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NANYANG
TECHNOLOGICAL
UNIVERSITY

**DEFINING THE ROLE OF THE PROGESTERONE RECEPTOR
AND PROGESTIN IN LUMINAL BREAST CANCER CELLS**

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LIST OF ABBREVIATIONS

AF-1	Activation Function 1
AF-2	Activation Function 2
AF-3	Activation Function 3
AR	Androgen Receptor
ATCC	American Type Culture Collection
BUS	B-upstream Segment
CBP	Creb-Binding Protein
<i>CDH1</i>	E-Cadherin gene
<i>CCNA2</i>	Cyclin A2 gene
<i>CCNB1</i>	Cyclin B1 gene
<i>CCND1</i>	Cyclin D1 gene
<i>CCNE</i>	Cyclin E1 gene
CDKs	Cyclin dependent kinases
CT	Cycle Threshold
CTE	COOH Terminal Extension
DBD	DNA Binding Domain
DCC-FCS	Dextran-Coated Charcoal Treated
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
E2	Estradiol 17 β
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
<i>ESR-1</i>	Estrogen receptor gene
ERE	Estrogen Responsive Elements
F	Phenylalanine

FCS	Fetal Calf Serum
GAPDH	Human Glyceraldehyde-3-Phosphate Dehydrogenase
HAT	Histone Acetyltransferases
HEPES	2-[4-(2-Hydroxyethyl) piperazin-1-yl] Ethanesulfonic acid
HER2	Human Epidermal Growth Factor Receptor-2
H	Hinge Region
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
HSP	Heat Shock Proteins
IF	Inhibitory Function Domain
K	Lysine
kDa	kilo Dalton
LBD	Ligand Binding Domain
LC3B	Microtubule-associated Protein 1 Light Chain 3
MAPK	Mitogen-activated Protein Kinases
mRNA	Messenger Ribonucleic acid
NaCl	Sodium Chloride
NCoR	Nuclear Receptor Co-repressor 1
NLS	Nuclear Localization Signal
NTD	N-terminal Domain
pcDNA	Vector Control
PCR	Polymerase Chain Reaction
PDVF	Polyvinylidene Difluoride
PEI	Polyethyleneimine
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKMT	Protein lysine Methyltransferases
PMSF	Phenyl-methane sulfonyl fluoride

PMTs	Posttranslational Modifications
PR	Progesterone Receptor
PRA	Progesterone Receptor isoform A
PRB	Progesterone Receptor isoform B
PRE	Progesterone Response Element
PRMT	Protein arginine Methyltransferases
PTEN	Phosphatase and Tensin homolog on chromosome 10
Q	Glutamine
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
R	Arginine
R5020	Synthetic Progestin promegestone
RB	Retinoblastoma protein
<i>Rb</i>	Retinoblastoma gene
SDS	Sodium Dodecyl Sulfate
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SUMO	Small Ubiquitin-like Modifier
TBST	Tris-Buffered Saline with Tween 20
WHI	Women's Health Initiative
Wt	Wild Type

ABSTRACT

Progesterone is a steroid hormone essential for the reproduction and the development of the mammary gland. Its effects are mediated by progesterone receptor (PR) which is induced by estrogen in most target tissues. In humans and mice, PR is expressed as two major isoforms: PRA and PRB. Although progesterone has been implicated in breast cancer its role in the development of breast cancer remains controversial. There is evidence for the increased risk of breast cancer incidence in post-menopausal women receiving estrogen plus progestin replacement therapy compared to estrogen therapy alone. However, progestin demonstrated therapeutic benefits for metastatic breast cancer. In laboratory studies, progestin can stimulate or inhibit cell growth depending on experimental conditions and cell models. It has been reported that progesterone inhibits estrogen-induced growth of ER and PR-positive luminal breast cancer cells MCF-7 in xenograft model and this is mediated by modulating the activity of ER. However, the role of PR in mediating the effect of progestin in this most commonly used ER and PR-positive model is controversial. This is partly due to the fact that PR expression is estrogen-dependent and the level of estrogen-induced PR is relatively low. As a result, the effect of progestin treatment is often marginal. In order to better understand PR-mediated effect of progestin in luminal breast cancer, the main aim of the study was to establish MCF-7 luminal breast cancer cells overexpressing PRB and define the molecular and cellular aspects of PRB-mediated effect of progestin in this MCF-7 cell model. We hypothesize that progestin exerts inhibitory effect on MCF-7 cells expressing PR independent estrogen. The study demonstrated that progestin R5020 causes drastic cell growth inhibition. More interestingly, R5020 completely abolished estrogen-induced cell growth. Furthermore, the R5020-treated cells in three dimensional (3D) Matrigel culture were unable to form proper mammospheres and almost the entire population underwent apoptosis after 1-2 weeks in 3D culture. Thus, progestin R5020 strongly suppresses the growth of luminal breast cancer cells when there is abundant expression of PRB. Taken together, our study demonstrated powerful anti-tumoral effect of R5020 on breast cancer cells expressing high levels of PR and future in vivo studies could lead to improved outcomes in breast cancer patients.

Chapter 1. Introduction

1 Introduction

Breast cancer is a complex disease that can be classified into several subtypes by using different clinical biomarkers. Currently, estrogen receptor (ER), human epidermal growth factor receptor-2 (HER2) and progesterone receptor (PR) status are available biomarkers for profiling of invasive breast carcinoma Chlebowski, Kuller et al. (2009). Based on the use of these markers, five distinct subtypes of morphologically similar breast cancers (luminal A, luminal B, normal breast-like, HER2-overexpressing, and basal-like) have been identified (Bauer, Brown et al. 2007). When compared to other subtypes, luminal breast tumors are considered to have a good prognosis since they express both estrogen and progesterone receptors. First line therapy for treatment of this kind of tumors is endocrine therapy (Hagan and Lange 2014). However, 40% of patients eventually relapse because they develop endocrine resistance to therapy (Ma, Wang et al. 2004). Study from 2011 from regional registry Singapore-Malaysia by analyzing distribution of histological tumor characteristics in 4058 patients with breast cancer exhibited that 54% of patients were PR-positive, compared to 46% PR-negative whereas in 1623 patients PR status was unknown. On the other hand, analysis of ER/PR combination status showed that 44% of patients were ER+/PR+, 12% ER+/PR-, 10%ER-/PR+ and 34% ER-/PR- status (Pathy, Yip et al. 2011).

Types of breast cancer:

- Luminal A: ER+, PR+, HER2-
- Luminal B: ER+, PR+, HER2+
- Basal like: ER-, PR-, HER2-
- HER2-Overexpressing ER-, PR-, HER2+
- Normal like breast cancer: ER+, PR+, HER2+, Ki-67^{LOW}

Figure 1.1 Molecular subtypes of breast cancer.

Adapted and modified from <https://www.lifelinecelltech.com/optimal-systems-for-breast-cancer-research>

Several studies imply that progesterone and its receptor have a significant role in the development of luminal breast cancer. However, progesterone has a controversial role in the breast cancer etiology because its effect can be stimulatory or inhibitory (Gill, Tilley et al. 1991, Pasqualini, Paris et al. 1998, Lange, Richer et al. 1999, Lin, Ng et al. 1999, Montazeri, Bouzari et al. 2015). Therefore, understanding the role of progesterone, as well as progesterone receptor, in the tumorigenesis may lead to the development of a new target for endocrine therapy.

1.1 Nuclear receptors

Nuclear receptors (NRs) are part of a large family of ligand-activated transcription factors (Tsai and O'Malley 1994, Glass and Rosenfeld 2000). Based on their different mechanisms of action and ligand properties, NRs superfamily can be classified into three groups (hormone, metabolic and orphan NRs) (Mangelsdorf, Thummel et al. 1995). These transcription factors directly regulate genes that are involved in numerous physiological processes, such as homeostasis, reproduction, development, and metabolism (Mangelsdorf, Thummel et al. 1995, Xiao, Wang et al. 2013). NRs exert their functions as monomers, homodimers, and heterodimers, and they are associated with chaperone protein family that provides appropriate intracellular localization by anchoring target protein in cytoplasm or nucleus (Echeverria and Picard 2010).

Members of the hormone receptor subfamily I include estrogen (ER α and ER β), androgen (AR), progesterone (PR), glucocorticoid (GR) and mineralocorticoid receptors (MR). Subtype I members are generally anchored with chaperone family proteins in the cytoplasm. Upon ligand binding, the receptor dimerizes and undergoes conformational changes, followed by receptor dissociation from chaperone complex. In the nucleus, the receptor binds to a specific location on DNA of target genes, termed as hormone responsive elements (HRE) (Aumais, Lee et al. 1996, Kumar and Thompson 1999, Kumar and Thompson 2003).

Metabolic receptors subfamily II, such as farnesoid X (FXR), liver X (LXR) and peroxisome proliferator-activated receptors (PPARs), bind to DNA as heterodimers with their obligate partner retinoid X receptor (RXR) (Li and L. Guo 2015).

Subfamily III, orphan receptors, include all NRs for which ligands have yet to be defined (Giguere 1999, Bonofiglio, Cione et al. 2011, Shen, Bai et al. 2011).

Nuclear receptors share a common structural and domain organization. The N-terminal domain is a highly variable region (VR, region A/B) and consists of the activation function 1 (AF-1). Central DNA-binding domain (DBD, region C) is highly conserved domain. Ligand binding domain (LBD, region E) is a moderately conserved domain of NRs. The hinge region (region D) is the domain that is linking DBD and LBD domains, and contains nuclear localization signal (NLS) (Mangelsdorf, Thummel et al. 1995, Kumar and Thompson 1999, Robinson-Rechavi, Garcia et al. 2003). The other activation functional domain AF-2 is present in LBD region (Gronemeyer and Laudet 1995).

Figure 1.2 Nuclear receptor superfamily is composed of conserved domain structure

NRS structure consists of an N-terminal activation domain, which is important for interactions with co-regulators (activators and repressors), a DNA-binding domain, a small hinge region and a C-terminal ligand binding domain that interacts with the ligands and transcriptional co-regulators Adapted and modified from Cell, Vol. 83, 835-839, December 15, 1995 (Mangelsdorf, Thummel et al. 1995). 2007 American Physiological Society Volume 31, Issue 1, January 2007, Pages 26-33 (Wierman 2007). Int J Environ Res Public Health. 2014 Aug 26;11(9):8709-42. doi: 10.3390/ijerph110908709 (Ng, Perkins et al. 2014)

NRs promote gene transcription mainly through classical gene transactivation and transrepression. These activities are modulated by transcriptional co-regulators that enable NRs to target gene promoters and to coordinate transcription (McKenna, Lanz et al. 1999, Bulynko and O'Malley 2011). Additionally, post-translational modifications can influence NR activities whereby they are targeted by other cellular pathways. One of the outcomes is activation of kinases in response to growth factors (Berrabah, Aumercier et al. 2011, Treuter and Venteclef 2011). Numerous human diseases are triggered by dysregulated NRs signaling (Sever and Glass 2013, Achermann, Schwabe et al. 2017). It has been shown that nuclear receptors, estrogen (ER) and progesterone (PR) play a significant role in development and progression of ovarian and breast cancer (Arnett-Mansfield, deFazio et al. 2001, Daniel, Hagan et al. 2011, Hevir, Trošt et al. 2011, Giulianelli, Vaqué et al. 2012).

1.2 Physiological function of Progesterone

Progesterone is a sex steroid hormone produced by ovaries in corpus luteum, and it has a pivotal role in growth and development of the normal mammary gland. Furthermore, progesterone has a significant role in other parts of the female reproductive system, such as the uterus, ovary, cervix and breast. Progesterone has been identified as an essential hormone for ovulation (Robker, Russell et al. 2000). Progesterone can have an inhibitory role in the uterus, whereas in the developing breast progesterone has a proliferative role (Conneely, Mulac-Jericevic et al. 2003). During the menstrual cycle, progesterone has an effect on endometrium. Changes in receptor abundance at various stages of the menstrual cycle suggest the important role of this hormone in regulation of menstrual cycle (LESSEY, KILLAM et al. 1988). Progesterone is very important for maintaining a healthy pregnancy by preventing myometrical contractility, and plays essential role from embryo implantation to parturition (Sitruk-Ware 1986, Morel, Roucher et al. 2016). Recently it has been reported that progesterone administered in luteal phase of cycle can reduce the risk of miscarriage (Ismail, Abbas et al. 2018). Furthermore, studies showed that progesterone might play significant role in regulation of insulin release and development of diabetes during the pregnancy (Picard, Wanatabe et al. 2002).

Progesterone also plays an important role in non-reproductive tissues, such as the brain (Graham and Clarke 1997, Lee, Jeong et al. 2006). Progesterone receptors have been found in heart and great vessels, and this indicated that this hormone might be functional in cardiovascular system

(Ingegno, Money et al. 1988). In addition, it has been reported that progesterone inhibits synthesis of bone collagen, whereas estrogens and androgens had small stimulatory or no effect on this process (Canalis and Raisz 1978). *In vitro* studies showed that progesterone and estrogen appear to have synergistic role in the treatment of osteoporosis (Seifert-Klauss and Prior 2010). In humans, progesterone might play role in immune system by activating G-protein in Jurkat cells or by inhibition of K⁺ channel in T cell line, which is mediated via membrane PR receptors (mPR) (Ehring, Kerschbaum et al. 1998, Dosiou, Hamilton et al. 2008). Furthermore, mPR receptors have been reported to play role in sperm capacitation and acrosomal reaction in human spermatozoa by modulating Ca²⁺ influx (Luconi, Francavilla et al. 2004, Aideé Saray and Mayel 2016).

1.3 Progesterone receptor

Progesterone receptor is a mediator of physiological effects of progesterone (Lange and Yee 2008, Hill, Roemer et al. 2012). Based on the studies on PR-Knockout (KO) mice, which exhibited defects in reproductive tissues, including ovulation failure, lack of mammary ductal branching and alveolar development, it is clear that PR is crucial for normal reproductive development (Lydon, DeMayo et al. 1995). PR belongs to the nuclear hormone receptor superfamily, together with thyroid receptors, other steroid hormones and retinoids (Evans 1988, Mangelsdorf, Thummel et al. 1995).

In humans and mice, PR is expressed as two major isoforms: PRA and PRB. These isoforms are products of the same gene on chromosome 11, but transcription starts from two different promoters (Kastner, Krust et al. 1990). They regulate different subsets of genes and play unique roles. Namely, PRB is essential for normal mammary gland development (Mulac-Jericevic, Lydon et al. 2003) while PRA is crucial for uterine development (Mulac-Jericevic, Mullinax et al. 2000).

These two isoforms of progesterone receptor are different in size and they share common domain architecture with other nuclear receptors, although with small differences. The most notable structural difference between these two isoforms is that PRB (116 kDa) has 164 amino-acids more than PRA (94 kDa) (Kastner, Krust et al. 1990, Giangrande, Pollio et al. 1997). Moreover, data suggest that PRB is stronger transactivator than PRA (Tung, Shen et al. 2001, Tung, Abdel-

Hafiz et al. 2006). Because of these isoform-specific functional differences, tissue responses to progesterone are affected by PRA:PRB ratio (Arnett-Mansfield, deFazio et al. 2001). PRA and PRB are expressed at similar levels in normal tissues implicating both isoforms play a role in response to progesterone, but the ratio is disrupted in cancerogenic tissue (De Amicis, Zupo et al. 2009). It has been reported that breast tumors with high PRA: PRB ratios predict more aggressive diseases (Graham, Yeates et al. 1995, Mote, Bartow et al. 2002, Graham, Yager et al. 2005, Rojas, May et al. 2017).

1.3.1 Functional domains of progesterone receptor

PR shares a similar domain structure with other nuclear receptors. Both isoforms have identical ligand-binding domain (LBD), highly conserved DNA-binding domain (DBD), hinge region (H) that contains nuclear localization signal (NLS) and the most variable is N-terminal domain (Figure 1.2) (Meyer, Quirin-Stricker et al. 1992, Conneely, Mulac-Jericevic et al. 2003, Obr and Edwards 2012).

Figure 1.3 Schematic representation of progesterone receptor isoforms PRB and PRA.

Progesterone receptor is composed of several functional domains: AF-1, AF-2, AF-3 (Activation Functions 1, 2 and 3), BUS (B-Upstream Segment), IF (Inhibitory Function domain), DBD (DNA binding domain), H (Hinge) and LBD (Ligand-Binding Domain). Adapted and modified from Breast Cancer Research 20024:182 (Gao and Nawaz 2002). Hum Reprod Update. 2005 May-Jun;11(3):293-307. Epub 2005 Mar 24 (Chabbert-Buffet, Meduri et al. 2005).

1.3.1.1 Amino terminal transactivation domain (NTD)

The NTD is a poorly conserved domain consisting of activation function domains (AF-1, AF-3) (Kumar and Thompson 2003). The AF-1 is a proline-rich region located between 456 and 546 amino acids. This 91 long amino acid region is sufficient for transcriptional activity when attached to DBD (Meyer, Quirin-Stricker et al. 1992). AF-1 has a role in the ligand-independent activity of PR, but can also synergize with AF-2 in the LBD domain, for ligand-dependent activity (McKenna, Lanz et al. 1999, Dong and Lye 2004).

The AF-3 is present only in the PRB isoform of progesterone receptor, in 164 amino acids long stretch, described as a B-upstream segment (BUS) (Dressing, Hagan et al. 2009). This segment is responsible for different transcriptional activities of PR isoforms because AF-3 can make scaffold with other two AF subdomains, and this interaction can be crucial for recruiting coactivators. Therefore, the presence of the AF-3 in PRB makes this isoform a stronger transactivator than PRA (Quiles, Millan-Arino et al. 2009). In addition, IF inhibitory action targets exclusively AF-1 and AF-2 (Hovland, Powell et al. 1998). The presence of AF-3 contributes to stronger transactivation activity of PRB as this domain is supposed to mask the inhibitory function at the NTD of PRA.

1.3.1.2 DNA binding domain (DBD)

The DBD is one of the most highly conserved domains among all nuclear receptors, and this domain is responsible for recognition of distinct domains on DNA near promoters of target genes (Evans 1988). Different parts of DBD are involved in two processes, dimerization of the receptor and recognition of response elements on DNA (Tsai and O'Malley 1994). Three α -helices are forming DBD. Helix 1 have so-called “P-box” that is responsible for DNA binding and contains one zinc finger. Helix 2 holds “D-box” with one zinc finger, and it is involved in dimerization of the receptor. Another α -helix is present at COOH terminal extension (CTE), and it is critical for monomeric DNA binding (Aranda and Pascual 2001).

1.3.1.3 Hinge region

The hinge is the poorly conserved region between DBD and LBD which contains nuclear localization signal (NLS) responsible for translocation of receptors from the cytoplasm to the

nucleus. This region is also involved in the interaction with some co-regulators (Aranda and Pascual 2001, Hill, Roemer et al. 2012).

1.3.1.4 Ligand binding domain (LBD)

The LBD is the main switch for the nuclear receptor, which affects nuclear receptor transcriptional activity upon ligand binding. This multifunctional domain is highly conserved and also mediates dimerization of receptor and interaction with heat-shock proteins. The LBD is formed by 12 α - helices and contains AF-2 (Wurtz, Bourguet et al. 1996). AF-2 is necessary for interaction with co-activators, and when the ligand is not present, AF-2 is in its active conformation, but it is blocked by corepressor molecules. Upon binding the ligand, co-repressors are released, and co-activators now can bind to AF-2 (Bourguet, Ruff et al. 1995).

1.4 Modes of PR action

1.4.1 Genomic progesterone signaling

Progesterone receptor is a transcriptional factor that regulates the expression of specific genes in various target tissues, including female reproductive organs. General PR signaling pathway is well established. Namely, when the ligand is not present, PR forms complexes with various heat shock proteins (HSP90 and HSP70) (Carson-Jurica, Lee et al. 1989, Lange 2008). Ligand binding promotes a conformational changes of the receptor, dissociation of heat shock proteins and dimerization of the receptor (Smith 1993). These rearrangements are followed by binding of progesterone receptor to the particular place in DNA named as progesterone response element (PRE) in close proximity to the promoter of target genes. Protein-protein interactions are important for signaling pathway because these interactions provide recruitment of co-activators and the establishment of transcription initiation complex (Leonhardt, Boonyaratankornkit et al. 2003). Posttranslational modification of progesterone receptor, including phosphorylation, acetylation and sumoylation are taking place after interaction with ligand (Lange, Shen et al. 2000, Hagan, Regan et al. 2011). PR also regulates the activity of other transcription factors through a mechanism known as tethering. For example, progesterone cross-talk with NF- κ B or Ap-1 transcription factors represses function of these factors (Bamberger, Bamberger et al. 1996, Kalkhoven, Wissink et al. 1996). In addition, phosphorylated PR can modulate transcriptional activation by tethering to SP-1 or STAT (Owen, Richer et al. 1998, Proietti, Salatino et al. 2005,

Faivre, Daniel et al. 2008). Then again, PR tethering interactions can occur in ligand-independent manner (Daniel, Knutson et al. 2009, Ward and Weigel 2009).

Figure 1.4 Schematic representation of genomic activation of progesterone receptor.

Ligand binding is followed by dissociation of HSP and conformational changes of protein. First, nuclear localization signal (NLS) becomes exposed, and then complex is transported to the nucleus, where it can bind specific place on DNA, named as PRE. This is followed by the recruitment of transcriptional machinery and co-regulatory complex. Adapted and modified from Science Direct, Volume 68, Issues 10–13 (November 2003) (Leonhardt, Boonyaratanakornkit et al. 2003).

1.4.2 Non-genomic progesterone signaling

Progesterone receptor is localized in cytoplasm, whereas in nucleus it fulfills its function (Reiner, Neumeister et al. 1990). In addition to the classical membrane-localized form of PR, mPR, there is another form of PR. This discovery revealed that progesterone could have different function in tissues where there is no classical PR (Dressing, Goldberg et al. 2011). Some of the non-genomic functions of progesterone include change of Ca^{2+} flux in human sperm (Blackmore, Neulen et al. 1991), and interactions with G proteins (Karteris, Zervou et al. 2006). Furthermore, ligand-bound progesterone receptor might interact with other transcription factors and change their function.

Steroid receptors can be activated by cross-talk with other signaling pathways in the absence of the hormones. Activation of unliganded SHRs is followed by phosphorylation of the receptor and, depending on activating signal, different co-regulators can be recruited (Karteris, Zervou et al. 2006, Carascossa, Dudek et al. 2010). Progesterone receptor can be activated by phosphorylation on Ser400 site by CDK2 after stimulation with growth factor (Pierson-Mullany and Lange 2004). Studies showed that elevated cyclic AMP (cAMP) activates protein kinase A (PKA), that further phosphorylates SRC at two phosphorylation sites and modulates chicken PR activity in ligand-independent manner (Rowan, Garrison et al. 2000). Our previous study demonstrated that a charged residue (K464) in the AF-1 region altered the activity of PR in a ligand-independent manner (Chung, Sze et al. 2014).

1.4.3 Posttranslational modifications (PTMs)

The expression and function of PRs are precisely regulated by PTMs, including phosphorylation, sumoylation, acetylation, and methylation (Hill, Roemer et al. 2012).

PR receptors are phosphoproteins, and thirteen serine residues and one threonine residue are modified by protein kinases within the NTD region. Phosphorylation of PR contributes to the stability and the localization of PR (Qiu and Lange 2003, Dressing, Hagan et al. 2009). Serine residues at positions 81, 162, 190 and 400 are constitutively phosphorylated in the absence of hormone (Lange and Yee 2008).

Sumoylation is a dynamic process that includes covalent attaching of Small Ubiquitin-like Modifier (SUMO) monomers to lysine residues of target proteins (Hagan, Regan et al. 2011). The tallying of SUMO monomers may control the stability of proteins, alter subcellular localization or the ability of proteins to interact with their partners (Verger, Perdomo et al. 2003). Residue Lys388 is a target for hormone-induced sumoylation in PR receptor, and this PTM represses transcriptional activity of the receptor (Daniel, Faivre et al. 2007).

Acetylation was initially identified in histones, where it has a key role in the regulation of gene expression through the modification of chromatin structure and making access to the basal transcription machinery (Allfrey, Faulkner et al. 1964). Acetylation occurs at lysine residues, by activation of histone acetyltransferases (HAT) (Spencer, Jenster et al. 1997). In nuclear receptors, acetylation can regulate diverse functions, and usually it takes place on the hinge

region that contains consensus sequence KXKK (Fu, Wang et al. 2004). The best-known mammalian acetyltransferases are p300 and CBP (Creb-Binding protein). Studies performed by our laboratory reported that Lys183 is a target for acetylation by p300 in PR and that this modification enhances PR activity at PRE promoter (Chung, Sze et al. 2014).

In addition to phosphorylation, sumoylation, and acetylation, methylation is supplemental PTM important for nuclear receptor function. Methylation is a major mechanism leading to the silencing of genes and it is mediated by methyltransferases, enzymes that transfer methyl group (CH₃) from S-adenosyl methionine methyl donors to lysine (K) and arginine (R) residues in target proteins (Ren, Liu et al. 2007). Abnormal methylation has been considered as one of the underlying mechanisms of tumorigenesis, development, and metastasis of various cancers (Ren, Liu et al. 2007). There are two types of methyltransferases, protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMT). Methylation is a reversible process and can have diverse effects on the function of nuclear receptors (Wu and Zhang 2009). Protein arginine methyltransferase-1 (PRMT1) specifically methylates ER α at Arginine-260. This methylation is crucial for mediating the extra-nuclear function of the receptor ER α that includes kinase signaling, and ER α methylation appears to be upregulated in a subset of breast cancer (Le Romancer, Treilleux et al. 2008).

1.5 Estrogen and estrogen receptor

There are three forms of estrogen: estrone (E1) is the principal form of estrogen during menopause, estradiol (E2) is the primary estrogenic hormone active in women before menopause and estriol (E3) form is the predominant form during pregnancy (Tulchinsky, Hobel et al. 1972, Pasqualini, Chetrite et al. 1996). E2 is responsible for many biological processes, including growth and development of the reproductive and cardiovascular tissues, as well as bones and the brain (Hall, Couse et al. 2001). The effects of E2 are mediated by both ER α and ER β . Upon binding to LBD, ER undergoes a conformational change and binds to the estrogen-responsive elements on DNA (Chauchereau, Amazit et al. 2003). Activation of ER stimulates the expression of target genes by recruiting coactivators (Balan, Wang et al. 2006). ER α and ER β are sharing the structural organization with other nuclear receptors and can form both homodimers and heterodimers. ER α and ER β are products of two different genes on chromosomes 6 and 14, respectively (Marino, Galluzzo et al. 2006). ER α is primarily expressed in the mammary gland,

uterus, gland, testis, heart and skeletal muscle, whereas ER β is expressed in the ovaries and prostate (Couse, Lindzey et al. 1997).

It has been reported that estrogen can induce breast cancer development *in vitro* and *in vivo* and that estrogens play an important role in the cancerogenesis (Feigelson and Henderson 1996, Zheng and Lin 2008). Studies showed that alteration of ER α in the mammary gland epithelial cells of mouse models resulted in development of mammary tumors after estradiol treatment (Tilli, Frech et al. 2003, Frech, Halama et al. 2005). In addition, studies showed that ER α and ER β have opposite effect on cell proliferation and tumor development in xenograft mouse models. While ER α promotes proliferation, expression of ER β in MCF-7 cells exerts antiproliferative effects and inhibition of tumor formation in mouse models after estradiol treatment (Paruthiyil, Parmar et al. 2004, Treeck, Lattrich et al. 2010).

Progesterone receptor expression is a clinically beneficial parameter for the assessment of the ER function, as PR is the target gene for ER. ER and PR are crucial for tumorogenesis, prognosis and the treatment of breast cancer. Estrogen and ER α have proliferative effect in breast cancer, therefore this receptor is the primary target for ER+ breast cancers. This kind of therapy is widely used and relatively successful in patients with ER+ breast cancer, but endocrine resistance occurs in up to one-third of all breast cancer patients. Better response to hormonal therapy has been observed in ER+ and PR+ breast cancer (Osborne, Yochmowitz et al. 1980, Bardou, Arpino et al. 2003). ER+ and PR-negative breast cancers exhibit more aggressive phenotype and are less responsive to endocrine therapy (Fisher, Redmond et al. 1988). The increased expression of PR in ER+ breast cancer is associated with better clinical outcome in breast cancer patients (Ravdin, Green et al. 1992).

1.6 Senescence

Senescence is the natural biological process and it has positive and negative roles. The importance of cellular senescence in cancer and aging was recognized long time ago, as in these two processes cells encounter severe cellular damage and dysfunctions. Cellular senescence represents proliferative arrest of cells that can be induced by different stressor stimuli. Despite the fact that cells are growth arrested, they remain metabolically active (Hayflick and Moorhead 1961, Faragher and Kipling 1998, Shay and Roninson 2004, Wiley and Campisi 2016). Cellular

senescence can be induced by shortening of telomeres in human fibroblasts (Harley, Futcher et al. 1990). Stress-induced senescence, termed as premature senescence, can be induced under conditions of nutrient, growth factor and oxygen depletion (Sherr and DePinho 2000, Chen, McMillan-Ward et al. 2008). Senescent cells display distinct biomarkers that differ them from dividing cells. These markers includes drastically changed morphology including large and flat phenotype, altered cytoskeleton features and enlarged nucleus (Kuilman, Michaloglou et al. 2010). Few studies demonstrated the role of senescent cells in several biological processes, such as embryogenesis, wound healing and instance tissue repair (Krizhanovsky, Xue et al. 2008, Storer, Mas et al. 2013, Eming, Martin et al. 2014, Yun, Davaapil et al. 2015). Senescence itself has been found to have tumor suppressive role, but inflammatory response associated with senescence plays a significant role in tumor progression (Campisi 2001, Rodier, Coppe et al. 2009, Freund, Orjalo et al. 2010).

Tumor suppressor proteins P53 and RB have been indicated as one of the most important signals for initiating senescence (Shay, Pereira-Smith et al. 1991, Qian and Chen 2013, Rufini, Tucci et al. 2013). RB tumor suppressor binds and inactivates E2F transcription factor activity. RB is a crucial regulator of E2F genes that drive cell cycle and important regulator of senescence (Campisi 2001, Narita, Nuñez et al. 2003). RB action is regulated by many post-translational processes and the most important one is phosphorylation (Takahashi, Ohtani et al. 2007). Cyclin-dependent kinases (CDKs) are responsible for phosphorylation of RB, and when this protein is in hyper-phosphorylated form, it losses capacity to bind and reinforce E2F activity. Expression of CDKs inhibitors, such as p21, blocks activity of CDKs. Then, RB is not phosphorylated and cells can progress to S phase of cell cycle (Sherr and McCormick , Cobrinik 2005, Dimova and Dyson 2005). Inactivation of tumor suppressor gene P53 by mutation is very common event in human cancers (Hollstein, Sidransky et al. 1991). Activation of P53 pathway induces expression of p21 (el-Deiry, Tokino et al. 1993). P21 is one of the best characterized proteins involved in induction of cellular senescence (Afshari, Nichols et al. 1996, Stein, Drullinger et al. 1999). Furthermore p21 represents the link between p53 and RB tumor suppressor pathways (Sherr and McCormick , Stein, Drullinger et al. 1999, Takahashi, Ohtani et al. 2007). p16 has been reported to have a more prominent role in maintaining cells in growth-arrested stage during the cellular senescence (Stein, Drullinger et al. 1999, Ben-Porath and Weinberg 2005). On the other hand, loss of p16

activity induces bypassing of senescent stage and resumes cell growth (Foster, Wong et al. 1998, Jarrard, Sarkar et al. 1999).

Figure 1.5 Activation of the senescence by p53 and p16 pathways.

Senescence induces the p53 pathway that includes induction of cyclin-dependent kinase (CDK) inhibitor p21. p16 pathway inhibits phosphorylation of retinoblastoma protein (RB) and suppresses transcription factor E2F. This leads to repression of genes included in cell cycle progression. Adapted from Nature Reviews Molecular Cell Biology volume 8, pages 729–740 (2007) (Campisi and d'Adda di Fagagna 2007).

1.7 NF- κ B pathway

The NF- κ B is a transcription factor complex that governs the expression of a large number of genes. This complex is composed of 5 members of Rel family: RelA (p65), RelB, c-Rel, NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), with p50/p65 heterodimer being the most common one (Hoffmann, Natoli et al. 2006). Members of this family can form homo- and heterodimers, and in inactivated form they are located in cytoplasm bound to I κ B inhibitors (Verma, Stevenson et al. 1995, Baeuerle and Baltimore 1996). Different stimuli can trigger this pathway through receptors, such as TNF receptor family (TNFR), various cytokine receptors (IL-1R), Toll-like receptors (TLRs), which leads to the activation of classical canonical pathway (Baldwin 1996

351). Upon ligand binding, I κ B kinase (IKK) complex is activated and phosphorylates I κ B α . I κ B α has an inhibitory function, and it is blocking nuclear localization signal on NF- κ B homo- or heterodimers. Upon phosphorylation, I κ B α is targeted for proteasomal degradation, and activated subunits of NF- κ B rapidly translocate to nucleus where they bind to specific regions on DNA of target genes. CD40, B-cell activation factor (BAFFR), receptor activator for nuclear factor kappa B (RANKL) are receptors that are mediating non-canonical stimulation of NF- κ B pathway. Activation of these receptors leads to activation of different type of kinase named as NF- kappa B inducing kinase (NIK). This kinase releases NF- κ B complex p52/RelB from inhibitor and enhances nuclear localization (Baldwin 1996, Hoesel and Schmid 2013, Liu, Zhang et al. 2017).

Figure 1.6 NF- κ B signaling pathways.

Canonical pathway includes activation of IKK kinase and p50/p65 heterodimer from inhibitor, and translocation to nucleus. Non-canonical pathway includes activation of NIK kinase, mediated by different receptors and translocation of p52/RelB heterodimer to nucleus. Adapted and modified from Trends in Immunology; Volume 25, Issue 6, 1 June 2004, Pages 280-288 (Bonizzi and Karin 2004).

In the background of the well-established connection between apoptosis and senescence is NF- κ B activation (Schuler and Green 2001). Studies showed that NF- κ B subunit p65 was more abundant in the chromatin of senescent fibroblasts as compared to that of young matching part (Chen, Williams et al. 2005). The growth arrest induced by p53/p21 signaling could be disabled by inhibition of NF- κ B signaling. This finding suggests that the NF- κ B pathway had a two-way role in the induction of SASP (Kuilman and Peeper 2009, Salminen, Kauppinen et al. 2012).

Studies have shown that NF- κ B pathway can promote chemoresistance, by inducing expression of anti-apoptotic genes, or chemosensitivity by inducing expression of the genes responsible for inducing senescence (Acosta, O'Loughlen et al. 2008, Kuilman, Michaloglou et al. 2008, Chien, Scuoppo et al. 2011, Rajaram, Buric et al. 2015). Activation of NF- κ B occurs in response to DNA damage. Subsequent production of chemokines plays an important role in reinforcing the cell arrest, modifying the microenvironment and activating immune surveillance of the senescent cells (Chien, Scuoppo et al. 2011).

1.8 Autophagy

Autophagy is a dynamic cellular process, which includes catabolic degradation of cellular organelles and proteins, in order to recycle building blocks, e.g. amino acids, in conditions like acute starvation, or to remove unnecessary macromolecules and pathogens from the cytosol (Mathew, Karantza-Wadsworth et al. 2007, Yao, Wang et al. 2012). Autophagy pathway starts with a formation of the autophagosome, a double-membrane-bound structure that degrades its target inside the membrane. These structures are then fusing with lysosomes, organelles that contain enzymes that can degrade all types of biological polymers (Kondo, Kanzawa et al. 2005). Three types of autophagy have been identified: microautophagy, chaperone-mediated autophagy and macroautophagy (henceforth referred to as autophagy) (Mizushima 2007). Microtubule-associated protein 1 light chain 3 (LC3) regulates expansion of phagophore (cytoplasmic material labeled for degradation with sequester membrane) and formation of autophagosome (double membrane vesicle) (Xie, Nair et al. 2008). Formation of autophagosome is followed by fusion with lysosomes when lysosomal enzymes are degrading inner membrane and the cargos of autophagosomes (Yorimitsu and Klionsky 2005, Xie and Klionsky 2007).

Figure 1.7 Schematic representation of autophagic progression.

Formation of phagophore around the cytoplasmic material labeled for degradation. LC3 induce expansion of phagophore and formation of autophagosomes. Autophagosome fuse with lysosome and lysosomal degradation of autophagosome cargo occurs, whereas LC3 is recycled. Adapted from, *Experimental & Molecular Medicine*, volume 44, pages 69–72 (2012) (Jing and Lim 2012).

It has been reported that autophagy pathway has a significant role in immunity and inflammation (Levine, Mizushima et al. 2011). Defects in autophagy have been associated with various diseases (Levine and Kroemer, 2008). The role of the autophagy in cancer is controversial. While some mechanisms of autophagy are able to promote hypoxia tolerance and tumor cell survival (Rouschop and Wouters 2009) autophagy also establishes an alternative pathway of cell death, autophagic cell death, in transformed and cancer cells (Chen, McMillan-Ward et al. 2008). Literature indicates that autophagy can be associated with delayed apoptosis or prolonged survival of noninvasive breast tumor cells (Pattingre, Tassa et al. 2005, Abedin, Wang et al. 2007). Beclin 1 deletion has been reported in numerous human breast cancers (Futreal, Söderkvist et al. 1992, Liang, Jackson et al. 1999). Autophagy plays a significant role in breast cancer cells response to endocrine therapy. Endocrine therapy resistance in estrogen receptor positive (ER+) breast cancer remains an important clinical problem. Studies showed that estrogen and progesterone may induce autophagy in bovine mammary epithelial cells by non-genomic pathway (Zielniok, Sobolewska et al. 2017). It has been reported that inhibition of

autophagy in some cancers, including breast cancer, resensitized the resistant cancer cells to the various therapeutic agents (Chen, Rehman et al. 2010). These studies emphasize a potential use of autophagy inhibitors combined with conventional chemotherapeutic drugs for early breast cancer treatment.

1.9 Progesterone, progestin and breast cancer and controversies

Clinical studies have shown that progesterone can promote carcinogenesis in breast tissue (Briskin 2013). Women's Health Initiative (WHI) reported that the combination of estrogen and progestin used in hormone replacement therapy (HRT) is associated with increased risk of breast cancer compared with estrogen alone (Chlebowski, Hendrix et al. 2003). Furthermore, the combined use of progestin and estrogen in HRT correlated to increased breast cancer occurrence along with a more aggressive disease phenotype in contrast to estrogen therapy alone (Stefanick, Anderson et al. 2006, Chlebowski et al. 2010, Lambrinoudaki 2014). The role of progesterone in breast cancer development remains controversial because this hormone can manifest both, proliferative and inhibitory effects on breast cancer tumorigenesis (Lange, Richer et al. 1999). This controversial effect of progesterone or progestins was demonstrated to be mediated by PR (Horwitz, Costlow et al. 1975, Horwitz 1978, Groshong, Owen et al. 1997, Levenson and Jordan 1997, Lange, Richer et al. 1999). The expression of PR is a clinically beneficial parameter for checking ER function (Hagan and Lange 2014). PR and ER expression are important guidelines for clinicians to predict the response to endocrine treatment for hormone-dependent breast cancers (Lin, Eng et al. 2001, Chlebowski, Kuller et al. , Hevir, Trošt et al. 2011, Briskin 2013). Progesterone and its synthetic forms (progestins) have been reported to stimulate or inhibit the growth of luminal breast cancer cells depending on the source of the cell lines and the experimental conditions (Horwitz 1978, Cho, Aronica et al. 1994, Groshong, Owen et al. 1997, Lin, Ng et al. 1999, Alkhalaf, El-Mowafy et al. 2002, Daniel, Hagan et al. 2011, Knutson and Lange 2014). Therefore, it is crucial to perform further studies regarding the potential adverse effects of progestin treatment.

Hormone-responsive luminal breast cancer cell lines MCF-7 and T47D are widely used for breast cancer research, and contradictory results were reported from different laboratories. MCF-7 is an ER and PR positive cell line, but the expression of PR is only detected upon stimulation

by estradiol (Horwitz 1978). On the other hand, T47D cells express both ER and PR constitutively (Sartorius, Groshong et al. 1994, Subik, Lee et al. 2010).

Progestins, such as promegestone (R5020) and medroxyprogesterone acetate (MPA), have been shown to either stimulate or inhibit the growth of T47D cells depending on the duration of exposure. This biphasic effect of the progestins on T47D was found to be mediated by PRB (Hissom and Moore 1987, Gill, Tilley et al. 1991, Groshong, Owen et al. 1997, Richer, Lange et al. 1998, Thuneke, Schulte et al. 2000, Proietti, Salatino et al. 2005, Skildum, Faivre et al. 2005). R5020 is synthetic progesterone commonly used in scientific studies of PR activity because of its known stability and slower metabolism compared to other progestins (Vignon, Bardon et al. 1983). Studies reported that R5020 had no effect on tumor growth in androgen receptor (AR)-positive, glucocorticoid receptor (GR)-positive but PR-negative breast tumors in animal model (Vignon, Bardon et al. 1983). Different studies showed that R5020 is exhibiting anti-estrogenic activity in T47D and R27 human breast cancer cell lines (Vignon, Bardon et al. 1983 453). In addition, R5020 treatment inhibited cell growth in T47D cells and estradiol treatment was not able to induce cell growth in the presence of R5020, yet the effect of R5020 was abolished by EGF treatment (Gill, Tilley et al. 1991). High concentrations of progesterone reportedly inhibited the growth of MCF-7 cells (Alkhalaf, El-Mowafy et al. 2002). Yet, progestins have also been implicated in the proliferation and pro-survival of luminal breast cancer cells (Daniel, Hagan et al. 2011). Studies showed that R5020 and progesterone decreased an uptake of E2 precursor estrone sulfate and its conversion to E2, and decreased concentration of E2 in R5020 treated cells compared to control treated cells (Hobkirk 1993, Pasqualini, Paris et al. 1998). Animal models provide better understanding of progesterone and PR mechanism of action and give us the significant insight into the development of normal human breast and hormone-dependent breast cancer (Kariagina, Aupperlee et al. 2007). Furthermore, R5020 was shown to attenuate the growth of estrogen-induced tumors of MCF-7 luciferase cells in the xenograft study (Mohammed, Russell et al. 2015). In mice model it has been reported that the PRB itself is sufficient to respond to progesterone in ovaries and uterus, whereas mammary gland development was abolished. However, lack of PRB resulted in defective mammary gland alveogenesis (Mulac-Jericevic, Lydon et al. 2003) It was proposed that PR could interfere with the proliferative effects of estrogen in ER α + breast tumors through the alterations in chromatin binding and target genes expression (D'Santos, Taylor et al. 2015, Mohammed, Russell et al.

2015, Carroll, Hickey et al. 2017). These studies in the T47D and the MCF-7 cell models collectively implied an importance of a functional PR in modulating the progression of ER α + breast carcinoma in response to estrogen stimulation. In tamoxifen resistant ZR-75-1 breast cancer cell line, E2 treatment stimulated cell growth and induced PR expression. On the other hand, MPA treatment inhibited cell growth (van den Berg, Martin et al. 1990). Prolonged incubation of ZR-75-1 cell line with R5020 inhibited mitogenic effect of estrogen, whereas addition of bovine insulin or antiprogesterin RU486 antagonized the effect of R5020 (Poulin, Dufour et al. 1989).

A better understanding of the role of progesterone, as well as the mechanism of action of the PR in the cancer pathogenesis, is necessary for the development of a new target for endocrine therapy. PR overexpression was studied in the MDA-MB-231 basal-like ER α and PR negative breast cancer cell line, where progestins were found to exert an anti-proliferative effect in PR positive breast cancer cells by acting on PR (Lin, Ng et al. 1999). However, the impact of PR in MCF-7, in the absence of E2 has not been fully defined yet. Herein, we aim to investigate the implication of PRB expression in MCF-7 cells.

1.10 Objectives of the study

There are many controversial issues regarding the role of progestins in breast cancer. The most contradictory is a proliferative response to progestins in luminal breast cancer cells. The effect of progestins on the cell growth depends on many factors, of which the most important are experimental conditions and source of cell lines. Namely, treatment with progestins can manifest proliferative and pro-survival activity in luminal breast cancer cells (Daniel, Hagan et al. 2011) or inhibit cell growth when a high dose is applied (Alkhalaf, El-Mowafy et al. 2002). MCF-7 and T47D hormone-dependent cell lines are the most frequently used *in vitro* cell culture models for assessment of the role of progestin against tumor cell proliferation. Different laboratories that were using these human breast cancer cell line models (characterized by a different expression of PR) published conflicting reports. We hypothesize that R5020 have inhibitory effect in PRB overexpressing MCF-7 cells. Therefore, the aim of this study was to determine cellular and molecular mechanisms of action of PRB overexpressing in MCF-7 luminal breast cancer cells. Progesterone receptor is therapeutically interesting because of interaction with other

transcriptional factors and as a possible second line therapy for hormone-dependent breast cancer.

Specific objectives of this study are to:

1. Examine the R5020 effect on cell growth of MCF-7 cells overexpressing PRB.
2. Cell cycle analysis in response to R5020 treatment in PRB stably transfected MCF-7 cells.
3. Investigation of the role of ER α and PRA in response to R5020 treatment in PRB overexpressing MCF-7 cells.
4. Mammosphere formation in MCF-7-PRB cells after treatment with R5020.
5. Study of R5020 role in inducing cellular senescence and autophagy.
6. Investigation of E-cadherin down-regulation after R5020 treatment in MCF-7-PRB cells.

Chapter 2. Material and methods

2 Material and methods

2.1 Chemicals and Reagents

Table 2.1 Chemicals and Reagents.

Chemicals	Manufacturer
10% Tween-20	Bio-Rad
10X-TRIS-glycine buffer	Bio-Rad
10X-TRIS-glycine-SDS buffer	Bio-Rad
2- Mercapthoethanol	Sigma Chemical Co (St. Louis, MO, USA)
2- Propanol	Sigma Chemical Co (St. Louis, MO, USA)
4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)	Merck
Acrylamide	Bio-Rad
Activated charcoal	Sigma Chemical Co (St. Louis, MO, USA)
Ammonium persulfate	Bio-Rad
Aprotinin	Sigma Chemical Co (St. Louis, MO, USA)
Dextran	Sigma Chemical Co (St. Louis, MO, USA)
Diethylpyrocarbonate DEPC	Sigma Chemical Co (St. Louis, MO, USA)
DMSO	Sigma Chemical Co (St. Louis, MO, USA)
Dulbecco's phosphate buffered saline (DPBS)	Sigma Chemical Co (St. Louis, MO, USA)
Estradiol	Sigma Chemical Co (St. Louis, MO, USA)
Ethanol 99.9%	Merck
Fetal calf serum FCS	Hyclone (ThermoScientific)
Formaldehyde (37%)	Sigma Chemical Co (St. Louis, MO, USA)
HCl	Merck
Isopropanol	Merck
Leupeptin	Sigma Chemical Co (St. Louis, MO, USA)
LipofectAMINE2000®	Invitrogen Inc. (Carlsbad, CA, USA)
Methanol	Merck
Millipore	Merck
Pepstatin A	Sigma Chemical Co (St. Louis, MO, USA)
Phenol: chloroform: isoamyl-ethanol	Sigma Chemical Co (St. Louis, MO, USA)
Phenylmethylsulphonylfluoride (PMSF),	Sigma Chemical Co (St. Louis, MO, USA)
Promegestone (R5020)	Perkin Elmer (Santa Clara, California, USA).
RNAse A	Sigma Chemical Co (St. Louis, MO, USA)
Sodium fluoride (NaF),	Sigma Chemical Co (St. Louis, MO, USA)
Sodium vanadate	Sigma Chemical Co (St. Louis, MO, USA)
SYBR Green I	Sigma Chemical Co (St. Louis, MO, USA)
Tetramethylethylenediamine (TEMED)	Bio-Red
Triton-X-100	Bio-Rad
TRIzol reagent	Invitrogen Inc. (Carlsbad, CA, USA)

2.2 Cell culture

MCF-7 and T47D cancer cell lines were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red, supplemented with 7.5% fetal calf serum (FCS) (Sigma-Aldrich, St, Louis, MO, USA) and 2 mM L-glutamine. Cells were incubated at 37 °C in humidified atmosphere of 5% CO₂ and 95% air. The stable cell line was cultured under same conditions, and the cell culture media was supplemented with 400 ng/ml Hygromycin. Cells were sub-cultured 1-2 times per week in dilution of 1:5. Cell culture work was done in sterile conditions.

MCF-7 cells are ER, PR positive cells, but PR expression is low. These cells were isolated from the pleural effusion of female human Caucasian with breast adenocarcinoma (Soule, Vazquez et al. 1973). This cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

T47D (Chauchereau, Amazit et al.) cells are ER, PR positive cell line, isolated from the pleural effusion of a female human with ductal carcinoma (Keydar, Chen et al. 1979).

2.2.1 Cell freezing

For long-term usage, cells were kept in liquid nitrogen storage at low temperature. Before freezing, cells were cultured in a 10 cm dish in conditions as described above, up to 80% confluency. Cells were detached by using 0.05% trypsin and transferred into falcon tube. Centrifugation at 800 rpm for 5 minutes at 4 °C was used to pellet the cells. The supernatant was removed, and cell pellet was re-suspended in 0.5-1 ml of growth medium. Freezing agent containing 85% growth and 15% DMSO was added in 1:1 ratio to the cell suspension. DMSO was used as a cryoprotectant to help to prevent damage of the cells at low temperatures. After that, cells were aliquot into freezing vials. Freezing vials were placed at -80 °C overnight. Next day, vials were transferred into liquid nitrogen storage.

2.2.2 Cells thawing

In order to prevent cell damage thawing of the cells from liquid nitrogen was done step by step. Cells were moved from liquid nitrogen to -80 °C freezer for 10-20 minutes. Cells were thawed in 37 °C water bath. The cells were moved from the cryovial into 10 cm dish. Growth medium was

added drop-wise while tilting the dish back and forth. Cell were incubated the overnight and the next day media was changed to remove freezing agents.

2.2.3 Hormone treatment

For the treatment of cells, we used promegestone R5020 (10 nM), and estradiol E2 (10 nM). R5020 is a synthetic progestin with similar effects to progesterone. In all experiments, ethanol (0.01%) was used as a control treatment.

Prior to the hormone, treatment cells were plated in the phenol- red-free medium supplemented with 5% dextran-coated charcoal-treated FCS (DCC-FCS) and 2 mM L-glutamine for 48 hours. After that, the dose of selected hormones was added. At day 3, 24 hours after the treatment or day 6, 96 hours after the treatment, cells were collected for further analysis.

2.2.4 Cell counting

To determine cell proliferation cells were plated in triplicates at density of 9×10^4 cells per clone in the 6-well dish. Cells were cultivated and stimulated with hormones, as previously described, for six days. After that, cells were collected by using 0.05% trypsin, inactivated by serum-containing DMEM with 7.5% FCS. The cells were centrifuged before counting at 800 rpm for 5 minutes, and the medium was discarded. The pellet was resuspended in a particular volume of medium and 50 ul of cells was taken and counted using hemocytometer.

The total number of cells was determined by the following formula:

$$\text{Total number of cells} = \frac{\text{Number counted using hemocytometer}}{4} \times 10000 \times \text{volume used to resuspend cells (ml)}$$

2.2.5 Anchorage-independent growth

2.2.5.1 Acini Formation Study

MCF-7 stably transfected with pcDNA or PRB were pre-treated with 10 nM R5020 for 72 hours. 40 ml of Matrigel® (Corning) were used to coat each well of a 8-chamber slide (Lab-Tek) and allowed to solidify at 37 °C. The cells were then re-seeded at density of 5000 cells onto the pre-coated 8-chamber slides in 2% Matrigel in culture medium supplemented with 10 nM R5020 or

0.01% Ethanol. The cells were cultured in their respective culture treatment mix for 15 days and medium was replaced on Days 3, 7 and 10. The formed acini structures were imaged on days 15 using inverted microscope Olympus IX_71 under 10x magnification.

On day 15, the Matrigel was digested with 1 mg/ml Dispase (Gibco) in DMEM at 37 °C for 1 hour to release the formed acini structures. The released acini structures were pelleted down at 350 r.c.f. for 10 minutes at 4 °C. The supernatant was removed and the pelleted acini were treated with 300 ml 0.05% Trypsin (Gibco) for 10 minutes in 37 °C water bath, followed by inactivation with 900 ml culture medium. Absolute number of live cells was quantified via cell counting using trypan-blue exclusion staining.

2.2.5.2 2% Matrigel cell growth

MCF-7 stably transfected with pcDNA or PRB were pre-treated with 10 nM R5020 for 72 hours. The cells were then re-seeded at density of 6250 cells into 24-wells in 2% Matrigel in culture medium supplemented with 10 nM R5020 in 0.01% Ethanol. The cells were cultured in their respective culture treatment mix for 15 days and medium was replaced on Days 3, 7 and 10. Live cells were imaged on day 15 using inverted microscope Olympus IX_71 under 10x magnification.

On day 15, the cells were fixed with 1% paraformaldehyde in PBS for 15 minutes at room temperature and washed twice with PBS. The cells were then stained with 0.1% crystal violet in 10% ethanol for 15 minutes at room temperature and washed with deionised water before air-drying. The stained cells were imaged using HP scanner G4050.

2.2.6 Cell cycle analysis

MCF-7 stable transfected cells were plated at a density of 10×10^4 in 6-well plates. In hormone-treated experiments, cells were plated in 5% DCC-FCS. Cells were collected using 0.05% trypsin, inactivated by serum containing DMEM. Cells were centrifuged at 1000 rpm for 5 minutes, and the medium was discarded. Then cells were stained with propidium iodide for 1 hour at 4 °C and analyzed by flow cytometry using BD LSRFortessa™ X-20 flow cytometer (BD Biosciences, USA).

2.2.6.1 BrdU cell cycle analysis

MCF-7 stable transfected PRB2 clone was plated at a density of 9×10^4 in 6-well plates. Cells were plated in phenol red-free DMEM containing 5% DCC-FCS and 2 mM L-glutamine for 48 hours prior to treatment with 10 nM R5020 and vehicle control (0.01% ethanol). 96 hours after treatment with R5020 BrdU at final concentration 20 μ M was added to all wells except for the unlabeled control well. Cells were incubated with BrdU for 30 minutes at 37 °C and 5% CO₂. Cells were collected by using 0.05% trypsin, inactivated by serum containing DMEM. Cells were centrifuged at 800 rpm for 5 minutes, and the supernatant was removed. Cells were washed with 1x PBS and again centrifuged at 800 rpm for 5 minutes, and the supernatant was removed. Cells were resuspended in 100 μ l of cold PBS. Cells were fixed by adding 70% ethanol and incubated overnight at 4 °C. The next day, fixed cells were centrifuged at 4000 rpm for 2 minutes, and the supernatant was discarded. 2 N HCl/Triton X-100 was used to denature DNA for 30 minutes at room temperature. A sample was neutralized for 2 minutes by using Na₂B₄O₇ (sodium- tetra borate) at pH 8.5. Then cells were washed with PBS/1% BSA. Cells were stained with anti-BrdU antibody at concentration of 0.5 μ g/ml in 0.5% Tween 20/1% BSA PBS for 30 minutes at room temperature. After removal of antibody, cells were resuspended in PBS containing 10 μ g/ml of propidium iodide, and exposed to 10 μ g/ml of RNAase A for 30 minutes in the dark. Samples were analyzed by flow cytometry using BD LSRFortessa™ X-20 flow cytometer (BD Biosciences, USA). Forward scatter (FSC), related for cell size, and side scatter (SSC), related to cell granularity, were used to eliminate cell debris and dead cells (P1) (Mullaney, Van Dilla et al. 1969, Nielsen, Larsen et al. 1982, Benson, McDougal et al. 1984, Reardon, Elliott et al. 2014). For single cells gating, we used these two parameters to compare cell size vs. cell signal (P2), and cell granularity vs. cell signal (P3). Furthermore, single cells gating (P3) was used for gating BrdU labeled cells (P4-P5) (Figure 2.1).

Figure 2.1 Cell cycle analysis using the BrdU FITC assay.

Dot plots of single cells gating.

2.2.7 Cell imaging

MCF-7 stable transfected cells were plated at a density of 6×10^4 in 6-well plates in phenol red-free DMEM for 48 hours prior to treatment with 10 nM R5020 or E2 10 nM and vehicle control (0.01% ethanol). The images were taken 96 hours after the treatment.

2.3 Transfection

2.3.1 Establishment of stable cell line

MCF-7 cells (ATCC) were plated in the 6-well plate, at a density 2.5×10^5 per dish, in phenol red-free, antibiotic-free medium DCC-FCS. The cells were transfected 24 hours after plating with pcDNA 3.1 (+/hygro expression vector (Invitrogen, Carlsbad, CA, USA)), and PRB using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and 8 hours after transfection medium was changed. The cells were split from the 6-well plate into two 100 mm dishes, 24 hours after transfection. Positive clones were selected by using selection medium (phenol red DMEM containing 400 μ l of hygromycin B antibiotic). A selection of positive clones was based on Western Blot analysis of PRB protein expression. Validation was performed three times before experiments with stable positive clones started.

2.3.2 Adenoviral transduction

MCF-7 stable clones were plated in 6-well plates in phenol-red free DMEM containing 5% DCC-FCS and 2 mM L-glutamine at a density of 2.5×10^5 cells per well for 24 hours hormone treatment experiments, and at 1×10^5 cells per well for 96 hours hormone treatment experiments. Within 48 hours of seeding, fresh medium was added to the cells. Cells were transduced with direct adding of adenovirus AD/ER α C3 of MOI 5 or AD/PRA of MOI 1 to the wells. 24 hours after transduction, cells were treated with hormones as described above.

2.3.3 Total RNA extraction

Total RNA was isolated using TRIzol reagent (Life Technologies, INC., Gaithersburg, MD, USA). PRB transfected MCF-7 cells were entirely lysed by passing several times through a 1 ml pipette. The RNA was separated from the rest of lysate by using chloroform: isoamyl-ethanol (24:1) (Sigma-Aldrich, Munich, Germany). RNA extraction was completed with acid- phenol: chloroform: isoamyl-ethanol (50:24:1) (Sigma-Aldrich, Munich, Germany). The extracted RNA was precipitated by using isopropyl alcohol and washed with 75% ethanol (in 0.2% DEPC water).

The isolated RNA was dissolved in 0.2% DEPC-treated water. Quality and concentration of RNA was assessed using a Nano- drop spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

2.4 Molecular Biology

2.4.1 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

1 μ g of total RNA was reverse transcribed to cDNA, using qScript cDNA Synthesis kit (Quanta BioSciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. qRT-PCR was performed according to manufacturer's instruction by using SYBR green master mix (KAPA Biosystems, Boston, MA, USA) and an ABI QuantStudio 6 Detection System (Applied Biosystems Inc., Foster City, CA, USA). The qRT-PCR for each gene was performed in triplicates. The cycling conditions are illustrated in Table 2.2. The fold changes were calculated by normalizing CT values of the gene with CT values of 36B4 gene. Primers used for qRT-PCR are listed in Table 2.3.

Table 2.2 Cycling conditions of qRT-PCR

No. of cycles	Temperature	Duration
Hold stage	50 °C	2 min
	95 °C	10 min
40 cycles	95 °C	30s
	60 °C	1 min
	72 °C	30s
Melt curve	95 °C	15s
	60 °C	1 min
	95 °C	15s

Table 2.3 Primers used for qRT-PCR.

Primer Name		Primer Sequence – 5' to 3'
CCNA2	Forward	TTATTGCTGGAGCTGCCTTT
	Reverse	CTCTGGTGGGTTGAGGAGAG
CCNB1	Forward	CACTTCCTTCGGAGAGCATC
	Reverse	CAGGTGCTGCATAACTGGAA
CCND1	Forward	TGGAGGTCTGCGAGGAACAGAA
	Reverse	TGCAGGCGGCTCTTTTCA
CCNE	Forward	TGTTGGCCACCTGTATTATCTGG
	Reverse	GTC ATG AAC ATA TCT GCT CTC C
Rb	Forward	CTTGCATGGCTCTCAGATTAC
	Reverse	AGAGGACAAGCAGATTCAAGGTG
p21	Forward	CTGCCCAAGCTCTACCTTCC
	Reverse	ACAGGTCCACATGGTCTTCC
IL-1α	Forward	AGATGCCTGAGATACCCAAAACC
	Reverse	CCAAGCACACCCAGTAGTCT
IL1β	Forward	TTCGACACATGGGATAACGAGG
	Reverse	TTTTTGCTGTGAGTCCCGGAG
IL-8	Forward	GGGCCAAGAGAATATCCGAAC
	Reverse	TGGATCCTGGCTAGCAGACTA
ICAM1	Forward	CAGGGAATATGCCCAAGCTA
	Reverse	GAACCATGATTGCACCACTG
LC3B	Forward	ACCATGCCGTCGGAGAAG
	Reverse	ATCGTTCTATTATCACCGGGATTTT
E-Cadherin	Forward	ATTTTCCCTCGACACCCGAT
	Reverse	TCCCAGGCGTAGACCAAGA
ERα	Forward	GGAGGGCAGGGGTGAA
	Reverse	GGCCAGGCTGTTCTTCTTAG
36B4	Forward	GATTGGCTACCCAACTGTTGCA
	Reverse	CAGGGGCAGCAGCCACAAAGGC

2.5 Biochemistry

2.5.1 Protein Lysate collection

Cells were lysed with cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM PMSF, 100 mM sodium fluoride, 0.2 M sodium molybdate and 1 mM sodium vanadate) and by scraping. Proteins in the supernatant were collected after centrifugation (13800 rpm, 4 °C for 12 minutes). Protein concentration was determined by PierceTM BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

2.5.2 Western Blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was used for the separation of protein samples based on their mass under denaturing conditions. For preparation for SDS-PAGE gels were composed of two layers. Different percentage of resolving gel 8%, 10%, 12% or 15% were used to separate proteins based on molecular weight (Table 2.4). Stacking gels of 4% were used constantly for Tris-Glycine SDS-PAGE analysis.

Table 2.4 Preparation for two gels for 1.5mm spacer of resolving and stacking Gels for Tris-Glycine SDS-PAGE analysis

Resolving Gel						Stacking Gel		
Final [conc]	Solutions	15%	12%	10%	8%	Final [conc]	Solutions	4%
	ddH ₂ O	1.627 ml	3.127 ml	4.177 ml	5.137 ml		ddH ₂ O	4.33 ml
0.375 M	1 M Tris pH 8.5	5.62 ml	5.625 ml	5.625 ml	5.625 ml	0.125 M	1 M Tris pH 6.8	750 ml
	30% Acrylamide	7.5 ml	6.0 ml	4.95 ml	3.99 ml		30% Acrylamide	800 ml
0.10%	10% SDS	150 ml	150 ml	150 ml	150 ml	0.10 %	10% SDS	60 ml
	10% APS	75 ml	75 ml	75 ml	75 ml		10% APS	40 ml
Fresh	TEMED	22.5 ml	22.5 ml	22.5 ml	22.5 ml	Fresh	TEMED	20 ml

Protein samples from the cells were boiled for 8 minutes before running SDS-PAGE. Depending on the gel percentage SDS-PAGE was performed by using constant voltage of 100 V or 90 V and then 120 V for 15% gels for 1.5-2.5 hours. Transfer of protein samples to a nitrocellulose

membrane or polyvinylidene difluoride (PVDF) membrane was done at constant voltage of 100 V for 2-2.5 hours. PDVF membrane was activated in methanol for 5 minutes prior usage. Membranes were blocked with 5% skimmed milk for 30 minutes or 2.5 BSA for 1 hour before overnight incubation with primary antibodies (Table 2.5).

Table 2.5 List of antibodies used for immunoblotting.

Antibody Name	Host	Dilution	Source
H190 Total PR	Rabbit	1:1000	Santa Cruz Biotechnology Inc., CA, USA
ERα	Mouse	1:1000	Santa Cruz Biotechnology Inc., CA, USA
Akt -phospho-S473	Rabbit	1:1000	Cell Signaling Technology
Pan Akt	Rabbit	1:000	Cell Signaling Technology
β-actin	Mouse	1:1000	Santa Cruz Biotechnology Inc., CA, USA
GAPDH	Mouse	1:20000	Ambion, USA
LC3B	Rabbit	1:1000	Cell Signaling Technology
Cyclin A2	Rabbit	1:1000	Cell Signaling Technology
Cyclin B1	Rabbit	1:1000	Cell Signaling Technology
Cyclin E1	Rabbit	1:1000	Cell Signaling Technology
Cyclin D1	Rabbit	1:1000	Cell Signaling Technology
pan IκBa	Rabbit	1:1000	Cell Signaling Technology
IκBa- phospho	Rabbit	1:1000	Cell Signaling Technology
P-65	Rabbit	1:1000	Cell Signaling Technology
p-65 phospho	Rabbit	1:1000	Cell Signaling Technology
E-cadherin	Mouse	1:1000	Cell Signaling Technology
β-tubulin	Mouse	1:1000	Cell Signaling Technology

The secondary antibodies, anti-mouse (1:1000) and anti-rabbit (1:2000) (GE Healthcare, UK), conjugated with HRP were used according to the class of the primary antibodies. Membranes were washed after incubation with primary and secondary antibodies 3x10 minutes with TBST buffer-Tris-Buffered Saline with Tween 20 (50 mM Tris, 5 M NaCl, 0.05% Tween 20). The proteins of interest were detected by using Millipore (Merck KGaA, Darmstadt, Germany) and by detecting chemiluminescence signal by exposure of the membranes to X-ray film.

2.5.3 Immunostaining

Immunofluorescence staining of cells is the method used to detect certain protein in the sample. We used this technique to detect specific proteins in MCF-7 stable clones with or without hormone treatment. This procedure involves several steps. In each 35 mm dish, we put coverslips

that were sterilized prior to experiment with 100% ethanol. Cells were seeded at density of 8×10^4 per well in 5% DCC-FCS growth medium, and starved for 48 hours prior to hormone treatment. Cells are treated with hormones as described above for 96 hours before proceeding with the method. Cells were fixed with 3.7% formaldehyde for 10 minutes. We permeabilized the cells with 0.2% Triton-X-100 for 10 minutes at room temperature, and then blocked them with 2% FBS for 2 hours. Primary antibody (anti-E-cadherin) was diluted with 2% FBS appropriately and added on parafilm when the coverslip was transferred onto the antibody with the cells facing the antibody. Coverslips were incubated in a humidified container at 4 °C overnight. The next day, secondary antibody was added in dark, and incubated at 37 °C for 2 hours. Coverslips were mounted on slides and dried overnight.

Antibodies diluted in FBS and used for immunostaining are:

- Phalloidin-FITC-conjugate 1:20;
- E-cadherin 1:200;
- DAPI 1:2000;
- Anti-mouse- DyLight594 1:250;
- Anti-rabbit 1:200.

2.6 Statistical analysis

Data were analyzed for significant differences using unpaired Student t-test ($p < 0.05$ was considered statistically significant), one-way or two-way ANOVA tests with 95% confidence interval using the program GraphPad Prism 6.

***Chapter 3. PRB overexpression in MCF-7
cells induces cell cycle arrest increased
senescence and autophagy in response to
progestin***

3 Results

3.1.1 Progestin affects cell growth in PR+ luminal breast cancer cells

As reviewed earlier, progestin has been reported to affect the proliferation of luminal breast cancer cells differently in different studies (Skildum, Faivre et al. 2005, Carnevale, Proietti et al. 2007, Boonyaratanakornkit, Bi et al. 2008, Daniel, Hagan et al. 2011). To understand how progestin could affect the growth of different luminal breast cancer cells under our laboratory conditions, we tested the effect of progestin R5020 on the growth of MCF-7 and T47D cells. R5020 is a synthetic progesterone agonist with little cross activity with other steroid receptors (Horwitz, Costlow et al. 1975). Cells were treated with 10 nM R5020 for 96 hours. We observed that R5020 did not have an impact on the growth of the MCF-7 cells (Figure 3.1a). On the other hand, the PR-positive (PR+) T47D cells responded to R5020 treatment with increased cell proliferation (Figure 3.1c), which is in accordance with previous studies (Hissom and Moore 1987, Richer, Lange et al. 1998, Proietti, Salatino et al. 2005, Skildum, Faivre et al. 2005, Faivre and Lange 2007), but different from other studies that demonstrated inhibitory effect of MPA and R5020 in T47D cells with extended treatment duration (Sartorius, Groshong et al. 1994, Groshong, Owen et al. 1997, Thuneke, Schulte et al. 2000). In contrast to our observation and those of others (Hissom and Moore 1987, Richer, Lange et al. 1998, Faivre and Lange 2007) several studies reported that R5020 treatment inhibited cell growth in T47D cells (Gill, Tilley et al. 1991, Dinda, Kodali-Gali et al. 1997, Hilton, Kalyuga et al. 2010), and that estradiol treatment was not able to induce cell growth in the presence of R5020 (Gill, Tilley et al. 1991).

It has been reported that R5020 could alter the function of ER α in luminal breast cancer cells (D'Santos, Taylor et al. 2015, Mohammed, Russell et al. 2015). Therefore, it is plausible that the observed growth (Figure 3.1a, Figure 3.1c) could be due to alterations in the hormone receptors' expression status induced by the long-term exposure of R5020 in these cells. Western blot analysis revealed that the PR expression in T47D was down-regulated in the presence of R5020 (Figure 3.1d). The lack of PR expression observed in the MCF-7 cells in the absence or presence of R5020 is in agreement with reports demonstrating the requirement of estrogen to induce PR expression in MCF-7 cells (Figure 3.1b) (Levenson and Jordan, 1997; Huan et al., 2014; Cho, Aronica et al., 1994). On the other hand, R5020 treatment did not affect the protein expression of

ER α in both cells lines (Figure 3.1b, Figure 3.1d). This suggests that the ER α expression might not partake in R5020-induced alterations and that the observed growth effects by R5020 could be solely mediated by PR.

Figure 3.1 The effect of progestin R5020 treatment on cell growth of luminal breast cancer cells.

Cells were starved in 5% dextran-coated charcoal treated FCS (DCC-FCS) for 48 hours before treatment with either 10 nM of progestin R5020 or vehicle control (0.01% ethanol) for 96 hours. (a) Cell growth in parental MCF-7 cell line in the presence of R5020. The data represents the mean value of triplicates $\pm$ SEM. *P<0.05, **P<0.01 compared to control treatment. (b) The expression of PR and ER α in parental MCF-7 cell line, after treatment with R5020. (c) Cell numbers of T47D cell line were determined by counting in a hemocytometer. The data represents mean value $\pm$ SEM of three independent experiments. (d) PRB and ER α protein levels were determined by Western blotting analysis. Membranes were probed with anti-PR (H-190), anti-ER α antibody and GAPDH as loading control.

3.1.2 R5020 inhibits cell growth in MCF-7 cells overexpressing PRB

MCF-7 cells are the most commonly used model for the study of hormone-dependent breast cancer. Since PR expression in MCF-7 cells requires the stimulation by estrogen (Cho, Aronica et al. 1994, Levenson and Jordan 1997, Huan, Wang et al. 2014), the levels of PR are normally very low in the absence of estrogen. This could account for the lack of significant effect of R5020 treatment. To understand the effect of PRB in response to progestin without the pretreatment with estrogen, which could have a confounding effect on the detection of progestin, we established stable overexpression of PRB in MCF-7 cells. Control cells were transfected with control vector (pcDNA3.1). The receptor's protein expression levels in the stably transfected cells were analyzed via immunoblotting analysis (Figure 3.2). Three clones of matching receptor expression levels were chosen for subsequent experiments. The PRB transfected cells are designated as MCF-7-PRB.

To elucidate the effect of progestin on cell growth in PRB stably transfected cells MCF-7-PRB, we measured the growth response of the individual clones of the vector-transfected and MCF-7-PRB cells following 96h R5020 treatment. We observed that progestin had significantly inhibited the growth by 2-3 folds in all three MCF-7-PRB clones while there was no significant effect in the vector-transfected cells (Figure 3.2b). Treatment with R5020 down-regulated the level of protein as a result of ubiquitination and proteasomal degradation of PR protein. It was observed that PRB exhibited an upshift in the gel in the MCF-7-PRB cells treated with R5020 (Figure 3.2c). This is consistent with the understanding that R5020 alters the gel mobility of PRB by PRB phosphorylation (Qiu and Lange 2003). Western blot analysis revealed that ER α levels were down-regulated in MCF-7-PRB cells in the presence of R5020 treatment. On the other hand, R5020 did not affect ER α levels in the vector-transfected MCF-7 cells (Figure 3.2c), which is consistent with the observation in parental MCF-7 cells (Figure b). Our finding is consistent with a previously reported decrease of ER α levels due to PRB overexpression in breast cancer cells (De Amicis, Zupo et al. 2009).

Figure 3.2 R5020 inhibits cell growth in MCF-7-PRB.

(a) PR expression in PRB stably transfected MCF-7 cells. Cell lysates of the three chosen clones collected at 24 hours after plating were subjected to Western blotting and probed with anti-PR antibody. GAPDH was used as a loading control. (b) Cells were seeded in medium containing 5% DCC-FSC medium for 48 hours before treatment with 10 nM R5020 in 0.01% ethanol for 96 hours. Cell growth of individual clones of stably transfected MCF-7 (pcDNA and PRB) in response to R5020. Cell numbers were quantified and the data is presented as absolute cell numbers, mean $\pm$ SEM, triplicates Asterisks indicated significance * P <0.05, ** P <0.01, *** P <0.001, **** P <0.001 compared to respective controls. (c) PR and ER α protein expression of the stably-transfected MCF-7 cells in the presence of R5020. Cell lysates of the three chosen clones were collected after treatment with 10 nM R5020 for 96 hours. The expression profiles were analysed by Western blotting and probed with anti-PR, anti-ER α and anti-GAPDH as a loading control.

3.1.3 R5020 affects the cells cycle of PRB stably transfected MCF-7 cells

Cell cycle dynamics is crucial for the coordination of growth and development of the cells in normal physiological condition, but also in cancer and other pathological conditions. The dynamic cell cycle progression is determined by four successive phases of cell cycle and circular pathway G1-S-G2-M-G1 where G1 and G2/M phases are crucial checkpoints that separate DNA replication and mitosis. Cell cycle checkpoints are internal control mechanisms that monitor the DNA replication and integrity of the chromosomes and providing cells surveillance mechanisms to ensure correct cell cycle phases progression (Tyson and Novak 2001, Tyson and Novak 2008, Gérard and Goldbeter 2014). In cancers these checkpoint control mechanisms are usually defective, and cells are arrested in G1 or G2/M phase of cell cycle (Evan and Vousden 2001, Kastan and Bartek 2004). Our data showed that R5020 treatment inhibited the growth of MCF-7 cells stably transfected with PRB, compared to the control vector (Figure 3.2b). It had been reported that the R5020 altered the cell cycle progression of PR-positive breast cancer cells by affecting them at different stages of the cell cycle (Musgrove, Lee et al. 1991, Groshong, Owen et al. 1997, Lin, Ng et al. 1999, Alkhalaf, El-Mowafy et al. 2002). Therefore, we postulate that the overexpression of PRB in the MCF-7 cells could have altered the cell cycle progression of the cells in response to R5020. Flow cytometry analysis was conducted to analyze how PRB overexpression would affect the cell cycle distribution upon R5020 treatment. To ensure that the growth inhibition by R5020 (Figure 3.2b) was not due to clonal differences in their cell cycle distribution, we analyzed the cell cycle distribution of all three clones of the pcDNA and PRB stably-transfected cells on the day of the plating (Figure 3.3a). Our data implied that there were no differences between the individual clones of the MCF-7-PRB cells and the clones of the MCF-7-pcDNA cells. Also, our data showed that there were no differences in PRB vs MCF-7-pcDNA cells in the ethanol treated groups. Previous studies have demonstrated that progesterone/progestins drove the transition of breast cancer cells through the first cell cycle. On the other hand, prolonged exposure attenuated their progression into the next cycle (Clarke & Sutherland, 1990, Groshong, Owen et al., 1997, Lamb et al., 2000, Lange, Richer et al., 1998). Therefore, we analyzed the cell cycle distribution of the clones 24 hours and 96 hours after R5020 treatment. All three phases of the cell cycle (G1, S and G2/M) of the MCF-

7-pcDNA clones were unaffected by presence of R5020 at both 24 and 96 hours after R5020 treatment (Figure 3.3b and c).

On the other hand, the overexpression of PRB was found to increase the percentage of MCF-7-PRB clones in the S-phase of the cell cycle 24 hours after treatment with R5020 (35%) as compared to the vehicle (ethanol)-treated cells (25-27%)(Figure 3.3b (ii)). R5020 treatment for 96 hours altered the cell cycle distribution of the MCF-7-PRB clones (Figure 3.3b (ii), Figure 3.3b (iii)). There was a decrease in the proportion of MCF-7-PRB cells in the S phase, from ~28% in the control vs <20% in the R5020-treated samples (Figure 3.3c (ii)). There was also a significant increase of MCF-7-PRB cells in the G2/M phase of the cell cycle upon R5020 stimulation (Figure 3.3c (iii)). Our findings suggest that prolonged treatment with R5020 promoted the cell cycle arrest of MCF-7-PRB cells in the G2/M phase. This arrest in the G2/M phase could explain the growth inhibition induced by R5020.

Figure 3.3 R5020 reduced the percentage of MCF-7-PRB cells in S phase of cell cycle 96 hours after the treatment

pcDNA and PRB stably transfected MCF-7 clones were starved in 5% DCC-FCS medium 48 hours prior the treatment with 10 nM R5020 in 0.01% ethanol for 24 and 96 hours. (a) The cell cycle distribution of the individual clones of the MCF-7 stably transfected with pcDNA vector and PRB on the day of cell seeding. The data is represented as percentages of cells in the G1 (i), S (ii) and G2/M (iii) phase of the cell cycle, mean $\pm$ SEM, triplicates. (b) The cell cycle distribution of the individual clones of the MCF-7-pcDNA and MCF-7-PRB cells in response to 24 hours treatment. The data is represented as the percentage of the cells in the G1 (i), S (ii) and G2/M (iii) phase of the cell cycle, mean $\pm$ SEM, triplicates. (c) The cell cycle distribution of the individual clones of the MCF-7-pcDNA and MCF-7-PRB MCF-7 cells in response to 96 hours treatment. The data is represented as the percentage of the cells in the G1 (i), S (ii) and G2/M (iii) phase of the cell cycle, mean $\pm$ SEM, triplicates. Asterisks indicate the statistical significance between R5020 against control (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001).

3.1.4 R5020 treated MCF-7-PRB cells show very little BrdU incorporation

To further analyze the effect of R5020 on DNA synthesis, we used a brominated analog of thymidine, 5-Bromo-2'-deoxyuridine (BrdU) incorporation assay (Beisker and Hittelman 1988, Campisi and d'Adda di Fagagna 2007), to examine whether R5020 inhibits DNA synthesis in MCF-7-PRB cells. MCF-7-PRB 2 were starved in DCC-FCS medium 48 hours before treatment with 10 nM R5020, and 96 hours after treatment cells were pulse labeled with 20 μ M BrdU for 30 minutes. Cells were then collected immediately (0 hours) or after 16 hours.

At 0 hour, only 3% of R5020-treated cells were labelled with BrdU, in contrast to 23% of control cells (Figure 3.4). 16h later, the number of BrdU-labeled control cells was increased to ~30% from 23%, which was an indication of cell division. But BrdU-labeling in R5020-treated cells remained similar to that of at time 0, indicating a complete lack of cell division (Figure 3.4b). Therefore, it appears that R5020 treatment for 96h in MCF-7-PRB cells almost completely abolished cell proliferation.

Figure 3.4 Flow cytometry analysis of PRB2 pulse labeled with BrdU at 0 and 16 hours time points.

MCF-7-PRB2 cells were grown in 5% DCC-FCS for 48 hours before they were treated with either 0.01% ethanol or 10 nM R5020 for 96 hours. The cells were then pulse labeled for 30 minutes with 20 μ M BrdU. At the designated time, the cells were collected, stained with FITC-labeled anti-BrdU antibody and PI. Cells were analyzed by flow cytometer. (a) Representative dot plot of 0h and 16h BrdU incorporation. (b) Quantitative analysis of BrdU incorporation in MCF-7-PRB2 cells. The data is represented as the percentage of the cells in S phase of the cell cycle, mean $\pm$ SEM, triplicates. Asterisks indicate the statistical significance between R5020 against control (** $P < 0.01$, **** $P < 0.0001$).

3.1.5 R5020 treatment induced irreversible cell growth inhibition in MCF-7-PRB cells

We have demonstrated that R5020 treatment for 96 hours almost completely inhibited cell growth in MCF-7-PRB cells (Figure 3.2). To elucidate if the observed cell growth inhibition is irreversible, we examined cell proliferation after withdrawal of R5020. After R5020 treatment for 96 hours, numbers of MCF-7-PRB cells treated with R5020 were ~70% and 40% of the vehicle-treated controls (Figure 3.5a). In the second set of samples, R5020 was removed and cells were cultured for additional 96 hours. Fresh medium was replaced every 48 hours after R5020 withdrawal to maintain cell proliferation (Figure 3.5). Despite removal of the medium containing R5020, the hormone-treated cells remained growth inhibited as compared to the controls. In contrast, the vehicle-treated cells tripled the number after an additional 96 hours of cultivation. This data suggests that R5020 induced irreversible cell growth inhibition.

Figure 3.5 R5020 induces irreversible cell growth inhibition in MCF-7-PRB cells

MCF-7-PRB2 and MCF-7-PRB3 cells were starved in 5% DCC-FCS for 48 hours before treatment with either 10 nM R5020 or 0.01% ethanol for 96 hours. (a) Cells were counted 96 hours after R5020 treatment. (b) 96 hours after treatment, R5020 was withdrawn and replaced with fresh medium which was changed every 48 hours for an additional 96 hours. Cell number was determined by counting on a hemocytometer. The data represents the mean value of triplicates $\pm$ SEM. Statistical significance was calculated by student t-test and asterisks indicated significance between R5020 vs Ctrl (* P < 0.05, ** P < 0.01, **** P < 0.0001).

3.1.6 PRA was not able to transrepress PRB-mediated growth inhibition in response to R5020 in the MCF-7-PRB cells

PR gene has two isoforms, PRB and PRA that are transcribed from the same gene, but different promoters (Kastner, Krust et al. 1990). The different PR isoforms have been reported to exhibit distinct ligand-dependent gene expression response in their target tissues and cells (Mulac-Jericevic, Mullinax et al. 2000, Conneely, Mulac-Jericevic et al. 2002). It has been shown that the combined transfection of PRA and PRB had diminished the progesterone response in choriocarcinoma (BeWo) cell line compared with the cells transfected only with PRB (Cho, Ryu et al. 2004, Wang, Lee et al. 2008). In addition, PRA was reported to exert an inhibitory effect on the function of PRB (Vegeto, Shahbaz et al. 1993, Giangrande and McDonnell 1999). Therefore, we hypothesized that the presence of PRA could inhibit the R5020-induced responses in MCF-7-PRB cells. To investigate this, we introduced PRA into MCF-7-pcDNA3 and MCF-7-PRB2 cells using adenoviral transfection with adenoviral vector (Ad) containing the PRA cDNA (Ad-PRA).

The levels of PRA protein in the transduced cells were analyzed via Western blotting (Figure 3.6a). Unexpectedly, R5020 treatment stimulated the expression of PRA in the adenoviral-transduced MCF-7-pcDNA3 cells. Akin to the Ad-PRA-transduced MCF-7-pcDNA3 cells, R5020 increased PRA expression in the Ad-PRA transduced MCF-7-PRB2 cells.

Notably, the presence of PRA in the MCF-7-pcDNA3 cells (Figure 3.6a) did not have an impact on the growth of the cells exposed to R5020. Intriguingly, the growth inhibitory effect of R5020 remained the same in the Ad-PRA transduced MCF-7-PRB2 cells (Figure 3.6b). Thus, the presence of PRA did not affect the growth response of the PRB-transfected MCF-7 cells, regardless of its initial PR status. Consistent with the reduced cell growth analysis, R5020 did not have an impact on the proportion of cells in the G1 phase of the stably-transfected MCF-7 cells, regardless of their PRB and/or PRA expression status. The percentage of cells in the S phase and G2/M phase of the MCF-7-PRB cells remained unchanged in the Ad-PRA transduced cells as compared to the Ad-ctrl (Figure 3.6c (ii) and (iii)), in the presence and absence of ligand treatment.

Figure 3.6. PRA does not inhibit PRB-mediated growth inhibition in response to R5020.

MCF-7-pcDNA3 and MCF-7-PRB2 were plated in 5% DCC-FCS medium. The cells were transduced with Ad-EV and Ad-PRA vectors at MOI1 for 24 hours prior to treatment with 10 nM R5020 in 0.01% ethanol for 96 hours. (a) Protein expression of total PRB and ERα in MCF-7 stably transfected clones MCF-7-pcDNA3 and MCF-7-PRB2 upon transduction in response to R5020. The protein expression was analyzed by Western blotting and probed with anti-PR, anti-ERα and anti-GAPDH as a loading control (c) The cell cycle distribution of the Ad-EV/PRA transduced MCF-7-pcDNA3 and MCF-7-PRB2 in response to R5020 treatment. The data is represented as the percentage of the cells in the G1 (i), S (ii) and G2/M (iii) phase of the cell cycle, mean ± SEM, triplicates. Asterisks indicate the statistical significance between R5020 against control (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

3.1.7 Overexpression of ER α in MCF-7-PRB MCF-7 cells did not reverse R5020-mediated growth inhibition.

The down-regulation of ER α by R5020 was reported to be mediated through the action of PRB at the transcription level (De Amicis, Zupo et al. 2009). However, the magnitude of the down-regulation in our study was much greater, possibly due to the overexpression of PRB. Since ER α -mediated estrogen activity is essential for MCF-7 growth, we wondered if R5020-induced growth inhibition was due to the loss of ER α expression in MCF-7-PRB cells. To determine the role of ER α in modulating R5020-induced cell growth inhibition, we overexpressed ER α in one of the MCF-7-PRB clones, PRB clone 2 (PRB2), using adenoviral transduction. Upon transduction with adenoviral vector containing ER α cDNA (Ad-ER α) for 16 hours, the growth of the cells was determined 96 hours after treatment with R5020 and compared against the adenoviral control vector (ctrl). The transduction of ER α into PRB2 cells did not alleviate the growth inhibitory effect of R5020, despite promoting the growth of MCF-7-PRB2 cells in the absence of R5020 (Figure 3.7a). The transduction of the Ad-ER α induced a robust expression of ER α in the MCF-7-PRB2 cells and this robust expression was maintained in the presence of R5020 (Figure 3.7b). This suggests that the growth inhibition by R5020 was not due to diminished expression of ER α .

Figure 3.7 The expression of ERα in MCF-7-PRB cells did not alleviate R5020-induced growth inhibition.

Cells were plated in DCC-FCS medium for 24 hours and then were transduced with direct adding of adenovirus control vector Ad-Control vector or Ad-ERα of MOI5 to the wells. 24 hours after transduction cells were treated with 10 nM R5020 for 96 hours. (a) Cell growth of ERα overexpression in MCF-7-PRB2 in response to R5020. MCF-7-PRB2 was transduced with Ad-ctrl or Ad-ERα of MOI5 for 16 hours prior to treatment with 10 nM R5020 in 0.01% ethanol for 96 hours. The number of cells was determined with a hemocytometer. The data represents the mean value of triplicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, compared to their respective control. (b) PR and ERα expression in Ad-ctrl and Ad-ERα, MCF-7-PRB2 in the presence of R5020. Cell lysates of the transduced cells were collected after treatment with 10 nM R5020 for 96 hours. The expression profiles were analysed by western blotting and probed with anti-PR, anti-ERα and anti-GAPDH as a loading control.

3.1.8 R5020 completely abolished estrogen-induced cell growth in MCF-7-PRB cells

It has been suggested that the cross-talk between PR and ERα could play a significant role in breast cancer cell response to steroid hormones (De Amicis, Zupo et al. 2009, Daniel, Gaviglio et al. 2015, Hegde, Kumar et al. 2016). Estrogen (E2) treatment of MCF-7 cancer cells is able to alter the expression of diverse genes and to regulate proliferation and phenotype modification of breast cancer cells (Huan, Wang et al. 2014). Furthermore, the overexpression of PRB was able to alter the expression of ERα target genes in MCF-7 cells in the absence of progesterone/progestins (Daniel, Gaviglio et al. 2015). It was also reported that R5020 marginally inhibited estrogen-induced growth in xenograft model. This effect was reported to be

due to the PR-mediated shifting of ER α binding to chromatin, but the study did not present the effect of R5020 on MCF-7 cells in cell culture (Mohammed, Russell et al. 2015). Therefore, we wondered if E2 stimulation would override the inhibitory effect of R5020 or vice versa in the MCF-7-PRB cells. To determine this, we treated MCF-7-PRB cells with E2 in the presence or absence of R5020. In this experiment, we used one of the three MCF-7-pcDNA clones and two of the MCF-7-PRB clones. Since all three MCF-7-pcDNA clones exhibited similar characteristics as parental MCF-7 cells in their response to R5020 (Figure 3.1a and 3.2b), one clone (MCF-7-pcDNA3) was randomly chosen for this study. On the other hand, MCF-7-PRB2 and MCF-7-PRB3 cell were selected based on their similar levels of expression of PRB. The cells were treated with 10 nM R5020 and/or 10 nM E2 for 96h and their cell numbers quantified (Figure 3.8a). As expected, E2 significantly increased the cell number of vector-transfected MCF-7 cells in the presence or absence of R5020, and R5020 alone had no effect on the growth of these cells (Figure 3.8a). Also, E2 significantly increased the cell number in MCF-7-PRB cells in the absence of R5020. However, R5020 completely abolished E2-induced cell growth in both clones (Figure 3.8a). This suggested that at high levels PRB is able to override the effect of estrogen completely. Western analysis was conducted to elucidate how E2 and R5020 treatment could affect the expression status of PRB and ER α in MCF-7-PRB cells (Figure 3.8b). PR was not detectable in MCF-7-pcDNA cells after E2 treatment. PRB level was decreased in MCF-7-PRB cells treated with R5020 with and without E2, but E2 treatment alone did not show effect on PRB protein level in vector-transfected controls cells and MCF-7-PRB cells. This is due to the assay sensitivity because E2 is known to induce PR expression. Importantly, ER α protein level in MCF-7 -PRB cells was drastically decreased 96 hours after R5020, E2 and R5020/E2 treatment. It is interesting to point out that E2 treatment downregulated ER α level to a lesser extent than R5020 treatment in MCF-7-PRB cells.

Figure 3.8 R5020 abolishes estrogen-induced growth in MCF-7-PRB cells.

Cells were plated in 5% DCC-FCS medium 48 hours before treatment with 0.01% ethanol control, 10 nM R5020 and/or 10 nM E2 for 96 hours before collection. (a) The effect of R5020 and/or E2 on cell growth of the MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3. The cell number was determined with a hemocytometer. The data represents the mean value of triplicates $\pm$ SEM. Asterisks indicate the statistical significance between R5020 against control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). (b) Protein expression of MCF-7 cells stably expressing vector control MCF-7-pcDNA 3 and MCF-7-PRB (clones 2 and 3). The lysates were analyzed by Western blotting and probed with anti-PR (H-190), anti-ER and anti-GAPDH as a loading control.

3.1.9 R5020 inhibits the survival and acini formation of MCF-7-PRB cells in 3 dimensional cell culture

The monolayer or two-dimensional (2D) culture has been widely used to study the biological mechanisms that are crucial for diverse cellular function including cell growth and differentiation. *In vitro* three-dimensional (3D) cell culture is the model used for simulating the *in vivo* biology organization and function of the cells in tissues in microenvironments that imitate conditions of living organs. Interaction between cells and extracellular matrix (ECM) is crucial for tissue function (Baker and Chen 2012). It has been reported that in 3D culture mammary epithelial cells are able to form organotypic acinar structures and to rebuilt basement membrane

(Petersen, Rønnov-Jessen et al. 1992). Normal animal cells are anchorage-dependent cells, and they can grow in monolayer in cell culture. The moment they thoroughly cover monolayer, they stop dividing due to the contact inhibition (Borowicz, Van Scoyk et al. 2014). On the other hand, aggressive cancer cells do not exhibit contact inhibition, and they continue growing on the top of monolayer forming clumps or foci (Mori, Chang et al. 2009). To further examine the inhibition of cell growth that we observed previously in monolayer (2D) culture, we investigated the effects of progestin R5020 on anchorage-independent growth in MCF-7-PRB cells. Cells were plated and pre-treated with R5020 for 72 hours. Then we re-plated cells in 2% or 50% Matrigel according to the manufacturer's protocol. The medium was changed on the days 3, 7 and 10 after re-plating.

In 2% Matrigel culture, vector-transfected control cells thrived very well with or without R5020 treatment. Vehicle-treated MCF-7-PRB cells also grew very well without R5020 treatment. MCF-7-PRB cells treated with R5020 for 72 hours appear to have survived initially after plating but gradually died (Figure 3.9a). The form of cell death under this condition is currently under investigation. After 2 weeks in culture, only a few clusters of cells were growing in R5020-treated cells and this accounted to less than 5% of the untreated controls. Similarly, vector-transfected cells formed typical acini under acini culture conditions. Vehicle-treated MCF-7-PRB cells also formed acini similarly to vector controls in acini formation assay. However, treatment of MCF-7-PRB cells with 10 nM R5020 abolished this ability of forming acini (Figure 3.9b).

Figure 3.9 R5020 inhibits anchorage-independent cell growth and completely abolishes formation of acini in Matrigel in MCF-7-PRB cells.

MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were pre-treated with 10 nM R5020 for 72 hours. The cells were then re-seeded in 2% Matrigel in culture medium treated with 10 nM R5020 in 0.01% ethanol. The cells were cultured in their respective culture treatment mix for 15 days and medium was replaced on days 3, 7 and 10. (a) R5020 effect on MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 MCF-7 cell growth in 2% Matrigel. The cells were then stained with 0.1% crystal violet. (b) R5020 effect on acini formation in MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3. MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were seeded onto Matrigel-coated chamber slide in 2% Matrigel. Cell numbers of the formed acini were determined by counting on a hemocytometer. Absolute number of live cells was quantified via cell counting using trypan-blue exclusion staining. Representative columns are means from three independent experiments $\pm$ SEM. (c) The formed acini structures were imaged on day 15 using inverted microscope Olympus IX_71.

It remains to be investigated why R5020-treated MCF-7-PRB cells in 2D culture appeared to be alive despite of growth inhibition, but died in 3D culture despite of receiving fresh medium every 3rd day.

We showed earlier that the R5020 treatment in MCF-7-PRB cells abolished estrogen-induced cell proliferation. We also investigated if R5020 similarly abolishes cell growth in 3D culture. 3D images were taken on the days 3, 8, 10 and 14 after plating. MCF-7-pcDNA vector control cells were able to grow well in 2% Matrigel and form acini in all treatments, and E2 treatment gave noticeable organoid structures. MCF-7-PRB3 cells were unable to form acini after treatment with R5020 with and without E2 treatment. Acini are organoid structures- sphere-shaped monolayers of epithelial cells that surround a central lumen (O'Brien, Zegers et al. 2002). In vehicle or E2-treated culture, acini were visible after 3 days but proper acini were not obvious in culture with R5020 or R5020+ E2 treatment. Similar observation was made in the subsequent days (day 8, day 10 and day 14) when there were very few acini (Figure 3.10b).

a)

Figure 3.10 R5020 treated cells are unable to survive and to form mammosphere in Matrigel with or without E2 treatment.

MCF-7-pcDNA3 and MCF-7-PRB3 cells were pre-treated with 10 nM R5020 and/or 10 nM E2 for 72 hours in 0.02% ethanol. The cells were then re-seeded in 2% Matrigel in culture medium treated with 10 nM R5020 or 0.01% ethanol. The cells were cultured in their respective culture treatment mix for 15 days and medium was replaced on days 3, 7 and 10. (a) R5020 and E2 effect on MCF-7 stably transfected cell growth in 2% Matrigel. The cells were then stained with 0.1% crystal violet. (b) R5020 and E2 effect on acini formation in MCF-7-pcDNA3 and MCF-7-PRB3. The formed acini structures were imaged by using microscope Olympus IX_71.

3.1.10 R5020 treatment induced senescence-like characteristics in MCF-7-PRB cells

We showed previously that in MCF-7-PRB cells R5020 treatment induced cell growth inhibition in 2D and 3D culture (Figure 3.2, Figure 3.9), and irreversible cell cycle arrest in 2D culture (Figure 3.3). Therefore, we were interested in investigating if R5020-treated cells exhibit senescence-like characteristics in the MCF-7-PRB cells. Cellular senescence is the processes defined by a loss of replicative capacity in previously proliferating cells (Hayflick and Moorhead 1961, Faragher and Kipling 1998, Shay and Roninson 2004, Wiley and Campisi 2016). Cells undergoing senescence display several characteristic morphological and biochemical features. Senescence is characterized by numerous biomarkers including an irreversible cell cycle arrest, with increased expression of cell cycle inhibitors and increase of senescence-associated β -galactosidase (Sa β -gal) activity. Senescent cells undergo morphological modifications where cells exhibit flat and sizeable cellular morphology, increased nuclear size and alter cytoskeletal element organization (Faragher and Kipling 1998, Kuilman, Michaloglou et al. 2010). Terminal cell cycle arrest of senescent cells was shown to require functional p53 and cyclin dependent kinase inhibitors including p21^{waf1/cip1} and p16^{INK4a} (el-Deiry, Tokino et al. 1993, Afshari, Nichols et al. 1996, Jarrard, Sarkar et al. 1999, Stein, Drullinger et al. 1999, Crescenzi, Palumbo et al. 2008). Although senescent cells are permanently growth-arrested, they remain metabolically active (Faragher and Kipling 1998, Bird, Ostler et al. 2003).

Progesterone and PRB have been found to play a significant role in inducing senescence in the ovarian cancers (Takahashi, Kato et al. , Dai, Wolf et al. 2002). However, the studies of progesterone and its receptor PRB on senescence breast cancer are scarce. It has been established that senescent cells are undergoing drastic morphological changes including cell enlargement and flattened shape (Chen, Tu et al. 2000, Cho, Ryu et al. 2004). We have observed that MCF-7-pcDNA3 cells maintained their morphology 96 hours after R5020 treatment. On the other hand, MCF-7-PRB cells treated with R5020 were flattened with large surface area and enlarged nuclei. Although the cell growth in MCF-7-PRB cells was inhibited by R5020 treatment, we did not observe any morphological feature associated with apoptosis in 2D culture (Figure 3.11).

Figure 3.11 MCF-7-PRB cells exhibit senescence-like phenotype 96h after R5020 treatment.

pcDNA3, PRB2 and PRB3 MCF-7 cells were plated in 5% DCC-FCS medium 48 hours before treatment with 10 nM R5020 in 0.01% ethanol. Live cells images were taken at 96 hours post-treatment. The images were acquired by inverted microscope Olympus IX₇₁.

To further characterize changes in cell morphology that accompany senescence, we investigated the arrangement of F-actin in the cytoskeletal structure. It has been reported that morphological changes in senescent cells are followed by the amplified formation of actin stress fibers (Wang and Gundersen 1984, Chen, Tu et al. 2000, Cho, Ryu et al. 2004). Drastic morphological change in MCF-7-PRB cells after R5020 treatment suggests that cytoskeletal proteins such as filamentous actin (F-actin) might play a role in this process. Immunostaining of F-actin showed an increase of actin stress fibers crossing the entire long axis in the cytoplasm of R5020 treated MCF-7-PRB cells compared to non-treated MCF-7-PRB and MCF-7-pcDNA cells (Figure 3.12).

Increase in intracellular granularity have been reported in senescence and autophagy (Sherwood, Rush et al. 1988, Paglin, Hollister et al. 2001, Monastyrsky and Klionsky 2006). To examine the granularity of the cells, FACS analysis was performed. FACS analysis revealed that R5020-treated MCF-7-PRB cells exhibited increased granularity (SSC) compared to vehicle treated control cells (Figure 3.13).

Figure 3.12 R5020 increases formation of F-actin stress fibers in MCF-7-PRB cells.

MCF-7-pcDNA3 and MCF-7-PRB2 cells were plated in 5% DCC-FCS medium 48 hours before treatment with 10 nM R5020 or 0.01% ethanol for 96 hours. Cells were then stained with FITC conjugated phalloidin and DAPI for F-actin and nuclei, respectively. The images were acquired by inverted microscope and Olympus IX_71.

Figure 3.13 R5020 treatment increases cell granularity of MCF-7-PRB cells.

MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were seeded in 5% DCC-FCS medium 48 hours before treatment with either 10 nM R5020 or 0.01% ethanol for 96 hours. Cells were stained with propidium iodide. Cell cycle analysis was performed by flow cytometry. Representative dot plots of cell size (FSC) and granularity (SSC) of one pcDNA and two PRB clones.

3.1.11 R5020 treated MCF-7-PRB cells exhibit elevated senescence-associated β -galactosidase (SA- β -gal) activity

Senescent cells express senescence-associated β -galactosidase (SA- β -gal) activity that can be detected by using chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) (Kurz, Decary et al. 2000, Gary and Kindell 2005).

To assess cellular senescence of MCF-7-pcDNA and MCF-7-PRB cells, SA- β -gal activity was examined 96 hours after R5020 treatment. β -Galactosidase staining was positive in MCF-7-PRB clones treated with R5020, compared to the vehicle control-treated samples, as well as in vehicle- and R5020-treated MCF-7-pcDNA clones. In all samples we noticed that small percentage of cells was stained (Figure 3.14). Positive staining in some samples could be

explained by serum-depleted conditions (four days in the same medium), or the cell confluence. Cell growth conditions, such as low serum and high cell density, were suggested to give positive staining with X-gal (Dimri, Lee et al. 1995, Gary and Kindell 2005). Nonetheless, R5020-treated cells show obvious increase in the amount of SA- β -gal staining (Figure 3.14)

Figure 3.14 R5020 induces senescence-associated beta-galactosidase staining (SA- β -gal) in MCF-7-PRB cells.

MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were plated in 5% DCC-FCS medium 48 hours prior to hormone treatment with 10 nM R5020 in 0.01% ethanol for 96 hours. β -gal activity was detected using SA- β -Gal staining kit and visualized by inverted microscope Olympus IX_71.

3.1.12 R5020 altered the expression of cyclins and CDKs in MCF-7-PRB cells

Our data revealed that R5020 strongly inhibited the growth of MCF-7-PRB cells mediated by PRB. In addition, these cells were accumulated in the G2/M phase of the cell cycle 96h after the treatment with R5020 (Figure 3.3c). The progression of cells through the cell cycle is tightly regulated by several cyclins and cyclin-dependent kinases (CDK) (Cordon-Cardo 1995, Besson,

Dowdy et al. 2008, Foster, Yellen et al. 2010). Progestins have been reported to play a role in the regulation of the cell cycle progression, altering the recruitment and formation of cyclins-CDK complexes (Musgrove, Swarbrick et al. 1998, Sweeney, Swarbrick et al. 1998, Faivre, Skildum et al. 2005, Boonyaratanakornkit, McGowan et al. 2007). In order to determine the underlying mechanism of R5020-derived cell growth arrest, we examined cyclins and CDKs expression profile of the MCF-7-PRB cells after stimulation with R5020 for 24 hours (Figure 3.15). The mRNA levels of cyclin A2 (*CCNA2*), cyclin B1 (*CCNB1*), cyclin D1 (*CCND1*) and cyclin E (*CCNE*) were found to be significantly upregulated by R5020 in the MCF-7-PRB cells 24h after the treatment (Figure 3.15). This is surprising as the gene expression pattern of the cyclins in response to R5020 is not consistent with its growth inhibitory effect.

Figure 3.15 R5020 treatment exerts different effects on mRNA of *CCNA2*, *CCNB1*, *CCND1*, *CCNE*.

MCF-7 cells stably transfected with pcDNA control vector and PRB, were plated in 5% DCC-FCS medium for 48 hours before treatment with 10 nM R5020 in 0.01% ethanol for 24 hours. Gene expression profiles of MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 in response to R5020 for 24 hours. Relative mRNA levels of cyclin A2 (*CCNA2*) (i), cyclin B1 (*CCNB1*) (ii), cyclin D1 (*CCND1*) (iii), and Cyclin E (*CCNE*) (iv). The data is represented as mean $\pm$ SEM, triplicates, in 3 independent experiments. Asterisks indicate the statistical significance between R5020 treatment and the control (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

Our next step was to analyze the expression of cyclins and cyclin-dependent protein kinases at the protein level by Western blotting analysis. Cyclins A2, B1, D1 and E2 were visibly increased 24 hours after R5020 treatment, whereas CDK2 and CDK4 protein levels were not altered. These results are consistent with their mRNA changes and the cell cycle-promoting effect of R5020 at

24-hour time point. Consistently, 96 hours upon R5020 treatment, protein levels of cyclins were changed according to R5020 growth inhibitory effect, with the exception of cyclin E1. We saw consistent decreases in protein levels of cyclins A2, B1 and D1. We also noted consistent and marked decreases in CDK2 and CDK4 protein levels, which was regulated by the interplay of cyclins and CDK inhibitors (King, Deshaies et al. 1996, Cayrol and Ducommun 1998, Ekholm and Reed 2000, Truman, Kitazono et al. 2001, Amanatullah, Zafonte et al. 2002). Surprisingly, R5020 treatment caused a remarkable increase of cyclin E1. This appears to be in conflict with R5020 growth-inhibitory effect. However, one study revealed that cyclin E/CDK2 activation or cyclin E overexpression can inhibit DNA synthesis while allowing G1/S phase transition and this is cell-context-dependent (Sgambato, Doki et al. 1997).

Figure 3.16 R5020 treatment exerts different effects on protein levels of cyclins A2, B1, E1 and D1.

MCF-7- pcDNA3 control vector, MCF-7-PRB2 and MCF-7-PRB3 were plated in 5% DCC-FCS medium for 48 hours before treatment with 10 nM R5020 in 0.01% ethanol for 24 hours and 96 hours. Protein expression profile of MCF-7 stably transfected cells in response to 24 hours and 96 hours treatment with R5020. Cell lysates were analyzed via Western blotting and probed with anti-PR, anti-cyclin A2, anti-cyclin B1, anti-cyclin D1, anti-cyclin E1, anti-CDK2, anti-CDK4 and anti-GAPDH as a loading control.

3.1.13 R5020 activates p53-p21 pathways in MCF-7-PRB cells

Cellular senescence is a multipart process that involves several vital molecules. Participation of p53-p21 and p16-Rb pathways in senescence have been well studied (Shay, Pereira-Smith et al. 1991, Stein, Drullinger et al. 1999, Acosta, O'Loughlen et al. 2008). Tumor suppressor P53 activity modulates cell cycle arrest and senescence by regulating downstream targets involved in regulation of these processes (Qian and Chen 2013, Rufini, Tucci et al. 2013). CDK inhibitor p21, which is a p53 target, induces G1 arrest and leads to cell senescence (el-Deiry, Tokino et al. 1993, el-Deiry, Harper et al. 1994, Kester, Sonneveld et al. 2003).

P16 is not expressed in MCF-7 cell line (Craig, Kim et al. 1998, Karimi-Busheri, Rasouli-Nia et al. 2010). Therefore, to further evaluate the changes of the biomarkers that accompany senescence, qRT-PCR and Western blotting were carried out to analyze the gene and protein expressions of biomarkers *p21* and *Rb*.

From the results we obtained, the control vector MCF-7-pcDNA cells did not respond to R5020 treatment by changing *p21* or *Rb* genes 24 hours after treatment (Figure 3.17). On the other hand, we observed that at the gene expression level *p21* showed tendency to be upregulated in MCF-7-PRB3 clone treated with R5020, although MCF-7-PRB2 clone did not respond in the same manner. mRNA level of *Rb* was increased at this time point in MCF-7-PRB cells (Figure 3.17a (ii)). Thereafter, mRNA levels of *p21* and *Rb* were significantly increased in MCF-7-PRB clones in response to R5020 treatment for 96 hours, whereas there was no change in vector-transfected control cells (Figure 3.17b).

Figure 3.17 R5020 regulates *p21* and *Rb* on mRNA level in MCF-7-PRB.

MCF-7-pcDNA3 control vector, MCF-7-PRB2 and MCF-7-PRB3 were plated in 5% DCC-FCS medium 48 hours before treatment with 10 nM R5020 in 0.01% ethanol for 24 hours and 96 hours. (a) Relative gene expression of *p21* (i) and *Rb* (ii) gene 24 hours after treatment (b) Relative gene expression of *p21* (i) and *Rb* (ii) gene 96 hours after treatment. Representative graph of 3 independent experiments. The data represents the mean value of triplicates $\pm$ SEM. Asterisks indicated significance between R5020 vs Ctrl (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Tumor suppressor P53 activity modulates cell cycle arrest and senescence by regulating downstream targets involved in these processes (Qian and Chen 2013, Rufini, Tucci et al. 2013). Activation of P53 by progestin leads to induction of *p21* gene (el-Deiry, Tokino et al. 1993, Kester, Sonneveld et al. 2003). We hypothesized that P53 protein might have a significant role in the induction of senescence in R5020-treated MCF-7-PRB cells by regulating *p21* gene expression. Hereafter, we investigated p21, P53 and RB protein levels 24 and 96 hours after R5020 treatment (Figure 3.18).

Total RB was decreased in MCF-7-PRB clones at both 24- and 96-hour time points. Changes of phospho-RB levels in these cells were not visible 24 hours after the treatment. R5020 treatment for 96 hours did not affect levels of phospho-RB in MCF-7-pcDNA3 clone, whereas in MCF-7-PRB cells phospho-RB levels were decreased compared to ethanol-treated samples, with specific upshift on the gel. It is worth pointing out that the level of phospho-RB 24 hours after treatments in all samples was comparably higher to samples treated for 96 hours. The p53 protein was up-regulated in MCF-7-PRB treated cells 24 hours post-treatment but decreased 96 hours after treatment (Figure 3.18a). However, there were inconsistent changes in p21 levels in the two PR-transfected clones. R5020 increased p21 protein level 96 hours after treatment in MCF-7-PRB2 clone but not in MCF-7-PRB3 clone. To clarify this discrepancy, a time course experiment was conducted to track the changes of p53 and p21 in response to R5020 treatment. Interestingly, R5020 increased p53 levels in vector-transfected control cells, which express little PR, at all tested time points although these changes were marginal. R5020 also increased p53 levels in the two clones of MCF-7-PRB at all the time points except for MCF-7-PRB3 at 96 hours when p53 was decreased. Interestingly, the increase of p21 follows a slight different time course. In vector control, p21 changes were small but discernible at 24h, 30h and 48 hours' time points. In MCF-7-PRB2, noticeable increases were observed at 48 and 96 hours. In MCF-7-PRB3, the increases were detected at 30h and 48h, but not at 96 hours. Therefore, it appears that p21 changes in MCF-7-PRB2 come later than that in MCF-7-PRB3 but lasted for a similar amount of time. This could be due to the specific cellular context that enables the p53-p21 program to take effect. But the time frame of p21 alterations in response to progestin is consistent with the study in T47D-B cells in which the p21 protein expression level reaches its peak around 36 hours after treatment (Groshong, Owen et al. 1997).

Figure 3.18 R5020 regulates p53, p21 and RB protein expressions in MCF-7-PRB cells.

MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were cultured in 5% DCC-FCS 48 hours prior the treatment with 10 nM in 0.01% ethanol. (a) Protein level expression of total RB, phospho-RB, p53 and p21 in MCF-7 stably transfected clones 24 hours and 96 hours after R5020 treatment. (b) Protein level expression of p53 and p21. The protein lysate were collected 24h, 30h, 48h and 96 hours after the R5020 treatment and protein expression level was determined by Western blotting. Antibodies probed H-190 (anti PR), anti-total RB, anti-phospho-RB, anti-p53, anti-p21 and a loading control anti-GAPDH.

3.1.14 R5020 induces senescence-associated secretory phenotype (SASP) in MCF-7-PRB cells

Senescent cells secrete a myriad of proteins associated with inflammation and oncogenesis that can alter the tissue microenvironment. These factors can have paracrine or autocrine activities, and this is known as the senescence-associated secretory phenotype (SASP) (Campisi 2001, Campisi and d'Adda di Fagagna 2007, Rodier, Coppe et al. 2009, Kuilman, Michaloglou et al. 2010). Secreted factors vary depending on the cell type and mostly include proinflammatory cytokines (IL-1 α , IL-1 β , IL-6, IL-8) and adhesion molecules, such as endothelial intercellular

adhesion molecule ICAM1 (Shelton, Chang et al. 1999, Kuilman, Michaloglou et al. 2008, Rodier, Coppe et al. 2009). These factors are known to promote epithelial-mesenchymal transition (EMT), invasiveness and have a significant role in recruiting immune cells to senescent tissue (Coppe, Patil et al. 2008, Kuilman and Peeper 2009). IL-6 is the most prominent cytokine of SASP and it has been shown to associate with oncogenic stress induced senescence (Ortiz-Montero, Londono-Vallejo et al. 2017, Nelson, Kucheryavenko et al. 2018). Both IL-1 α and IL-1 β are upregulated in senescence of endothelial cells and fibroblasts (Maier, Voulalas et al. 1990, Uekawa, Nishikimi et al. 2004). In SASP of fibroblast, cell surface-bound IL-1 α is a critical protein which maintain senescence-associated IL-6/IL-8 cytokine secretion (Orjalo, Bhaumik et al. 2009).

It has been established that MCF-7 cells, as ER α positive cancer cells, express low or undetectable IL-6 and IL-8 (Conze, Weiss et al. 2001, Sansone, Storci et al. 2007, Sasser, Sullivan et al. 2007). The IL-1 family of cytokines has been found to inhibit cell growth, cause cell cycle arrest and down-regulation of ER α in MCF-7 breast cancer cell line (Paciotti and Tamarkin 1988, Sgagias, Kasid et al. 1991).

By using qRT-PCR, we discovered a marked increase in expression of genes encoding pro-inflammatory cytokines interleukin *IL-1 α* and *IL-1 β* 24h and 96h after the R5020 treatment, (Figure 3.19). Several studies have reported that progesterone suppresses IL-8 production (Ito, Imada et al. 1994, Loudon, Elliott et al. 2003, Horie, Harada et al. 2005, Leo, Wang et al. 2005). We observed a downregulation of *IL-8* in MCF-7-PRB cells 24h after R5020 treatment which corresponds to the reported study (Figure 3.19a (iii)). Interestingly, R5020 was able to induce expression of *IL-8* in MCF-7-PRB cells 96h after treatment (Figure 3.19b (iii)) although luminal breast cancer cells do not express *IL-8* constitutively (Jiang, Yang et al. 2000, Chavey, Bibeau et al. 2007, Sasser, Sullivan et al. 2007). Same as *IL-8*, *ICAM1* was downregulated at 24 hours (Figure 3.19a (iv)) and upregulated at 96 hours (Figure 3.19b (iv)). We also examined *IL-6* expression, however, it was undetectable (data not shown). Taken together, our data suggests that R5020 might induce SASP in MCF-7-PRB cells.

Figure 3.19 R5020 induces gene expression of cytokines *IL-1α*, *IL-1β*, *IL-8* and *ICAM1* in MCF-7-PRB cells.

MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were plated in 5% DCC-FCS medium 48h before treatment with 0.01% ethanol vehicle control or 10 nM R5020. (a) Relative gene expression of *IL-1α* (i), *IL-1β* (ii) *IL-8* (iii) and *ICAM1* (iv) 24 hours after the R5020 treatment. (b) Relative gene expression of *IL-1α* (i), *IL-1β* (ii) *IL-8* (iii) and *ICAM1* (iv) 96 hours after the treatment with 10 nM R5020. Representative graphs from three independent experiments. The data represents the mean value of triplicates $\pm$ SEM. Statistical significance was calculated by t-test and asterisks indicate significance between R5020 vs Ctrl (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$).

NF- κ B signaling network regulates various genes including cytokines, growth factors and genes that controls cell-cell, and cell-ECM adhesion important for development and branching of the

mammary gland (Brantley, Chen et al. 2001). The NF- κ B pathway in tumor is often activated as a result of the exposure to the inflammatory microenvironment and secreted cytokines (Schottelius and Dinter 2006). Considerable amount of research has revealed that NF- κ B is the major signaling pathway which stimulates the appearance of SASP (Chien, Scuoppo et al. 2011, Salminen, Kauppinen et al. 2012). Since we observed an upregulation of secreted factors of SASP by R5020 in MCF-7-PRB cells, we sought to investigate the role of the NF- κ B signaling R5020-induced senescence. NF- κ B activity is regulated through selective phosphorylation and degradation of IKK catalytic subunit I κ B α kinase (Chen, Egan et al. 2003, Borowicz, Van Scoyk et al. 2014) and subunit p65. Therefore, further analysis of the hormone-induced responses and NF- κ B pathway activity included evaluation of these critical proteins. Phospho-p65 (S536) level in MCF-7-PRB cells was efficiently upregulated 24 hours after treatment without any effect on the control vector. Phospho-p65 was slightly downregulated 96 hours after treatment in MCF-7-pcDNA3 and MCF-7-PRB2, while in MCF-7-PRB3 we noticed upregulation of this protein. Total-p65 protein expression level did not show obvious changes in all clones 24 and 96 hours after treatment.

However, based on the phosphorylation blot of I κ B α 24 and 96 hours after adding R5020, we showed that the level of phospho-I κ B α protein was upregulated 96 hours after the treatment with R5020. The protein level of total I κ B α and phospho-I κ B α was upregulated in the first 24 hours after treatment; whereas we did not detect the change of total I κ B α protein expression level in samples treated for 96h (Figure 3.20a). To confirm that R5020 treatment is inducing I κ B α phosphorylation we performed time course Western blot analysis (Figure 3.20b). In addition, densitometrical analysis has been done to confirm that R5020 treatment was explicitly inducing phosphorylation of p65 and I κ B α , and that the change in phospho-protein expression was not due to differences in total protein, we performed densitometrical analysis including samples from three independent experiments and we calculated ratio p-p65/p65 and p-I κ B α /I κ B α at 24 and 96 hours. P-p65/p65 ratio revealed induced phosphorylation of the p65 protein in MCF-7-PRB treated samples at both time points; whereas we did not observed any changes in control vector MCF-7-pcDNA3 with and without treatment (Figure 3.20c). This suggests that R5020 induces the activation of NF- κ B at both 24h and 96h time points. Consistently with the phenomenon of NF- κ B activation, p-I κ B α /I κ B α ratio after 96 hours was increased (Figure 3.20d (ii)).

Figure 3.20 R5020 activate NF-κB pathway in MCF-7-PRB cells.

Cells were plated in 5% DCC-FCS medium 48 hours before treatment with 10 nM R5020 (a) Protein level expression of PR, total-p65, phospho-p65, total-IκBα, and p-IκBα 24 and 96 hours after treatment. (b) Time course of protein level expression of PR, total-p65, phospho-p65, total-IκBα, and p-IκBα were analyzed by Western blotting using antibodies against p65, phospho-p65, IκBα, and p-IκBα. Protein levels were normalized to GAPDH. (c) p-p65/p65 ratio was calculated 24 hours (i) and 96 hours after treatment (ii). (d) p-IκBα/IκBα ratio was calculated 24 hours (i) and 96 hours (ii) after R5020 after treatment. Levels of total and p-phospho proteins were normalized to corresponding loading control GAPDH. The data represents the mean value of triplicates $\pm$ SEM. Statistical significance was calculated by t-test and asterisks indicate significance between R5020 vs Ctrl (*P<0.05, **P<0.01, ***P<0.001, ****P<0.001).

3.1.15 R5020 significantly decreases the protein levels of E-cadherin in MCF-7-PRB cells

Cancer progression is characterized by process of EMT, where the cells are losing cell-cell or cell-extracellular matrix junction and they gain capacity to migrate to other tissues (Thiery 2002, Kalluri and Weinberg 2009). Cell adhesion molecules have multiple functions including regulation of intra and inter-cellular signaling (Freemont and Hoyland 1996). These molecules are pivotal for regulation of processes as cell growth, immune and inflammatory response, differentiation, cell motility, wound healing and alteration of adhesion properties of the molecules that play crucial role in cancer progression (Smit and Peeper 2010). Broadly, cell adhesion molecules and adhesion processes are reported to be tumor suppressive, and they inhibit metastatic cascade in tumors (Fawcett and Harris 1992, Okegawa, Li et al. 2002). It has been reported that E-cadherin is tumor-suppressive epithelial adhesion molecule, and that suppression of this molecule is common in breast, prostate, liver and lung cancers (Vleminckx, Vakaet et al. 1991, Birchmeier and Behrens 1994, Christofori and Semb 1999). Progesterone induces cellular differentiation in MDA-MB-231 breast cancer cells transfected with PR and E-cadherin level in these cells is decreased due to EMT (Lin, Jin et al. 2003).

To evaluate the effect of R5020 treatment on E-cadherin in stably transfected MCF-7 cell line we examined protein expressions in these cells 24 and 96 hours after the R5020 treatment. Our data indicated that levels of E-cadherin decreased in all MCF-7-PRB clones treated with R5020 for 24 hours, although the decrease of the protein was not as significant as 96 hours after treatment (Figure 3.21). To confirm E-cadherin down-regulation 96 hours after the R5020 treatment, we performed immunostaining in MCF-7-pcDNA3 and MCF-7-PRB2 cells. It was obvious that in vehicle treated MCF-7-PRB samples, E-cadherin was located on cell-cell junctions, but after R5020 treatment the level of protein was drastically decreased and we observed that E-cadherin was delocalized (Figure 3.21b). Our results also showed a down-regulation of ER α in MCF-7-PRB cells after R5020 treatment (Figure 3.21a). Studies revealed that down-regulation of E-cadherin can be mediated by estrogen in breast cancer cells (Oesterreich, Deng et al. 2003). There are two possible pathways that can lead to down-regulation of E-cadherin by R5020:

through the direct effect of R5020 on PR, or through ER α . Therefore, we hypothesized that ER α could be involved in E-cadherin down-regulation in ligand-independent manner.

Figure 3.21 R5020 treatment significantly decreases protein levels of E-cadherin in MCF-7-PRB cells.

Cells were plated in 5% DCC-FCS 48 hours before treatment. Cells underwent vehicle control or 10 nM R5020 treatment 24 hours and 96 hours before harvest. (a) Cell lysates of MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were collected and PRB, ER α and E-Cadherin level were analyzed using Western blotting, GAPDH was used as a loading control. (b) MCF-7-pcDNA3 and MCF-7-PRB2 cells were treated for 96 hours and cells were incubated with E-cadherin and stained with anti-mouse-conjugated Dylight594 and for nuclei (DAPI). Images were acquired by inverted microscope Olympus IX₇₁.

To evaluate if E-cadherin downregulation is mediated by ER α , we performed qRT-PCR of E-cadherin and ER α in MCF-7-PRB clones. E-cadherin mRNA (*CDHI*) level was significantly downregulated in MCF-7-PRB cells 6 hours after R5020 treatment (Figure 3.22a). ER α (*ESR-1*) gene expression showed the same response as E-Cadherin to R5020 treatment (Figure 3.22b).

Figure 3.22 R5020 treatment downregulates mRNA levels of E-cadherin and ER α in MCF-7-PRB cells.

MCF-7-PRB2 and MCF-7-PRB3 MCF-7 cells were plated in 5% DCC-FCS medium 48 hours before treatment with 10 nM R5020 in 0.01% ethanol for 6 hours. (a) Relative gene expression of *CDHI*. (b) Relative gene expression of *ESR-1*. The data represents the mean value of triplicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to respective controls (representative graph of 3 independent experiments, triplicates).

To investigate whether downregulation of ER α leads to E-cadherin down-regulation, our next step was to overexpress ER α in MCF-7-PRB cells. We showed that despite ER α overexpression, E-cadherin was downregulated after R5020 treatment (Figure 3.23a). Our investigation of morphological change in these cells demonstrated that even with overexpressed ER α cell morphology was changed after treatment (Figure 3.23b). R5020-treated cells exhibited enlarged, flattened phenotype. Since R5020-induced down regulation of E-cadherin mRNA occurs as early as 6 hours after treatment and ER α overexpression did interrupt this effect, it is plausible that R5020 directly modulate E-cadherin via PRB without involvement of ER α .

Figure 3.23 Overexpression of ER α in MCF-7-PRB cells does not rescue the loss of E-cadherin protein and changes in morphology of the cells induced by R5020.

MCF-7-PRB2 cells were plated in 5% DCC-FCS medium. At 24 hours cells were transduced with direct adding of adenovirus control vector Ad-control vector or Ad-ER α of MOI5 to the wells. 24 hours after transduction, cells were treated with 10 nM R5020 for 96 hours. (a) Protein expression of E-Cadherin, PRB and ER α of MCF-7-PRB2 cells transduced with Ad-control vector or Ad-ER α were analyzed by Western blotting. GAPDH served as a loading control. (b) Live cells were imaged 96 hours after R5020 treatment using inverted microscope Olympus IX_71.

To test hypothesis that E-cadherin downregulation is directly mediated through PRB in response to R5020 treatment, we examined E-cadherin protein expression in cells with changed PRA:PRB ratio via adenoviral transduction. Notably, E-cadherin protein level was not decreased in MCF-7-pcDNA transfected with PRA, whereas in MCF-7-PRB control and MCF-7-PRB transduced with PRA, E-cadherin downregulation was observed upon R5020 treatment (Figure 3.24a). In PRA

transduced MCF-7-pcDNA cells, there was no morphological change following R5020 treatment. However, in MCF-7-PRB cells, as well as in cells with PRA overexpression, cell flattening and spreading was observed when cells were treated with R5020 for 96 hours (Figure 3.24b).

Figure 3.24 Changed in PRA:PRB ratio in MCF-7 stably transfected cells is unable to trans-repress PRB-mediated senescence-like phenotype and rescue E-Cadherin downregulation induced by R5020.

Cells were transduced with direct adding of adenovirus control vector Ad or Ad-PRA of MOI1 24 hours after plating. 24 hours after the transduction, cells were treated with 10 nM R5020 for 96 hours. (a) Protein expression of E-cadherin, PRB and ERα in MCF-7-pcDNA3 and MCF-7-PRB2 MCF-7 cells transduced with Ad or Ad-PRA was analyzed by Western blotting and probed with anti-PR (H-190), anti- E-Cadherin, anti-ERα antibody and anti-GAPDH as a loading control. (b) Live cells were visualized and acquired by using inverted Olympus IX_71.

We have previously shown that R5020 treatment abolished E2-induced growth and acini formation in MCF-7-PRB cells. Our next goal was to evaluate if E2 treatment would affect

R5020-induced decrease of E-cadherin 96h after treatment. Notably, in MCF-7-pcDNA samples E-cadherin exhibited slight increase after treatment with R5020 and R5020+E2. On the other hand, E-cadherin was significantly downregulated in samples treated with R5020 in the presence or absence of E2. Treatment with E2 did not lead to morphological change compared to controls, whereas combination of R5020 and E2 induced cell spreading, consistent with the senescence-like phenotype. Thus, R5020 can override the effect of E2 in all samples examined so far (Figure 3.25b).

Figure 3.25 R5020 treatment down-regulates E-Cadherin in MCF-7-PRB cells with and without E2 treatment.

Cells were starved in 5% DCC-FCS for 48h before treatment with 10 nM R5020 and/or 10 nM E2 for 96 hours. (a) Protein expression of PRB, E-cadherin and ER α in MCF-7-pcDNA3, MCF-7-PRB2 and MCF-7-PRB3 cells were analyzed by Western blotting and probed with anti-PR (H-190), anti-E-Cadherin, anti-ER α antibody and anti- GAPDH as a loading control. (b) MCF-7-PRB3 cells exhibit senescent like morphological change 96 hours after R5020 treatment in both presence and absence of E2. Live cells were visualized and acquired by using microscope Olympus IX_71.

3.1.16 R5020 induces autophagy in MCF-7-PRB cells

It has been described earlier that R5020 induced senescence-like phenotype in MCF-7-PRB cells along with the production of proinflammatory factors. We aimed to understand the underlying mechanism responsible for the induction of senescence. We postulated that autophagy might be associated with the efficient production of SASP factors and activity of senescent cells (Young, Narita 2009). Autophagy is a dynamic cellular process, which includes catabolic degradation of cellular organelles and proteins in order to recycle building blocks (e.g. amino acids) for reuse in the synthesis of new components in conditions like acute starvation, or to remove unnecessary macromolecules and pathogens from cytosol (Mathew, Karantza-Wadsworth et al. 2007, Yao, Wang et al. 2012). Additionally, studies have shown that ligand activated PRB induces autophagy in MCF-7 and T47D cell lines by attenuating PI3K/Akt pathway (Young, Narita et al. 2009, Barth, Glick et al. 2010, De Amicis, Guido et al. 2014, De Amicis, Guido et al. 2016). Hence, in order to elucidate the influence of progestin R5020 treatment on cell transition from proliferative to the arrested stage, our next step was to test whether autophagy plays a role in the cell's response to progestin treatment.

It has been demonstrated that microtubule-associated proteins 1A/1B light chain 3B (LC3B I and LC3B II) are essential autophagy proteins and are often used as consistent markers for observing autophagy (Mizushima 2004, Mizushima and Yoshimori 2007, Tanida, Ueno et al. 2008, Barth, Glick et al. 2010). The amount of LC3B-II, as detected by immunoblot, is considered to be marker of autophagy, while the latter is named as autophagy flux (Kabeya, Mizushima et al. 2000). Therefore, we investigated the effect of progestin R5020 treatment on LC3B protein expression in MCF-7 stable cells. Obtained results revealed that there was a slight decrease in LC3B-I and LC3B-II protein expression in vector-transfected control cells after 24 hours and remained unchanged after 96 hours of R5020 treatment. In contrast, MCF-7-PRB cells exhibited remarkable increases in LC3B-I and LC3B-II protein expression levels 24 and 96 hours following R5020 treatment (Figure 3.26a). This data suggests involvement of PRB in mediating autophagy after R5020 treatment. Since we observed variations in LC3B protein expression after treatment, we aimed to quantify two different forms of this protein via densitometry analysis. Densitometry analysis showed a slightly increase of LC3B II protein level 24 hours after

treatment, whereas 96 hours after treatment there is significant increased level of this protein (Figure 3.26b (i)) and 96 hours after R5020 treatment (Figure 3.26b (ii)).

Figure 3.26 R5020 induces autophagy in MCF-7-PRB cells.

Cells were plated in 5% DCC-FCS medium 48 hours before hormone treatment with 10 nM R5020 for 24 and 96 hours. (a) The lysate was collected 24 and 96 hours after treatment. Lysates were analyzed by Western blotting using antibodies against LC3B. Protein levels were normalized to GAPDH. Representative blot of three independent experiments is shown. (b) R5020-induced level of LC3B II at 24 hours (i) and 96 hours (ii) R5020 treatment. Levels of LC3B II protein was normalized to corresponding GAPDH protein level. The data represents the mean value of triplicates $\pm$ SEM. Statistical significance was calculated by t-test and asterisks indicate significance between R5020 vs Ctrl (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.001).

Since our data indicated an increase in the LC3B protein expression levels, we hypothesized that R5020 might exert a direct effect on the genomic level of autophagy-related protein LC3B. To evaluate whether R5020 treatment can directly modulate LC3B gene expression, we tested gene expression level 24 and 96 hours after hormonal treatment. Using real-time PCR, we found that within 24 hours of hormone treatment there was a marked increase in expression of genes encoding LC3B protein (Figure 3.27a). 96 hours after R5020 treatment, LC3B mRNA level in

PRB expressing cells was notably upregulated whereas in control vector cells we did not observe any change (Figure 3.27b).

Figure 3.27 R5020 treatment upregulates LC3B gene expression.

MCF-7 cells stably transfected with vector control pcDNA and PRB were plated in 5% DCC-FCS medium 48 hours before hormone treatment with 10 nM R5020 for 24 and 96 hours. (a) Relative L3B gene expression 24 hours after R5020 treatment. (b) Relative LC3B gene expression 96 hours after R5020 treatment. The data is represented as the mean value of triplicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to respective control (Three independent experiments, triplicates). The data represents the mean value of triplicates $\pm$ SEM. Statistical significance was calculated by t-test and asterisks indicate significance between R5020 vs Ctrl (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$).

To determine if the increase in LC3B protein levels is due to autophagy flux, or because autophagy degradation is blocked, we used an inhibitor of lysosomal activity (Figure 3.28). The level of LC3B II protein has been analyzed by densitometry. In MCF-7-pcDNA3 cells without inhibitor LC3B II protein level was unchanged, while with lysosomal inhibitor increased LC3B II was observed 24 hours (Figure 3.28b (i)) and 96h after hormonal treatment (Figure 3.28b (ii)). In MCF-7-PRB cells there was no difference in the LC3B II protein level without lysosomal inhibitor 24 hours after hormonal treatment (Figure 3.28b (i)). However, in MCF-7-PRB with inhibitor LC3B II was increased 96 hours following R5020 treatment (Figure 3.28b (ii)).

Recent studies reported that the progesterone-mediated modulation of autophagy involves Akt activation which results in autophagy inhibition (De Amicis, Guido et al. 2014). Based on that, we investigated Akt protein level expression in our samples. There was no accumulation of Akt

proteins 24 hours after treatment with and without lysosomal inhibitor in MCF-7-pcDNA cells. In MCF-7-PRB samples treated with R5020 there was a decreased level of Akt. On the other hand, in hormone-treated MCF-7-PRB cells with and without inhibitor there was a slight decrease. Phospho-Akt protein level did not change 24 or 96 hours after treatment in MCF-7-pcDNA samples, although we saw the accumulation of this protein after application of lysosomal inhibitor. In MCF-7-PRB cells after the treatment there was a slightly increased level of phospho-Akt, but with inhibitor the difference was not noticeable (Figure 3.28a). As we did not see the difference in the expression of this protein when we used lysosomal inhibition with and without treatment, we performed densitometry analysis only for samples with and without R5020 treatment to confirm our finding. We observed that protein levels of total Akt protein remain unchanged in MCF-7-pcDNA cells with and without R5020 treatment. On the other hand, in MCF-7-PRB cells with treatment there was a decreased level of this protein. p-Akt (S473) protein level was not changed in control vector, but in MCF-7-PRB treated cells we observed an increased level of this protein, and that correlated with our previous observations. Densitometry analysis 96h after treatment followed the same pattern of expression of these proteins. We also calculated phospho-Akt/Akt ratio to see if Akt pathway was activated by R5020 treatment. This analysis indicated that Akt protein was activated in MCF-7-PRB treated with R5020 for 24h (Figure 3.28c (i)) and 96 hours (Figure 3.28c (ii)). This data correlates with our previous finding that autophagy flux is decreased in PRB R5020-treated samples.

Figure 3.28 R5020 treatment induces autophagy in MCF-7-PRB cells treated with lysosomal inhibitor.

MCF-7-pcDNA3 and MCF-7-PRB2 cells were plated in 5% DCC-FCS 48h before hormone treatment with 10 nM R5020 for 24 and 96 hours. The cells were treated with lysosomal inhibitor Leupeptin and NH₄Cl for 6 hour prior to lysate collection. (a) The lysate was collected 24 and 96 hours after treatment. Lysates were analyzed by Western blotting using anti-LC3B antibody anti-Akt and anti-phospho-Akt (S473). Protein levels were normalized to GAPDH. Representative blot is shown. (b) LC3B II protein level 24 hours (i) and 96 hours (ii) after R5020 treatment. (c) p-Akt/Akt ratio in MCF-7-PRB 24 hours (i) and 96 hours (ii) after R5020 treatment. The data is represented as the mean value of triplicates $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to respective control (Three independent experiments, triplicates).

3.2 Discussion

Breast cancer can be considered as an endocrine disease. Nearly two-thirds of breast cancers are ER+ and PR+ and anti-estrogen or aromatase inhibitors are the frontline endocrine therapies. However, the majority of the responders develop endocrine resistance. Progestin had been used as a second-line endocrine therapy for metastatic breast cancer with a clinical benefit similar to that of aromatase inhibitor (Buzdar, Douma et al. 2001). In the last 15 years, progesterone and progestins are emerging as a primary driver of mammary development and breast carcinogenesis. Clinical trials of hormone replacement therapy (HRT) in post-menopausal women concluded that progestin, and not estrogen, increases the risk of breast cancer (Beral 2003, Chlebowski, Anderson et al. 2010). This is consistent with the understanding that progesterone, not estrogen, induces the expansion of adult mammary stem cells in mice model (Joshi, Jackson et al. 2010). Laboratory studies also reported conflicting results that suggest both pro- or anti-tumoral effects depending on the cell line and experimental conditions (Groshong, Owen et al. 1997, Lin, Eng et al. 2001, Chen, Hardy et al. 2011, Obr and Edwards 2012). This reflects the complex nature of PR biology. Further understanding of the mechanism of PR action under different settings will contribute to the understanding of the complexity of PR signaling.

There are various breast cell models for the study of PR function. The most commonly used are the ER- and PR-positive MCF-7 cells which express PR in response to estrogen stimulation, and T47D cells which are ER- and PR-positive and express PR independent of estrogen as a result of genetic alteration (Horwitz 1978, Sartorius, Groshong et al. 1994, Subik, Lee et al. 2010). In theory, MCF-7 cells are the most representative of the clinical ER- and PR-positive breast cancer because PR expression is the result of estrogen stimulation of ER activity. But there are two perceived limitations for studying the mechanism of PR activity. First, estrogen is needed to induce PR and the prior presence of estrogen is a confounding factor to clearly define progestin response. Second, the level of PR is relatively low and the responses to progestin are often in low magnitude and can be difficult to detect or conclude. To circumvent these limitations, we decided to establish overexpression of PRB, which is a stronger transactivator than PRA, in MCF-7 cells to study the response to of progestin.

3.2.1 R5020 induces irreversible cell cycle arrest through regulation of cyclins and CDKs

We demonstrated that three MCF-7 clones overexpressing PRB exhibited biphasic response to R5020. The treatment for 24 hours increased cell cycle progression and S-fraction. After 96 hours, R5020 drastically and irreversibly inhibited cell growth. This biphasic regulation of cell growth by progestin is consistent with that reported in T47D cells where cells first exhibited accelerated cell growth, and then are arrested in G1 phase of cell cycle (Groshong, Owen et al. 1997, Thuneke, Schulte et al. 2000). R5020 treatment enhanced S phase entry and induced expression of cyclin D1 in T47D cells overexpressing PRB through activating MAPK cascade (Skildum, Faivre et al. 2005). To further investigate cell growth inhibition we performed cell cycle flow cytometry study in MCF-7 stably transfected cells. This experiment and cell cycle analysis showed that all 3 clones are having the same response to R5020 treatment. Although PRB1 expression is much lower than the other clones, its PRB level is still much higher than the levels in the parental or vector-transfected MCF-7 cells in estrogen-deprived medium. The observation is a confirmation that PRB has intrinsic growth-inhibitory effect even at relative low levels. We observed that 24 hours after R5020 was added to the culture, more of the PRB transfected cells were in the proliferative status, in S phase of the cell cycle, compared to the ethanol-treated cell (Figure 3.3b (ii)). In contrast, 96 hours after the treatment, the proportion of cells in S phase of the cell cycle was reduced, while it was increased in G2/M phase (Figure 3.3c (iii)). Therefore, we postulated that cells were arrested in the cell cycle, at two checkpoints, G1 phase and G2 phase of cell cycle. We took into the consideration the previous finding which suggested that after progestin treatment T47D-YB cells were able to complete one round of cell cycle, but prolonged hormonal treatment induced changes that affected cell cycle kinetics and proliferation rate (Groshong, Owen et al. 1997, Reed 1997, Thuneke, Schulte et al. 2000).

Although R5020 was shown to induce cell cycle arrest at G2/M phase, the magnitude of the increase in G2/M appears to be moderate in light of the massive and irreversible inhibition of cell growth. BrdU incorporation assay of the MCF-7-PRB cells treated for 96 hours revealed that most of R5020-treated cells were unable to incorporate BrdU despite of the seemingly moderate decrease of S-phase cells.

To examine the mechanism of R5020's effect on inhibition of cell growth, we investigated the effect of R5020 on regulators of cell cycle progression. Two main checkpoints for cell cycle proliferation are G0/G1 and G2/M. Therefore, cyclins and CDKs that are included in the regulation of cellular progression through these two checkpoints were our primary target to investigate. Cyclins are a group of large and various family of structurally related proteins that promote activity and cell proliferation (Miller and Cross 2001, Truman, Kitazono et al. 2001). Cyclin-dependent kinases (CDKs) are also essential proteins involved in the regulation of cell cycle. During the cell cycle, expression of CDKs remains constant and CDK kinase activity is regulated by interaction with two groups of proteins: cyclins and CDK inhibitors (Ekholm and Reed 2000, Miller and Cross 2001, Truman, Kitazono et al. 2001). R5020's effect on cyclins and CDKs are consistent with its biphasic effect on cell growth. R5020 treatment for 24 hours resulted in increased mRNA expression of cyclins A2, B1, D1 and E, and increased protein levels of most of these cyclins. In contrast, decreased levels of cyclins A2, B1 and D1 and CDK2 and CDK4 were observed in R5020-treated cells after 96 hours when the cells were growth-arrested by R5020 treatment. It is interesting because these cyclins are involved in various phases of the cell cycle. It could be postulated that the growth inhibition that we saw in R5020 treated cells was due to the regulation of these cyclins/CDKs at the protein level. It is plausible that R5020-induced growth arrest by affecting all the phases of the cell cycle because of the inhibition of the cyclins and CDKs. On the other hand, down-regulated protein levels of cyclins A2 and B1, which are crucial for initiating and maintaining mitotic state of the cells (Akli, Zheng et al. 2004, Mullers, Silva Cascales et al. 2014), are consistent with the accumulation of G2/M phase cells in response to treatment with R5020 for 96 hours.

It is intriguing that R5020-induced growth inhibition after 96 hours is associated with marked up-regulation of cyclin E protein. It is known that mRNA level of CCNE is upregulated during G1-S transition when it is reaching the peak, and during the S phase of cell cycle, mRNA of CCNE is decreased (Reed 1997, Ekholm and Reed 2000). CCNE/CDK activity is therefore important for G1/S phase transition (Reed 1997) On the other hand, overexpression of cyclin E has also been reported to inhibit cell cycle (Lindahl, Landberg et al. 2004). For example, it has been reported that progesterone induces cell growth inhibition in the MCF-7 cell overexpressing cyclin E1 (Montazeri, Bouzari et al. 2015) but it is unlikely that to be the case with endogenous cyclin E. On the other hand, increased p21 levels in R5020-treated cells could inhibit cyclin

E/CDK2 activity as has been suggested for T47D cells treated with progestin (Musgrove, Swarbrick et al. 1998). With the increased activity of p21 together with decreased CDK2, increased cyclin E probably would not take much effect. Nonetheless, it is puzzling what is controlling the activity of this cyclin E and future study is needed to shed more light on this matter.

Taking all data together, after analyzing cell growth, cell cycle kinetics and expression of cyclins and CDKs, we proposed that our cells were arrested in two phases of the cell cycle. It is plausible that cells were able to complete one cell cycle 24 hours after treatment, but according to our data following the exposure to continuous treatment cells were arrested in G2/M phase or according to the down regulation of various cyclins and CDKs in other phases as well. What remains to be addressed is how these cyclins and CDKs are regulated at different time points and if the complex mechanism of regulation is accountable for the conflicting responses to progestin under different experimental conditions and in different laboratories. R5020 completely abolished estrogen-induced cell growth

It was suggested that the cross-talks between PR and ER α could play a significant role in the cell line's response to steroid hormones (De Amicis, Zupo et al. 2009, Daniel, Gaviglio et al. 2015, Hegde, Kumar et al. 2016). Furthermore, the overexpression of PRB could alter the expression of ER α target genes in MCF-7 cells in the absence of progesterone/progestins (Daniel, Gaviglio et al. 2015). Breast cancer can be characterized by expression of PR and ER, and PR expression can forecast the response to endocrine therapies targeting ER, partially because ER regulates PR expression (Musgrove and Sutherland 2009). Herein, it is plausible that cross-talk between PRB and unliganded ER α can affect cell growth in MCF-7-PRB cells. Our finding that R5020 down-regulate ER α in stable PRB cells (Figure 3.2c) is consistent with a study that reported decrease in ER α levels due to the PRB overexpression in breast cancer cells (De Amicis, Zupo et al. 2009). Therefore, we originally hypothesized that unliganded ER α cross-talk with PRB may play a role in the growth-inhibitory effect of R5020 after prolonged treatment. Our study showed that unliganded ER α did not affect the response of MCF-7-PRB cell to R5020 treatment. We also showed that R5020 completely abolished E2-induced cell proliferation in MCF-7-PRB cells in both 2D and 3D culture. It has been reported that progestin inhibited estrogen-induced tumor growth in MCF-7 tumor xenograft model (Mohammed, Russell et al. 2015). Analysis of global

genomic binding by ER and PR in response to estrogen or estrogen plus progestin revealed that progestin reshaped ER cistrome by shifting ER to different chromatin locus. The relationship between these progestin-programmed ER binding locus and PR binding locus was not clear in MCF-7 cells because there was little PR without estrogen treatment. In T47D cell, however, the 99% of the ER cistrome induced by progestin overlaps with that of PR. Interestingly, motifs of the reprogrammed ER α sites are not estrogen responsive elements but progesterone responsive elements. This suggests that liganded PRB recruited ER α to its target gene locus. The functional significance of this ER α and PR co-localization on the chromatin is not understood. In our study, ER α was rapidly downregulated by R5020 so co-localization with PRB is not quite relevant. But it is possible to study if there is a co-localization at the early time point (e.g. 3 hours) before ER α down-regulation and with PRB overexpression. It is not clear at this point if the down regulation of ER α is important for the inhibitory effect of R5020 because our study did not test the effect of ER α overexpression on R5020-induced inhibition of estrogen's effect.

Interestingly, unliganded ER α affected cell growth in ethanol treated cells (Figure 3.7a). ER α seems (although not statistically significant) to exhibit ligand independent activity in MCF-7 cells and ligand independent-ER α activity is particularly related to genes responsible for regulation of cell growth and maintenance of epithelial phenotype (Caizzi, Ferrero et al. 2014). It has been reported that unliganded PRB could form a complex with ER/PELP1 and activate target genes to enhance cell proliferation in ER and PRB positive cells treated with estradiol (Daniel, Gaviglio et al. 2015).

The relative expression of PRA/PRB ratio is often changed in human breast cancers. Higher PRA/PRB ratio is known to be associated with the aggressiveness of the tumor (Hopp, Weiss et al. 2004). Ligand induced transcriptional activity of two PR isoforms have non-overlapping target genes (Graham, Yeates et al. 1995, Richer, Jacobsen et al. 2002, Jacobsen, Richer et al. 2003). The activity of PRA can be predominant over PRB (Graham, Yager et al. 2005). It is surprising that pcDNA-transfected cells overexpressing PRA did not exhibit any effect following hormonal treatment. Furthermore, we demonstrated that overexpression of PRA in our MCF-7 stably transfected PRB cells was not able to abolish the effect of R5020 on the cell growth (Figure 3.6). Interestingly, we observed increased protein level of PRA in MCF-7-pcDNA/PRA cells after R5020 treatment. The upper PRA band is phosphorylated form after R5020 treatment.

Therefore we postulated that PRA was not able to inhibit function of PRB and this finding is not in agreement with the reported PRA inhibitory effects on PRB (Vegeto, Shahbaz et al. 1993, Giangrande, Pollio et al. 1997). Therefore, we concluded that PRB receptor is a principal mediator for R5020 response in MCF-7 luminal cells. We are not sure if the transient transfection of PRA is not sufficient to exert its predominant effect. Future experiment with stably transfected MCF-7 PRA+ cell line could confirm our findings.

3.2.2 R5020 induced cell senescence and SASP through p53 and NF- κ B

Cellular senescence is the process that has been described long time ago when scientist showed that primary human diploid fibroblasts stop proliferating after some time and this had been followed by cell cycle growth arrest (Hayflick and Moorhead 1961). Cellular senescence is characterized as irreversible cell growth arrest (Takahashi, Ohtani et al. 2007). Senescence has also been characterized as potent tumor suppressor mechanism (Campisi 2001, Braig and Schmitt 2006, Coppe, Patil et al. 2008, Krizhanovsky, Xue et al. 2008). Although, cells are growth arrested they remain metabolically active (Coppe, Patil et al. 2008, Rodier, Coppe et al. 2009). Additional characteristics of senescence are changed morphology with large, flattened cells (Kuilman, Michaloglou et al. 2010) and secretory phenotype when senescent cells are able to secrete various cytokines and chemokines have been described. We demonstrated that R5020 induces these senescence-like phenotypes including irreversible cell cycle arrest and morphological changes in MCF-7-PRB cells where cells exhibit large, flattened phenotype, with an enlarged nucleus (Figure 3.11). Drastically changed cell morphology in the R5020-treated MCF-7-PRB cells was associated with the increased actin stress fibers formation. Stress fibers exhibited thin, elongated phenotype and showed tendency to be anchored in the membrane (Figure 3.12) as multiple studies have shown before (Wang and Gundersen 1984, Cho, Ryu et al. 2004). R5020-treated MCF-PRB cells also exhibited several changes and markers characteristic for cell senescence. First, intracellular granularity have been reported to be increased in senescence and autophagy (Sherwood, Rush et al. 1988, Paglin, Hollister et al. 2001, Monastyrska and Klionsky 2006). We demonstrated increase in the cell granularity in MCF-7-PRB cells 96 hours after R5020 treatment. We postulated that this could be a consequence of formation of vesicles and lysosomal vacuoles that might be involved in processes of autophagy that mediates transformation of proliferative cell to arrested stage. Second, β -galactosidase

staining is an established marker for cell senescence. β -galactosidase is a eukaryotic lysosomal enzyme with a broad spectrum of natural and synthetic substrates. As a lysosomal component, normal environment for this enzyme to be active in endothelial cell culture is acidic at pH 4-4.5 (van der Loo, Fenton et al. 1998). Sa β -gal activity at pH 6.0 ensures that lysosomal β -galactosidase activity is suppressed and that most non-senescent cells will not be stained (Dimri, Lee et al. 1995, Lee, Han et al. 2006). We validated increased Sa β -gal activity in MCF-7-PRB treated cells, which confirmed our hypothesis that R5020 treatment induces senescence in MCF-7-PRB cells (Figure 3.14). Although we saw some positive staining in control cells, based on previous reports we postulated that this staining might come from serum depleted condition, as well as the confluence of the cells (Dimri, Lee et al. 1995, Gary and Kindell 2005). Third, cell senescence is associated with activation of p53-p21 axis. Induction of p53-dependent senescence has a crucial role in controlling tumor progression *in vivo* (Chen, Trotman et al. 2005). Studies showed that prolonged exposure to progestin can activate p53 protein in human breast and endometrial tumor cells (Kester, Sonneveld et al. 2003). Furthermore, p21 is downstream of p53 and a mediator of p53-induced G1 arrest (el-Deiry, Harper et al. 1994). p21 is one of the main proteins responsible for senescence-like growth arrest (LaBaer, Garrett et al. 1997, Stein, Drullinger et al. 1999). Previous reports showed that p53 induced cell growth arrest is mediated through induction of p21 protein and at mRNA level (el-Deiry, Tokino et al. 1993, el-Deiry, Harper et al. 1994).

Studies also showed that progesterone is involved in regulation of p21 (Xiong, Hannon et al. 1993, Peter and Herskowitz 1994). Our study showed that *p21* mRNA expression was not induced by R5020 treatment in MCF-7-PRB cells 24 hours after R5020 treatment (Figure 3.17a (i)). On the other hand, prolonged exposure to R5020 increased gene expression of this CDK inhibitor (Figure 3.17b (i)). We showed that 48 hours after treatment p21 protein exhibited the highest expression in both MCF-7-PRB clones. Thus, we postulated that *p21* is indirectly induced by R5020 through p53. This is supported by the evidence that the upregulation of p53 tumor suppressor protein occurred earlier than that of p21 at 24, 30 and 48 hours following R5020 treatment of MCF-7-PRB cells. 96 hours after R5020 treatment, protein level of p53 was decreased (Figure 3.18). These observations are in agreement with study where it has been shown that exposure to R5020 for four days led to decreased level of p53 in T47D cells (Hurd, Khattree et al. 1995). Taken altogether, we postulated that p53 plays a significant role in R5020-

induced cell senescence by inducing gene expression of *p21* in MCF-7-PRB cells. As we have just two time point for quantification of mRNA level, further studies should include different time points, as well as *p53* gene expression level.

Senescent cells are growth arrested and yet are metabolically active. Senescent cells are able to secrete different molecules as cytokines, chemokines and growth factors that can affect cell microenvironment drastically. Previous finding has demonstrated that SASP is induced by increasing mRNA levels of cytokines (Rodier, Coppe et al. 2009, Freund, Orjalo et al. 2010). IL-1 and IL-6 have been found to inhibit cell growth of MCF-7 cells (Paciotti and Tamarkin 1988, Sgagias, Kasid et al. 1991, Danforth and Sgagias 1993). We presented evidence to suggest that R5020 also induced SASP phenotype. mRNA expression of cytokines *IL-1 α* and *IL-1 β* was increased in MCF-7-PRB cells 24 hours after treatment (Figure 3.19a (i) and (ii)). In addition, gene expression levels of *IL-8* and *ICAM1* were downregulated in the first 24 hours after the treatment but were significantly increased 96 hours after the treatment (Figure 3.19b). It has been reported that in PR+ but ER- breast cancer cells *IL-8* gene is directly down-regulated by progesterone (Leo, Wang et al. 2005). IL-1 was reported to be upstream regulator of IL-6 and IL-8 (Orjalo, Bhaumik et al. 2009). These cytokines can act in the paracrine and autocrine manner and can have a cytostatic or promitogenic function (Kuilman, Michaloglou et al. 2008, Kuilman, Michaloglou et al. 2010). This data implied that IL-1 family of cytokines might be primary inflammatory mediator of R5020/PRB response in stably transfected MCF-7 cells. Cell surface IL-1 α has been shown to be a crucial regulator for secretion of proinflammatory cytokines IL-6 and IL-8 that also reinforce the cell growth arrest (Orjalo, Bhaumik et al. 2009). In human mast cells, IL-1 β promotes secretion of IL-8 (Kim, Lee et al. 2010). Study has demonstrated that progesterone and MPA reinforced IL-1 to induce IL-8 expression (Arici, MacDonald et al. 1996). It is plausible that upregulation of *IL-8* mRNA level 96 hours after R5020 treatment is mediated by the initial upregulation of *IL-1* cytokines. Induction of ICAM1 can also be stimulated by IL-1 β (Myers, Wertheimer et al. 1992, Wong and Dorovini-Zis 1992). Hence we speculate that the upregulation of *IL-1 α* and *IL-1 β* by R5020 in turn induces gene expression of both *IL-8* and *ICAM1*.

Ligand activated progesterone receptor can interact with other transcriptional factors and can modulate transcription of some genes. One of the transcription factors that can be modulated by

PR/ligand activation is NF- κ B pathway (Bamberger, Bamberger et al. 1996, Hsu, Yang et al. 2014, Hsu, Yang et al. 2015). Activation of NF- κ B pathway by a stimulus from cell environment triggers an immune response in cells by inducing cytokine production and enhancing recruitment of inflammatory cells. This pathway has an essential role in linking inflammation with cancer development and progression (Karin 2006, Karin, Lawrence et al. 2006). NF- κ B has also been identified as signaling pathway that promotes senescence (Rovillain, Mansfield et al. 2011). NF- κ B is a nuclear factor and can interact with other nuclear factors such as progesterone receptor. It is known that there is a mutual repression of PR and RelA (p65) subunit of NF- κ B (Kalkhoven, Wissink et al. 1996). Studies also showed that ligand-activated PR modulates NF- κ B activity by tethering to promoters of other genes (Kobayashi, Stice et al. 2010).

To explain secretion of proinflammatory factors and SASP in PRB treated cells, we hypothesized that NF- κ B pathway might play a significant role in R5020-induced response. The induction of cytokines such as *IL-1 α* and *IL-1 β* by R5020 could be the result of the activation of NF- κ B pathway. P65 and I κ B α proteins are central mediators of NF- κ B pathway (Brasier 2006). When unstimulated, NF- κ B proteins are sequestered in the cytoplasm by I κ B α which masks the nuclear localization signal of NF- κ B. NF- κ B activity is induced when I κ B α is phosphorylated by I κ B α kinase IKK, which leads to I κ B α degradation via proteasome pathway. IKK α phosphorylation of p65 at S536 site allows nuclear translocation and activation of NF- κ B (Kisselev and Goldberg 2001, Goldberg and Rock 2002, Christian, Smith et al. 2016). The results in this study revealed that p65 is phosphorylated 24 and 96h hours after R5020 treatment (Figure 3.20a). There is also a trend of increase of p65phospho/p65 ratio at both time points, suggesting p65 activation. Future study using immunostaining will confirm the nuclear translocation of p65. Consistent with p65 activation is the increased p-I κ B α phosphorylation at both 24 and 96 hours' time points but R5020 also stimulated an increase of I κ B α protein at 24h, complicating the interpretation of the results. This evident increase of I κ B α protein at early time indicates a direct effect of R5020 and may partly contribute to the decrease of *IL-8* and *ICAM1* at 24 hours by partly interfering with the activation of p65.

Taking all data together, we propose that *IL-1 α* and *IL-1 β* genes are directly induced by PR-mediated effect of R5020 at 24 hours. At the same time, R5020-induced expression of I κ B α exerts inhibitory effect on p65, resulting in a moderate decrease of the expression of *IL-8* and

ICAM1. On the other hand, rising levels of these two cytokines exert paracrine effect on p65 by phosphorylating I κ B α (Reber, Vermeulen et al. 2009), leading to a greater activation of p65. This results in the up-regulation of *IL-8* and *ICAM1* and even the greater upregulation of *IL-1 α* and *IL-1 β* 96 hours post R5020 treatment. Future study will verify if *IL-1 α* and *IL-1 β* are direct target genes of PR.

Cell-cell and cell-ECM adhesions are mediated by many proteins, including E-cadherin, N-cadherin, claudins, nectins (Nelson 2008). E-cadherin is an important cell adhesion molecule, and modification of this protein during cancer progression plays a crucial role in the development of metastatic and invasive properties of cancer (Baranwal and Alahari 2009). Anoikis is an apoptotic cell death in detached cell, caused by disruption of cell-ECM interaction (Frisch and Francis 1994). Our studies revealed different response of stably transfected MCF-7 cells to R5020 treatment in 2D and 3D culture. In 2D culture cell growth was inhibited in MCF-7-PRB 96 hours after R5020 treatment, but indicators of apoptosis process were not observed. On the other hand, in 3D culture MCF-7-PRB R5020-treated cells were dying progressively and were not able to form acini in Matrigel. We think this is due to the downregulation of E-cadherin which impairs cell-cell contact in 3D culture to a greater extent than in 2D culture. Furthermore, E-cadherin could be direct target of PRB/R5020 response in MCF-7 cells overexpressing PRB because the induction of E-cadherin mRNA was apparent 6 hours post treatment.

3.2.3 R5020 up-regulated the expression of LC3B and autophagy

Previous studies have reported that progestin stimulates the autophagic process in breast cancer cells MCF-7 and T47D (De Amicis, Guido et al. 2014). Different studies reported different mechanisms. In MCF-7 cells, progestin enhanced the expression of tumor suppressor gene PTEN through direct interaction with its enhancer. This resulted in the suppression of PI3K/AKT pathway and enhanced expression of autophagic activator UVRAG (De Amicis, Guido et al. 2014). In another study, progesterone induced autophagy in both MCF-7 and T47D cells (De Amicis, Guido et al. 2016). This was associated with an increase of beclin (a master autophagic regulator) expression and phosphorylation of BCL-XL. Beclin is known to be associated with BCL-CL through its BH3-like domain and this results in the inactivation of beclin (Maiuri, Le Toumelin et al. 2007, Oberstein, Jeffrey et al. 2007). BCL-XL phosphorylation results in the dissociation of beclin 1 and its activation. In our study, we found that R5020 induces the

expression of LC3B at 24 hours and even more so at 96 hours. This leads to the increase of both LC3B-I and LC3B-II proteins. Based on classical definition of autophagy activation, the ratio of LC3B-II and LC3B-I is supposed to be increased. However, we observed a decrease of LC3B-II/LC3B-I ratio at both time points. This is likely due to a greater increase of LC3B-I level as a result of increased gene expression of LC3B. Therefore, R5020 did stimulate autophagy by increasing both the total protein level of LC3B and its lipidation. This is supported by the autophagic flux assay in which inhibition with lysosomal inhibitors resulted in an accumulation of LC3B-II. Akt phosphorylation is known to inhibit autophagy (Degtyarev, De Mazière et al. 2008). Our data regarding Akt activation is, again, complex. On the one hand, there is an increased Akt phosphorylation 24 and 96 hours after treatment with R5020. On the other hand, there is an obvious down regulation of total Akt proteins at 96h when the increase of both LC3B-I and LC3B-II form is prominent. Further studies are required to clarify this contradictory phenomenon.

Taken together, our study showed that progestin is able to trigger autophagy at the transcription level by inducing LC3B gene expression. It is not clear whether this transcriptional regulation is a direct effect of PRB or indirect effect mediated by other transcription factors.

Taken all data together we propose signaling pathway for the effect of R5020 in PRB overexpressing MCF-7 cells after treatment with R5020 (Figure 3.29).

Figure 3.29 Schematic representation of signaling pathways for the effect of R5020 in luminal breast cancer MCF-7 overexpressing PRB cells.

R5020 caused irreversible cell cycle arrest and cell senescence in cells with abundant PRB. This can be concluded by induction of p53 and p21, reductions of cell cycle regulators (cyclins and CDKs), the increase of β-Gal staining and induction of SASP as well as activation of the NF-κB signaling components and the autophagic regulators.

Chapter 4. Conclusions

4 Conclusions

Progestin is known to be involved in the regulation of normal breast and breast cancer development but its precise role has been controversial. The controversy reflects the complexity of PR biology. MCF-7 cells are the most commonly used model for the study of the biology of ER- and PR-positive breast cancers, but the lack of PR expression in the absence of estrogen makes it difficult to define the function of PR. This study used PRB overexpressing MCF-7 cells to elucidate the biology of PR and revealed three important findings. First, long term progestin treatment (beyond 96h) caused irreversible cell cycle arrest and cell senescence in cells with abundant PRB. This can be concluded by many markers we studied, including the induction of p53 and p21, reductions of many cell cycle regulators (cyclins A1, B1, D1, CDK2 and CDK4), the increase of β -Gal staining and induction of SASP (increased expression of cytokines such as IL- α , IL-1 β , IL-8 and ICAM1). Second, with PRB overexpression, progestin could completely abolish the effect of estrogen. This may be related to the down-regulation of ER but could also be due to the functional interaction with ER. This suggests an interesting therapeutic opportunity for ER and PR positive breast cancers with high levels of PR. Third, the biphasic effect of R5020 suggests that the growth-inhibitory effect of R5020 is mediated through indirect effect of PRB and cross talk with other signalling molecules in the cell. This is supported by the biphasic regulation of many molecules, including the cell cycle regulator, the NF- κ B signalling components and the autophagic regulators. It is plausible that the induction of p53 and p21, and reductions of many cell cycle regulators (cyclins A1, B1, D1, CDK2 and CDK4), as well as the induction of SASP (increased expression of cytokines such as IL- α , IL-1 β , IL-8 and ICAM1) supported R5020-induced irreversible cell cycle arrest and cell senescence phenotype. The better understanding of the link between the primary effect and the secondary effect of R5020 would be useful in explaining the conflicting reports of progestin effects in breast cancer cells.

Chapter 5. Future work

5 Future Work

5.1 Verification of the discrepancies between the 2 MCF-7-PRB clones

Our findings revealed that long term exposure to R5020 inhibited the growth of MCF-7 cells overexpressing PRB. However, we had observed slight variations in some of the proteins and genes expression profiles between the two MCF-7-PRB clones studied in this thesis. This finding implicates the possibility of clonal differences complicating the elucidation of the mechanism behind PRB-mediated growth inhibition by R5020. This could be due the variation in the properties of different clones. Therefore, we propose to validate these discrepancies using a mixed population of MCF-7 cells overexpressed PRB via a lentiviral transduction delivery system that we have recently established.

5.2 Implications of R5020-induced G2/M cell cycle arrest

We had demonstrated that the extended treatment with R5020 induced accumulation of MCF-7-PRB cells in the G2/M phase of the cell cycle, as well as a reduction in the proportion of cells in the S phase fraction. Arrest in G2/M has been demonstrated to increase the sensitivity of cancer cells towards autophagy or apoptosis (Strasser-Wozak, Hartmann et al. 1998, Weir, Selvendiran et al. 2007, Wang, Zhang et al. 2016). Based on our data, we infer that R5020 could potentially enhance the sensitivity of MCF-7 towards chemotherapeutic drugs, which could promote the cell death response in breast cancer cells. In order to investigate this possibility, we sought to treat MCF-7-PRB cells with different doses and types of chemotherapeutic drugs and investigate their death responses.

We showed that incorporation of BrdU was reduced in MCF-7-PRB cells treated with R5020. We can use this technique to define the time point when cells are entering the cell-arrested stage by collecting cells every 6 hours post-treatment with R5020. We can use BrdU for immunostaining assay together with Ki-67 protein. The Ki-67 protein is expressed in all phases of the cell cycle, including mitosis, but is absent from resting cells (G0), and this feature makes this protein a useful biomarker for determining the specific phase of cell cycle in a given cell population. To evaluate cyclins, CDK and CDKs inhibitors interaction in the regulation of the cell growth in MCF-7-PRB cell line, we propose to perform co-immunoprecipitation of cell

lysates from MCF-7 stably transfected clones using anti-CDK and anti-cyclin antibodies and to test presence of CDK inhibitor p21 in these complexes 24 and 96 hours after treatment.

It has been reported that cells 'slip' to the G1 phase of cell cycle after mitosis, although chromosome segregation and cytokinesis processes were not conclusive. These slippage can induce cell death or cell cycle arrest in G1 phase of cell cycle (Cheng and Crasta 2017). The mitotic slippage leads to polyploidy and increased number of micronuclei (Elhajouji, Cunha et al. 1998). While performing immunostaining experiments we have noticed an increase in number of micronuclei in R5020 treated MCF-7-PRB cells, compared to control. Therefore, we propose quantification of micronuclei and further investigation if this phenomena could be a part of mitotic slippage by technique described in the report (Fenech 2000).

Our preliminary results with Ad-PRA transduced MCF-7-PRB cells revealed that altered PRA:PRB expression did not change R5020-induced response of the MCF-7-PRB cells. Accordingly, our next goal is to establish PRA overexpressing MCF-7 cell line and to evaluate the effect of R5020 on this cell line. This study will conclude if there are differences in PRB and PRA activity in different breast cancer cell lines, as it has been proposed by other studies (Graham and Clarke 2002).

5.3 Implications of R5020-induced SASP

Senescent cells establish SASP through secretion of chemokines, growth factors and chromatin remodeling factors (Coppé, Patil et al. 2008) and induce malignant phenotypes in neighboring cells (Krtolica, Parrinello et al. 2001, Campisi 2005).

Secreted factors, such as IL-1 α and IL-1 β , are potent proinflammatory cytokines that enhance cell migration under inflammatory conditions (Lin, Kuo et al. 2009, Rider, Carmi et al. 2011). Study has demonstrated that secreted IL-1 β , and to a lesser extent IL-1 α , are required for *in vivo* angiogenesis and invasiveness of different tumor cells (Voronov, Shouval et al. 2003). The induction of gene expression of proinflammatory cytokines, including IL-1 α , IL-1 β and IL8, in R5020-treated MCF-7-PRB cells in our study suggests that MCF-7-PRB cells display SASP. It would be interesting to investigate how the SASP in R5020-treated MCF-7-PRB affects the microenvironment and the nearby cells. Wound healing assay, transwell migration and invasion assay using immune cells and tumor cells in response to conditioned media of R5020-treated MCF-7-PRB cells will help us to address the function of SASP of these cells.

5.4 Role of p53 in R5020-induced senescence

The regulation of p53 protein by R5020 treatment in MCF-7-PRB cells demonstrated in our study suggests that p53/21 pathway plays a role in R5020-induced senescence. We hypothesize that p53 is the critical molecule that governs the senescence induced by R5020 in MCF-7-PRB cells. We will test our hypothesis by knocking down p53 or use viral onco-protein E6 to interfere with p53 function in these cells. In addition, p53 has been reported to be anchored in cytoplasm by cytoskeletal proteins in senescent cells and this anchorage in cytoplasm might prevent p53-induced apoptosis (Nishio and Inoue 2005). Examination of p53 subcellular localization by using PAb240 antibody that specifically recognizes a conformation variant will help us to understand how PRB affects the distribution and function of p53.

5.5 Mechanism of R5020-induced autophagy

Autophagy can both activate and inhibit senescence. Recently, it has been identified as a new effector mechanism for senescence (Young, Narita et al. 2009, Glick, Barth et al. 2010, De Amicis, Guido et al. 2016). Based on current data, we postulate that autophagy could mediate senescence induced by R5020 by driving cells from proliferating to arresting stage. To test this hypothesis, we will examine if the blockage of autophagy process at early stage using inhibitor 3-methyladenine (3-MA) affects the transition from active to senescent stage in R5020-treated MCF-7-PRB.

Although we demonstrated an induction of autophagy by R5020, the mechanism of autophagy is yet to be investigated. Mammalian target of rapamycin (mTOR) is very well defined in autophagy process, and mTOR expression is regulated by the PI3K/AKT pathway (Jung, Ro et al. 2010). Investigation of mTOR pathway might give us valuable insights of how R5020 and PRB regulate autophagy machinery in luminal breast cancer cells. Autophagy was reportedly mediated by PRB induction of PTEN and the subsequent inhibition of AKT activation (De Amicis, Guido et al. 2014). Thus, study of R5020-regulated PTEN expression might give us insightful information how Akt is regulating autophagy process in our stably transfected cell line.

Chapter 6. References

6 References

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