently known which of the kinases reside in domains permanently and or which are transient components, homing in to phosphorylate proteins only under specific conditions. Even permanent residents may not always be active, as most kinases need themselves to be activated.

Protein kinase C (PKC) has long been recognized to affect epithelial and endothelial barriers. Protein Kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases. Expression of PKCs is widely varied with different cell types expressing different family members. PKCs preferentially phosphorylate substrates that contain a serine/threonine in the context of adjacent amino acids, with different isoforms preferring particular amino acids that surround the phosphorylation site (Nishikawa, Toker, Johannes, Songyang, & Cantley, 1997; Fujii et al., 2004).

PKC isoforms are classified into three subgroups, based on the presence or absence of functional membrane-binding domains in their N-terminal regulatory domains. These are known as the conventional PKCs (cPKCs; comprise PKC α, βI/βII and γ isoforms), the novel PKCs (nPKCs; comprise δ, ε, η and θ) and the atypical PKCs (aPKCs; comprise PKC ζ and ι). These isoforms differ in their mechanism of action, subcellular distribution, substrate type and expression. The cPKCs are activated by both DAG, which binds to the C1 domain, and Ca^{2+}, which binds to the C2 domain (Figure 1.9). After Ca^{2+} signaling, the binding of DAG to the C1 domain increases the affinity of PKCs for membrane lipids, inducing conformational changes that lead to a catalytically competent form. Phorbol esters bind to the same site as DAG and mimic its activation (Giogi et al., 2010; Oancea & Meyer, 1998; Gopalakrishna & Jaken, 2000). Novel PKCs are independent of Ca^{2+} and activated directly by DAG (Giogi et al., 2010). Recent studies indicate the existence of another model in which cellular stimulation results in inducible phosphorylation at some of the three sites in PKCs (Saito, Kikkawa, & Nishizuka, 2002; Thannickal & Fanburg, 2000; Chiarugi & Buricchi, 2007). Atypical enzyme (aPKC) activation takes place independently of calcium or DAG, but they may be activated by other PKCs (Zugaza, Sinnett-Smith, Van Lint, & Rozengurt, 1996; Paolucci & Rozengurt, 1999; Nishizuka, 1992; 1995).

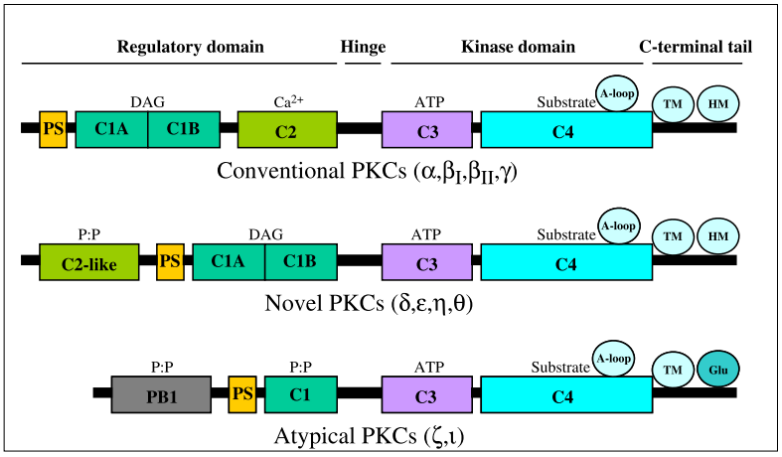


Figure 1.8 Structure of PKC isoenzymes (Freeley, Kelleher, & Long, 2011).

All PKC isoforms share a similar overall structure in that they consist of an N-terminal regulatory domain that is coupled to a highly conserved C-terminal kinase domain. The N-terminal regulatory domain functions to tether the enzyme to various locations in the cell such as the plasma membrane by one or more modules (C1, C2, PB1 or phosphatidylserine-binding domains) when engaged by lipid second messengers or other interacting proteins, and to negatively regulate enzymatic activity.

Under physiological conditions, PKC activation occurs in response to various growth factors. Furthermore, series of phosphorylations regulate PKC activation (Parekh, Ziegler, & Parker, 2000). Mutational analysis of predicted phosphorylation sites (Cazaubon, Bornancin, & Parker, 1994; Orr & Newton, 1994) and mass spectrometry (Tsutakawa, Medzihradszky, Flint, Burlingame, & Koshland Jr., 1995; Keranen, Dutil, & Newton, 1995) that three key phosphorylation sites were identified on the C-terminus that were important for PKC function. These sites are known as the activation-loop (A-loop), the turn-motif (TM) and the hydrophobic-motif (HM). While all three sites are conserved in cPKCs and nPKCs, the aPKCs do not contain an HM residue but instead possess a negatively charged glutamic acid, perhaps mimicking a constitutively phosphorylated state. In the absence of activating cofactors, such as Ca^{2+} and DAG, PKCs are maintained in an inactive conformation by binding of the PS to the substrate-binding cavity (Giogi et al., 2010; Newton, 2003).

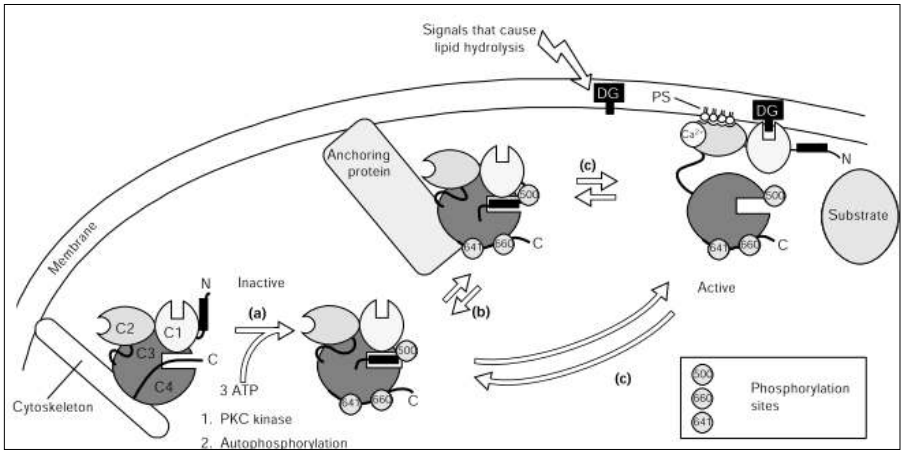


Figure 1.9 Model for the regulation of PKC by phosphorylation, targeting proteins, and cofactors (Newton, 1997).

The activation of PKCs was showed in Figured 1.9. In general, newly synthesized PKC associates with the cytoskeleton, in a conformation that exposes the activation loop which is represented by a gap in C4, and tethers the carboxyl terminus (C) near the active site. Phosphorylation by a putative PKC on the exposed activation loop (at Thr500) correctly aligns residues for catalysis, allowing autophosphorylation at two carboxy-terminal positions (Thr641 and Ser660). Phosphorylation at Thr641 locks the kinase in a catalytically competent conformation. Subsequent phosphorylation at the second carboxy-terminal site (Ser660) releases mature protein kinase C into the cytosol. Phospholipase C (PLC) when activated by growth factor receptors, hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate DAG and inositol trisphosphate (IP_3), which subsequently mobilize intracellular calcium (Steinberg, 2008). While removal of the N-terminus located pseudosubstrate sequence which binds to the substrate-binding cavity in the C-terminus and blocks catalytic activity, from the kinase domain occurs lipid second messengers bind to the regulatory domain, resulting in substrate binding and phosphorylation (Dutil & Newton, 2000). This step depends on PKC isoenzyme.

PKC isozymes play key roles in many of the signaling pathways that control cellular growth, proliferation, differentiation and cell death (Steinberg, 2008; Newton, 2010). They respond to a variety of external stimulators, including hormones, growth factors,

and other membrane receptor ligands. PKC isozymes can act as therapeutic targets for several diseases, such as cardiovascular diseases (e.g., atherosclerosis, myocardial fibrosis, cardiac hypertrophy, and hypertension) (Churchill, Budas, Vallentin, Koyanagi, & Mochly-Rosen, 2008; Palaniyandi, Sun, Ferreira, & Mochly-Rosen, 2009), immune and inflammatory diseases (e.g., asthma, arthritis, and hepatitis) (Lee, Duan, & Tan, 2008; Boschelli, 2009), neurological diseases (e.g., Alzheimer's disease and bipolar disorder) (Zarate & Manji, 2009; Pascale, Amadio, Govoni, & Battaini, 2007), and metabolic disorders (e.g., obesity, insulin resistance, hyperglycemia, and hyper-cholesterolemia) (Farese & Sajan, 2012; Clarke & Dodson, 2007; Das Evcimen & King, 2007). Further, significant work has also explored the activation, mechanism, and function of PKC isoenzymes in the development and progression of multiple types of cancer. Furthermore, the expression patterns and functions of PKC isozymes in cancer cells largely depend on the type of cancer being investigated; however, the mechanism is not clear.

1.2.5.2 Palmitoylation

Palmitoylation is one of a group of lipid modifications (collectively termed lipidation) which is mediated by polytopic membrane proteins (Fukata, M., Fukata, Y., Adesnik, Nicoll, & Bredt, 2004; Mitchell, Vasudevan, Linder, & Deschenes, 2006), implying that cellular palmitoylation reactions occur at the cytosol–membrane interface. There are ~23 putative S-palmitoyl transferases in mammals, characterized by the presence of a DHHC (aspartate-histidine-histidine-cysteine) motif within a ~50 amino acid cysteine-rich domains. The large number of these DHHC proteins locates to distinct membrane compartments (Ohno, Kihara, Sano, & Igarashi, 2006) implies that the cellular palmitoylation machinery is a highly regulated and coordinated system.

Di-cysteine palmitoylation motifs are conserved throughout the claudin and tetraspanin proteins. It is clear that palmitoylation functions to mediate stable membrane attachment of soluble proteins. Analyses using different proteins and systems have revealed that palmitoylation has many distinct effects on modified

proteins including regulating protein trafficking, protein stability, membrane microlocalization, and protein–protein interactions (Resh, 2006a, b; Greaves and Chamberlain, 2007; Linder and Deschenes, 2007; Greaves et al., 2009b; Noritake et al., 2009). TJ localization of some claudins can be promoted by palmitoylation. Two palmitoylation sites were identified within the cytoplasmic loop and two further sites within the cytoplasmic COOH terminus, close to the fourth transmembrane region in claudin-14 (Van Itallie, Gambling, Carson, & Anderson, 2005). Palmitoylation also occurs in claudin-1, -2, -3 and -4 (Butt et al., 2011). Tetraspanins, such as CD9 and CD81, are also palmitoylated which is critical to their function as membrane scaffolding proteins and organization of higher order membrane domains (Hemler, 2005).

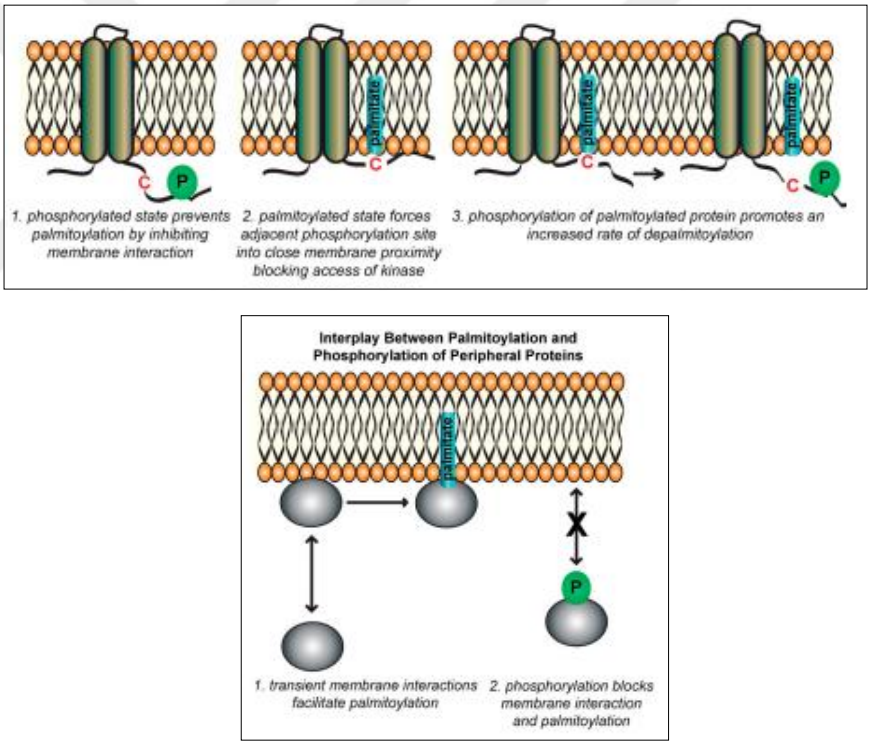


Figure 1.10 Interplay between palmitoylation and phosphorylation (Salaun, Greaves, & Chamberlain, 2010).

Regulation of phosphorylation by palmitoylation was identified many years ago for the β2-adrenergic G protein–coupled receptor (Moffett, Mouillac, Bonin, & Bouvier, 1993). One potential mechanism is membrane insertion of palmitoylated cysteines

restricting access of protein kinases to the adjacent phosphorylation sites. Vice-versa, regulation palmitoylation of by phosphorylation was showed between the STREX variant of BK potassium channels and PKA (Tian et al., 2001). A recent study also reported that the STREX variant contains palmitoylated cysteines adjacent to the PKA site that regulate plasma membrane binding of the cytosolic C terminus (Tian et al., 2008).

1.2.5.3 Cholesterol

Tetraspanins are the largest family of transmembrane proteins in mammals for which detailed structural information about the intramembrane domain remains unavailable. This lack of information, together with a limited understanding of the structural relationship between the intramembrane domain and the extracellular region of the protein, has greatly hindered efforts to understand the molecular action mechanisms of these proteins. Direct binding of tetraspanins to cholesterol has been demonstrated (Charrin et al., 2003), accounting for the partial recovery of TEMs in insoluble fractions of sucrose gradients under certain conditions (Claas, Stipp & Hemler, 2001). Digitonin extraction, which precipitates membrane cholesterol, seems to precipitate tetraspanin oligomers, thus revealing direct tetraspanin–partner associations (Serru et al., 1999; Charrin et al., 2003). Consistently, cholesterol depletion has varying effects on TEM function. Zimmerman et al. (2016) also showed that the crystal structure of human CD81, a tetraspanin full-length and extracellular helices converge at the inner leaflet to create an intramembrane pocket bound with cholesterol within the cavity. Cholesterol binding modulates modulate CD81 activity in cells, suggesting a potential mechanism for regulation of tetraspanin function (Zimmerman et al., 2016).

1.2.6 Incorporation in Specialized Domains of Plasma Membrane

Structural and compositional gradients of plasma membrane proteins in epithelial cells which constitute several epithelial surface domains, give the ability of individual cells to differentiate their plasma membrane to form specialized domains with distinct

protein and lipid compositions, also is crucial for many cell biological processes, including cell adhesion, signaling, directed migration, asymmetric cell division and cell fate determination, the development of neuronal networks, and the development of functional epithelial and endothelial barriers (Nelson, 2003; Wodarz & Näthke, 2007).

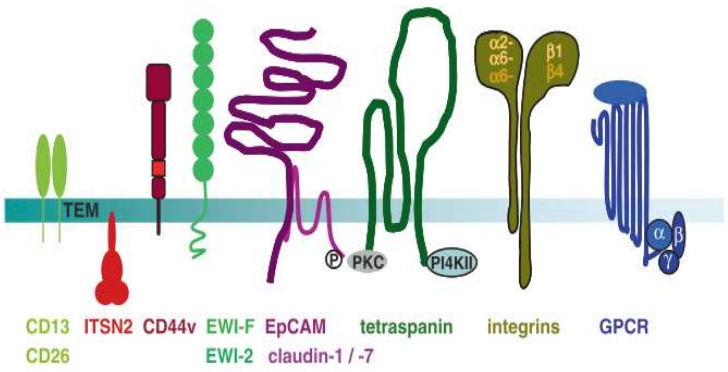


Figure 1.11 Tetraspanins and associated molecules (Zöller, 2010).

The members of tetraspanin family form lateral associations with multiple partner proteins and with each other in a dynamic assembly in tetraspanin-enriched microdomains (TEMs), described as the tetraspanin web (Rubinstein et al., 1996). TEMs play an important role in cell-cell interactions and in cell-fusion events. The associations between tetraspanins with their nontetraspanin partner molecules in TEMs are referred as primary interactions in which a single tetraspanin molecule can form different partnerships in different cell types (Boucheix & Rubinstein, 2001). Partnerships formed with the extracellular or the intracellular domains of the interacting molecules are highly stoichiometric, are highly avid and retained after lysis in harsh detergents (such as 1% NP-40), and are direct, as demonstrated by chemical crosslinking (Wu, Medina, & Sun, 1995; Xu et al., 2004). The associations between members of the tetraspanins family have been referred to as secondary interactions which palmitoylation is necessary for the maintenance of tetraspanin-tetraspanin interactions (Rubinstein et al., 1996). These secondary associations which are not stoichiometric are not disrupted by mild detergents (1% Brij 96 or 1% Brij 97) and can be augmented by the presence of divalent cations (Charrin et al., 2002). The indirect

associations of tetraspanins with additional proteins, which are referred as tertiary interactions are not disrupted in milder detergents, such as 1% CHAPS. These all interactions cluster in TEMs and enable lateral dynamic organization in the membrane and the cross-talk with intracellular signaling and cytoskeletal structures.

Epithelia and endothelia typically express multiple claudin isoforms in a tissue specific manner, suggesting the potential of claudin isoforms to intermix in tight junction strands. The interactions between different classes of claudins have the potential to control barrier permeability through the formation of tight junctions composed of multiple claudins. Claudins interact in either laterally in the plane of the membrane (heteromeric interactions) or head to head binding between adjacent cells (heterotypic interactions) (Findley & Koval, 2009).

1.3 Phytochemicals as Anti-Cancer Agents

Cancer development is a multistage and multifactorial process involving a series of events affecting proto-oncogenes or tumor-suppressor genes, and generally occurs over an extended period (Jones & Baylin, 2002). During this process, accumulation of genetic and epigenetic alterations leads to the progressive transformation of a normal cell into a malignant cell, offering the opportunities to intervene its development (Beliveau & Gingras, 2007). Although chemotherapeutic drugs are some of the most important treatments for cancer, often cancer cannot be entirely cured and may be accompanied by unexpected adverse side-effects that can limit their efficacy (Payne, James, & Weiss, 2006; Rybak, Mukherjea, Jajoo, & Ramkumar, 2009). There is an urgent need to find some safer cancer therapeutic and preventive agents (Yoon & Baek, 2005) which the use of foods or food ingredients for chemotherapeutic purposes has drawn the interest of scientists, in part because these foods are generally recognized as safe (GRAS). Epidemiological studies have suggested that specific dietary consumption patterns are associated with risk of developing cancer at several sites. In particular, high consumption of plant-based foods including fruits, vegetables and legumes (Block, Patterson, & Subar, 1992; Steinmetz & Potter, 1991) appeared to be protective. However, other studies have revealed that rather than a single bioactive

compound, the antioxidant and anticancer activities are often exerted by complex mixtures through complementary and overlapping mechanisms (Fernandez-Panchon, Villano, Troncoso, & Garcia-Parrilla, 2008; Liu, 2004). The properties of plants led to the development of a promising approach to cancer prevention, termed "chemoprevention," which uses natural or synthetic substances or their combination to block, retard, or reverse the development and progression of carcinogenesis (Atawodi et al., 2009).

Table 1.3 Representative anti-cancer mechanisms of selected phytochemicals derived from different foods (Wang, Meckling, Marcone, Kakuda, & Tsao, 2011).

Examples of Mechanism	**Phytochemical Representatives**	**Major Plant Derived Dietary Source**	**Tumor Cell Line**	**Reference**
Antioxidant effects: Scavenging free radicals or inducing antioxidant enzymes (glutathione peroxidase, superoxide dismutase)	Secoisolariciresinol diglucoside (SDG)	Flax, sunflower, sesame and pumpkin seeds	Not applicable	Niemyer and Metzler, 2003
Modulation of protein kinase and lipid kinase signalling pathways	Luteolin	Celery, green pepper	Human hepatoma HepG2 cells	Liu et al., 2011
Inhibiton of cyclooxygenase-2 (COX-2) enzymatic activity	Resveratrol	Grapes, peanut, blueberries, cranberries and lingonberries	Human ovarian cancer OVCAR-3 cells	Lin et al., 2011
	Fisetin	Onion, cucumber, apple and strawberry	Human colon cancer HT29 cells	Suh et al., 2009
	β-carotene	Mangoes, papayas, carrots, yams, spinach and kales	Human colon cancer LS-174 cells	Palozza et al., 2005

Table 1.3 continued.

Examples of Mechanism	Phytochemical Representatives	Major Plant Derived Dietary Source	Tumor Cell Line	Reference
Phytoestrogonic effects	Daidzin and genistin	Soybeans	Human estrogen receptor positive (ER+) breast cancer MCF-7 cells	Hwang et al., 2006
	Resveratrol	Grapes, peanut, blueberries, cranberries and lingonberries	Human androgen-sensitive prostate cancer LNCaP cells	Kuwajerwala et al., 2002
Effect on nuclear transcription factor NF-kB	Sulforaphane	Cruciferous vegetables such as cabbage, kale, broccoli, cauliflowers	Human leukemia cells (U937, THP-1, HL60, K562), human prostate carcinoma PC-3 cells, human breast carcinoma MCF-7 cells, human hepatocellular carcinoma HepG2 cells	Moon et al., 2009
Induction of phase I or phase II metabolizing enzymes	Glucosinolates and their enzymatic metabolites isothiocyanates	Cruciferous vegetable e.g. cauliflower, kale cabbage, broccoli	Not applicable	Talalay and Fahey, 2001

Table 1.3 continued.

Examples of Mechanism	Phytochemical Representatives	Major Plant Derived Dietary Source	Tumor Cell Line	Reference
Induction of cell cycle arrest	Epigallocatechin gallate (EGCG)	Green tea and black tea	Human breast cancer MDA-MB-231 cells	Thangapazham et al., 2007
	EGCG	Green tea and black tea	Human hepatocellular carcinoma HepG2 cells	Kuo and Lin, 2003
	Gallic acid	Blackberry, raspberry, mongo peels, walnut and white tea	Human promyelocytic leukemia HL- 60 cells	Madlener et al., 2007
	Resveratrol	Grapes, peanut, blueberries, cranberries and lingonberries	Human colon carcinoma HT-29 cells	Liang et al., 2003
Inhibition of matrix metalloproteinases (MMP)-2, MMP-9	EGCG	Green tea and black tea	Human breast cancer MDA-MB-231 cells	Thangapazham et al., 2007
	Proanthocyanidin	Cocoa, grape skin, cranberry	Human prostate tumor DU145cells	Neto et al., 2006

1.3.1 Biological Activities of Flavonoids in Cancer

Plants produce a vast and diverse assortment of organic compounds. The substances which do not appear to participate directly in growth and development, traditionally referred to as secondary metabolites. Based on their biosynthetic origins, there are three broad categories of plant secondary metabolites as natural products; terpenes and terpenoids (~25,000 types), alkaloids (~12,000 types), and polyphenolics (~8,000 types). Among the active constituents of extracts from herbs, apples, tea, grape, red wine, and other fruits and vegetables (Ververidis et al., 2007), Polyphenolic compounds constitute one of the most numerous groups in the plant kingdom and can be divided into various classes on the basis of their molecular structure, with

flavonoids which is the most common group of plant phenylpropanoids and have been used worldwide as traditional medicines for thousands of years (Rathee, Chaudhary, Rathee, Rathee, Kumar, & Kohli, 2009).

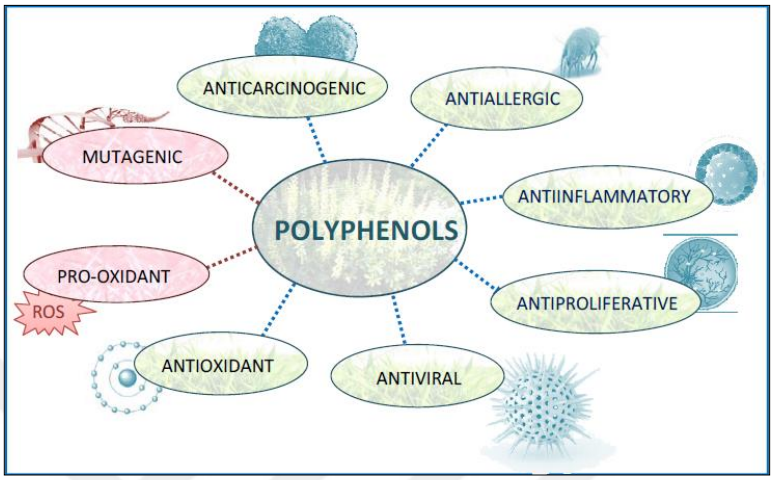


Figure 1.12 The different roles of polyphenols.

Flavonoids are further divided into subclasses based on the connection of an aromatic ring to the heterocyclic ring. The six major subclasses of flavonoids include the flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, myricetin), flavanones (e.g., naringenin, hesperidin), catechins or flavanols (e.g., epicatechin, gallocatechin), anthocyanidins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein).

Due to the fact that carcinogenesis is a multistage process with an accumulation of genetic and epigenetic alterations, targets for chemoprevention could be multiple and could vary from the initiation phase, to the promotion phase, and then to the progression phase (Manson, 2003). Since flavonoids display a vast array of cellular effects, they can affect the overall process of carcinogenesis by several mechanisms, including,

- inhibition of DNA topoisomerase I/II activity (Salti et al., 2000; Snyder & Gillies, 2003),

- decrease or increase in reactive oxygen species (Chun, Chang, Choi, Kim, & Ku, 2005),
- DNA oxidation and fragmentation (Musonda & Chipman, 1998; Abalea et al., 1999; Yi, Fischer, Krewer, & Akoh, 2005),
- regulation of heat-shock-protein expressions (Jakubowicz-Gil, Paduch, & Kandefer-Szerszen, 2005),
- cell cycle arrest (Gupta, Hussain, & Mukhtar, 2003; Lee, Kim, Park, Chung, & Jang, 2003; Yin, Giuliano, Law, & Van Herle, 2001; Ong et al., 2004),
- modulation of survival/proliferation pathways (Spencer, Rice-Evans, & Williams, 2003; Gong, Li, Nedeljkovic-Kurepa, & Sarkar, 2003; Park & Seol, 2002; Lee et al., 2001; Masuda, Suzui, & Weinstein, 2001; Kuo & Lin, 2003; Lee et al., 2005; Frey & Singletary, 2003; Siddiqui, Adhami, Afaq, Ahmad, & Mukhtar, 2004; Shimizu, Deguchi, Lim, Moriwaki, & Kopelovich, 2005; Selvendiran et al., 2006; Lee et al., 2004; Kaneuchi et al., 2003),
- release of cytochrome c with subsequent activation of caspase–9 and caspase– (Ong et al., 2004; Spencer et al., 2003; Masuda et al., 2001; Shimizu et al., 2005; Selvendiran et al., 2006; Michels et al., 2005; Wang, Lin-Shiau, & Lin, 1999),
- increased levels of caspase–8 and t-Bid (Selvendiran et al., 2006; Michels et al., 2005),
- down-regulation of Bcl–2 and Bcl-x L expression and enhanced expression of Bax and Bak (Park & Seol, 2002; Lee et al., 2001; Masuda et al., 2001; Kuo & Lin, 2003; Lee et al., 2005; Selvendiran et al., 2006),
- increased tissue (t) plasminogen (PA) and u-PA (Abou-Agag et al., 2001),
- decreased vascular endothelial growth factor (VEGF) (Selvendiran et al., 2006; Kaneuchi et al., 2003; Trompezinski, Denis, Schmitt, & Viac, 2003; Bagchi, Sen, Bagchi, & Atalay, 2004; Kim et al., 2002),
- decreased metalloprotease (MMP) levels (Lee et al., 2004; Kaneuchi et al., 2003; Singh et al., 2002; Annabi et al., 2002; Toi et al., 2003, Ramos, Alia, Bravo, & Goya, 2005, Yang & Landau, 2000; Brusselmans, de Schrijver, Heyns, Verhoeven, & Swinnen, 2003; Chen, Z., Schella, Hob, & Chen, K., 1998),
- increase in migration-inhibitory factor (MIF) (Chung et al., 2003),

- modulation of nuclear factor nB (NF-κB) (Gong et al., 2003).

The study of flavanoids as potential chemopreventers, or for chemotherapy, is assuming increasing importance, considering its high toxicity for cancer cells, along with its relatively scarce effects on normal, non-transformed cells (Lugli et al., 2009).

1.3.1.1 Antioxidant Capacity

The best described property of almost every group of flavonoids is their capacity to act as antioxidants. The antioxidant activities depend upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability (Kelly, Anthony, & Dennis, 2002; Pandey, Mishra, A. K., & Mishra, A., 2012). The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS (Cao, Sofic, & Prior, 1997). Mechanisms of antioxidant action can include (Mahomoodally, Gurib-Fakim, & Subratty, 2005) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; (Pandey, 2007) scavenging ROS; and (Dixon, Dey, & Lamb, 1983) upregulation or protection of antioxidant defenses (Halliwell & Gutteridge, 1998; Mishra, Kumar, & Pandey, 2013).

The normal redox state of biological tissues can be disturbed by highly reactive free radicals which are molecules or large molecular fractions containing one or more unpaired electrons. The most significant type of reactive free radicals generated in living systems is reactive oxygen species (ROS), derived from oxygen such as superoxide anion ($O_2O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). ROS can be formed during the normal course of metabolism (Boveris & Chance, 1973; Norberg & Arner, 2001; Valko, Rhodes, Moncol, Izakovic, & Mazura, 2006). They can also be caused by stress and exogenous factors such as smoking, radiation and environmental pollutants.

Table 1.4 The major oxygen-free radicals and their metabolites (Cimino, Esposito, Ammendola, & Russom, 1997; Halliwell, 1990; Valko et al., 2006).

ROS/RNS	Main Sources	Characteristics	Enzymatic defence systems	Product(s)
Superoxide anion ($O_2^{\bullet -}$)	Mitochondria Activated phagocytes Xanthine oxidase Cytochrome P450	Not highly reactive Cannot penetrate membranes	Superoxide dismutase (SOD)	$H_2O_2+O_2$
Hydrogen peroxide (H_2O_2)	From $O_2^{\bullet -}$ via SOD Activated macrophages Cytochrome P450 Xanthine oxidase Microsomes and peroxisomes	Not a free radical Can penetrate membranes; an intermediate in the production of more reactive ROS Intracellular signaling molecule	Glutathione peroxidase (GPx)	$H_2O+GSSG$
Hydroxyl radical ($^{\cdot}OH$)	From $O_2\bullet-$ and H_2O_2 via transition metals (Fe^{2+} or Cu^{+})	Highly reactive Strong reactivity with biomolecules Extremely damaging to biological system	Catalases	H_2O+O_2
Nitric oxide ($NO^{\cdot}$)	Nitric oxide synthases (iNOS)	Reactive nitrogen species (RNS) NO+O2•−=peroxynitrite (OONO−) (highly cytotoxic) Can penetrate membranes An important oxidative biological signaling molecule	Protein S-nitrosothiols (Protein SH)	Protein-SNO+RSH

At physiological concentrations, ROS play an important role in the modulation of several signaling networks regulating cell function. On the other hand, living cells have developed multiple antioxidant defence mechanisms to regulate overall ROS levels and protect themselves from ROS-induced damage (Rhee, 2006). These defence systems include some antioxidants produced in the body (endogenous) and others obtained from the diet (exogenous). The endogenous ROS-scavenging defence system includes enzymatic antioxidants such as superoxide dismutases (SOD), glutathione peroxidase (GSH-Px) and catalases and nonenzymatic antioxidants peroxiredoxins (PRDXs), glutaredoxins, thioredoxins (TRXs) and glutathione (Gibellini et al., 2010). Also, exogenous dietary antioxidants are needed for diminishing the cumulative effects of oxidative damage over the life span (Wayner et al., 1987; Halliwell, 1994). The ROS levels that exceed the antioxidant capacity of the cell result in oxidative stress. Oxidative stress disturbs the normal redox state that all cells favor, and therefore due to their high reactivity, ROS may attack biological macromolecules (DNA, lipids, proteins) inducing oxidation and causing membrane damage, enzyme inactivation and DNA damage (Halliwell & Gutteridge, 1999; Valko, Izakovic, Mazur, & Rhode, 2004). As a consequence, oxidative stress has been considered a contributing factor in the development of many human chronic diseases including inflammation, atherosclerosis, angiogenesis, aging, and cancer (Bonomini, Tengattini, Fabiano, Bianchi, & Rezzani, 2008; Christian & Kunal, 2009; Franco, Schoneveld, Georgakilas, & Panayiotidis, 2008; Valko et al., 2006). ROS increase the rate of mutagenicity, which leads to DNA damage and chromosomal instability, thereby potentiating cancer progression (Radisky, et al., 2005; Samper, Nicholls, & Melov, 2003). High ROS levels lead to a severe increase in chromosome breaks and fragments, end-to-end fusions (dicentrics, Robertsonian-like fusions, rings), and translocations (Szatrowski & Nathan, 1991; Toyokuni, Okamoto, Yodoi, & Hiai, 1995). One of the main features of cancer cells, when compared to the normal ones, is a persistent pro-oxidative state that can lead to intrinsic oxidative stress. Cancer cells have evolved mechanisms to protect themselves from intrinsic oxidative stress and have developed a sophisticated adaptation system that essentially involves the rearrangement of the antioxidant functions and the upregulation of pro-survival molecules (Farber & Rubin, 1991). In cancer cells, ROS may promote cell survival and proliferation, thus contributing to

cancer development (Burdon, 1995). ROS can coordinate a variety of redox-sensitive transcription factors, such as NFκB, Nrf2, HIF, and p53 (Trachootham, Lu, Ogasawara, Nilsa, & Huang, 2008). A moderate increase of ROS often leads to NFκB activation, with subsequent induction of genes encoding for proteins that inhibit apoptosis, including Bcl-xL, cellular inhibitors of apoptosis (cIAPs), FLICE inhibitory protein (FLIP L), Gadd45, and TNFR-associated factors (TRAF)-1 and -2 (Karin & Lin, 2002).

The idea that ROS-driven oxidative stress initiates cancer and drives progression has fueled a long-standing interest in the use of exogenous dietary antioxidants to prevent cancer. Recently, Liu, et al in the *in vivo* study using rats, found that quercetin increases the production of antioxidant enzyme activity, such as GSH-Px, GR, SOD and CAT, resulted in prevention of lipid peroxidation and preserved membrane integrity (Liu, Guo, Chu, & Lu, 2014). Also, certain flavonoids have potential capacity to chelate trace metal ions, Fe^{2+} and Cu^{+} which play a vital role in ROS formation (Maleśev & Kunti, 2007). Myricetin and quercetin strongly inhibit TRX reductase (Lu et al., 2006). Flavonoids also modulate other detoxifying enzymes, including cycloxygenase (Laughton, Evans, Moroney, Hoult, & Halliwell, 1991), inducible nitric oxide synthase (Raso, Meli, Di Carlo, Pacilio, & Di Carlo, 2001), monoxygenase, NADH-oxidase, and GSH reductase (Elliott, Scheiber, Thomas, & Pardini, 1992).

1.3.1.2 Apoptosis

The cell homeostasis is regulated by different mechanism included proliferation, growth arrest, and apoptosis (Story & Kodym, 1998). Cancer is more accurately described as being the product of malfunctions within the regulation of the cell cycle, such that injured or mutated cells which are normally killed are allowed to progress through the cell cycle, accumulating mutations. In response to a range of stresses in cell homeostasis, well-organized control mechanisms which cause cells to undergo cell-cycle arrest or apoptosis, are shown to exist. Cell-cycle arrest and apoptosis allow

cells to pause in the cell cycle either temporarily or permanently and the elimination of abnormal cells that are proliferating aberrantly or that have suffered DNA damage.

Apoptosis provides a physiological mechanism for the elimination of abnormal cells, and induced programmed cell death could have beneficial effects on carcinogenesis. It has been demonstrated that flavonoids can trigger apoptosis (Watson, Cai, & Jones, 2000; Ramos, et al., 2005), although the regulation and induction of the apoptotic process by these natural products remain to be elucidated. Some dietary anticancer agents also generate ROS to trigger cell program death (apoptosis) instead of against ROS-induced DNA damage. Interestingly, these agents are also tumor-cell specific and do not occur in normal cells (Antosiewicz, Ziolkowski, Kar, Powolny, & Singh, 2008).

Large varieties of different stimuli are able to initiate programmed cell death by apoptosis signalling pathways. There are three main apoptotic pathways: the extrinsic pathway which is triggered with death receptors expressed on the cell surface, the intrinsic pathway mediated by molecules released from the mitochondria and the third pathway activated by granzmes (Igney & Krammer, 2002; Elmore, 2007).

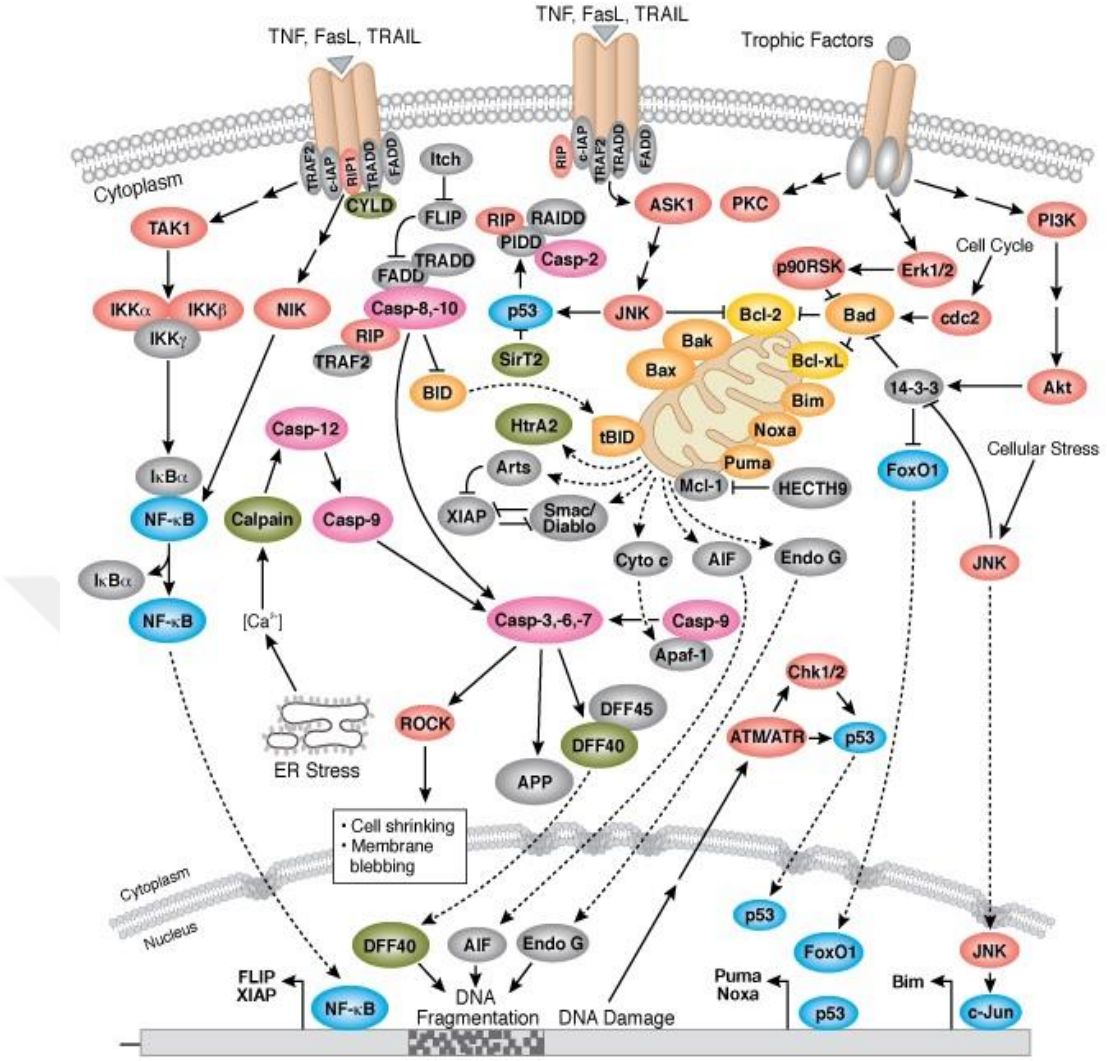


Figure 1.13 The apoptotic pathways.

1.3.1.2.1 The Extrinsic Pathway. Death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley, Killeen, & Lenardo, 2001) which share similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain” (Ashkenazi and Dixit, 1998), playing a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. Cell death signals, originated at the plasma membrane where an extracellular ligand (e.g., FasL or TNF-α) binds to its cell surface transmembrane “death receptor” (e.g., Fas receptor or TNF receptor), induce oligomerization of the receptor. This, in turn, promotes clustering of proteins that bind to the intracellular domain of the receptor (e.g., FADD, or Fas-associated death domain-containing

protein) and rescruits adapter protein TRADD binding with FADD and RIP (Xiong & Goeddel, 1995; Wajant, 2002). FADD and TRADD associate with the prodomain of initiator caspases (e.g., caspase-8 or -10) to promote their dimerization and activation and a death-inducing signaling complex (DISC) is formed (Kischkel et al., 1995). As initiator caspases, caspases-8 and -10 are activated; active caspase-8/-10 can then directly cleave and activate execution caspases, such as caspase-3.

1.3.1.2.2 The Intrinsic Pathway. Diverse non-receptor-mediated stimuli which initiate the intrinsic pathway may act in either a positive or negative fashion and produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. Negative signals such as the absence of certain growth factors, hormones and cytokines can lead to failure of suppression of death programs, thereby triggering apoptosis. The stimuli that act in a positive fashion are radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli change the inner mitochondrial membrane and result in an opening of the mitochondrial permeability transition (MPT) pore and loss of the mitochondrial transmembrane potential. Two main groups of normally sequestered pro-apoptotic proteins are released from the intermembrane space into the cytosol (Saelens et al., 2004). The first group molecules, released from the intermembrane space include cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi (Du, Fang, Li, Y., Li, L., & Wang, 2000; Garrido et al., 2005). Cytochrome c interacts with the adaptor protein, Apaf-1 (apoptotic protease activating factor-1) and the formed heptameric backbone of the apoptosome complex recruits and activates caspase-9 through dimerization (Zou, Henzel, Liu, Lutschg, & Wang, 1997; Acehan et al., 2002; Boatright et al., 2003).

1.3.1.2.3 Execution Phase. Both the extrinsic and intrinsic pathways end at the point of the execution phase, the final pathway of apoptosis. This phase begins with the activation of the execution caspases. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7, executioner caspases cleave various substrates including cytokeratins, PARP, the plasma membrane

cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others. The cleavages cause the morphological and biochemical changes seen in apoptotic cells (Slee, Adrain, & Martin, 2001). The most important of the executioner caspases is caspase-3 which is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10).

The last step in apoptosis is the phagocytic uptake. The hallmark of this phase is phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments (Fadok, de Cathelineau, Daleke, Henson, & Bratton, 2001). Early and efficient uptake with no release of cellular constituents results in essentially no inflammatory response.

1.3.1.3 Cell Cycle

DNA is routinely endangered by endogenous and exogenous insults. The mechanisms that cells have developed to cope with this constant attack on the DNA are elegant. DNA-repair processes don't work perfect and a variety of different repair mechanisms are needed due to the fact that there are various types of DNA lesion that can occur. In addition to directly repairing DNA breaks or adducts, cells respond to DNA damage by arresting cell-cycle progression or by undergoing programmed cell death.

The classic definition of cancer is "uncontrolled cell division". There are fundamental alterations in the genetic control of cell division, resulting in an unrestrained cell proliferation and soon forming large masses of rapidly growing cells (tumors). In response DNA damage occurred in a variety of ways during cancer progression, cell cycle checkpoints can be activated in G1 phase, in S phase and at the G2/M transition (Kastan & Bartek, 2004; Bartek & Lukas, 2007). The response differs depending on induced checkpoints which allow cells to arrest cell cycle. While in normal cells, the products of proto-oncogenes act at different levels along the pathways that stimulate cell proliferation, mutated proto-oncogenes or oncogenes can promote tumour growth. Also, inactivation of tumour suppressor genes results in dysfunction

of proteins that normally inhibit cell cycle progression. Cancer cells are often defective in these checkpoint mechanisms (Curtin, 2012). Such defects very likely contribute to neoplastic transformation and progression by coupling genetic instability with resistance to apoptotic cell death.

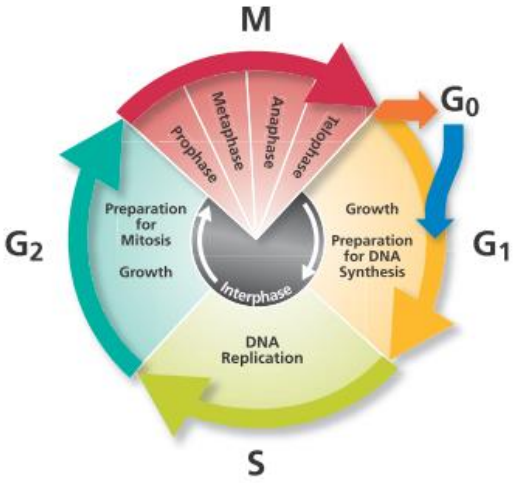


Figure 1.14 The cell cycle.

The cell cycle has four sequential phases: G0/G1, S, G2, and M. G1 is actually the most important regulatory phase in the cell cycle. It is in this phase that the cell decides whether to irreversibly continue the cell cycle through mitosis, or to enter G0, a quiescent phase during which the cell can function but not divide. Once the cell has passed the restriction point, proteins synthesized in late G1 initiate the DNA replication machinery of S-phase. G2 serves as a buffer to ensure the completion of DNA synthesis before the cell physically divides in mitosis. Mitosis (nuclear division) is comprised of four substages during which specific events occur to separate daughter chromosomes from the parent's and enclose them in a new cell. The two gap phases, G0/G1 and G2 provide time for the cell to grow and double the mass of their proteins and organelles. They are also used by the cells to monitor internal and external conditions before proceeding with the next phase of cell cycle. The cell's passage through cell cycle is controlled by a host of different regulatory proteins.

DNA damage is also used to cure cancer. Radiation therapy and many chemo-therapeutic agents that currently used to treat malignancies target the DNA, including radiation therapy and. Unrepaired DNA damage arrests cell cycle and then induce apoptosis. Therefore, from the perspective of cancer, DNA damage causes the disease, it is used to treat the disease, and it is responsible for the toxicity of therapies for cancer.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Cell Lines

The cell lines used were normal ovarian surface epithelial (OSE), A2780, OVCAR–3 and SKOV–3. Normal ovarian surface epithelial (OSE) cell line was purchased from Abm-Good. It was immortalized by SV–40 transfections. A2780, OVCAR–3 and SKOV–3 ovarian cancer cell lines were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

2.2 Cell Growth Conditions

A2780 and OVCAR–3, SKOV–3 and OSE cell lines were grown in RPMI, McCoy's 5A and Prigrow growth mediums, respectively. All growth mediums contain 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated in CO_2 incubator, conditioned to 37 ^oC and 5% CO_2 levels.

2.3 The Preparation of Cell Lysates

The cells, at confluence about 80-90% were washed with cold phosphate buffered saline (PBS). The cells were pelleted after centrifuging at 2,000 rpm for 5 minutes. The pellets were lysed in the lysis buffer (150 mM Tris, pH 8.0, 150 mM NaCl) including the appropriate detergent (1% Triton X-100, Brij 96 and CHAPS) and supplemented with protease and phosphatase inhibitors. After sonication, the lysates were incubated in the lysis buffer on ice for 15 minutes and centrifuged at 14,000 rpm for 20 minutes at 4 ^oC. The supernatants were used for western blotting and immunoprecipitation.

2.4 Protein Determination

The protein levels were determined with BCA assay. The supernatans were diluted and mixed with working solution mixture (90 A solution and 1 B solution of the assay kit). The mixtures were incubated at 37 ^{o}C for 30 minutes and then cooled to room temperature. The absorbance was read at 562 nm. BSA standard was used for calibration.

2.5 Immunoprecipitation

For immunoprecipitation, cells were washed with cold PBS and lysed in lysis buffer, mentioned in the section "The preparations of cell lysates". After centrifuging at 14,000 rpm for 20 minutes, the protein levels of the supernatants were calculated by BCA protein assay. The supernatants containing about 500 μg protein were incubated with specific primer antibodies of the studied molecules. After overnight incubation at 4 ^{o}C by rolling, the Protein A/G beads were added and incubated for 1 hour at 4 ^{o}C. The immune complexes were collected by centrifuging at 14,000 rpm for 20 minutes 4 ^{o}C. The pellets were rinsed three times with lysis buffer. The cleared pellets were incubated in SDS-PAGE loading buffer at 95 ^{o}C for 20 minutes and maintained in the solubilized form by centrifuging.

2.6 Western Blot Analysis

After the last centrifugation step, the supernatants were mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and equal amounts of total protein were loaded onto SDS-PAGE gels. SDS-PAGE (Laemmli, 1970) was performed using a 10–12% gel and the fractionated proteins were electroblotted (Towbin et al., 1979) onto nitrocellulose membranes. After blocking with 5% BSA or skim milk powder in TBS with 0.1% Tween 20 (TBST), membranes were incubated with against mono- or polyclonal antibodies of studied molecules. The membranes were washed in TBST and further incubated with HRP-conjugated secondary antibodies. The ECL Western blot analysis system was used to detect the

antibodies. Autoradiographs were quantified using ImageJ software, and the relative intensity of the signals was normalized to the loading control. The results shown are the means ± SEM of at least three independent experiments.

2.7 Immunoflorescence Staining

Cells were grown on glass coverslips and fixed for 15 min in 4% paraformaldehyde (PFA) at room temperature. Cells were permeabilized in 0.1% Triton X-100 in PFA for 5 min at room temperature and washed in PBST (PBS with 0.1% Tween 20). After blocking nonspecific antibody binding with 1% bovine serum albumin (BSA) in PBST, cells were incubated for 1 h at 37 ºC with antibodies against studied molecules and washed in PBST. Thereafter, they were incubated with secondary antibodies conjugated with AlexaFluor 488 and 594 for 1 h at 37 ºC. Cells were washed in PBST and then cell nucleus was stained with DAPI for 5 min at room temperature. At the last step, cells were mounted upside down on the microscope slide in mounting medium to prevent photobleaching. The three-dimensional localization of studied molecules was assessed with confocal laser scanning and immunoflorescence microscopy.

2.8 Treatment of Protein Kinase C Activator and Inhibitors

For studying the effects of PKCs on the interactions, 70-80% confluent cells were incubated with 100 nm of PKC activator, PMA for 30 minutes at 37 ºC. 5 μM of PKC delta inhibitor, Rottlerin and PKC eta pseudosubstrate were treated for 2 hours at 37 ºC.

2.9 Inhibition of Palmitoylation, Cross-linking and Cholesterol Depletion

70-80% confluent cells were incubated 50 μM of palmitoylation inhibitor, bromopalmitate (BP) for 20 hours at 37 ºC. Membrane permeable and impermeable cross linking agents, DSP and DTSSP were treated at 1 mM for 30 minutes at 37 ºC. After treatments, cells were lysed in RIPA buffer. For cholesterol depletion

experiments, cells were incubated with 25 mM MβCD for 30 minutes at 37 ºC or cells were lysed in lysis buffer containing saponin. The totol cholesterol and cholesterol esters were measured as indicated in the kit (Biovision). The fluorescence intensity was read at Ex/Em 535/587 nm.

2.10 Cell Viability Assay

Cell viability was assessed by MTT assay as described previously (Mosmann, 1983). Cells in the logarithmic growth phase were plated in 96-well plates at a density of 7500 cells/well and treated for 24 h with different concentrations (0.25-50 μM) of flavonoids (Apigenin, luteolin, myricetin, fisetin, quercetin, naringenin, epigallocatechingallate, epicatechin and catechin). At the end of the treatment, 100 μL of MTT (5 mg/mL) was added to each well and the cells were cultured for another 4 h. The formazan crystals were dissolved in DMSO, and the absorbance of each well was measured at 540 nm on a microplate reader. The viability of control cells was set as 100%, and that of the treated cells was expressed in percentages relative to the viability of the control cells. The IC_{50} values were determined by curve fitting.

2.11 Measurement of Intracellular Reactive Oxygen Species (ROS)

70-80% confluent cells were detached by trypsinisation and counted by hematocytometry. 25,000 cells per well were seeded in a dark, clear bottom 96-well microplate and allowed cells to adhere overnight. After remove the media, each well was washed with 100 μL of 1X Buffer. 100 μL of diluted DCFDA solution was added to stain cells and incubated for 45 minutes at 37°C in the dark. DCFDA Solution was removed and each well was washed with 100 μL of 1X Buffer or 1X PBS. The flavonoids were diluted in 1X Buffer and 100 μL of diluted compounds were added into each well. After 5 hours, fluorescence intensity of each well was measured immediately at E_x/E_m= 485/535 nm by fluorometric microplate reader.

2.12 Determination of Intracellular Oxidative Products

Thiobarbituric Acid-Reactive Substances (TBARS) Assay: Harvested 5 x10^6 cells were sonicated in 200 µL ice-cold PBS and placed 100 µL cell lysate into a labeled 1.5 mL micro-centrifuge tube. After adding 200 µL ice cold 10% TCA to the 100 µL of each sample, the samples were incubated for 5 minutes on ice. The samples were centrifuged 5 min at 14,000 rpm and then 200 µL of each clear supernatant into a new labeled tube. After mixing with 200 µL TBA Reagent, they were vortexed and incubated at 100°C for 60 min. They were cooled down to room temperature. After mixing centrifuge tubes, 100 µL in duplicate from each tube were load to wells of a black flat-bottom 96-well plate and read fluorescence intensity (λex/em = 560 nm/585 nm) on a plate reader. In this study, an MDA standard was used to construct a standard curve.

Protein Carbonyl Assay: The protein samples of the cells treated with flavonoids dissolved in dH_2O and sonicated to disrupt the cells. 100 µl of sample containing approximately 0.5 - 2 mg protein per assay was used. 100 µl DNPH was added to each sample, vortexed and incubated for 10 min at room temperature. Into each sample, 30 µl of TCA added, vortexed, placed on ice for 5 min, spinned at maximum speed for 2 min. After supernatant was removed and discarded without disturbing pellet. 500 µl of cold acetone was added into each tube and washed the pellet. Then, 30 seconds in a sonicating bath is typically sufficient to effectively disperse the pellets. The samples were place at -20°C for 5 min and then centrifuged for 2 min and carefully removed the acetone. 200 µl of Guanidine solution was added and the pellet was sonicated briefly. Most proteins will be resolubilized easily at this point. The samples were spinned very briefly to pellet any unsolubilized material. 100 µl of each sample was transfered to the 96-well plate and measured OD at ~ 375 nm in a microplate reader.

2.13 Measurement of Caspase-3 and -9 Activities

Caspase-3 and 9 activities were measured as indicated in accordance with the manufacturer's protocol of Caspase-3 Assay Kit (abcam) and Caspase-9 Assay Kit

(abcam). The fluorescence intensity fluorescence of AFC was measured at Ex/Em=400/505 nm.

2.14 Apoptosis Assessment by Annexin V/PI Dual Staining

In order to evaluate the apoptosis, phosphatidylserine (PS) exposure was analyzed using Annexin V and PI dual staining assay. Annexin V-FITC Apoptosis Detection Kit (Cell Signaling) was used in accordance with the manufacturer's protocol. After treatment of cells with apigenin, luteolin and myricetin, the cells at 10^5 to 10^6 cells/ml were harvested, washed in cold PBS and resuspended in 1X Annexin V Binding Buffer. 96 µl cell suspension was aliquotted in an assay tube, and 1 µl Annexin V-FITC Conjugate and 12.5 µl Propidium Iodide (PI) Solution was added into each cell suspension. After incubation for 10 min on ice in the dark, the cell suspension was diluted to a final volume of 250 µl/assay with ice-cold, 1X Annexin V Binding Buffer and then immediately analysed by flow cytometry.

2.15 Cell Cycle Analysis

The OVCAR-3 and SKOV-3 cells (50–60% confluent) were treated with apigenin, fisetin and myricetin for 48 h in complete medium. The cells were trypsinized thereafter, washed twice with cold PBS and centrifuged. The cell pellet was resuspended in 1 ml cold PBS to which cold ethanol (4 ml) was added and the cells were incubated overnight at 4°C. The cells were centrifuged at 1100 rpm for 5 min, pellet washed twice with cold PBS, suspended in 200 µl PBS containing 0.1% Triton X-100 and incubated with 20 µl RNase and 20 µl PI solution for 15 min. Flow cytometry was performed with a FACScan (Becton Dickinson, Franklin Lakes, NJ). A minimum of 10,000 cells per sample were collected and the DNA histograms were further analysed.

2.16 Boyden Chamber Invasion Assay

The ability of ovarian cancer cells to pass through 8 μm pore polycarbonate filters coated with Collagen was measured by Boyden chamber invasion assay. Briefly, OVCAR-3 and SKOV-3 cells were seeded on the upper chamber at a density of 1×10^5 cells/well in 250 μl of serum-free medium and then were treated with 20 μm of apigenin, myricetin and luteolin for 48 h. Medium containing 10% FBS was applied to the lower chamber as chemoattractant. At the end of incubation, the cells/media in the upper surface of the membrane were carefully removed by pipetting out, and the insert was placed into a clean well containing 225 μL of prewarmed Cell Detachment Solution. After incubation for 30 minutes at 37°C, the cells were dislodged completely from underside by gently tilting the invasion chamber plate back and forth several times during incubation. 75 μL of this Lysis Buffer/ CyQuant GR Dye Solution was added into each well containing 225 μL cell detachment solution with the cells that invaded through the collagen-coated membrane and then incubated for 15 minutes at room temperature. 200 μL of the mixture was transferred into a black flat-bottom 96-well plate and read with a fluorescence plate reader using 480/520 nm filter set.

CHAPTER 3
RESULTS AND DISCUSSION

The thesis was presented under two main headings: the mechanisms of ovarian cancer progression and the chemoprevention treatments with flavonoids.

3.1 The Mechanisms of Ovarian Cancer Progression

The first objective of the thesis is to examine the changes in levels of cell adhesion and membrane molecules involved in cancer progression and metastasis and to determine whether and which of the molecules associate each other in ovarian cancer. Then, these interactions were characterized and studied in detail.

3.1.1 The Expression of EpCAM, Claudin Isoforms, Tetraspanins and PKC Isoenzymes

A2780 ovarian cancer cell line was obtained from EACCC. The cells were isolated from the primary tumors in the ovaries. OVCAR-3 and SKOV-3 cell lines, obtained from ECACC were isolated from the peritoneal solid tumors and ascites in peritoneal cavity, respectively. Therefore, they have metastasized character. But the difference is their morphological characters which OVCAR-3 cells have both epithelial and mesenchymal morphology, and SKOV-3 cells have only mesenchymal morphology. After the disruption of inclusion cysts which formed in the early step of EOC, single cells or free-floating multicellular aggregates shed to survive through peritoneal cavity via the flow of physological fluids. The single cells which gain mesenchymal features with epithelial-mesenchymal transition (EMT) attach as secondary lesions on the surfaces of the abdominal cavity or organs, including the omentum, intestines, genital organs, spleen, and urinary bladder. The attached tumor cells form multiple solid nodules undergo mesenchymal-epithelial transition (MET), but this transition is also incomplete. Multicellular aggregates as spheroids grow anchorage-independent in the ascites and still have mesenchymal features.

The expression patterns of EpCAM, claudin-1, -3, -4 and -7, E-cadherin, tetraspanins CD82 and CD9, PKC isoenzymes, PKC α and β were investigated by western blotting compared within ovarian cancer cell lines, A2780, OVCAR-3 and SKOV-3. In order to solubilise all molecules from plasma membrane, we used different buffers, shown in Table 3.1.

Table 3.1 The constituents of the buffers

Buffer 1	**Buffer 2**	**Buffer 3**	**Buffer 4**
(RIPA buffer) 50 mM Tris, pH 8.0 containing 150 mM NaCl 1% Triton X-100 0.1% SDS 0.5% sodiumdeoxycholate	50 mM Tris, pH 8.0 containing 150 mM NaCl 1% Triton X-100	50 mM Tris, pH 8.0 containing 150 mM NaCl 1% Brij 97	50 mM Tris, pH 8.0 containing 150 mM NaCl 1% CHAPS

The results showed that Buffer 1 and Buffer 2 were the best buffers to isolate the studied molecules, even which have four transmembrane domains (Figure 3.1). Buffer 2 was selected for the further studies of coimmunoprecipitation which nonreduced conditions were needed. When compared the protein levels of studied molecules in lysate and supernatants, after lysis in different buffers and 14,000 rpm centrifugation, a considerable amount of the proteins was recovered in the supernatants, which implie that the proteins completely were solubilised.

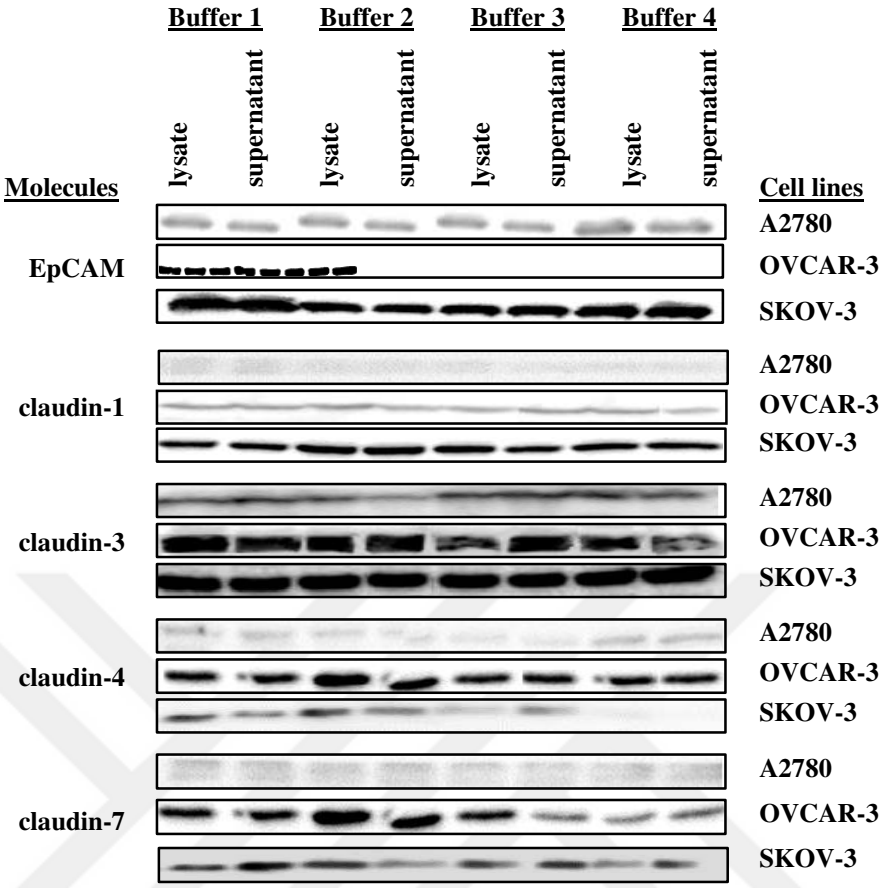


Figure 3.1 The expressions of EpCAM and claudin isoforms in A2780, OVCAR-3 and SKOV-3 cells.

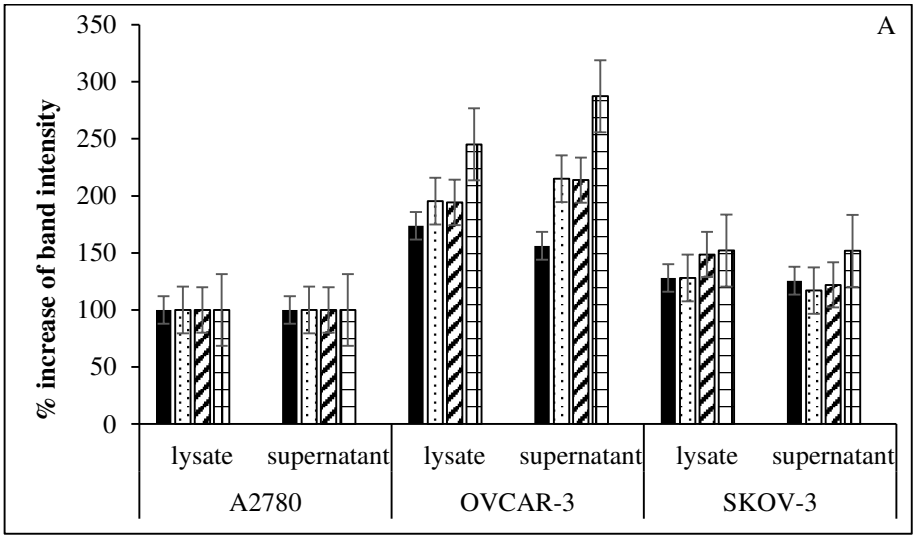


Figure 3.2 The relative intensity of EpCAM (A), claudin-1 (B), -3 (C), -4 (D) and 7 (E) band signals in A2780, OVCAR-3 and SKOV-3 cells lysed in Buffer 1 (solid bar), Buffer 2 (dotted bar), Buffer 3 (striped bar) and Buffer 4 (chequered bar).

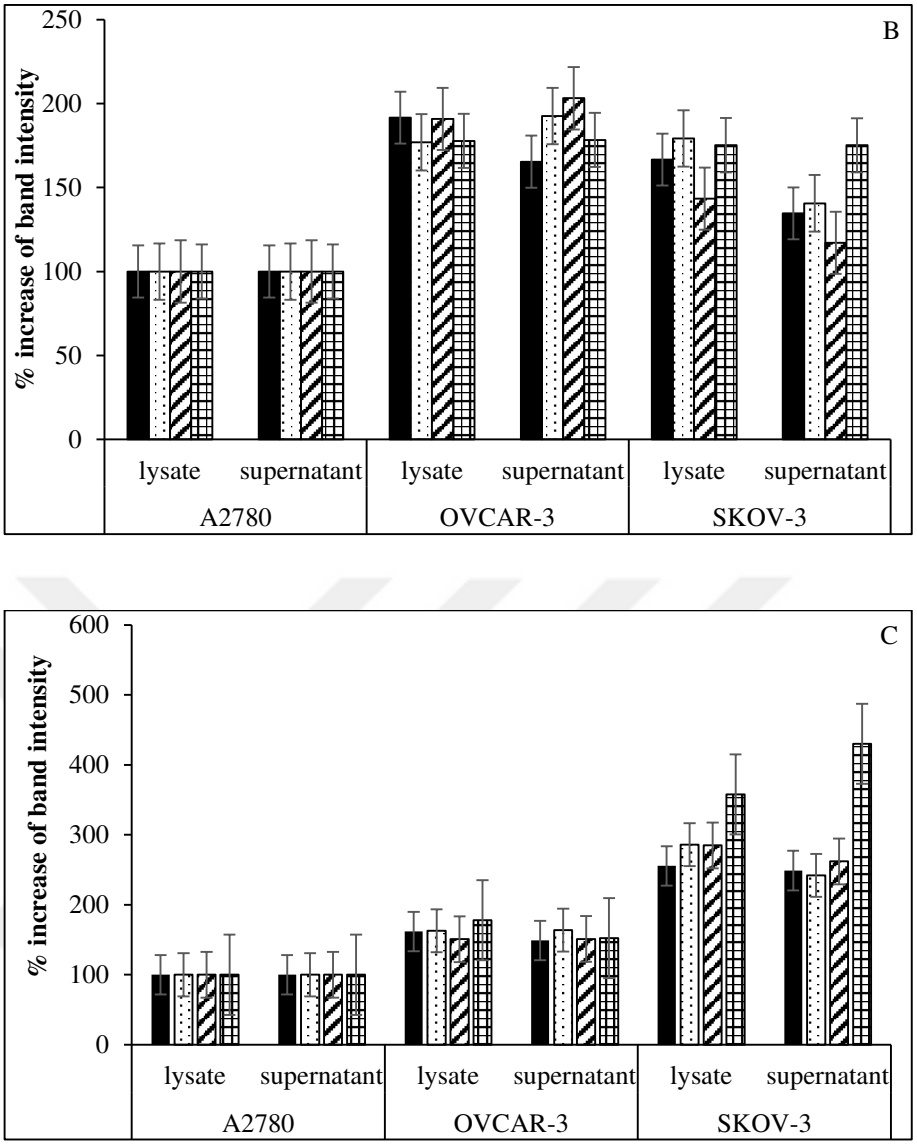


Figure 3.2 continued.

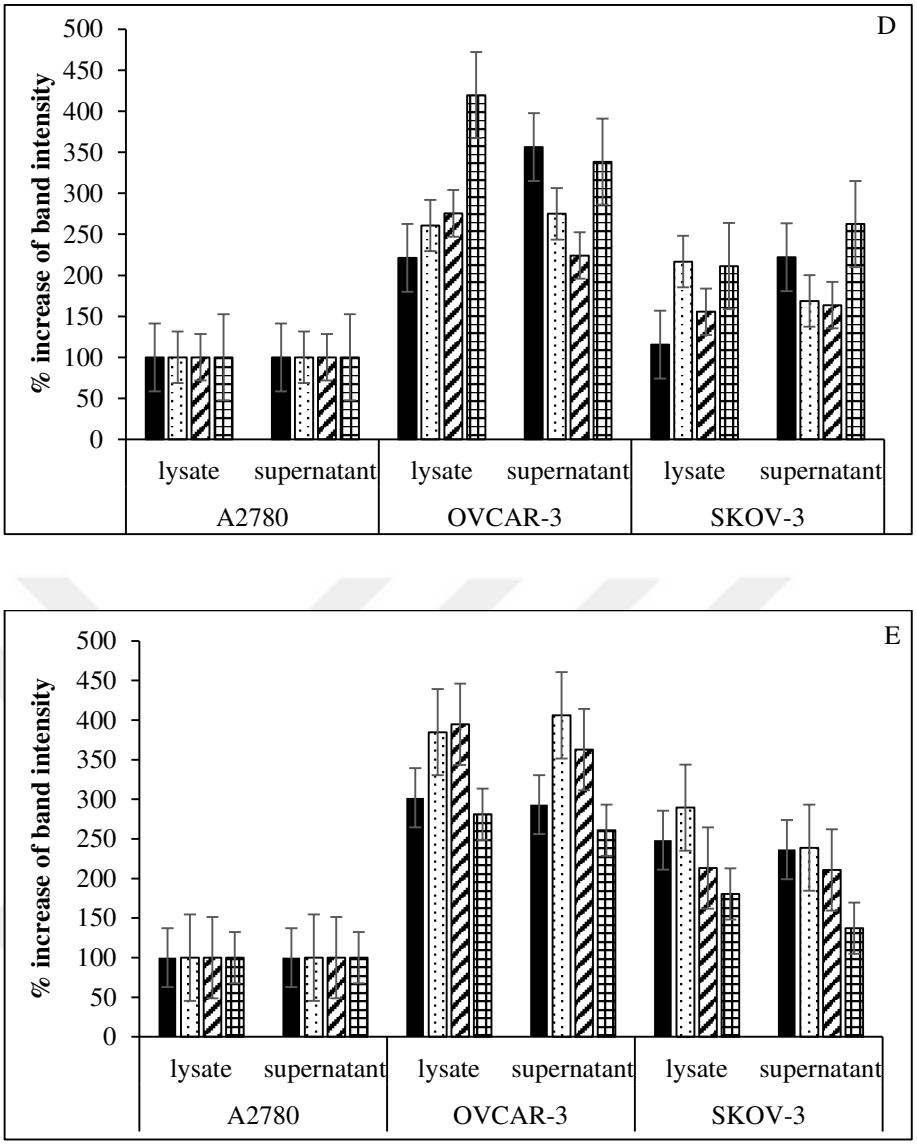


Figure 3.2 continued.

The levels of all studied molecules in supernatants of the cells which were lysed in Buffer 2 were compared in OSE, A2780, OVCAR-3 and SKOV-3 cell lines. Ovarian surface epithelial (OSE) cells did not express epithelial markers such as EpCAM and claudins. The results of EpCAM and claudins in OSE cells were not demonstrated because no bands were obtained. The levels of EpCAM and claudin isoforms were also lower in A2780 cells which differentiate from normal ovarian surface epithelial. When compared with A2780, EpCAM, claudin–1, -3, -4 and -7 were upregulated in OVCAR–3 and SKOV–3 cells (Figure 3.1 and 3.2). Van der Gun et al. (2011) also showed A2780 cell line as EpCAM-negative line, SKOV3 with an intermediate

EpCAM and OVCAR3 with a high EpCAM. The upregulated expressions of EpCAM and all studied claudins in OVCAR–3 cells also showed that OVCAR–3 cells had much more epithelial character than A2780 and SKOV–3 cells. Uncontrolled expression of the molecules such as EpCAM and claudins contributes to increased risk because cell adhesions mechanically link the cells and are critical for the maintenance of tissue integrity and also controlling uncontrolled differentiation and invasion.

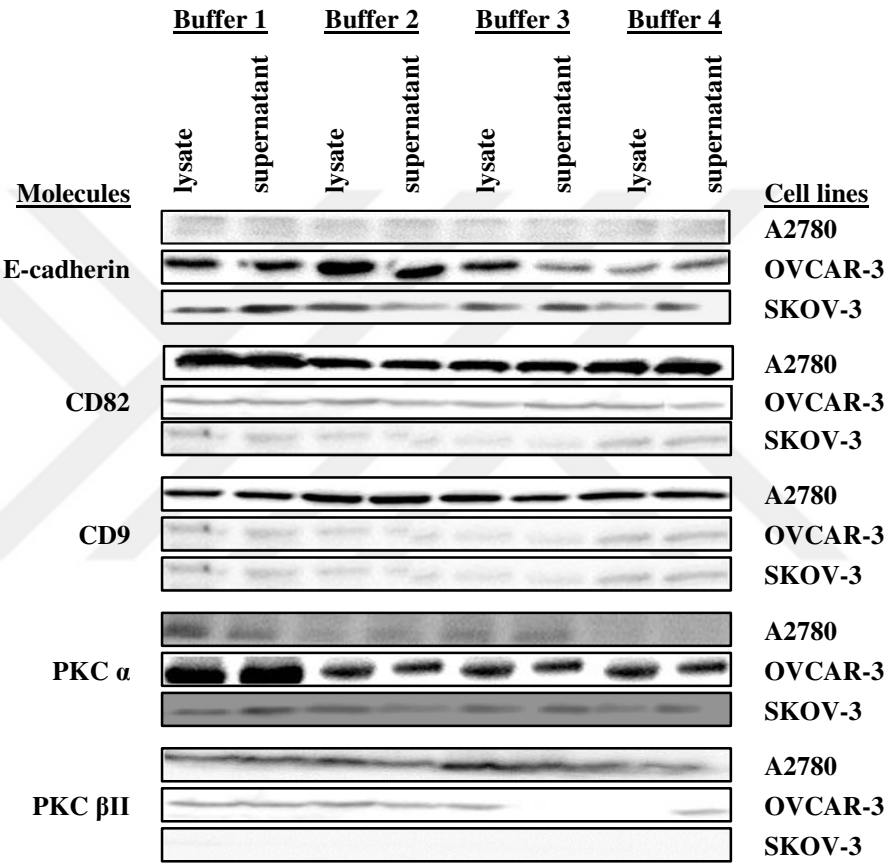


Figure 3.3 The expressions of E-cadherin, tetraspanins and PKC isoenzymes in A2780, OVCAR-3 and SKOV-3 cells.

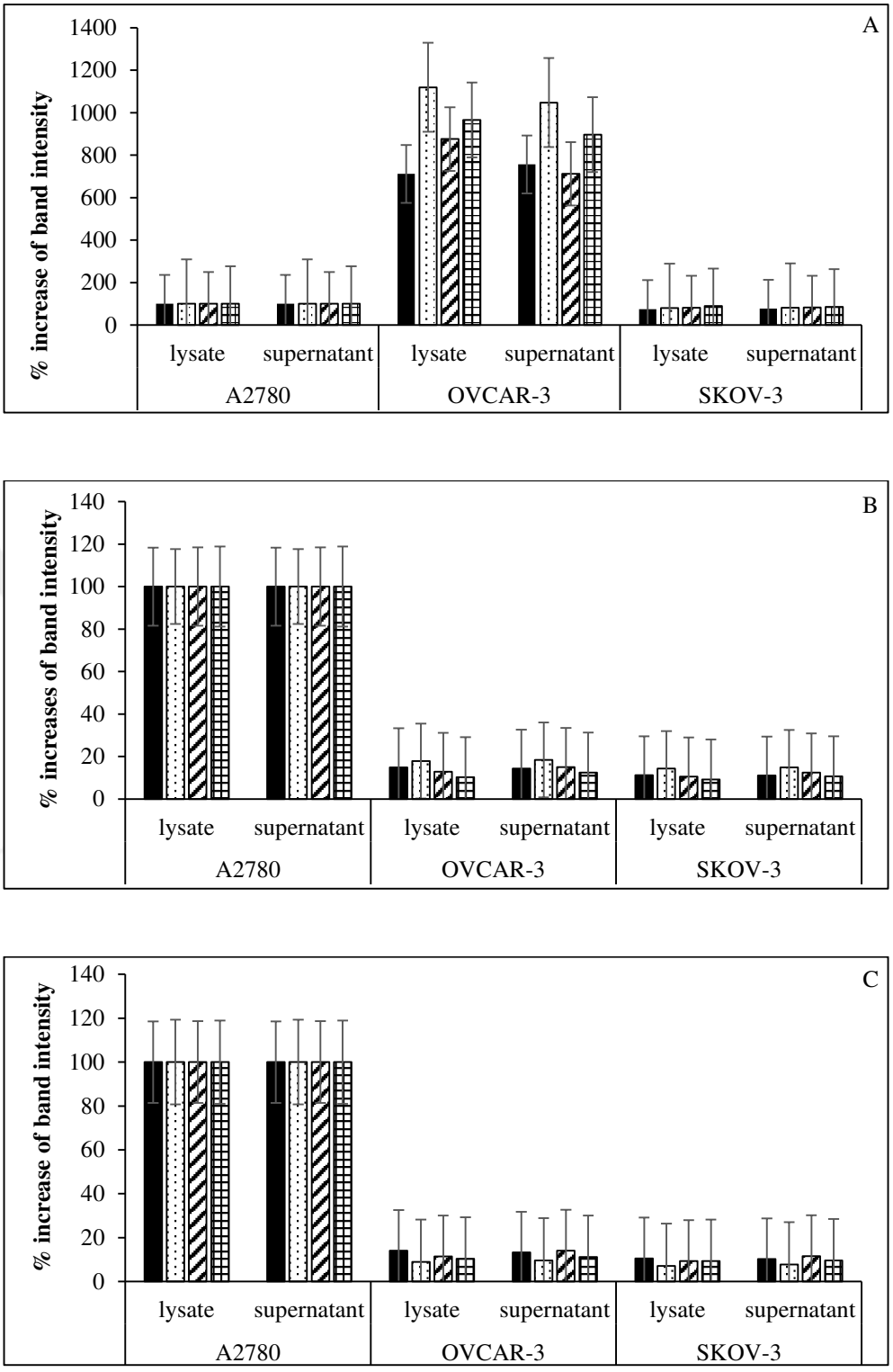


Figure 3.4 The relative intensity of E-cadherin (A), CD9 (B) and CD82 (C), PKC α (D) and βII (E) band signals in A2780, OVCAR-3 and SKOV-3 cells lysed in Buffer 1 (solid bar), Buffer 2 (dotted bar), Buffer 3 (striped bar) and Buffer 4 (chequered bar).

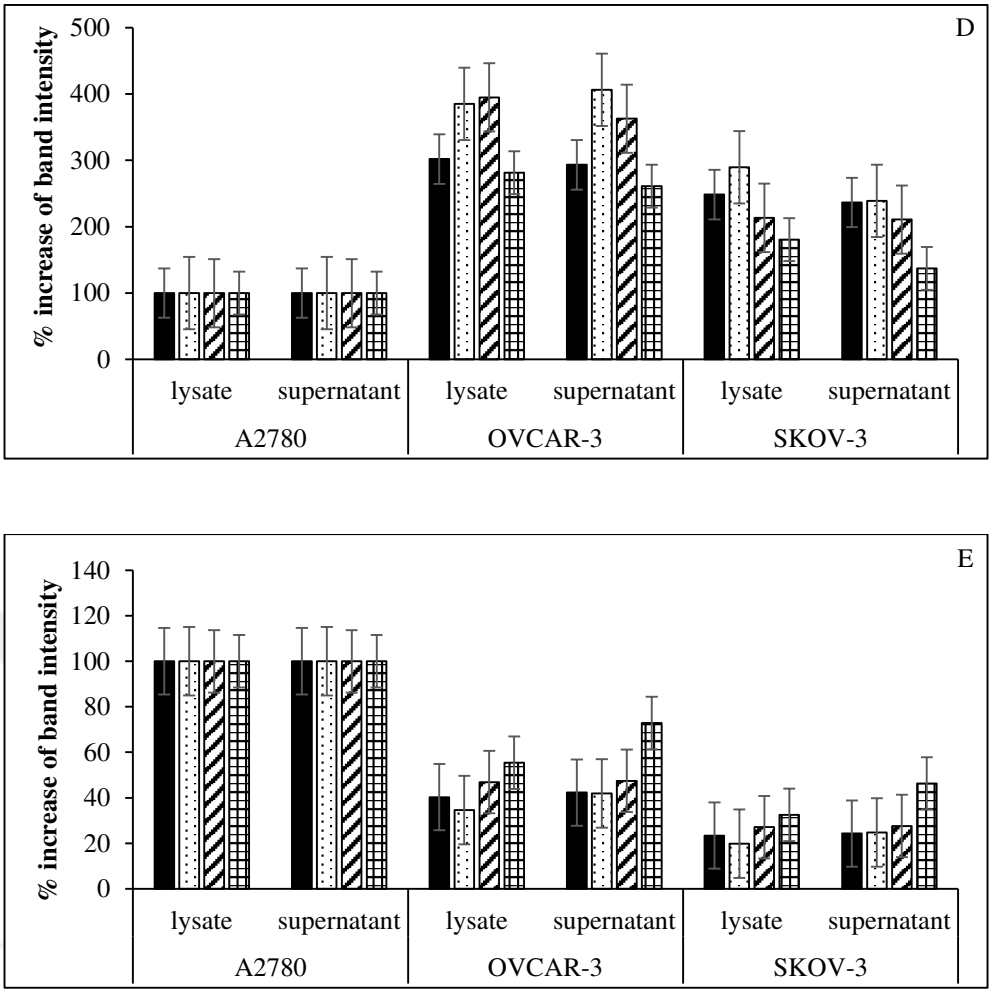


Figure 3.4 continued.

OSE cells did not express E-cadherin (data not shown). The absence of E-cadherin in these cells may be facilitate a primitive differentiation state which occurs in OSE cells covering over the ovary and this features helps OSE cells becoming highly migratory so that they can fill the large wound that is generated during oocyte release (Auersperg, Wong, Choi, Kang, & Leung, 2001). Similar to EpCAM and claudins expressions, E-cadherin was expressed in OVCAR-3 and SKOV-3 cells while this protein was very low in A2780 (Figure 3.3. and 3.4). Davidson et al. (2000) also showed that E-cadherin expression in ovarian carcinoma effusions significantly higher than compared to patient-matched primary carcinomas. Furthermore, higher expression levels of E-cadherin in OVCAR-3 cells than SKOV-3 demonstrated that

OVCAR-3 cells obtained from solid metastasized tumors underwent EMT in contrast to SKOV-3 cells obtained from ascites.

CD82 and CD9 were downregulated in OVCAR-3 and SKOV-3 cells compared to A2780 (Figure 3.3. and 3.4). Similar to the results, Houle et al. (2002) and Schindl et al. (2001) showed CD82 and CD9 downregulation in the ovarian cancer. The results also proved cancer metastasis suppressor role of CD82 and CD9, clinically connected to the progression, invasion, and metastasis of various malignancies, including prostate, breast, cervix, colon, and skin cancers (Dong et al., 1995; Liu et al., 2001; Lombardi et al., 1999; Takaoka et al., 1998; Yang et al., 2001). Similarly, CD82 expression was frequently downregulated or lost in poorly differentiated cancers or at the advanced stage of cancers (Dong et al., 1996; Ueda et al., 1996; Uchida et al., 1999; Yu et al., 1997; Yang et al., 2000).

The interactions of these cell adhesion molecules at *in situ* location is determined by posttranslational modifications including glycosylation, lipidation, ubiquitination, acetylation, proteolysis, and phosphorylation (Clark et al., 2005). Among them, phosphorylation of these molecules is essential for intracellular signalling pathway. As an enzyme responsible for phosphorylation, Protein kinase C (PKC) participates in variety of cancer cells (Hsu, Ding, Spencer, & Núñez, 1998). PKC-mediated phosphorylation of numerous protein substrates affected wide range of biological processes, including induction of cellular proliferation and differentiation, activation of nuclear transcription factors and cell surface receptors, and tumour promotion. Some studies indicated that overexpression of PKC α resulted in phosphorylation of P-gp170 and a decrease intracellular drug accumulation and therefore, increased expression of the multidrug resistance (MDR) phenotype (Gravitt et al., 1994; Chakrabarty & Huang, 1996; Masanek, Stammler, & Volm, 2002). When compared with A2780 cells, upregulated levels of PKCα in OVCAR-3 and SKOV-3 cells (Figure 3.3. and 3.4) obtained from chemotherapeutic drug resistant patient showed coherence with the studies of Gravitt et al. (1994), Chakrabarty et al. (1996) and Masanek et al. (2002). In contrast, PKC βII downregulated in OVCAR-3 and SKOV-3 cells compared with A2780 (Figure 3.3. and 3.4). Likewise, Dowling et al. (2016) showed that

colorectal cancer patients with low levels of this isozyme were advanced stage of cancer. All results showed that PKC βII acts as a tumour suppressor in ovarian and colorectal cancer (Masanek et al., 2002; Dowling et al, 2016). In contrast, the studies in breast cancer showed the pro-tumorigenic role of PKC βII (Wallace et al. 2014; Ali et al., 2009).

3.1.2 The Complex between EpCAM, Claudin Isoforms and Tetraspanins

Tumor cells settle the large array of functions to invade into the surrounding tissue and metastize to distant organs. For this settlement, they need cooperative activities between the tumor and surrounding tissue, but also between individual molecules in the tumor cell itself. There is ample evidence for the complex of four molecules normally overexpressed in colorectal cancer, promoting cancer progression (Kuhn et al., 2007). The complex between these four molecules, but not the individual molecules, was responsible in the cancer progression and also drug resistance. This complex includes CD44v6, of which expression by itself revealed conflicting results on colorectal cancer progression, two cell-cell adhesion molecules (EpCAM and claudin-7) that should hamper metastasis formation, and a tetraspanin (CO-029) known to act in concert with associated molecules (Kuhn et al., 2007).

Starting from the hypothesis that the complex between EpCAM, claudin isoforms and tetraspanins might influence ovarian cancer progression, using coimmunoprecipitation, we first evaluated whether and which of the molecules associate each other in A2780, OVCAR-3 and SKOV-3 cells. The difference between the characters of ovarian cancer cells lines demonstrated the functions of complex in ovarian cancer progression and metastasis.

3.1.2.1 Coimmunoprecipitation and Western blotting

Coimmunoprecipitation (Co-IP) is a popular technique to identify physiologically relevant protein-protein interactions by using target protein-specific antibodies to indirectly capture proteins that are bound to a specific target protein. These protein

complexes can then be analyzed to identify new binding partners. To determine which molecules were interacted each other in the ovarian cancer cells, the protein-protein complexes were isolated by using target protein-specific antibodies and analysed by western blotting.

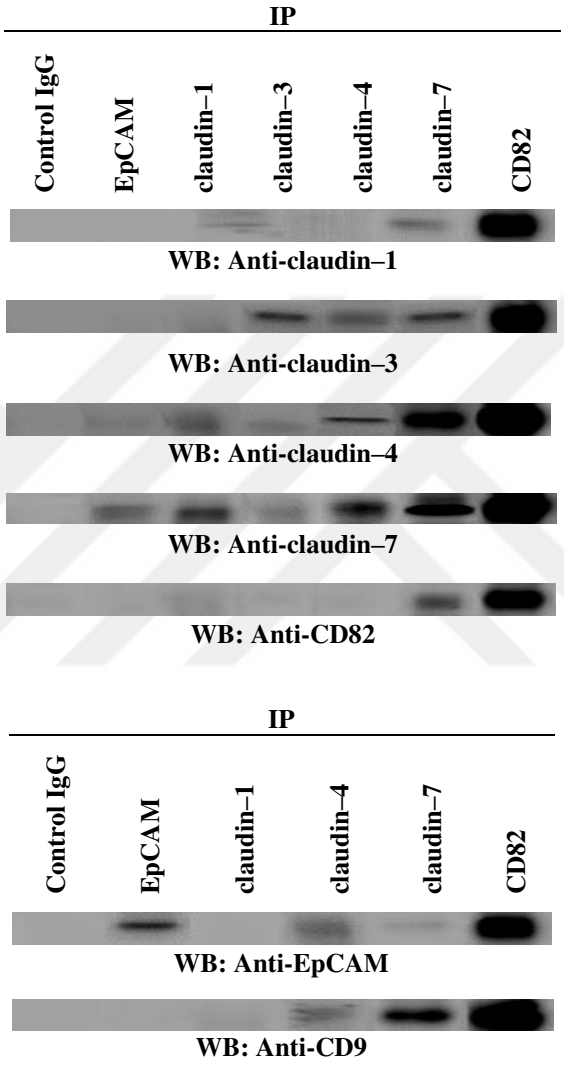


Figure 3.5 The interactions between all studied molecules in A2780 cells.

Figure 3.5 showed whether and which of EpCAM, claudin-1, -3, -4, and -7, CD82 and CD9 were interacted each other in A2780 cells. EpCAM and CD9 coimmunoprecipitated with claudin-4 and vice-versa. Claudin-3, -4 and -7 showed homo- and heterophilic interactions with claudin-4. Claudin-7 immunoprecipitates contained EpCAM, claudin-1, -3, -4 and -7, CD82 suggesting claudins interacted at

the low levels with the other studied molecules, although claudin levels are so low as to be absent. EpCAM, claudin-1, -3, -4 and -7 and CD9 immunoprecipitated in the CD82 immunoprecipitates. It also showed that CD82, located in the tetraspanin-enriched microdomains (TEMs) acted as scaffold to regulate the interactions.

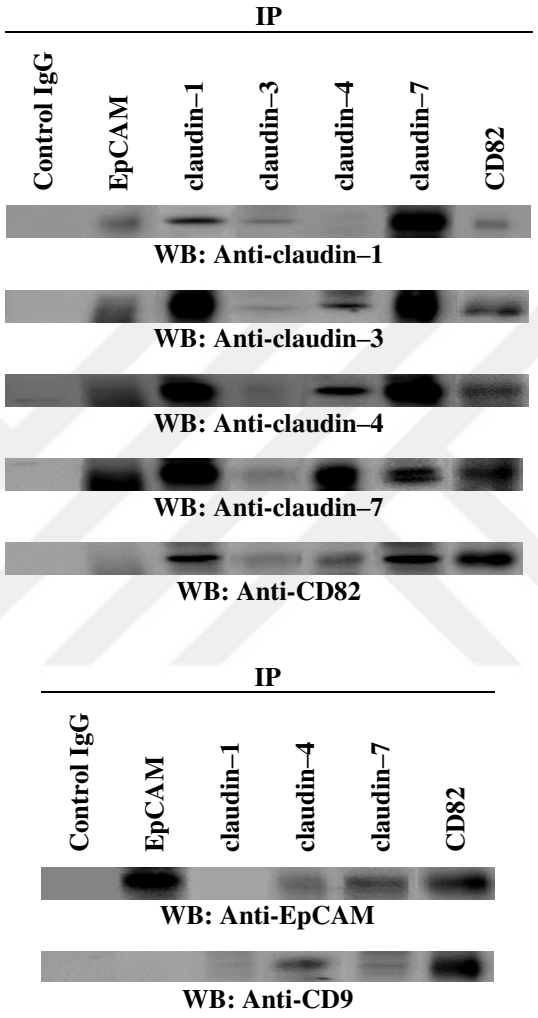


Figure 3.6 The interactions between all studied molecules in OVCAR-3 cells.

EpCAM coimmunoprecipitated with claudin-1, -3, -4, -7 and CD82 in OVCAR-3 cells and vice-versa (Figure 3.6). Heterophilic interactions between claudin-4 and claudin-7 were determined. The amounts of claudin-1 were at the low levels in claudin-4 immunoprecipitates while claudin-3 was significantly higher. Similar to A2780, CD82 as scaffolding molecule collected all studies molecules in CD82 immunoprecipitates of OVCAR-3 cells.

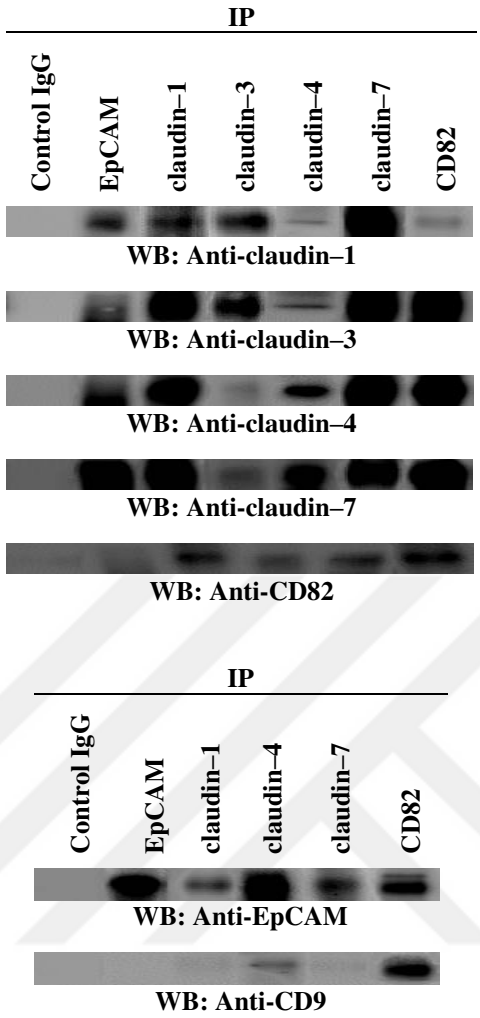


Figure 3.7 The interactions between all studied molecules in SKOV-3 cells.

As seen in Figure 3.7, in SKOV-3 cells, EpCAM immunoprecipitated in claudin-1, -3, -4, -7 and CD82 immunoprecipitates. Claudin-1 interacted with both claudin-4 and -7, and vice-versa. Also, claudin-4 immunoprecipitates contained claudin-7.

In general, claudin-4 and -7 coimmunoprecipitated, albeit weakly with EpCAM in A2780 cells. Instead, in the metastazing OVCAR-3 and SKOV-3 cells which expressed EpCAM, claudin-4 and -7 higher than A2780, claudin-4 and -7 strongly coimmunoprecipitated with EpCAM. Vice versa, anti–EpCAM precipitated claudin-4 and -7. OVCAR-3 and SKOV-3 cells also expressed EpCAM, claudin-4 and -7 at high levels compared with A2780 cells. These complexes were also stable after lysis with

the strong detergent Triton X-100, showing the direct and strong interactions between these molecules. Tetraspanin, CD82 even at low levels in OVCAR-3 and SKOV-3 tethered the other interacted molecules. The molecules in CD82 immunoprecipitates did not change depending on the cell line character. CD82 immunoprecipitates contained claudin-4 and claudin-7, higher in SKOV-3 cells. The results showed that CD82, even at low levels supported the location of EpCAM-claudins complexes in TEMs. In addition to EpCAM and claudins, homophilic and heterophilic interactions were shown between claudin isoforms. Claudin-1 did not coimmunoprecipitated or hardly immunoprecitated with claudin-4 and -7 in A2780 cells. Instead, immunoprecitates of claudin-1 in OVCAR-3 and SKOV-3 cells contained claudin-4 and -7, together with upregulated expressions of claudin-4 and -7. The coimmuprecipitations of claudin-1, -4 and 7 with CD82 demonstrated that these complexes were also located in TEMs.

3.1.2.2 Colocalization and Immunoflorescence

To prove the determined interactions in OVCAR-3 and SKOV-3 cells, EpCAM, claudin-4 and claudin-7 as well as the colocalizations were detected by immunofloresans, using conjugated to fluorescent dyes with a fluorescence microscope.

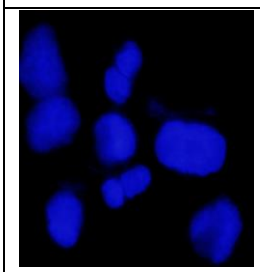
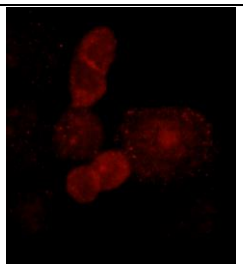
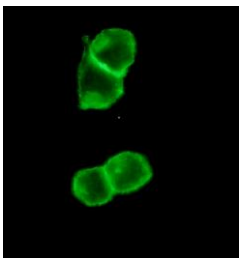
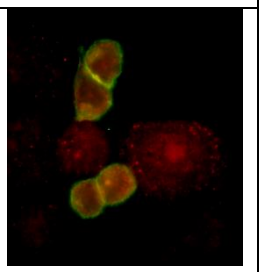
Nucleus (DAPI)	**EpCAM** (Alexa-594)	**claudin-4** (Alexa-488)	**Merge** (Alexa-594/Alexa-488)
			

Figure 3.8 The colocalization of EpCAM and claudin-4 in OVCAR-3 cells.

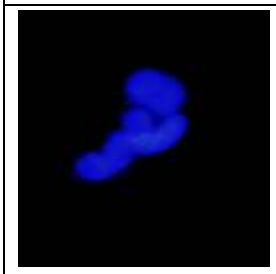
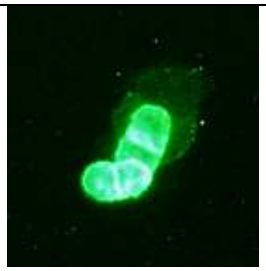
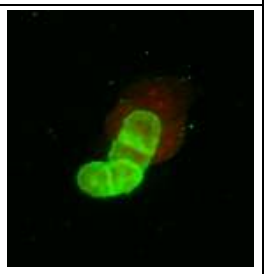
Nucleus (DAPI)	**EpCAM** (Alexa-594)	**claudin-7** (Alexa-488)	**Merge** (DAPI/Alexa-594/Alexa-488)
			

Figure 3.9 The colocalization of EpCAM and claudin-7 in OVCAR-3 cells.

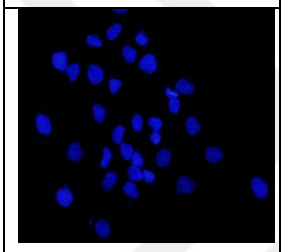
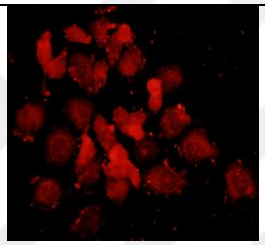
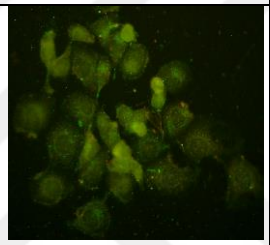
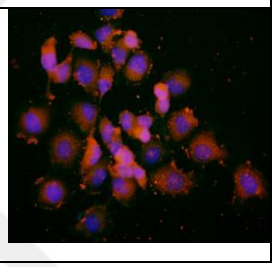
Nucleus (DAPI)	**EpCAM** (Alexa-594)	**CD82** (Alexa-488)	**Merge** (DAPI/Alexa-594/Alexa-488)
			

Figure 3.10 The colocalization of EpCAM and CD82 in OVCAR-3 cells.

As seen in Figure 3.8-10, the results obtained by coimmunoprecipitation demonstrated by colocalization by immunofloresans, showing that EpCAM and claudin-4 interacted in OVCAR-3 cells.

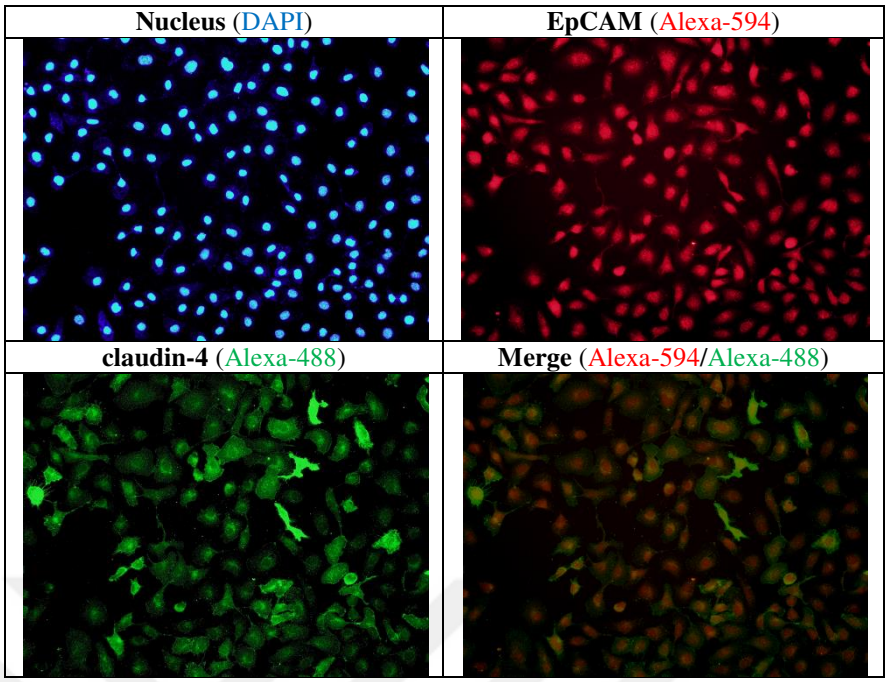


Figure 3.11 The colocalization of EpCAM and claudin-4 in SKOV-3 cells.

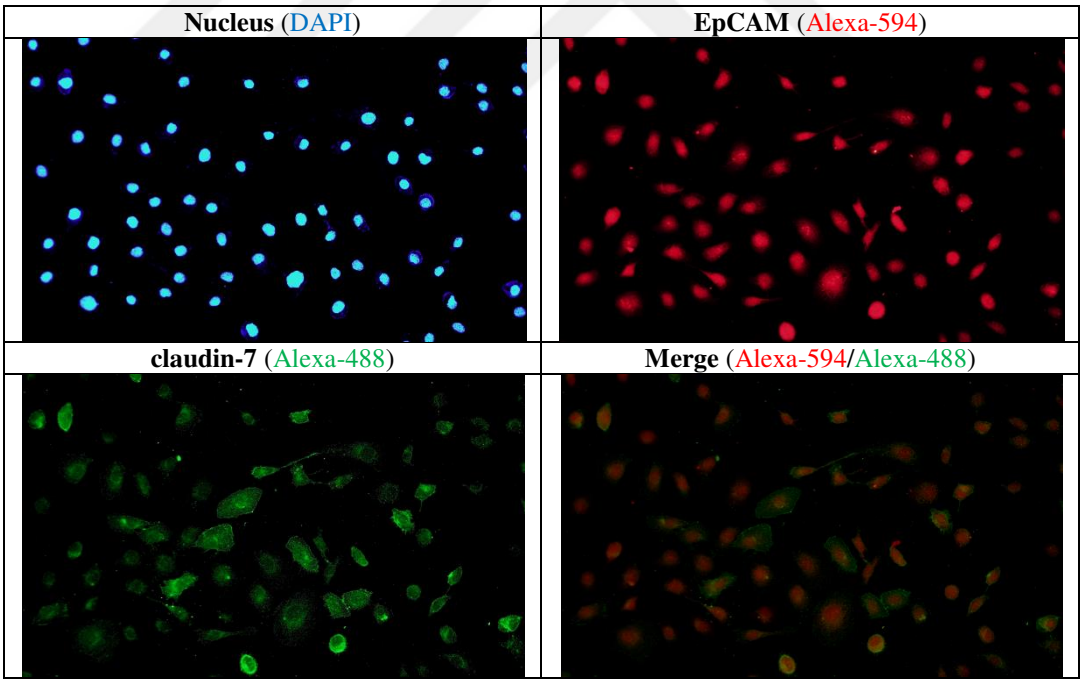


Figure 3.12 The colocalization of EpCAM and claudin-7 in SKOV-3 cells.

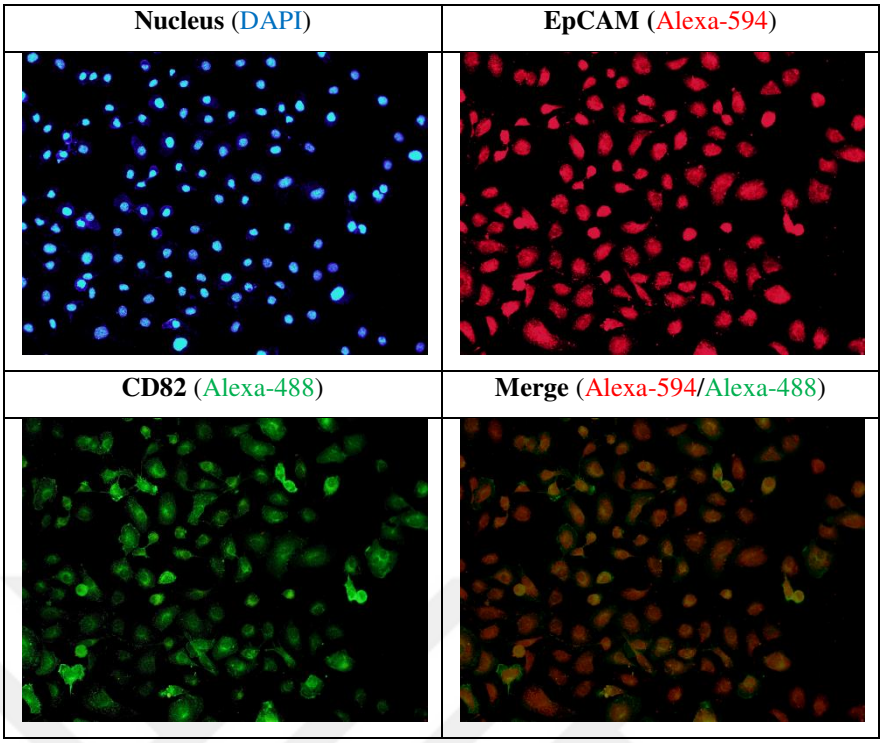


Figure 3.13 The colocalization of EpCAM and CD82 in SKOV-3 cells.

Figure 3.11-13 showed that EpCAM, claudin-4 and-7 and CD82 colocalizated in the membrane of SKOV-3 cells.

3.1.3 The Characterization of Interactions between EpCAM, Claudin Isoforms and Tetraspanins

Post-translational modifications, the regions in extracellular and/or intracellular domains of the molecules and the other membrane component, cholesterol impact the formation and regulation of the interactions between studied molecules. We evaluated the effects of palmitoylation, crosslinking of intra- and exta-cellular domains and cholesterol on the interactions.

Many transmembrane proteins, not only tetraspanins but also integrins, claudins, G protein-coupled receptors, glutamate receptors, amino acid permeases, and soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins undergo palmitoylation (Percherancier et al., 2001; Yang et al., 2004; Keller et al., 2004;

Hayashi, Rumbaugh, & Huganir, 2005; Valdez-Taubas & Pelham, 2005; Roth et al., 2006; Kovalenko, Xiuwei, Kolesnikova, & Hemler, 2007). Palmitoylation serves to regulate subcellular trafficking and/or protein degradation (Percherancier et al., 2001; Hayashi et al., 2005; Valdez-Taubas and Pelham, 2005), the assembly and/or maintenance of TEMs, tetraspanin subcellular distribution, stability during biosynthesis, and cell signaling, motility, and morphology (Berditchevski, Odintsova, Sawada, & Gilbert, 2002; Charrin et al., 2002; Yang et al., 2002; Zhou, Liu, Reddivari, & Zhang, 2004; Cherukuri et al., 2004; Yang et al., 2006). In addition to tetraspanins, the region of the cytoplasmic tail just after the fourth transmembrane domain, as well as the region of the intracellular loop just after the second transmembrane domain, contain conserved pairs of cysteines in claudins have been shown to be palmitoylated. Inhibition of palmitoylation impaired the ability of claudins to localize correctly at the tight junction, suggesting an important role of palmitoylation in protein trafficking (Van Itallie et al., 2005). Also, many proteins within TEMs, including tetraspanins are extensively and irreversibly palmitoylated through the action of recently characterized "DHHC" family of thiol-directed protein acyltransferases (PATs) which add 16-carbon fatty acid, palmitic acid to intracellular cysteines of various cytoplasmic and transmembrane proteins (Politis, Roth, & Davis, 2005; Mitchell et al., 2006; Ohno et al., 2006). The cytoplasmic Asp-His-His-Cys (DHHC) motif may play a central role in the transfer of palmitate to substrate.

Signal transduction pathways are highly complex protein interaction networks displaying transient and semi-stable association of a series of proteins. Where purified proteins are available, chemical crosslinking is the ideal strategy for demonstration of protein-protein interactions. If two proteins physically interact with each other, they can be covalently cross-linked by using bifunctional reagents containing reactive end groups that react with functional groups—such as primary amines and sulfhydryls—of amino acid residues. Also, after treatment of intact cells with cross-linkers, protein-protein interactions persisted despite the stringent detergent conditions. The formation of crosslinks between two distinct proteins is a direct and convincing evidence of their close proximity. In addition to information on the identity of the interacting proteins, crosslinking experiments can reveal the regions of contact between them.

DSP is a water-insoluble, homobifunctional N-hydroxysuccimide ester (NHS-ester) and DTSSP is its water-soluble analog (Figure 3.14). While DSP is lipophilic and membrane-permeable and therefore is useful for intracellular region conjugation, DTSSP can be used for crosslinking of extracellular regions.

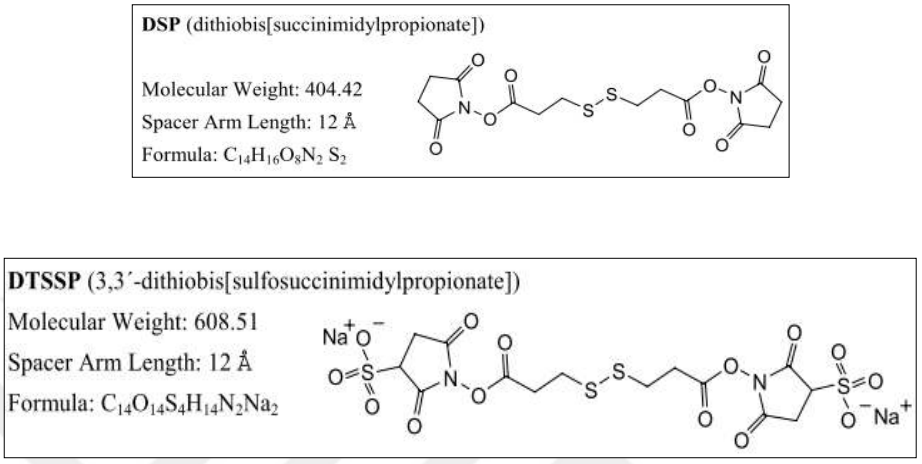


Figure 3.14 The structure of DSP and DTSSP.

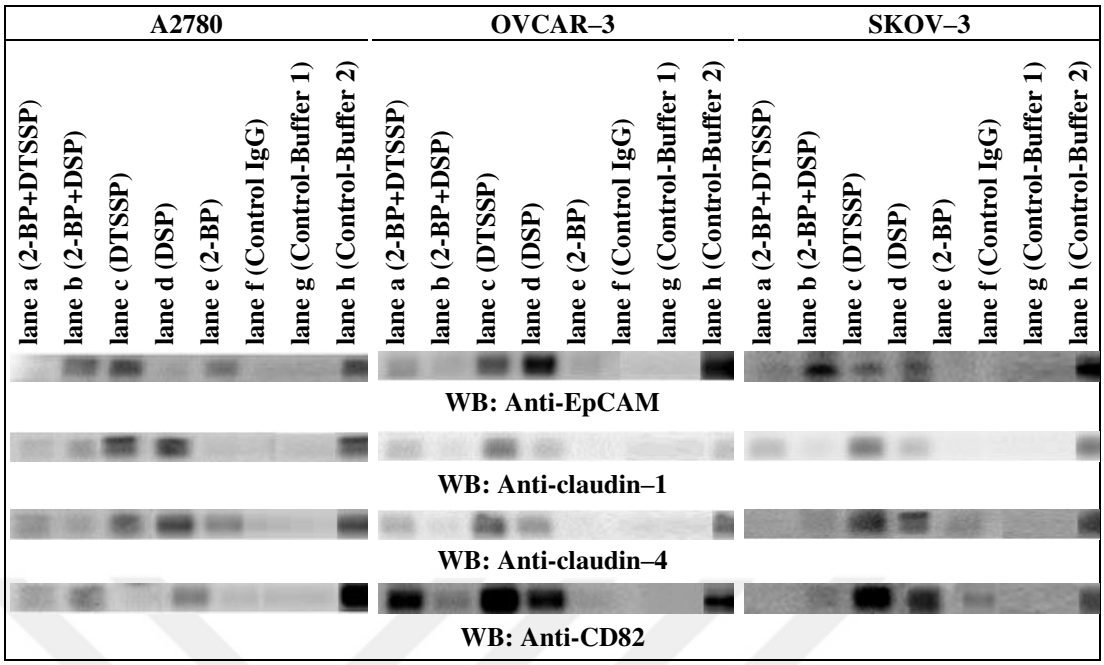


Figure 3.15 After DSP, DTSSP, 2-BP, 2-BP plus DSP, 2-BP plus DTSSP treatments, EpCAM, claudin-1 and -4 and CD82 interactions in CD82 immunoprecipitates of A2780, OVCAR-3 and SKOV-3 cells.

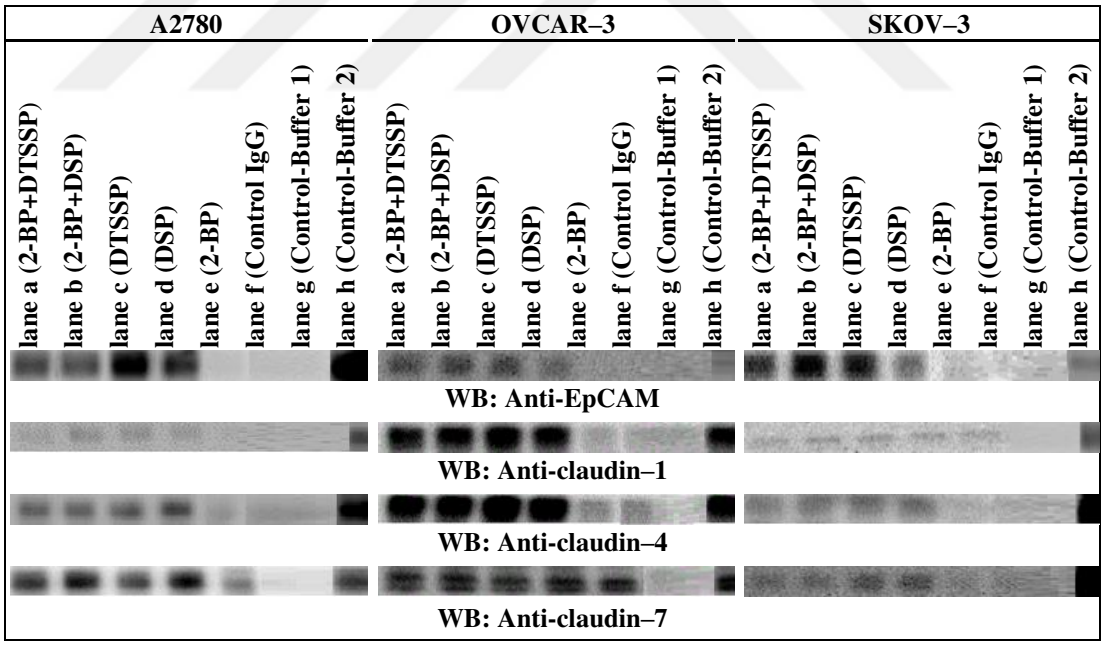


Figure 3.16 After DSP, DTSSP, 2-BP, 2-BP plus DSP, 2-BP plus DTSSP treatments, EpCAM, claudin-1, -4 and -7 interactions in claudin-7 immunoprecipitates of A2780, OVCAR-3 and SKOV-3 cells.

The palmitate analog, 2-bromopalmitate (2-BP) is an electrophilic α-brominated fatty acid which is the most commonly used to inhibit palmitoylation in cells. As seen in Figure 3.15, after treatment of intact A2780, OVCAR-3 and SKOV-3 cells with 2-BP, the interactions of EpCAM, claudin-1 and -4 with CD82 were examined in CD82 immunoprecipitates. The interactions of EpCAM with CD82 in A2780, OVCAR-3 and SKOV-3 cells were greatly destroyed compared with untreated control samples, lysed in Buffer 2 run in parallel (lanes e). In addition, CD82 also did not coimmunoprecipitated with claudin-1 and -4 in OVCAR3 and SKOV-3 cells, treated with 2-BP (lanes e). Similarly, after 2-BP treatment, EpCAM, claudin-1 and -4 molecules were not determined in the claudin-7 immunoprecipitates of A2780, OVCAR-3 and SKOV-3 cells (lanes e). The results showed that palmitoylation regions may be directly responsible of the interactions between EpCAM, claudin isoforms and CD82.

In both conditions which DSP and DTSSP were used for crosslinking of intracellular and extracellular domains (lanes c and d), the interactions of EpCAM, claudin-1 and -4 with CD82 in CD82 immunoprecipitates were conserved in OVCAR-3 and SKOV-3 cells, even lysed in the reduced lysis buffer (Buffer 1) compared with untreated control samples (Figure 3.15). The results showed convincing evidence of their close proximity in OVCAR-3 and SKOV-3 cells by both intracellular and extracellular interaction domains. In contrast to OVCAR-3 and SKOV-3 cells, the interactions between EpCAM and CD82 in A2780 cells were lost when lysed in the Buffer 1, even DSP treatment (lane d) while EpCAM maintained association with CD82 under relatively harsh detergent conditions of Buffer 1 only when cells were treated with DTSSP (lane c). These results suggested that CD82 and EpCAM interact via extracellular domains; vice versa, claudin-1 and -4 interactions with CD82 need intracellular domains. Similarly, with the results of OVCAR-3 and SKOV-3, the interactions of EpCAM, claudin-1 and -4 with CD82 in CD82 immunoprecipitates were conserved in A2780 cells treated with DSP (lane d), but they were lost under DTSSP treated conditions (lane c).

Both crosslinking of intra- and extracellular domains and palmitoylation may be responsible in the stable interactions of studied molecules because palmitoylation by membrane-bound DHHC proteins affects the close proximity of interaction between molecules for promotion of stable membrane associations (Shahinian and Silvius, 1995). Therefore, to evaluate the effects of palmitoylation-mediated membrane association on the crosslinking domains, A2780, OVCAR-3 and SKOV-3 cells were treated first 2-BP and then DSP or DTSSP (Figure 3.15 and 16). When palmitoylation was firstly inhibited, the effects of DSP and DTSSP on the CD82-EpCAM interactions reversed in A2780 cells (lanes a and b). The results demonstrated that palmitoylation domains were more important than the intracellular and extracellular interaction domains. 2-BP plus DSP treatment (lane b) in A2780 cells reduced the band density of CD82-claudin-1 and CD82-claudin-4 interactions compared with single 2-BP (lane e) and DSP treatments (lane d). In contrast, the interactions between EpCAM and claudin-1 with CD82 of A2780 cells increased after 2-BP plus DTSSP treatment (lane a). While CD82-claudin-1 interactions were lost in OVCAR-3 cells treated with 2-BP, 2-BP plus DSP and 2-BP plus DTSSP treatments were recovered the interactions. Similarly, claudin-4 levels in CD82 immunoprecipitates were not obviously altered in OVCAR-3 cells after 2-BP plus DSP (lane b) and 2-BP plus DTSSP treatments (lane a). DSP and DTSSP treatment after inhibiton of palmitoylation did not recover the interactions of EpCAM, claudin-1 and -4 with CD82, because of the significantly disruption of the interaction with 2-BP treatment. It showed that palmitoylation state may be force interaction domains into close interaction proximity. In A2780, OVCAR-3 and SKOV-3 cells treated with 2-BP plus DSP and DTSSP, the levels of EpCAM, claudin-1 and -4 associated with claudin-7 did not alter compared with the cells treated with DSP and DTSSP, suggesting that the interaction domains instead of palmitoylation domains may be important for the formation and stabilization of the interactions.

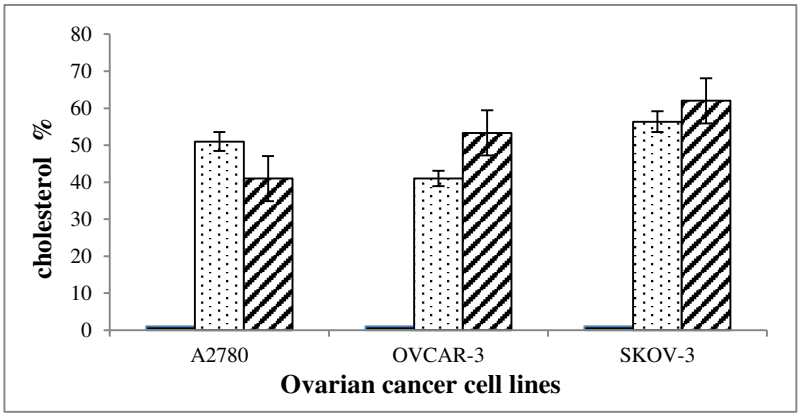


Figure 3.17 The cholesterol levels after MβCD treatment and lysis in saponin containing buffer (solid bar, untreated controls; dotted bar, MβCD treated cells; striped bar, the cells lysed in saponin containing buffer).

By interacting with each other, the tetraspanins assemble a network of interactions in tetraspanin-enriched microdomains (TEMs). In TEMs, palmitoylated tetraspanins bind to membrane cholesterol and are anchored to the actin cytoskeleton via direct binding to ERM linkers, and connect to specific intracellular signalling cascades. Partial depletion of cholesterol typically leads to a loss of protein localization in plasma membrane domains (Harder, Scheiffele, Verkade, & Simons, 1998). β-Cyclodextrins, class of heptasaccharides and saponin commonly used to selectively remove and sequestrate cholesterol from cellular membranes, respectively (Yancey et al., 1996; Charrin et al., 2003).

While MβCD was added in the growth medium of the A2780, OVCAR-3 and SKOV-3 cells to solubilize cholesterol more efficiently, saponin was used in combination with lysis buffer. Cholesterol was measured in the growth medium following MβCD treatment and in the pellet after at 14,000 rpm centrifugation of the lysate obtained from cell lysis in the buffer combined with saponin. 40–60% of total cholesterol typically removed depending on the cell lines (Figure 3.17).

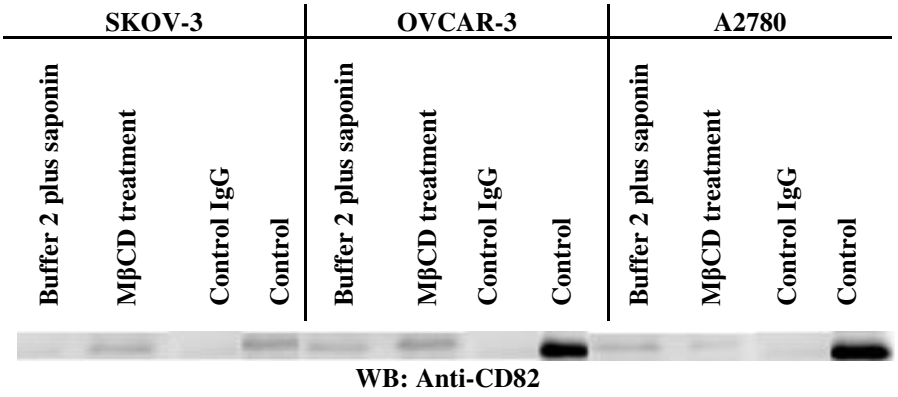


Figure 3.18 CD82 levels in CD82 immunoprecipitates of A2780, OVCAR-3 and SKOV-3 cells.

After MβCD treatment of intact cells, CD82-CD82 homophilic interactions were greatly diminished compared with the untreated control samples (Figure 3.18). When the cholesterol sequestration agent, saponin was used in combination with Buffer 2, it completely disrupted the interactions between CD82 molecules. We also investigated CD151 in CD82 immunoprecipitates, but we did not obtain any band, showing that cholesterol depletion completely destroyed CD82-CD151 interactions. Charrin et al. demonstrated that tetraspanin proteins bind to cholesterol and that this interaction requires palmitoylation of tetraspanin cysteine residues using photoactivatable cholesterol (Charrin et al., 2003). The relationships between CD82 and ganglioside/cholesterol strongly suggest that the functional interaction of TEMs and membrane rafts play a role in the motility-inhibitory function of CD82 (Xu et al., 2009).

3.1.4 The Claudin Interactions are Regulated by PKCs

When considering regulatory mechanisms which determine assembly, remodeling/modulation, and dynamics of cell adhesion, regulation occurs on various levels, such as regulation of transcription, post-translational modification, interaction with scaffolding proteins, and homo- or heterodimeric interactions. The activity and interactions of a protein at *in situ* location is determined by posttranslational modifications. Phosphorylation of tyrosine, serine, or threonine residues of several plasma membrane proteins can affect the protein which it increases the affinity for

another protein, inhibit a protein-protein interaction, or can activate enzymatic activity. PKC is a family of phospholipid-dependent serine/threonine kinases and play key roles in many of the signaling pathways that control cellular growth, proliferation, differentiation and cell death (Steinberg, 2008; Newton, 2010). Upon activation and translocation, PKCs associate closely with several different tetraspanin proteins. Therefore, the molecules which already constitutively associated with tetraspanin proteins become linked to PKC and these associated molecules are phosphorylated in a PKC-dependent manner.

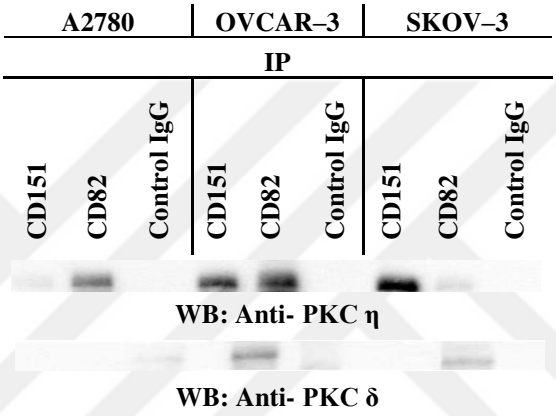


Figure 3.19 The interactions of PKC η and PKC δ with CD151 and CD82 in A2780, OVCAR-3 and SKOV-3 cells.

Starting from the consequences that PKCs associate closely with several different tetraspanin proteins upon activation and translocation, we first evaluated which PKC isoforms were located with associated tetraspanins by western blotting of PKC α, β1, δ, and η in CD82 and CD51 immunoprecipitates after activation of PKC isoforms with PMA. In the CD82 and CD151 coimmunoprecipitates of A2780, OVCAR-3 and SKOV-3 cells, PKC α and β1 were not observed. The CD82 and CD151 coimmunoprecipitated with PKC η and PKC δ at lower levels in OVCAR-3 and SKOV-3 cells (Figure 3.19). In A2780 cells, the interactions between CD151 and PKC η were not determined. The further studies continued with PKC η and PKC δ.

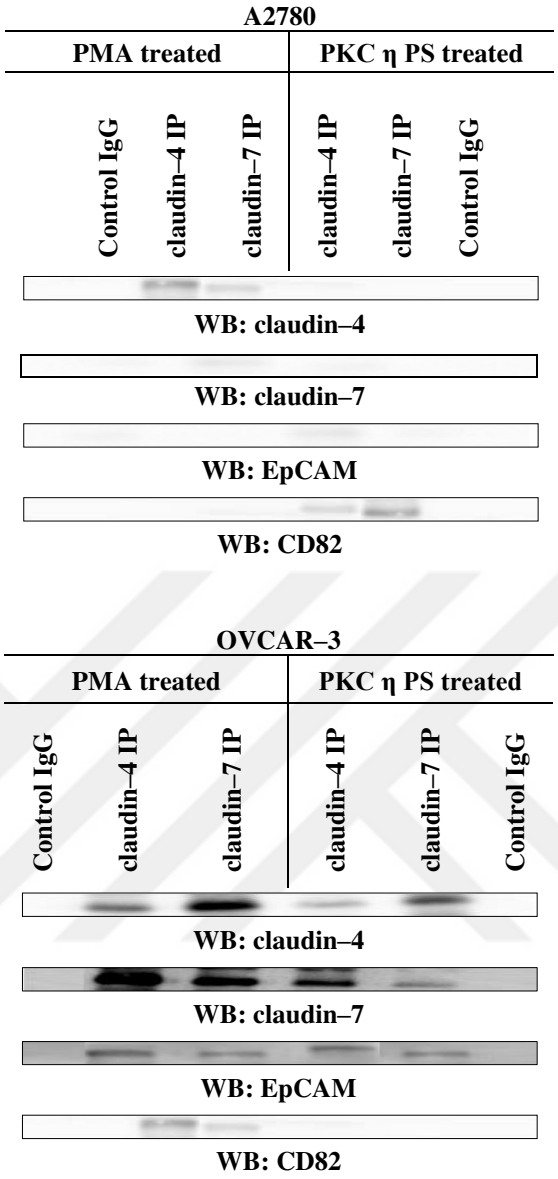


Figure 3.20 Claudin-4 and -7, EpCAM and CD82 in claudin-4 and -7 immunoprecipitates of SKOV-3 cells treated with PMA and PKC η PS.

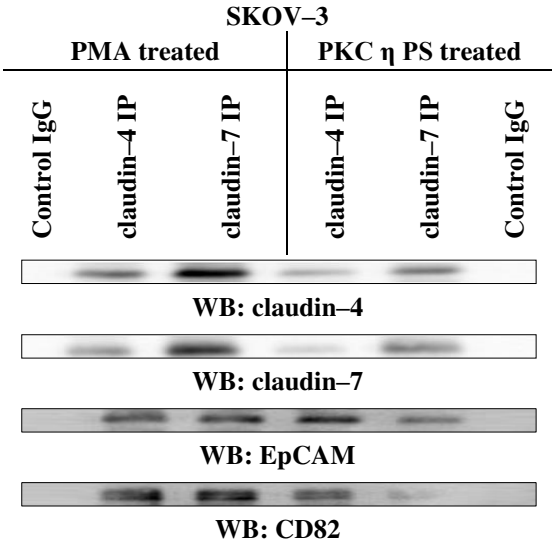


Figure 3.20 continued.

Figure 3.20 showed the interactions of claudin-4 and claudin-7 with claudin-4, -7, EpCAM and CD82 after PKC η PS treatment compared with PMA treatment. PKC η Pseudosubstrate (PS) specifically inhibits PKC η. Without activated PKC η, immunoprecipitations of claudin-7 from A2780 cells yielded no associated claudin-4. When compared with PMA treatment, the amounts of claudin-7, -4 and CD82 were significantly decreased in claudin-4 immunoprecipitates of OVCAR-3 and SKOV-3 cells after PKC η PS treatment. Similiarly, the interactions between claudin-7, -4 and CD82 with claudin-7 were diminished with PKC η inhibition. Unexpectedly, claudin-4 or claudin-7 interactions with EpCAM did not change after PKC η PS treatment.

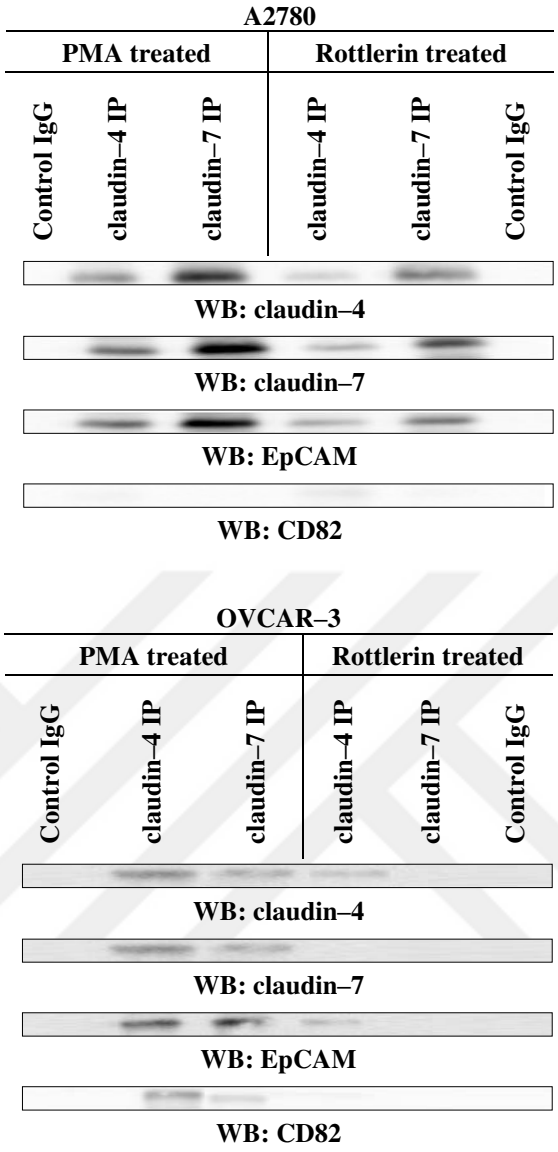


Figure 3.21 Claudin-4 and -7, EpCAM and CD82 in claudin-4 and -7 immunoprecipitates of SKOV-3 cells treated with PMA and Rottlerin.

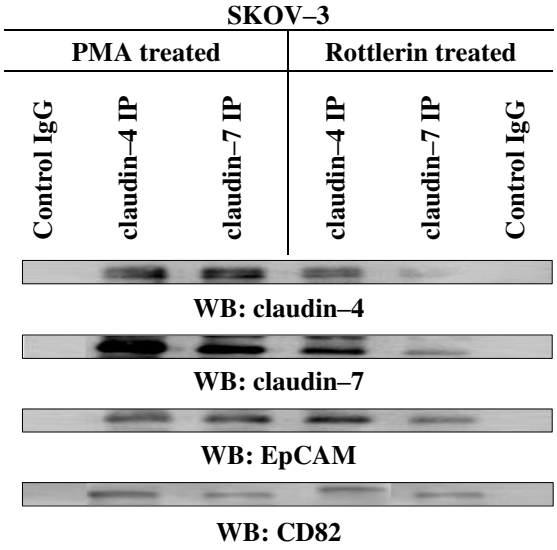


Figure 3.21 continued.

Figure 3.21 showed the interactions of claudin-4 and claudin-7 with claudin, -7, EpCAM and CD82 after Rottlerin treatment compared with PMA treatment. Rottlerin specifically inhibits PKC δ. When compared with PMA treatment, the amounts of claudin-4, -7 and EpCAM in the claudin-4 and claudin-7 immunoprecipitates decreased after rottlerin treatment in A2780 cells. In OVCAR-3 cells treated with rottlerin, interactions between claudin-7 and claudin-4 and also the amounts of EpCAM in the claudin-7 immunoprecipitates were completely lost, suggesting that phosphorylated claudin-7 is the most important for the complex formation between claudin-4, -7 and EpCAM. Also, after PKC δ inhibition with rottlerin, the claudin-4 and EpCAM amounts in claudin-4 immunoprecipitates were significantly decreased. In contrast, PKC δ inhibition decreased the amounts of claudin-4, -7 and CD82 in claudin-4 and -7 immunoprecipitates.

When all results were summed, phosphorylation, especially by PKC δ an η was important for the interactions between claudin-4, -7 and EpCAM, showing phosphorylated one of the studied molecules acts as scaffolding for the others.

3.2 The Chemoprevention Treatments with Flavonoids

We have three reasons for the selection of flavonoids for this study:

1. To develop effective chemopreventive approaches for target cancer type, a promising strategy is to take advantage of the biochemical differences between cancer cells and their normal counterparts. In this respect, agents that are capable of inducing selective signalling pathways related with carcinogenesis mechanism are receiving considerable attention in developing novel cancer preventive approaches. Also, due to the fact that carcinogenesis is a multistage process with an accumulation of genetic and epigenetic alterations, targets for chemoprevention could be multiple.

2. Although using chemotherapeutic drugs are some of the most important treatments for cancer, often cancer cannot be entirely cured and may be accompanied by unexpected adverse side-effects that can limit their efficacy (Payne, James, & Weiss, 2006; Rybak, Mukherjea, Jajoo, & Ramkumar, 2009). There is an urgent need to find some safer cancer therapeutic and preventive agents (Yoon & Baek, 2005). Based on the epidemiological studies, higher consumption of fruits and vegetables reduce cancer risk (Block, Patterson, & Subar, 1992; Steinmetz & Potter, 1991).

3. Ovarian cancer represents an excellent candidate disease for chemoprevention because of its high prevalence and presumed long latency period between initiation and the achievement of a fully malignant disease. Chemoprevention is use of dietary agent that may block, retard, or reverse the development and progression of carcinogenesis.

Starting from the hypothesis the multiple chemoprevention roles of flavonoids, we evaluated which of these roles are the effective on the ovarian cancer.

3.2.1 The Cytotoxicity of Flavonoids

Apigenin, luteolin, myricetin, fisetin, quercetin, kaempferol, catechin, epicatechin, epigallocatechin gallate and naringenin are the most common flavonoids, is widely distributed in many fruits and vegetables including parsley, onions, orange, tea, chamomile, wheat sprouts and in some seasonings. The effects of these flavonoids on the viability of A2780, OVCAR-3 and SKOV-3 cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cytotoxic activity study was performed by testing the drug in the concentration range of 0-100 μM in A2780, OVCAR-3 and SKOV-3 cells. Cytotoxicity was expressed as the inhibitory potency (IC_{50}), which was the concentration of flavonoids required to decrease the cell viability by 50%. Table 3.2 shows the IC_{50} values of these compounds after 24 h treatment.

Table 3.2 The IC values (μM) of flavonoids for OSE, A2780, OVCAR-3 and SKOV-3 cells

	Cell lines (IC_{50}) (μM)			
	A2780	**OVCAR-3**	**SKOV-3**	**OSE**
Myricetin	184	32	3.3	>500
Apigenin	538	75	8	200
Luteolin	137	100	30	250
Fisetin	141	92	100	
Naringenin	81	>500	375	
Kaempferol	>500	>500	50	
Quercetin	240	100	25	
Catechin	>500	>500	>500	
Epicatechin	>500	320	>500	
Epigallocatechin gallate (EGCG)	427	79.6	>500	

Apigenin, luteolin and myricetin treatment to A2780, OVCAR-3 and SKOV-3 cells resulted in a slight decrease in cell viability. To learn whether flavonoids can distinguish between cancerous cells and normal cells, we also tested apigenin, luteolin and myricetin in an immortalised normal ovarian epithelial cell line, OSE. These

flavonoids showed cytotoxity in a perfect manner and high concentrations of flavonoids treatment did not exhibit more pronounced cytotoxicity in OSE-SV40 cells than A2780, OVCAR-3 and SKOV-3 cells. A striking observation from these data was that the SKOV-3 cells were more sensitive to apigenin, luteolin and myricetin-mediated loss of viability, which occurred at lower doses and was much more pronounced than in the OSE, A2780 and OVCAR-3 cells. These observations suggested a selective response of these flavonoids to metastatic ovarian cancer cells compared to primary ovarian cancer and normal counterparts.

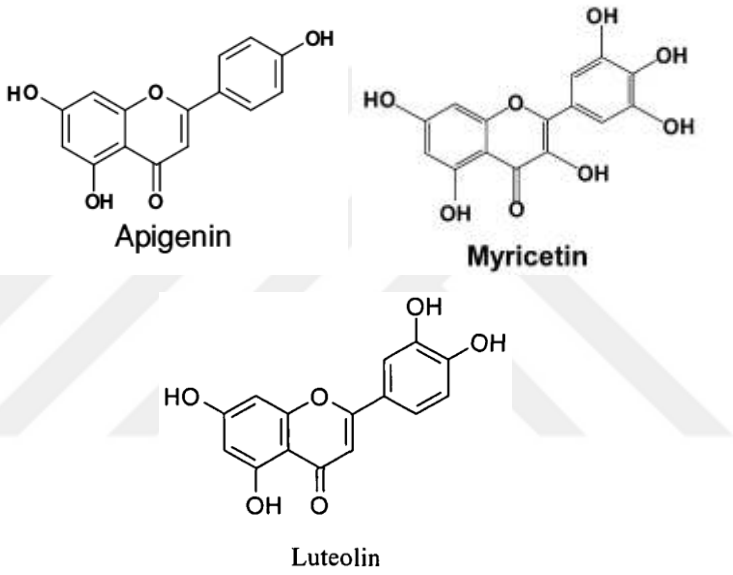


Figure 3.22 The structures of apigenin, luteolin and myricetin.

Since apigenin, luteolin and myricetin (Figure 3.22) were more pronounced effective at lower doses than the other investigated flavonoids, we continued our further studies with these flavonoids. In addition, the cytotoxic responses of A2780, OVCAR-3 and SKOV-3 cells to these flavonoids were almost different from each other, therefore to evaluate their effects on ovarian cancer progression, we continued with A2780, OVCAR-3 and SKOV-3 cell lines for the further studies.

3.2.2 The Effects of Flavonoids on the ROS Formation

ROS is known to be an important element in the induction of apoptosis. Therefore, to know whether apigenin, luteolin and myricetin stimulate ROS formation in A2780, OVCAR-3 and SKOV-3 cells, we used H_2CFDA-derived fluorochrome as an indicator of ROS accumulation.

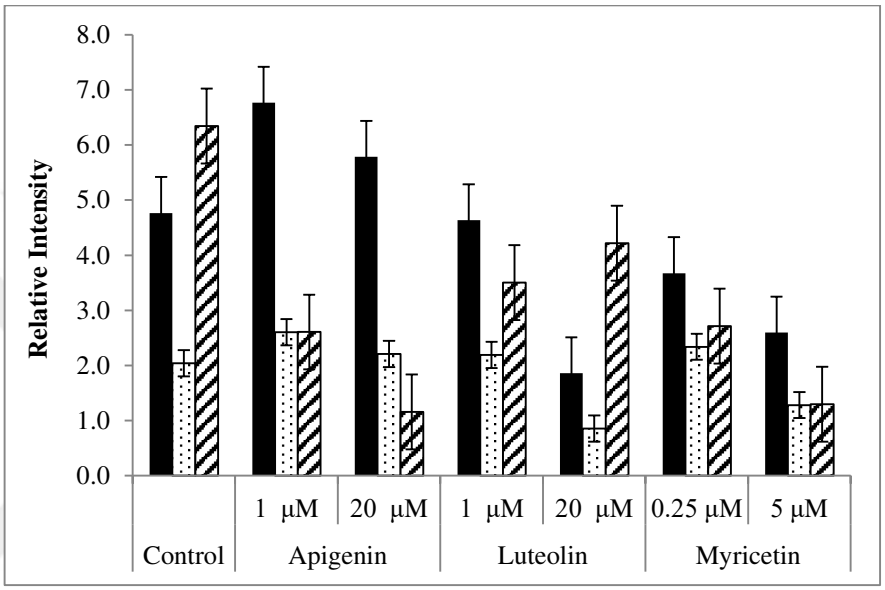


Figure 3.23 Intracellular ROS formation of A2780 (solid bar), OVCAR-3 (dotted bar) and SKOV-3 (striped bar) cells after apigenin, myricetin and luteolin treatment.

After treated with these flavonoids, time-dependent alterations of ROS production were observed as early as 4 h after flavonoid treatment (Figure 3.23). In A2780 cells (solid bar), quantitative analysis showed that apigenin (1 μM), luteolin (1 μM) and myricetin (0.25 μM) at the low concentrations significantly induced ROS production. At the highest concentrations (20 μM for apigenin and luteolin; 5 μM for myricetin), these flavonoids caused a dramatic decrease in ROS, but still higher than untreated control cells. As shown in Figure 3.21, the intracellular ROS levels were significantly increased in OVCAR-3 cells (dotted bar) treated with apigenin and myricetin at low concentrations (1 and 0.25 μM) while luteolin did not alter ROS production. Although 20 μM of apigenin decreased ROS production and it was similar to ROS intensity of untreated OVCAR-3 cells, luteolin and myricetin treatment at the highest

concentrations caused to dramatic decreases in ROS intensity, lower than untreated control cells. In contrast, ROS production of untreated SKOV-3 cells (striped bar) was quite higher than A2780 and OVCAR-3 cells. When compared with untreated control cells, apigenin and myricetin treatment significantly exhibited concentration-dependent decreases in ROS production. While ROS intensity of SKOV-3 cells treated with luteolin significantly decreased at 1 μM, luteolin treatment at 20 μM resulted in the slightly increased intensity compared with the cells treated with 1 μM of luteolin and it was still too low than untreated control cells. In coherence with our results, luteolin increased ROS production and induced apoptosis in HepG2 hepatocarcinoma cells (Hwang et al., 2011). You et al. also showed gallic acid-induced ROS production and –apoptosis in HeLa cervical cancer cells (You, Moon, Han, & Park, 2010). Also, apigenin treatment to prostate cancer cells exhibited a concentration dependent increase in ROS intensity (Shukla & Gupta, 2008).

3.2.3 The Effects of Flavonoids on the Lipid Peroxidation and Protein Oxidation

ROS-driven oxidative stress initiates cancer and drives progression has fueled a long-standing interest in the use of exogenous dietary antioxidants to prevent cancer. The flavonoids exert these effects as antioxidants, chelators of divalent cations and free radical scavengers and thus may be involved in preventing free radical mediated cytotoxicity, lipid peroxidation and protein carbonyl formation. We further investigated the cytoplasmic concentrations of MDA as a biomarker for lipid peroxidation and protein carbonyl were measured in A2780, OVCAR-3 and SKOV-3 cells treated with apigenin, luteolin and myricetin for 24 hours.

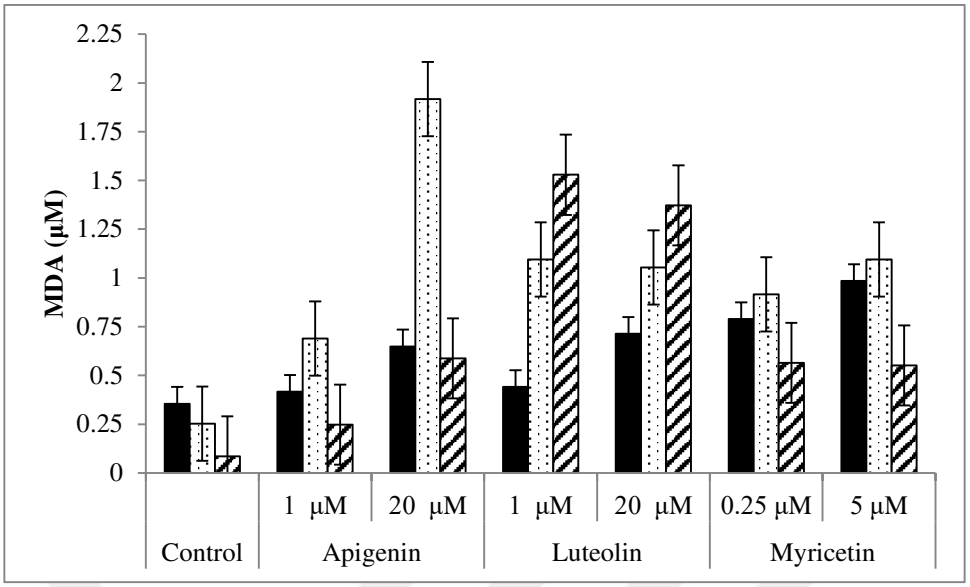


Figure 3.24 Intracellular MDA concentrations of A2780 (solid bar), OVCAR-3 (dotted bar) and SKOV-3 (striped bar) cells after apigenin, myricetin and luteolin treatment.

Figure 3.24 showed that in all three cell lines, MDA levels significantly increased after apigenin, luteolin and myricetin treatments compared with untreated controls of each cell lines. The MDA levels suggest that ROS levels may increase with prolonged flavonoid treatments after 4 hours when the cells were incubated with flavonoids for intracellular ROS analysis.

ROS-driven oxidative stress disturbs the normal redox state and due to their high reactivity, ROS may attack biological macromolecules such as DNA, lipids, proteins inducing oxidation. When ROS attacks to the proteins, proteins are oxidized and result in the protein carbonyls. We also measured protein carbonyl amounts in A2780, OVCAR-3 and SKOV-3 cells treated with apinenin, luteolin and myricetin at different concentrations after 24 hours.

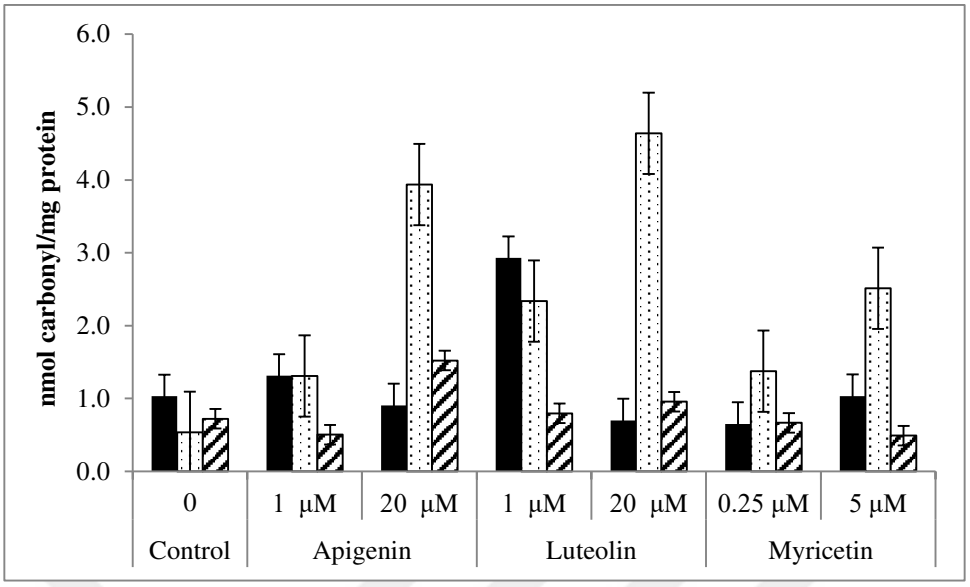


Figure 3.25 Intracellular protein carbonyl amounts of A2780 (solid bar), OVCAR-3 (dotted bar) and SKOV-3 (striped bar) cells after apigenin, myricetin and luteolin treatment.

In A2780 cells (solid bar), intracellular protein carbonyl amounts increased after apigenin and luteolin treatments at 1 μM, proving high protein oxidation driven by high ROS levels (Figure 3.25). After 20 μM of apigenin and luteolin treatments, protein carbonyl amounts decreased with the decreased ROS levels. Myricetin at low concentration caused a dramatic decrease in intracellular protein carbonyl amounts while at high concentration, it increased. Figure 3.21 also showed that apigenin, luteolin and myricetin treatments resulted in the concentration-dependent increases in the intracellular protein carbonyl amounts of OVCAR-3 cells (dotted bar), correlated with the increased MDA amounts and ROS levels. In SKOV-3 cells (striped bar), the increased concentration of myricetin caused to the decreased intracellular protein carbonyl amounts. A significant increase in the intracellular protein carbonyl amounts was found with the concentrations of 1 and 20 μM of luteolin.

3.2.4 The Effects of Flavonoids on the Caspase-3 and -9 Activities

Apoptosis, or Type I cell death occurs through extrinsic or intrinsic pathway in response to death-inducing stimuli. In extrinsic pathway, death receptor and ligand interacts, resulting in the caspase-8 activation and then execution enzyme, caspase-3

activation while the intracellular stimulants activate caspase-9 via different mechanisms and then caspase-9 activates caspase-3 in the intrinsic pathway. In response to death-inducing stimuli, an early response of apoptotic cell death is the induction of caspases, which play a crucial role in dismantling cellular infrastructure.

To test our hypothesis that apigenin, luteolin and myricetin induces apoptosis in ovarian cancer cells, we first analysed the activity of key apoptosis execution enzymes, caspase 3 which is activated by both extrinsic and intrinsic pathway and resulted in cell death, in all three studied ovarian cancer cell lines after flavonoid treatment at different concentrations for 24 h.

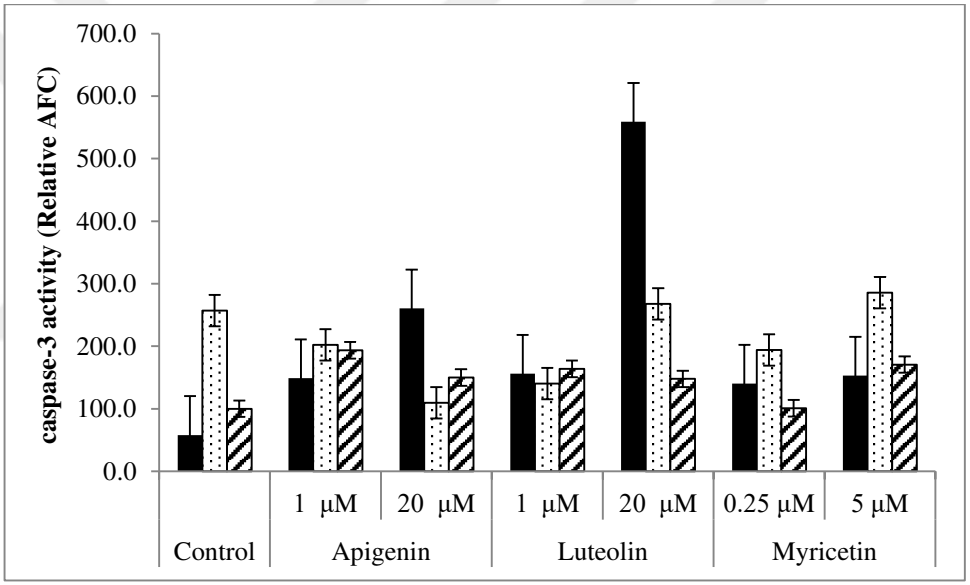


Figure 3.26 Caspase-3 activity of A2780 (solid bar), OVCAR-3 (dotted bar) and SKOV-3 (striped bar) cells after apigenin, myricetin and luteolin treatment.

The results showed that apigenin, luteolin and myricetin treatement significantly induced caspase-3 activity in concentration-dependent manner in A2780 cells (solid bar) (Figure 3.26). The cell viability inhibition induced by two flavonoids, apigenin and luteolin paralleled their abilities to induce caspase-3 activation. Also, in OVCAR-3 cells (dotted bar) treated with apigenin, caspase-3 activity significantly decreased with the increased apigenin concentration. Luteolin and myricetin at the highest concentrations significantly induced caspase-3 activity, but the low concentrations of

these flavonoids were not enough to induce caspase-3 activity. Apigenin and luteolin were effective at the low concentrations in SKOV-3 cells (striped bar). Myricetin treatment exhibited a concentration-dependent increase in caspase-3 activity. It is also interesting to notice that the increases in caspase-3 activity of OVCAR-3 and SKOV-3 cells are relatively small in ovarian cancer cells. This is within our anticipated range, because a strong effect is normally not expected from a natural dietary component. However, this apoptotic effect, although small in size, is affirmative, quick, and only requires a low concentration that is only slightly above the amount normally physiologically available. The negative correlation between ROS production and caspase-3 activity in the cells treated with flavonoid at different concentrations, especially in OVCAR-3 and SKOV-3 cells suggested that high concentrations of the studied flavonoids can scavenger intracellular ROS and caspase-3 can be activated by different ways. We therefore sought to investigate the mode of flavonoid-induced cell death by assaying for the activity of caspase-9.

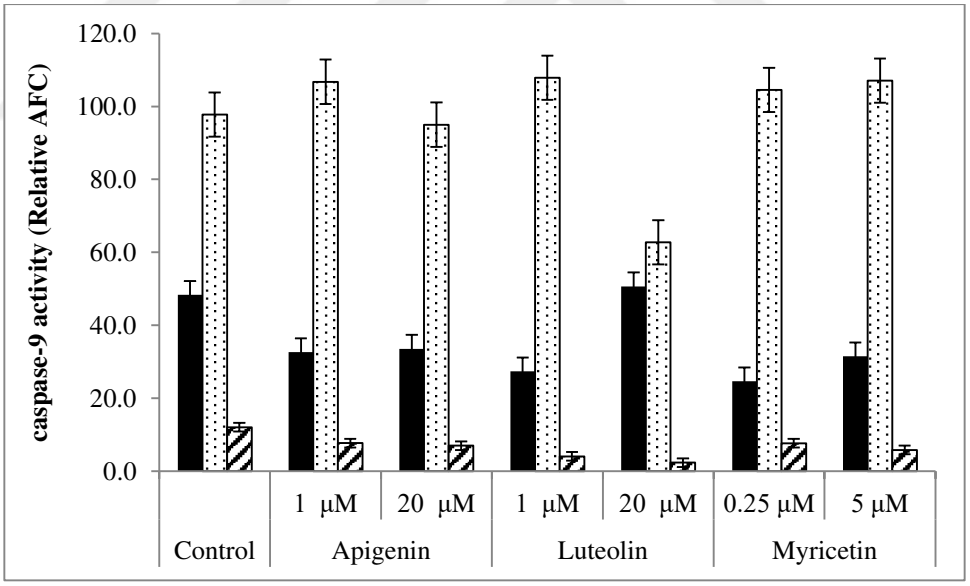


Figure 3.27 Caspase-9 activity of A2780 (solid bar), OVCAR-3 (dotted bar) and SKOV-3 (striped bar) cells after apigenin, myricetin and luteolin treatment.

As seen in Figure 3.27, Apigenin, luteolin and myricetin treatments resulted in the significant decreases in caspase-9 activity of A2780 cells (solid bar) compared to untreated control cells. In OVCAR-3 cells (dotted bar), caspase-9 activity increased in

concentration-dependent with myricetin treatment. In contrast, apigenin and luteolin as shown in A2780, significantly decreased caspase-9 activity. Caspase-9 activity shoed negative correlation with apigenin, luteolin and myricetin concentrations in SKOV-3 cells (striped bar). These decreases may be explained by the decreases in ROS production after flavonoid treatments.

3.2.5 The Effects of Flavonoids on the Annexin-V/PI Dual Staining

The appearance of phosphatidylserine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell is an early event in apoptosis, and can be used to detect and measure apoptosis. During apoptosis, PS is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V has a strong, Ca^{2+}-dependent affinity for PS and therefore can be used as a probe for detecting apoptosis. Propidium Iodide is used in conjunction with labelled Annexin V. The cell membrane integrity excludes Propidium Iodide in viable and apoptotic cells, whereas necrotic cells are permeable to Propidium Iodide. Thus, dual parameter FACS analysis allows for the discrimination between viable, apoptotic and necrotic cells.

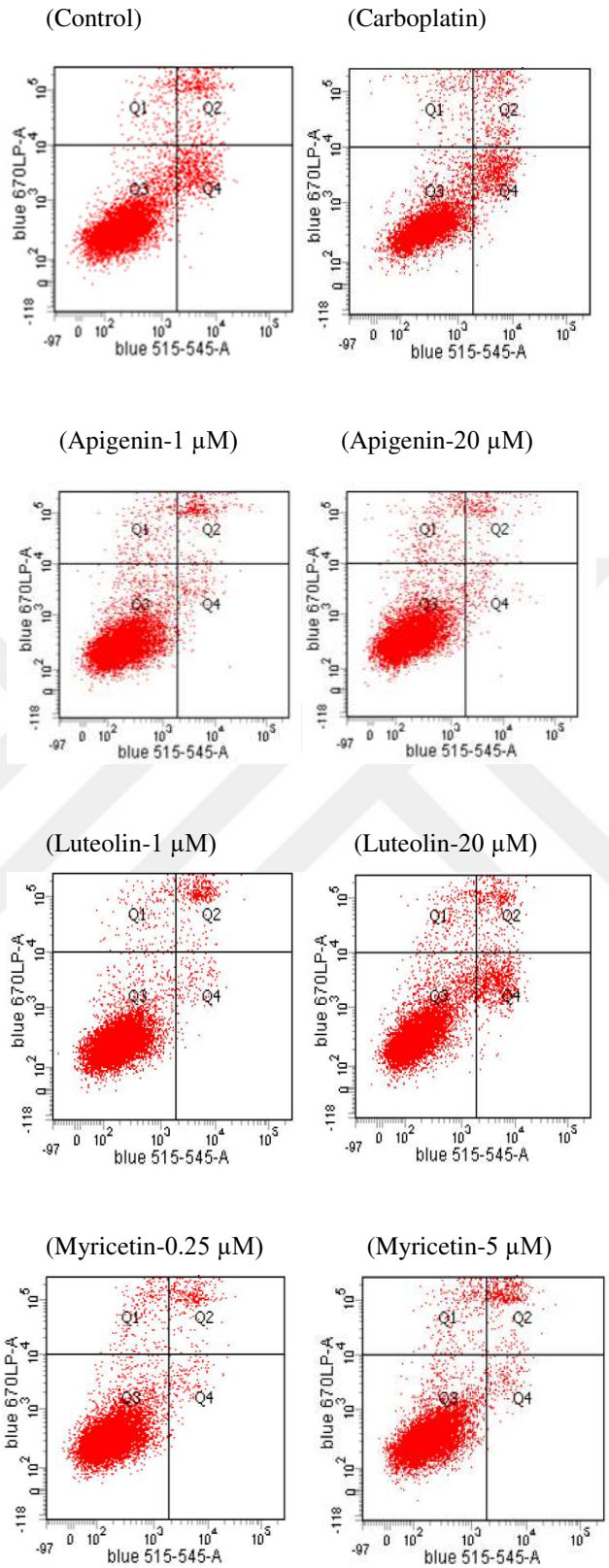


Figure 3.28 Flow cytometry for the dual staining of Annexin V and PI stained OVCAR-3 cells after treatments.

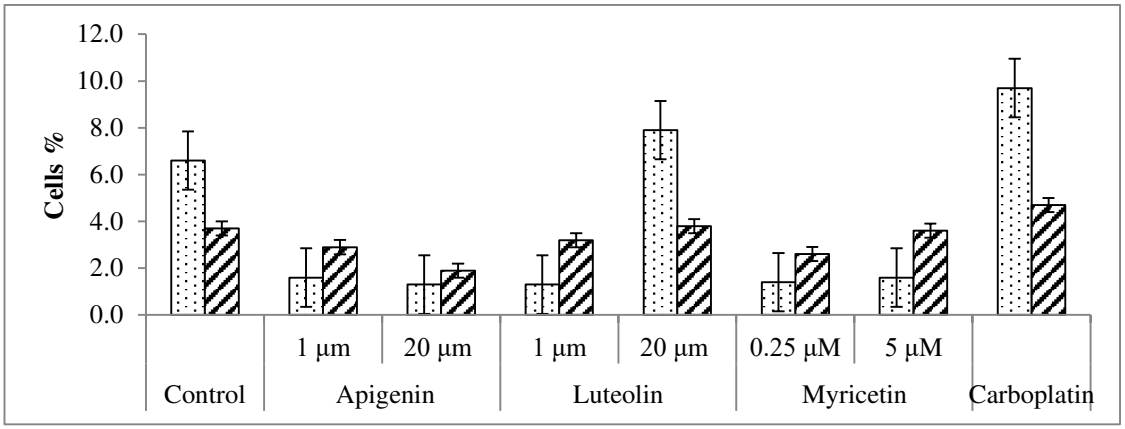


Figure 3.29 Representative histograms showing early apoptotic (dotted bar) and late apoptotic/necrotic (striped bar) cells by flow cytometry analysis of Annexin V-FITC/PI stained OVCAR-3 cells.

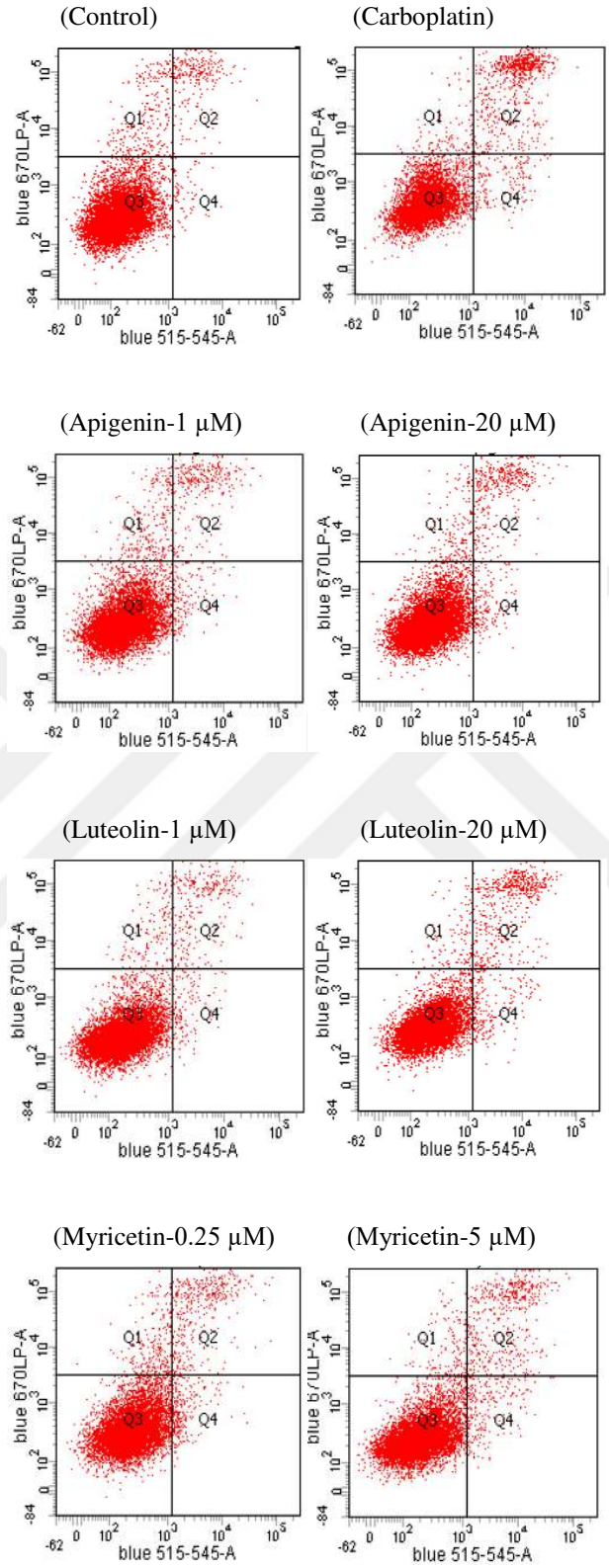


Figure 3.30 Flow cytometry for the dual staining of Annexin V and PI stained SKOV-3 cells after treatments.

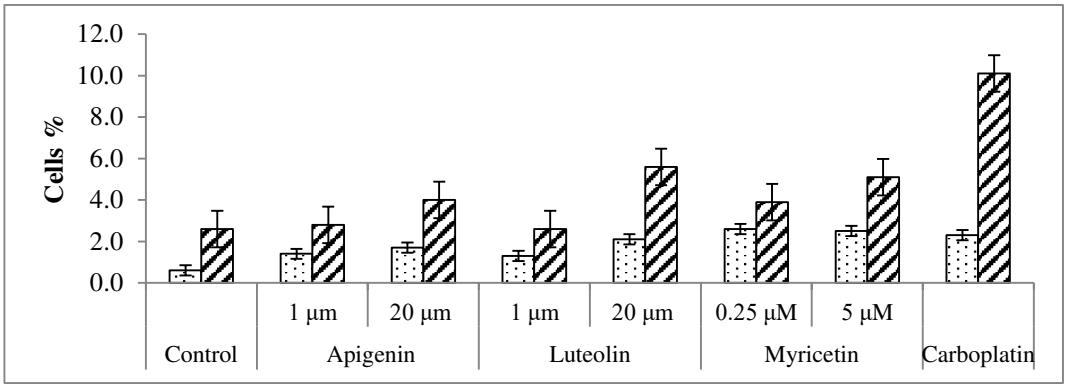


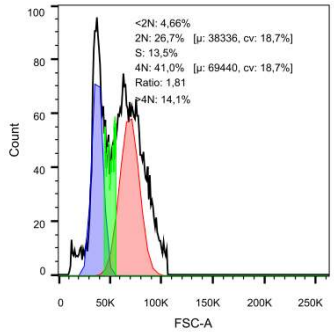
Figure 3.31 Representative histograms showing early apoptotic (dotted bar) and late apoptotic/necrotic (striped bar) cells by flow cytometry analysis of Annexin V-FITC/PI stained SKOV-3 cells.

Figure 3.28 and 3.29 showed that flavonoid treatments did not induce apoptosis in OVCAR-3 cells. In contrast, apoptotic cells in early and late phases significantly increased in SKOV-3 cells.

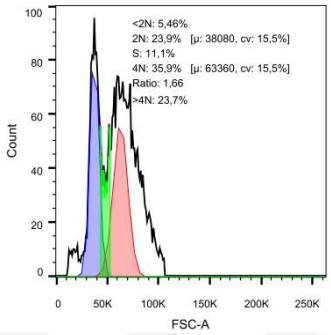
3.2.6 The Effects of Flavonoids on the Cell Cycle Arrest

To assess whether flavonoid-induced controlling of the cells is mediated via alterations in cell cycle, we evaluated the effect of apigenin, luteolin and myricetin on cell cycle distribution. We performed DNA cell cycle analysis using OVCAR-3 and SKOV-3 cells.

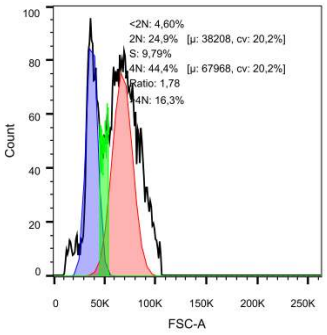
(Control)



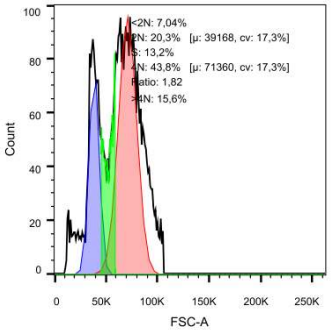
(Apigenin-1 µM)



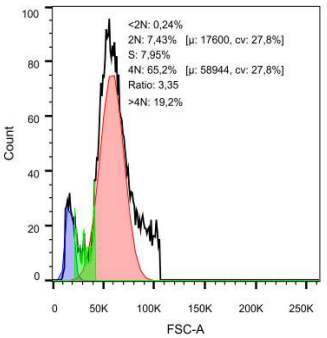
(Apigenin-20 µM)



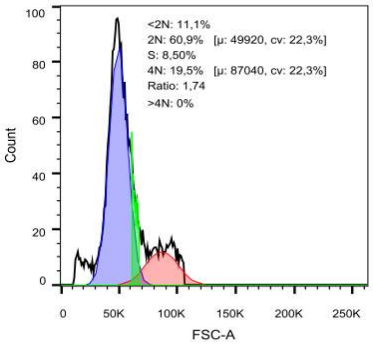
(Luteolin-1 µM)



(Luteolin-20 µM)



(Myricetin-0.25 µM)



(Myricetin-5 µM)

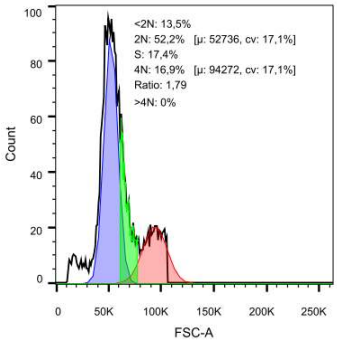


Figure 3.32 The distribution of OVCAR-3 cells in the different phases of the cell cycle after treatments.

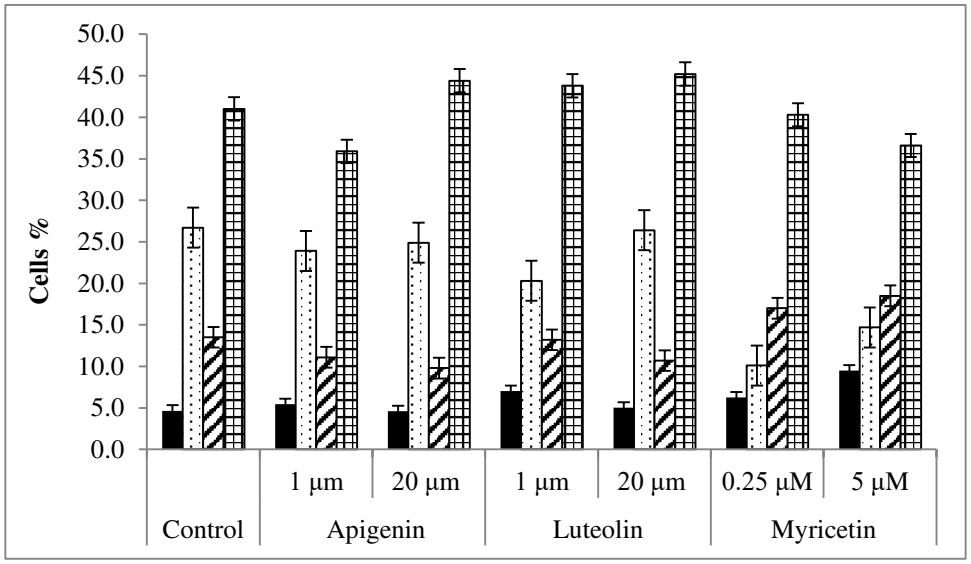


Figure 3.33 Representative histograms showing sub-G1 (solid bar), G0/G1 (dotted bar), S (striped bar) and G2/M (chequered bar) phases of cell cycle by flow cytometry analysis of PI stained OVCAR-3 cells.

As seen in Figure 3.32 and 3.33, myricetin treatment increased the number of OVCAR-3 cells in S phase and decreased in G0/G1 phase, showing myricetin arrested cell cycle in S phase. 20 µM of Apigenin and luteolin induced G2/M phase arrest in OVCAR-3 cells.

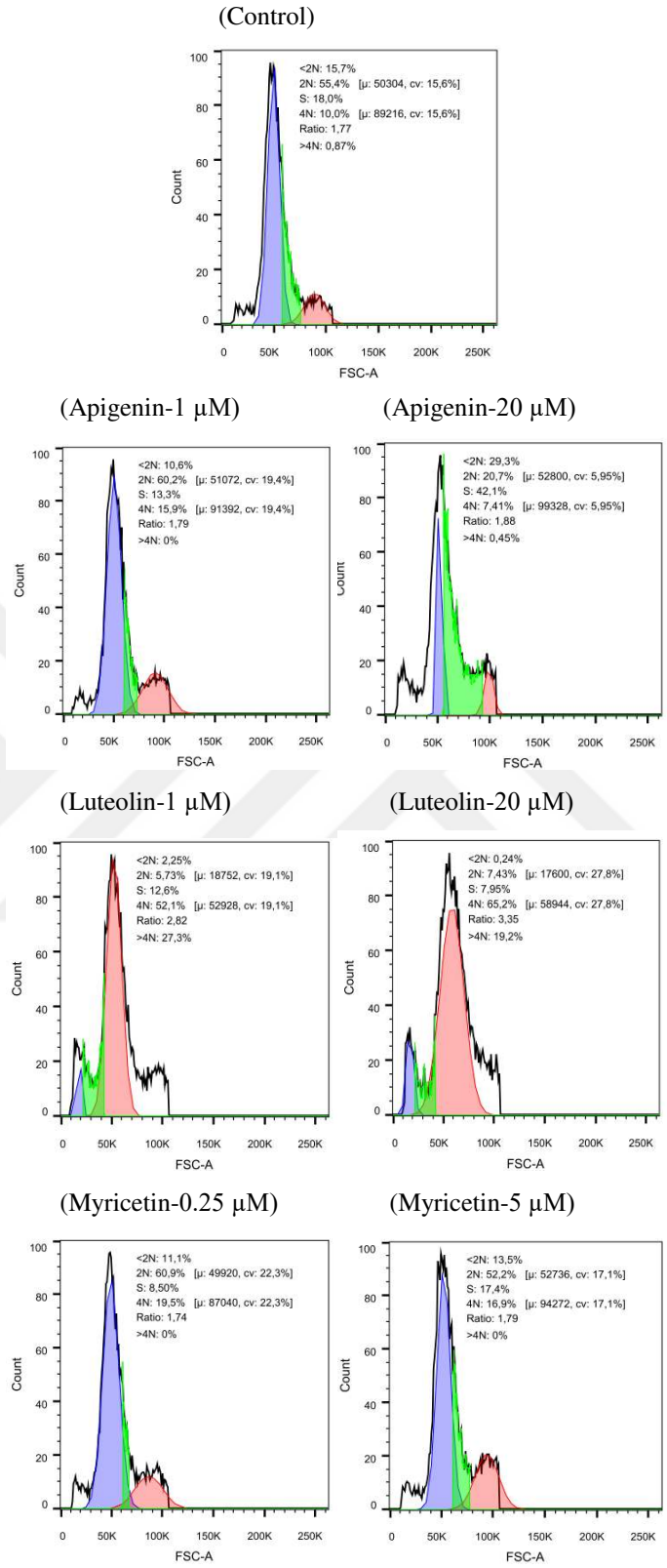


Figure 3.34 The distribution of SKOV-3 cells in the different phases of the cell cycle after treatments.

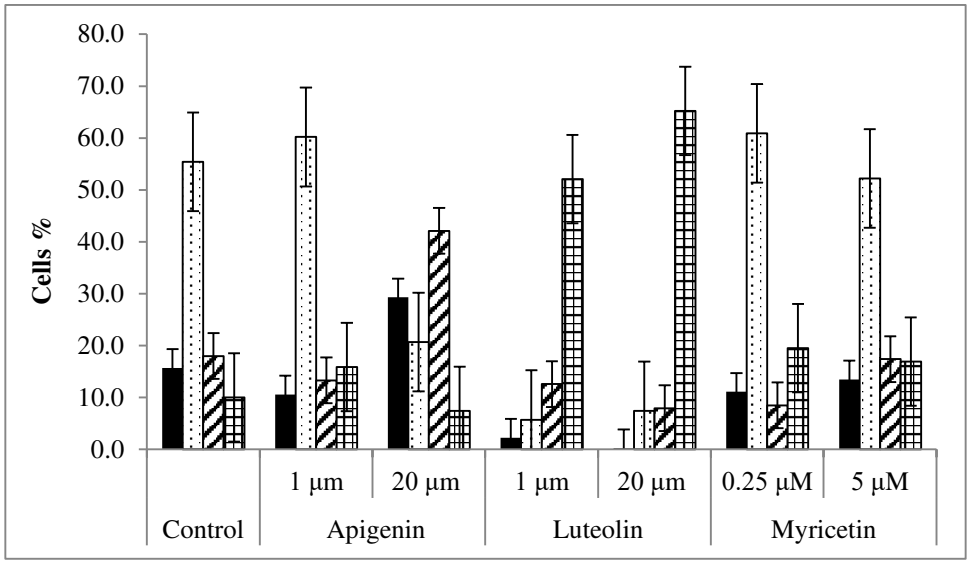


Figure 3.35 Representative histograms showing sub-G1 (solid bar), G0/G1 (dotted bar), S (striped bar) and G2/M (chequered bar) phases of cell cycle by flow cytometry analysis of PI stained SKOV-3 cells.

In SKOV-3 cells, low and high concentration of apigenin had different effect. 1 µM of apigenin induced G0/G1 and G2/M phase arrest whereas 20 µM of apigenin stopped cell cycle at S phase (Figure 3.34 and 3.35).

3.2.7 The Effects of Flavonoids on the Invasion

Cell motility is considered to be an important determinant of the metastatic potential of cancer cells. The effect of apigenin, luteolin and myricetin on the motility of OVCAR-3 and SKOV-3 cancer cells was examined using a cell invasion assay. Figure 3.36 showed fluroscence intensity of cells depending on the cell numbers of OVCAR-3 and SKOV-3.

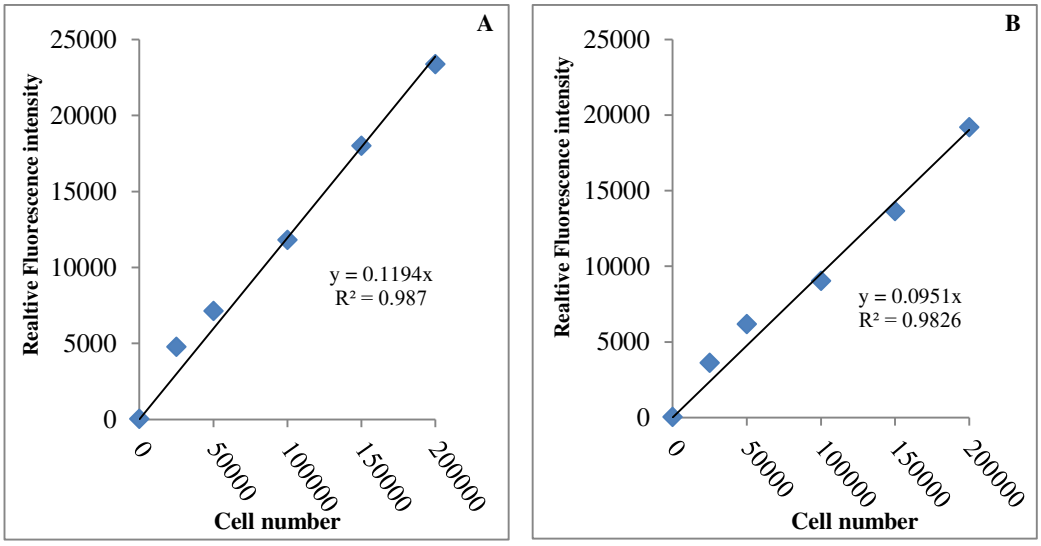


Figure 3.36 The standard curves of CyQuant GR Dye fluorescence intensity depending on the OVCAR-3 (A) and SKOV-3 (B) cell numbers.

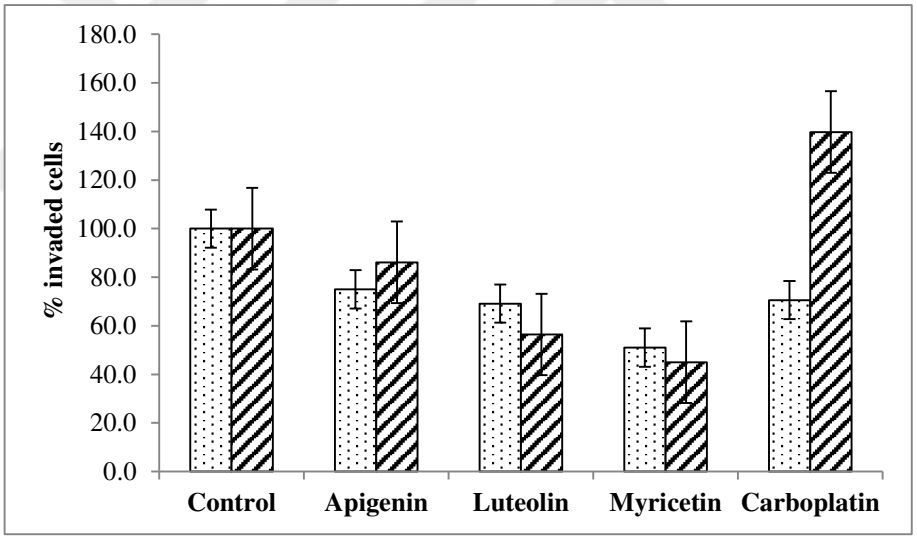


Figure 3.37 Effects of apigenin, luteolin and myricetin on OVCAR-3 (dotted bar) and SKOV-3 (striped bar) cell invasion.

During tumor metastasis, the cells must pass through the ECM. To evaluate whether These studied flavonoids affected OVCAR-3 and SKOV-3 cell invasion, cell invasion assays were carried out in a Transwell chamber coated with collagen. Treatment of OVCAR-3 and SKOV-3 cells with apigenin, luteolin and myricetin led to a significant

concentration-dependent decrease in the cell invasion rate, as compared with the control (Figure 3.37).

CHAPTER FOUR
CONCLUSION

Approximately 90% of primary malignant ovarian tumors are epithelial (carcinomas), and are thought by most investigators to arise from the ovarian surface epithelium (OSE) or more likely from surface epithelial inclusion cysts. OSE produces not only epithelial (laminin and collagen IV) but also mesenchymal (collagen types I and III) components of extracellular matrix, and it modulates from an epithelial to a mesenchymal morphology in response to a variety of environmental cues. The differentiation of EOCs is more complex, unlike carcinomas in most other organs in which epithelial cells become less differentiated in the course of neoplastic progression than the epithelium from which they arise. Tumor cells settle the large array of functions to invade into the surrounding tissue and metastize to distant organs. For this settlement, they need cooperative activities between individual molecules in the tumor cell itself. Starting from the hypothesis that the complex between EpCAM, claudin isoforms and tetraspanins might influence ovarian cancer progression, we first evaluated whether and which of the molecules associate each other in A2780, OVCAR-3 and SKOV-3 cells. In general, claudin-4 and -7 coimmunoprecipitated, albeit weakly with EpCAM in A2780 cells. Instead, in the metastazing OVCAR-3 and SKOV-3 cells which expressed EpCAM, claudin-4 and -7 higher than A2780, claudin-4 and -7 strongly coimmunoprecipitated with EpCAM. Vice versa, anti–EpCAM precipitated claudin-4 and -7. OVCAR-3 and SKOV-3 cells also expressed EpCAM, claudin-4 and -7 at high levels compared with A2780 cells. These complexes were also stable after lysis with the strong detergent Triton X-100, showing the direct and strong interactions between these molecules. Tetraspanin, CD82 even at low levels in OVCAR-3 and SKOV-3 tethered the other interacted molecules. Immunoprecitates of claudin-1 in OVCAR-3 and SKOV-3 cells contained claudin-4 and -7, together with upregulated expressions of claudin-4 and -7. The coimmuprecipitations of claudin-1, -4 and 7 with CD82 demonstrated that these complexes were also located in TEMs. Colocalization studies by immunoflorescence also proved the interactions. They were also characterized by how palmitoylation domains, crosslinking of intra- and extra-cellular domains and cholesterol interactions affect the determined interactions. PKC

activation and inhibition showed tha phosphorylated claudins regulated the interactions.

Ovarian cancer represents an excellent candidate disease for chemoprevention because of its high prevalence and presumed long latency period between initiation and the achievement of a fully malignant disease. Chemoprevention is use of dietary agent that may block, retard, or reverse the development and progression of carcinogenesis. Also, cancer cannot be entirely cured and may be accompanied by unexpected adverse side-effects of using chemotherapeutic drugs.

Because of the urgent need to find some safer cancer therapeutic and preventive agents, and the consequences of the epidemiological studies, showing higher consumption of fruits and vegetables reduce cancer risk, which one of the multiple chemoprevention roles are the effective on the ovarian cancer were evaluated. The cytotoxicity studies of different flavonoids showed that especially apigenin, luteolin and myricetin treatments resulted in a slight decrease in cell viability. Also, high concentrations of flavonoids treatment did not exhibit more pronounced cytotoxicity in OSE-SV40 cells than A2780, OVCAR-3 and SKOV-3 cells. These results suggest that apigenin, luteolin and myricetin may be safer than the using chemotherapeutic drugs. They were also altered intracellular ROS production, MDA concentration are protein carbonyl amounts. These studied flavonoids arrested cell cycle at the different phases and decreased ovarian cancer cell invasion.

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