INVESTIGATION OF NOVEL RNAi AND NANOPARTICLE APPROACHES FOR THEIR ANTI-PROLIFERATIVE AND DRUG-SENSITIZING EFFECTS IN BREAST CANCER

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By
Ermira Jahja
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By Ermira Jahja

August 2017

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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Abstract

INVESTIGATION OF NOVEL RNAi AND NANOPARTICLE APPROACHES FOR THEIR ANTI-PROLIFERATIVE AND DRUG-SENSITIZING EFFECTS IN BREAST CANCER

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Ph.D. in Molecular Biology and Genetics

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August 2017

Drug resistivity remains a major challenge in treating different cancer types. Among several strategies adapted to increase drug sensitivity in breast cancer cells, in the present thesis I studied an RNAi molecule targeting cholinergic receptor nicotinic alpha 5 subunit (CHRNA5) and a red-emitting oligomer nanoparticle, the two agents which I experimentally identified as negative regulators of cell proliferation.

Cholinergic signaling is implicated in several different pathologies including cancer. Nicotinic acetylcholine receptors (nAChRs) are shown to be involved in regulation of cell proliferation, however they are mainly studied as mediators of nicotinic activity. CHRNA5 subunit has been shown to have roles in acetylcholine (ACh) production/stability, drug addiction and susceptibility to lung cancer. Few studies of lung and gastric cancers as well as high throughput RNAi screens show CHRNA5 as a modulator of cell proliferation. In the present study multiple CHRNA5 isoforms were cloned from MCF7 breast cancer cells (ER positive, TP53 positive) as in the case of lung cancer; moreover, a significant antimitotic effect of CHRNA5 RNAi application was demonstrated in MCF7 breast cancer cells. Similar effect of CHRNA5 silencing was only partially observed in BT-20 and MDA-MB-231 cells (ER negative, P53 mutant), yet in a seeding density-dependent manner. For the first time in literature the transcriptomic changes associated with CHRNA5 RNAi in the MCF7 cells were studied by microarrays from which differentially expressed gene lists were used to obtain the affected pathways. Additional assays confirmed the

reduction in cell viability, DNA synthesis, G1 growth arrest, and changes in cytoskeleton complementing the microarray studies. Use of camptothecin (CPT) and doxorubicin (DOXO) in the absence or presence of CHRNA5 siRNA in MCF7, led to identification of CHRNA5's role in drug sensitivity. Comparisons between CHRNA5 siRNA and public microarray datasets revealed common genes/networks between topoisomerase (TOPO)/cyclin-dependent kinase (CDK) inhibitors and CHRNA5 depletion profile in MCF7 cells. mRNA-miRNA network analysis of differentially expressed common gene sets between TOPO inhibitors and CHRNA5 RNAi treatment identified potential common regulatory miRNAs.

In an independent study the anti-cancer as well as drug sensitivity associated effects of a novel CB7-capped, red-emitting conjugated oligomer nanoparticle (Red-CON) were characterized in MCF7 and MDA-MB-231 cells. Red-CON in its encapsulated form exhibited low toxicity and good efficacy as a drug delivery system. This nanoparticle formulation might serve well for future clinical and less toxic chemotherapeutic regimens.

Keywords: Breast cancer, CHRNA5, proliferation, motility, drug resistance, nanoparticle.

Özet

MEME KANSERİNDE ÖZGÜN RNAi VE NANOPARTİKÜL YAKLAŞIMLARININ ANTİ-PROLİFERATİF VE İLAÇ-HASSASİYETİNE YÖNELİK ETKİLERİNİN İNCELENMESİ

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Kanser tedavisinde ilaç özdirenci önemli bir sorundur. Bu tez kapsamında meme kanserinde ilaç hassasiyetini arttırmak için uyarlanmış bazı stratejilerin arasından, deneysel olarak hücre proliferasyonunun negatif regülatörü olduğunu bulguladığım iki molekülü, kolinerjik reseptör nikotinik alfa 5 altbirimini (CHRNA5) hedef alan RNAi ve kırmızı ışık yayan oligomer nanopartiküllerini, meme kanser hücrelerinde çalıştım.

Kolinerjik sinyalizasyon kanser dahil birçok değişik patolojide önemli yer tutar. Nikotinik asetilkolin receptörleri (nAChRs), hücre proliferasyonun regülasyonundaki rolleri gösterilmiş olmakla birlikte genelde, nikotinik aktivitenin aracıları olarak görülmüştür. CHRNA5 altbiriminin asetilkolin (ACH) üretimi/stabilitesi, ilaç bağımlılığı ve akciğer kanserinin duyarlılığında rol oynadığını gösterilmiştir. Daha önce akciğer ve gastrik kanserler üzerinde yapılan bazı çalışmalarda, ve yüksek çıktılı RNAi verileri ile, CHRNA5'in hücre proliferasyonunun da modülatörü olduğu gösterilmiştir. Bu tez çalışmasında ise, akciğer kanserinde olduğu gibi, MCF7 (ER pozitif, P53 positif) meme kanser hücrelerinden de birden fazla CHRNA5 izoformu klonlanmış olup MCF7 meme kanser hücrelerinde CHRNA5 RNAi uygulamasının antimitotik etki gösterdiği bulgulanmıştır. CHRNA RNAi'ın etkisi, BT-20 ve MDA-MB-231 (ER negatif, P53 mutant) hücrelerinde ise hücre ekim yoğunluğuna bağlı olarak kısmen görülmüştür. Literaturde ilk defa bu tez çalışması ile, MCF7 hücrelerinde CHRNA5 RNAi ile ilgili transkriptomik değişikliklerin profili cizilmiş, farklı ifade edilen genlerin listesi ve etkilenen yollaklar belirlenmiştir. Ek tahliller,

hücre yaşayabilirliği ve DNA sentezinde azaltma ve G1'de hücre siklusunda tıkanmanın yanısıra hücre iskeletinde de değişiklikler olduğunu göstermiştir. Kamptotesin (CPT) ve doxorubisin (DOXO), CHRNA5 siRNA'nın varlığı ya da yokluğunda MCF7 hücrelerinde çalışılmış olup bulgular, CHRNA5'in ilaç hassasiyetindeki rölünü göstermektedir. CHRNA5 siRNA profilinin halka açık microarray verileri ile karşılaştırılması sonucunda topoisomeraz (TOPO)/CDK inhibitor ve CHRNA5 RNAi profilleri arasındaki anlamlı ortak genler ve gen ağları ortaya çıkarılmıştır. TOPO inhibitörü ilaçlar ve CHRNA RNAi uygulamasında ortak etkilenen genlerin mRNA-miRNA ağ analizi ise potansiyel ortak regülatör miRNA'ların tespit edilmesine yol açmıştır.

Bağımsız bir diğer çalışmada yeni CB7-ekli, kırmızı ışık yayan konjuge oligomer nanopartikül (Red-CON), MCF7 ve MDA-MB-231 hücrelerinde, anti-kanser ve ilaç duyarlılığı açısından karakterize edilmiştir. Red-CON kapsüllenmiş formunda kullanıldığında ilaç dağıtma sistemi olarak düşük toksisite ve iyi etkinlik göstermiştir. Bu nanopartikül formulasyonun, gelecekteki klinik ve toksiksistesi düşük kemoterapi rejimlerinde faydalı olabileceği düşünülmektedir.

Anahtar sözcükler: Meme kanseri, CHRNA5, proliferasyon, motilite, ilaç direnci, nanopartikül

To my family …

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Abbreviations

CHRNA5 Cholinergic receptor nicotinic apha 5
TOPO Topoisomerase
DOXO Doxorubicin
CPT Camptothecin
NP Nanoparticle
CON conjugated oligomer-based nanoparticles
CB7 Cucurbituril 7
ER Estrogen receptor
SCC Squamous cell carcinoma
SERD Selective estrogen receptor downregulators
ERP Enhanced Retention and Permeability
NMJ Neuromuscular junction
SSB Single strand break
ACh Acetylcholine
ChAT Choline acetyltransferase
AChE Acetylcholinesterase
nAChR Nicotinic acetylcholine receptor
PR Progresterone receptor
CNS Central nervous system
RMA Robust multi-array average
TNBC Tripple negative breast cancer
ECM Extracellular matrix
HER2 Human epidermal growth factor receptor 2
TR Transfection reagent

Chapter 1

Introduction

1.1 Breast cancer treatment and resistance

1.1.1 General background on breast cancer treatment

Breast cancer therapies have undergone revolutionary progress, especially during the last twenty years, due to better understanding of breast cancer biology. The work of Halsted and colleagues [1] has shown that breast cancer is a disease which can result in metastasis through the lymphatic system. This implied the necessity of breast cancer surgery and radiation therapy as curative means. Another evolutionary finding was by Sir George Beatson who has shown that many breast cancers in premenopausal women could regress if ovaries were removed [2]. Discovery of estrogen receptor (ER) in 1960 was followed by the finding that ER could be measured and therapeutic response could be predicted [3]. Thus numerous ER or estrogen targets were subsequently developed. The first selective ER targeting agent was Tamoxifen; the first molecularly targeting drug in oncology. However, it was found that not all ER-positive breast cancers responded to endocrine manipulation while ER-negative tumors were totally out of the tamoxifen spectrum [4]. Human epidermal growth factor receptor 2 (HER2) over-expression (major cause of breast cancer patient mortality) on the other side was followed by the production trastuzumab (humanized monoclonal antibody), development of other monoclonal antibodies and small molecule receptor kinase inhibitors, all of which targeted HER2 protein [5]. The finding that some breast cancer patients lacked both PR, ER expression and HER2 overexpression (nowadays defined as “triple-negative”), made breast cancer research focus on the treatment of patients not benefiting from either of the therapies. These cancers were characterized by high proliferation, poor prognosis, low differentiation and high metastatic potential, leading to therapeutic uncertainity [6].

Apart from therapeutic approaches following the ER and HER2 status of breast tumors, there are other means of cancer treatment which focus on targeting the general hallmarks of cancer biology, such as angiogenesis; defined as new blood vessel formation within/around tumor mass. Bevacizumab (a humanized monoclonal antibody), targets VEGF/VEGFR entity as a major player in vascularization however, still only a subgroup of breast cancer population benefit from this treatment. Yet it is not possible to determine which group will benefit or not from this drug, making it a non-targeted therapy [7].

Current treatment of breast cancer depends on its subtype, based on the oncogenic signaling pathway that derives it. Surgery, radiotherapy, hormonal therapy and chemotherapy are the current options for breast cancer treatment, separately or in combination [8]. The current chemotherapeutic agents for breast cancer include taxanes (i.e., docetaxel, paclitaxel), anthracyclines (i.e., doxorubicin, epirubicin) [9], anti-metabolites (i.e., capecitabine, gemcitabine) [10], microtubule inhibitors and/or stabilizers (i.e., vinorelbine, ixabepilone) [11] and platinum-based antineoplastic drugs (i.e., cisplatin, carboplatin) [12].

Contemporary breast cancer research has improved our understanding of breast cancer as being not a single disease; rather a mixture of diseases with heterogeneous histology. Thus breast cancer outcomes have improved due to treatments which include improved old and new approaches. Nowadays most oncologists focus on personalized therapy by providing the right treatment, at the right time, to the right tumor type. Moreover, new therapeutic ways of attacking cancer cells more precisely, use drug combinations (sometimes included in one formulation), with less side effects to normal tissues [13].

1.1.1.1 Treatment of estrogen receptor (ER)-positive patients

ER-positivity includes the most prevalent invasive type of breast cancers, showing variable clinical outcomes and responses to anticancer therapy [14]. The current standard treatments of ER+ breast cancers are based on using aromatase inhibitors, selective estrogen receptor downregulators (SERDs) and tamoxifen [15]. Aromatase inhibitors (such as anastrozole and letrozole) are drugs which lower the estrogen

production by targeting the aromatase enzyme that produces it. SERDs are agents that inhibit ERapha expression, while tamoxifen is an ER modulator which antagonizes the receptor function in breast tissue [15]. Luteinizing hormone-releasing hormone (LHRH) agonists, as chemicals which prevent ovaries to produce estrogen and progesterone, are important for progesterone receptor (PR)-positive breast cancer therapy [16]. All of the above mentioned drugs enter in the class of 'endocrine/hormonal therapy'. However, endocrine therapy is characterized more by inducing a reversible dormant state to breast cancer cells rather than causing cell death [17].

1.1.1.2 Treatment of human epidermal growth factor 2 receptor (HER2)-positive patients

HER2 positivity cover 15-20% of early stage breast cancer patients, and is associated with increased tumor aggressiveness, low disease-free and overall survival, as well as increased breast metastasis rates [18]. Current treatment of HER2-positive breast cancer patients include small molecule inhibitors, monoclonal antibodies and antibody drug conjugates. In addition, combinational therapy shows better disease outcomes when compared to monotherapies. For instance, the addition of trastuzumab to neoadjuvant (pre-operative) chemotherapy doubles the probability of obtaining a complete pathological response and reduces the relapse risk [19]. Administration of aromatase inhibitors in combination to HER2-based therapy significantly improves treatment outcome in hormone-receptor positive women. Anti-HER2 vaccination is also a developing approach of assisting HER2-overexpressing tumors, with likeliness to improve treatment and avoid the side effects of current therapies [20]. Systemic chemotherapy on the other side, is applied for women having higher proliferative tumors [21].

1.1.1.3 Treatment of triple negative breast cancer (TNBC) patients

TNBC includes 10-20% of invasive breast cancers. It encompasses different subtypes; basal-like, normal breast-like, claudin-low, BRCA1-deficient breast tumors, all of which are more frequently seen in younger African-American women [22]. Due to the high heterogeneity of TNBC and to the absence of ER, PR

expression and HER2 amplification, there has been no targeted therapy available for this subgroup of patients, which caused an increasing demand for personalized therapy. Currently, chemotherapy is the only approved treatment for TNBCs, which serve solely for pain relief and prolonging survival. It includes anthracyclines, taxanes, anti-metabolites and microtubule inhibitors/stabilizers, as single-agent regiments or combined therapy [23]. Retrospective studies have shown that TNBCs are more sensitive and responsive to chemotherapeutic agents (probably due to their high proliferation rate) and increased neoadjuvant response as compared to other tumors [24]. Similar to other breast cancer subtypes, Doxorubicin- and Paclitaxel-based regiments are the standard treatments for TNBCs [25]. They are administered either alone or in a combinatorial way (especially when quick response is necessary). TNBCs, which are highly associated with BRCA-1 gene mutations, are quite sensitive to platinum agents as well [25]. Recent research on TNBC treatment includes a large phase III clinical study of targeted therapy using Bevacizumab (anti-angiogenic agent against VEGF), which might be given in the future as an adjuvant in addition to chemotherapy [26]. Vaccines (as a means of immunotherapy) are also under investigation for treating metastatic TNBCs [23].

1.1.2 Drug resistance in breast cancer patients

1.1.2.1 Molecular mechanisms of drug resistance

Development of cancer cell resistance toward anticancer agents is a major challenge in cancer treatment. Cancers may consist of cells with stem cell-like properties and/or with intrinsic ability to resist therapies [27] due to: loss of target protein expression after continuous therapy [28], induction of G0 phase [29], overexpression of some proteins related to drug efflux and metabolism [30], upregulation of some pro-apoptotic genes, activation of prosurvival signaling [31], and regulation of DNA repair mechanisms [32]. Moreover, these stem cells are suggested to posses self-renewal and tumor initiation abilities, which make cancer treatment even more difficult. Due to molecular heterogeneity of tumors, drug resistance can also arise from a small population of cells acquiring resistance as a selection mechanism [33]. In addition to tumor-derived cells, tumor microenvironment (consisting of ECM,

fibroblasts, immune cells and blood vessels in solid tumors) might also contribute to drug resistance by providing growth, resistance and refuge to metastatic cancer cells [34]. More interestingly, same microenvironment might render the tumor cells more responsive to other agents (named “synthetic lethality”), which seems to be cancer- and therapy type-specific [35].

1.1.2.2 Breast cancer subtype-specific drug resistance

Drug resistance in advanced breast cancer is the cause of incurability, however, early-stage breast cancer resistance is of great concern due to the possibility of being cured. For the ER+ breast cancers, endocrine therapy resistance is categorized either as intrinsic (not showing any response to endocrine therapy) or acquired (developing resistance after a response phase) [36]. Resistance to hormonal therapy seems to be agent-specific; after resistance developed from one drug, another endocrine therapy approach might show effectiveness [37]. Mechanisms of drug resistance in HER2+ patients on the other hand, involve absence of the extracellular binding domain [38], loss of HER2 amplification during treatment [39], activation of other HER-family receptors as a compensatory mechanism [40], and deregulation of several signaling pathways [41] . Combining of HER2 inhibitors (which show synergistic effects) is a strategy followed in order to overcome drug resistance [42].

1.1.3 Topoisomerase enzymes as anti-cancer drug targeting

1.1.3.1 Topoisomerase mode of action

During DNA replication and transcription process, double helix DNA needs to be uncoiled however, its structure restricts DNA of a free rotation inside cell nucleus. Thus, strand separation by helicase enzymes induces the formation of positive supercoiling at the front side and negative supercoiling at the back side of DNA replication and transcription [43]. The positive supercoiling (if not processed), stalls DNA replication and transcription progress, while supercoiling at the back side might induce development of abnormal DNA structure, which might be an obstacle for normal DNA functioning [44]. During normal DNA replication and transcription

process, DNA tension is avoided with the help of large proteins/enzymes named as ‘topoisomerases’ [45].

Human genome encodes six topoisomerases, which are classified either as Type I and Type II. During their catalytic functions, Type I enzymes make a single cleavage in one of the DNA strands, while type II topoisomerases cleave both strands [46]. Both enzyme groups similarly perform DNA cut by a nucleophilic attack and subsequent binding of their catalytic residue to the phosphate end of the phosphodiester backbone takes place [44]. After the relief of supercoiling by topoisomerization, DNA is religated and its sequence if left unchanged. One of the topoisomerase subtypes;TOP1mt, shows similar activity to topoisomerase 1 in uncoiling of the mitochondrial DNA [47].

1.1.3.2 Topoisomerase inhibitors

Topoisomerases are further categorized into subtypes according to their mode of activity. Apart from their role in DNA relaxation during transcription and translation, they also show some other functions (i.e., as splicing factors) [48, 49]. Except topoisomerase IA and IIB, other subtypes are already clinical therapeutic targets [44].

Topoisomerase (TOPO) inhibitors of the mammalian enzymes bind to DNA-topoisomerase complex and block the DNA religation step of the enzyme-catalytic action, resulting in single or double strand breaks [50] (Figure 1.1).

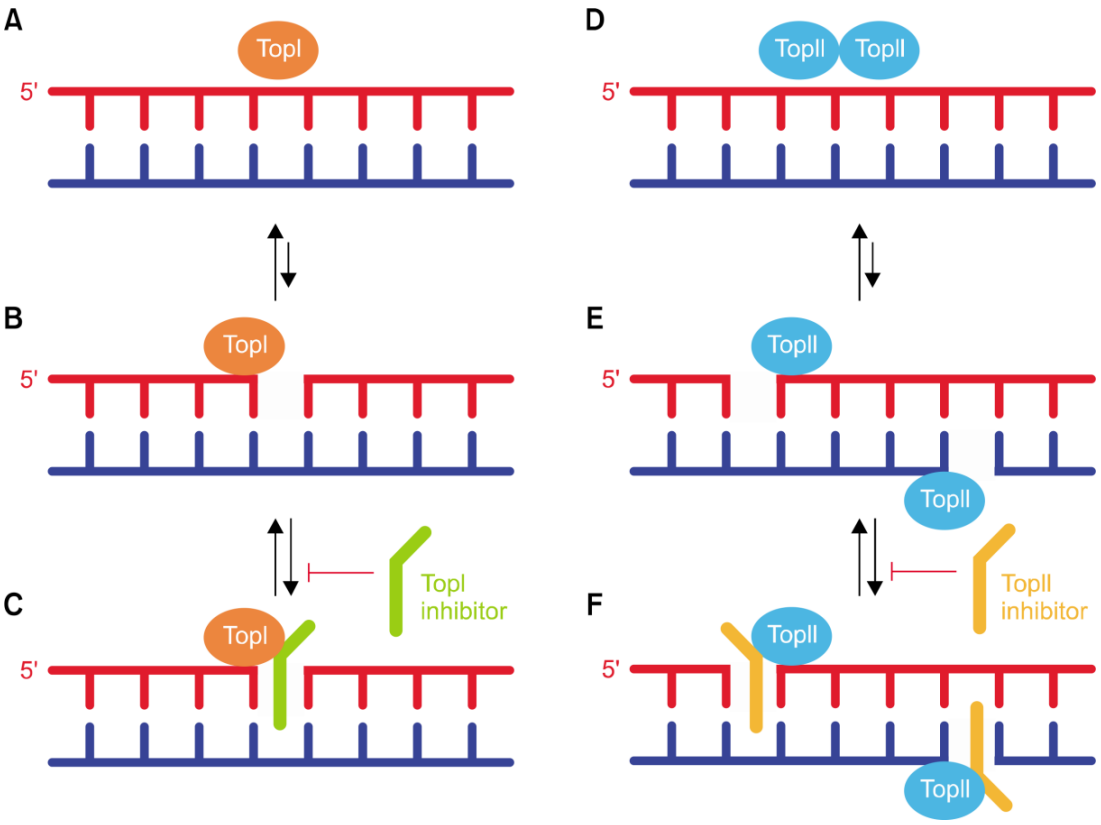

Figure 1.1: Activity of topoisomerase enzymes and their inhibitors.

Schematic representation of topoisomerase enzymes and TOPO inhibitors of class I and II. Arrows indicate the reversible processes of DNA cleavage and relegation during normal enzyme activity. DNA damage is induced when **A-C)** TOPO I inhibotors bind to topoisomerase I and **D-F)** TOPO II ihibitors bind to topoisomerase II enzymes. This figure was reprinted with permission from ScienceCentral. Young Ho Seo, Dual Inhibitors Against Topoisomerases and Histone Deacetylases, (2015) Journal of Cancer Prevention, https://www.e-sciencecentral.org/articles/SC000010991.

1.1.3.3 Topoisomerase I inhibitors

Topoisomerase 1 inhibitors include the camptothecin (CPT) and non-camptothecin types of drugs. CPT was first discovered by M. E. Wall and M. C. Wani (1966) from the wood bark of the Chinese tree Camptotheca acuminate, in an attempt to find anticancer drugs by screening of the natural products [51]. Few of the camptothecin derivatives are already approved for clinical use, i.e., topotecan and irinotecan [52]. Although having a wide range of application as anticancer drugs, CPT derivatives show limitations in usage due to their chemical instability at physiological pH, caused by the E-ring presence in their molecular structure [52]. Additional CPT derivatives have been designed to improve the clinical tolerability however could not overcome the chemical instability of camptothecins [47]. Non-camptothecin synthetic topoisomerase I drugs such as indenoisoquinolines, dibenzonaphthyridinones and

aromathecins have recently been developed to improve drug stability and to form more stable cleavage complexes [53, 54].

1.1.3.4 Topoisomerase II inhibitors

Topoisomerase II inhibitors poison Top2α and Top2β after which a TOP2-DNA cleavable complex is formed, named as 'TOP2cc', which is considered as a barrier to the normal progression of replication and transcription process [55]. TOPII inhibitors not only cause DNA double strand breaks (DSB), but also single strand breaks (SSB) [56]. They are classified into two major groups. The first group includes the most clinically used 'poisons' which elevate the level of TOP2-DNA covalent bond, such etoposide, doxorubicin and mitoxantrone [57]. The second class consists of 'catalytic inhibitors' which block the catalytic activity of TOPII but do not increase the TOP2-DNA complex levels, such as bisdioxopiperazines [57]. Doxorubicin, a TOP2 poison, was firstly isolated from Streptomyces peucetius species [58]. It is an anthracycline antibiotic and at the same time widely used for many kind of malignancies like childhood solid tumors, breast cancer and leukemias. However it came up as being toxic also to normal cells due to the production of reactive oxygen species (ROS), and causing of cardiotoxicity [59].

1.1.4 Camptothecin and doxorubicin in breast cancer research and treatment

1.1.4.1 Camptothecin mode of action in breast cancer cell lines

Chemotherapy is the dominant approach in treatment of cancer and usage of conventional drugs/their derivatives is still the most used medication means. In breast cancer therapy related research, there are several studies showing camptothecin and doxorubicin effects on breast cancer cell lines. A 2005 study conducted by Lamparska et al., showed that camptothecin exposure to MCF7 cells resulted in apoptosis within a short time period (60min) while in the long run of 24h drug administration, continuous development of autophagy occurred at a slower rate [60]. Moreover, BID knockdown in CPT treated MCF7 cells, induced a shift from apoptosis to autophagy, suggesting that BID might serve as a molecular switch in the way of cell decision between the two

cell death mechanisms [60]. In another study conducted by the same research group, electron microscopy imaging of 6h CPT administration in MCF7 cells revealed a heterogeneous population of cells having both apoptotic- and autophagy- related morphological features [61]. MCF7 cells treated 8-16h by 0.15 uM CPT accumulated p53 protein in the nuclei of cells, with a rapid increase of ~20 fold in S-phase cells [62]. In another study, characterization of apoptotic response to CPT of several breast cancer cell lines revealed that MCF7 (p53 wild type) was among the resistant ones, indicating that p53 might not be required for CPT-induced apoptosis [63]. P53-independent effect of CPT was also observed in multiple breast cancer cell lines in which CPT induced degradation of WRN, a helicase enzyme having role in DNA repair, genome stability and cellular senescence [64].

1.1.4.2 Doxorubicin mode of action in breast cancer cell lines

Similar to CPT, doxorubicin (DOXO) studies show not a single mechanism of breast cancer cell death induced by this drug. Doxorubicin-resistant MDA-MB-231 cell line, when exposed to an autophagy (shown to negatively associate with drug resistance) inhibitory molecule, became more sensitive to doxorubicin treatment and the cell death mode shifted from apoptosis to necrosis [65]. Doxorubicin resistance of MDA-MB-231 cell line has been linked to NF-kB expression which is also a metastasis inducer [66]. Restoration of p53 in this cell line showed impairment of NF-kB expression induced by doxorubicin, implying p53-dependent cytotoxicity of this drug [66]. On the other hand, MCF7 cells treated with doxorubicin showed upregulation of p21 level and increases in cellular senescence [67]. However, another study conducted in both MCF7 and MDA-MB-231 cell lines showed p53-status independent effect of doxorubicin in inducing apoptosis [68]. Doxorubicin uptake and drug response in MCF7 cells was correlated with cellular fluidity [69] as well as with diverse drug formulations to improve its biopharmaceutical and physicochemical properties [70, 71]. In order to increase efficacy and overcome resistivity of breast cancer cells to chemotherapeutic effect of doxorubicin, several studies have been conducted including combined treatments of this drug with other molecules. Combination of estrogen and doxorubicin showed enhanced apoptotic action of the drug on estrogen-independent MCF7 cells (MCF7/LS) via suppression of NF-kB signaling [72]. Sensitization of several breast cancer cell lines to

doxorubicin was similarly increased upon co-treatment with proteasome inhibitor [73]. In addition, many combinatorial treatments with natural products (reported from *in vitro* and *in vivo* studies), showed reduced cardiotoxic effects and/or higher drug efficacy of doxorubicin [74-76].

1.1.5 Nanomedicine in breast cancer treatment

1.1.5.1 Properties of nanoparticles used in cancer treatment

Nanotechnology has contributed vastly to the chemotherapeutic drug delivery system. Contemporary cancer treatment studies focus on the preparation of the drug formulations which are less toxic to normal tissues and which specifically target tumor cells [77]. Novel drug carriers are developed for delivering of already existing as well as newly designed drug molecules and at the same time for overcoming the pharmacological and physiological barriers of old therapies. Small size formulations of drug encapsulating complexes, increase in their drug loading efficiencies, as well as combination of different drugs/molecules in one complex are the characteristics of good nanoparticles (NPs) [78]. Such designs are also expected to show improved drug uptake and specificity to cancer cells, decrease resistivity to chemotherapeutic agents, lower toxicity to normal cells and increase efficacy in cancer therapy [79]. Since the gaps between the endothelial cells in the tumor vasculature surrounding cancer cells range from 100nm to 780nm, NPs for drug delivery are accordingly designed in nm size [80]. NPs are particularly coated with hydrophobic molecules to prevent binding to plasma proteins and gain favourable clearance properties from circulatory system [81]. Construction of NPs include usage of different materials (i.e., polymer, lipid, metal, ceramic), size and shapes (i.e., liposomes, spheres, emulsions, nanotubes), depending on their intended use [80]. NPs are further categorized into organic (e.g., liposomes, polymeric, micelles, etc.) and inorganic (e.g., silica, gold, iron oxide, etc.). In addition, such drug-encapsulating NPs should also be degradable after targeting cancer cells [80]. Drug targeting approach might be passive (passively entering the tumor interstitium and cell) [82], active (based on molecular recognition of cancer cells, i.e., being coupled to a ligand) [83] or a combination of the two [84]. In addition to the above mentioned advantages of

nanocapsules, recently designed NPs show sustained drug release, enhanced retention and permeability (EPR) in cancer cells as well as imaging functions [85]. EPR effect is the gold standard for newly designed NP-based formulations. Due to their molecular weight higher than 40-50kDa, macromolecular systems show selective accumulation of drug complexes in tumor site as well retention for longer periods [86]. This is due to higher vascular density around solid tumors with large gaps between endothelial cells [86]. Given these advantages, NPs have long been considered as having the potential to dramatically change clinical treatments by improving the present therapies or introducing new ones.

1.1.5.2 Usage of Conjugated Polymer Nanoparticles for medical applications

Conjugated polymer nanoparticles (CPNs) are another class of nanoparticles, conjugated polymer (CP) counterpart of which, is produced by one of the polymerization reactions (e.g., oxidative polymerization, Heck coupling, etc.) using different types of conjugated polymers (PF, PPV, PPE, PT). Their properties are significantly affected by the surface charges and functional groups [87]. CPNs are characterized by striking abilities of light-harvesting and light-emitting properties which make them very useful and multifunctional expecially for fluorescent targeted imaging, diagnosis, gene therapy and drug delivery [88]. Moreover, recently designed multifunctional nanoparticles show high photostability, low cytotoxicity and quantum yield, good biocompatibility, small size and ability to sensitize reactive oxygen species. Their imagining capabilities have shown importance in monitoring the delivery process by tracking the assembly and dissasambly of their cargo (e.g., oligonucleotides) [89].Thus, CPNs have been recently used to kill microorganisms or tumor cells as well as to label cells *in vitro* and *in vivo* [90]. Furthermore, conjugated oligomer-based nanoparticles (CONs) are a class of NPs given less focus in literature, but having comparable or even more advantages over CPNs. For instance, CONs show comparable cellular uptake and higher fluorescent quantum yields as well as faster release of their cargo compared to their polymeric counterparts [91]. CONs have also shown promising results as oligonucleotide nanocarriers for future drug delivery usage [92]. However, cellular toxicity is a major concern in CON design due to their high positive charge. To improve cellular compatibility, hydrophobic moieties are incorporated in the side chains of the nanoparticle

complexes, such as Cucurbituril (CB); a macrocycle composed of glycoluril units, having a hydrophobic cavity and two hydrophilic portals [93]. For instance, Pennakalathil et al., (2014) introduced a CB7-capped, red-emitting CON (Red-CON) which showed to be pH-responsive and possess both cellular imaging and drug delivery potential [94] (Figure 1.2). The oligomeric nanoparticle composed of amine groups, showed precipitation in human blood serum, however when capped by CB7 (a water soluable CB), it became non-precipitative in blood serum, at least for a 24h incubation. In addition, CB7-capping only mildly increased the oligomer NP size, keeping its size-dependent properties unchanged [94]. As an example of CONs' potential advantages, a very recent study has presented a multifunctional fluorescent oligomer with very promising results in both nonionizing real-time imaging of sentinel lymph node (SLN) (for early detection of breast cancer metastasis) and photothermal therapy (PTT) (for specific killing of metastatic cells) [95].

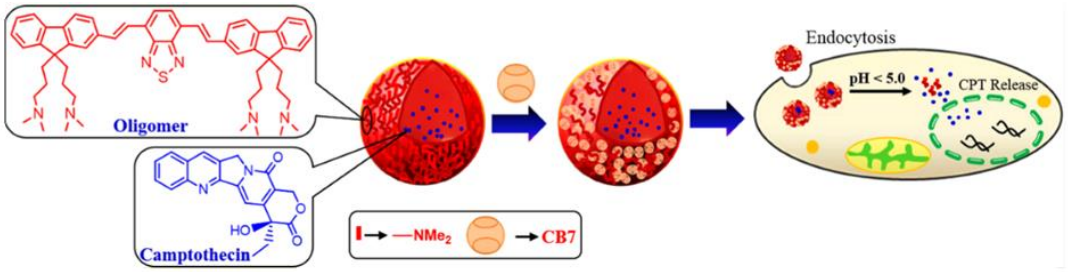

Figure 1.2: Structure of CB7-capped Red-CON.

Illustrative representation of Red-CON structural composition, capping as well as potential cellular internalization and drug delivery abilities. This figure was reprinted with permission from ACS publications. Copyright (2014) American Chemical Society. Pennakalathil et al., Red Emitting, Cucurbituril-Capped, pH-Responsive Conjugated Oligomer-Based Nanoparticles for Drug Delivery and Cellular Imaging, (2014) Biomacromolecules.

1.1.5.3 Combinatorial treatments using nanoparticles as drug carriers

Co-delivery of doxorubicin and other chemotherapeutic drugs in liposome encapsulated formulations is reported in several recent *in vivo* mice studies [78, 96], as well as patients of phase II and phase III trials [97, 98]. Newly developed liposomes can transport and release active drugs also by using the pathophysiology of tumor microenvironment as well as by targeting cancer cell-specific receptors, i.e., estrogen-anchored [99] and pH-sensitive formulations [99, 100].

Drug formulations designed for targeting of a specific aberrant pathway in tumor cells have increased interest in the last years. In addition, due to very effective survival mechanisms developed by cells to resist drug toxicity, RNAi (using small interfering RNA; siRNA) approach is an alternative in treating tumors. siRNA therapy (either alone or in combination to other molecules) is performed using non-viral carriers such as NP-based formulations. There are studies showing siRNA librariy screens including hundreds of siRNA candidates against cell cycle proteins in combination with conventional anticancer drugs for breast cancer therapy usage, in drug-resistant and -sensitive breast cancer cell lines and mouse xenografts [101-103].

After extensive research on NP-based drug formulations, some of them are now FDA approved either for therapeutic or diagnostic purpose. Cancer nanoparticle medicines are now used to treat different cancer types and stages [104]. In addition, intravenously delivered organic nanoparticle formulations show substantial success in gene therapy applications and delivery of small molecule drugs for cancer treatment [105]. Most of the approved nanoparticle drugs include liposomal encapsulations of anticancer drugs [105]. The first liposomal cancer nanomedicine to be approved by FDA (1995) was Doxil (PEGylated liposomal doxorubicin) [106], followed by other drugs such as DaunoXome (liposomal daunorubicin) [107], Myocet (non-PEGylated Doxorubicin) [108] and Abraxane (albumin-bound paclitaxel NP) [109]; all of which, compared to the free drug delivery process, posses the ability to preferentially accumulate in the tumor microenvironment due to their enhanced retention and permeability (ERP) [105]. In addition, several nanoparticles are now used as imaging agents, i.e., Feridex and Resovist (imaging of liver lesions) [110], Ferumoxtran (imaging of lymph node metastasis) [111], Optison and SonoVue (as ultrasound contrast agent) [112]. Current human trials are on the way to approve several other formulations, majority of which will be used for cancer treatment [105].

1.2 siRNAs as breast cancer candidate drugs

1.2.1 General background on RNAi machinery

A vast majority of human genome encodes for genes which are not translated to protein. Only 1.2% of genome is translated to protein [113]. Around 60 years from now, the first non-coding RNA (tRNA) was identified and its primary structure was characterized [114]. After 20 years, uridine-rich small nuclear RNAs (snRNA) were discovered [115]. These RNAs, in association with ribonucleoprotein particle (RNP), were later shown to be involved in spicing process of pre-mRNA. Most of the small nucleolar RNA (snoRNAs) interestingly were found to be processed from released introns [116]. Around 15 years later, Wightman et al. showed the regulatory mechanism of lin-14 gene (involved in the development of C. elegans) [117], after which two other studies directed by Ambros and Ruvkun, found lin-14 gene product to be a non-coding RNA of 22 nucleotides which regulated its own gene expression by binding to the 3' UTR [118, 119]. In year 2000, Pasquinelli et al., identified a small temporal RNA involved in C.elegans development, which was also conserved in humans [120]. This brought extensive discoveries of other small regulatory RNAs in humans and model organisms, now termed as 'microRNAs'. Together with these small-RNA class entities, long non-coding RNAs (lncRNAs) which size ranges up to several kilobases, are on investigation [121].

1.2.1.1 RNAi molecules

A microRNA (miRNA) molecule is a 21-25 nucleotides long non-coding RNA strand which is byproduct of a larger nuclear RNA precursor molecule. In brief, miRNAs are synthesized from the DNA strand by RNA polymerase II as pri-miRNAs, after which (while still in nucleus) they are processed by Drosha proteins into a hairpin structure called as 'pre-miRNA' and are then transported to cytoplasm [122]. Afterwards, pre-miRNAs are cleaved by Dicer protein which render the now called 'mature miRNA' or 'siRNA' in an ~21-25 nucleotide, 3' overhangs containing structure. Single-strand miRNAs then bind to nucleoprotein complex RISC (Figure 1.3) and either in perfect or partial complementarity with certain mRNA strands, induce the degradation of the transcript or their translation repression, respectively [122].

Small interfering RNAs (siRNAs) are the canonical affectors of RNAi mechanism which initially were recognized as exogenous elements involved in the defense mechanisms of plants [123]. They are double stranded, long and perfectly based-paired RNA strands which are either introduced from nucleus to cytoplasm as such or enter cell from the outside environment [124] (Figure 1.3). Endogenous small-interfering RNAs (endo-siRNAs) are mainly transcribed from the processed pseudogenes (DNA copies with no introns, reversed transcribed from their parental gene transcipts) [125], but also can originate from the diced genomic transcripts, convergent RNA transcripts or from natural sense-antisense transcript pairing [126]. Similar to miRNAs, endo-siRNAs are processed by Dicer after loaded into Ago-2 and form the RISC complex. During the RNAi pathway, the single strand siRNA guides RISC complex to the target transcript where it binds with perfect complementarity. The slicer activity of this complex induces a phosphodiester bond breakage of the target RNA; 10-11 nucleotides from its 5' base-paired start sit [127]. After this, other nuclease enzymes bind and complete the target transcript degradation process. RISC is now released and ready to cleave other targets [127]. Apart from the perfect complementarity, many siRNAs bind imperfectly to targets, similarly to miRNAs. Most of these imperfectly matches include their 'off-targets' binding events [128]. The 2-8 bases in the 5' region of RNAi molecules include the seed-region; the primary target-recognition site of siRNA. The degree of siRNA off-target gene silencing is determined by the strength of base-pairing between its seed region and the off-target transcript, affected also by other auxiliary factors [129].

Exogenously transfected RNAi molecules on the other hand, are nucleic acid molecules directly transfected to cells either as synthetic double strands, endonuclease-prepared or as short hairpin RNA strand (shRNA) precursor [130] (Figure 1.3). siRNA treatments are not 100% efficient and only transiently downregulate their target gene expression. However, for studying the short term effects of gene knockdown this method is sufficient and even advantageous, especially for targeting of essential genes [130]. RNAi screening has held an important role in identifying new genes as well as resolving many questions on biological networks and processes such as cell viability, cell morphology, drug resistance, etc [131].

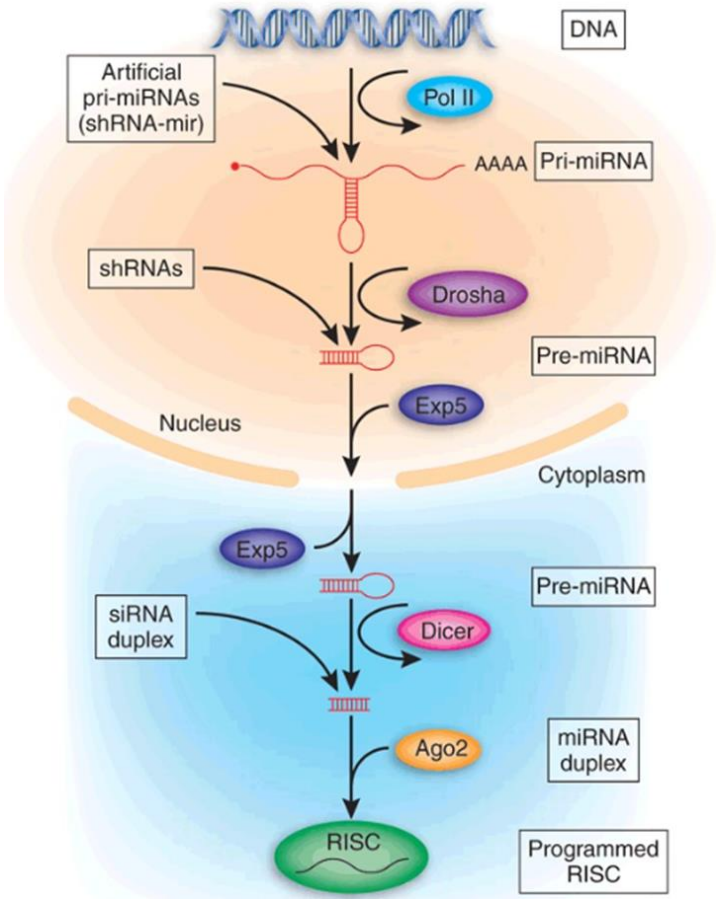

Figure 1.3: RNAi machinery.

The mechanism of processing endogenous and exogenous RNAi molecules by the cell. This figure was reprinted with permission from Nature Publishing Group. Cullen, RNAi the natural way, (2005) Nature Genetics.

1.2.2 RNAi delivery methods

1.2.2.1 RNAi transfer systems

Gene delivery of RNAi is used either in research procedures for downregulating the target gene or for gene therapy purpose. Two of the main nucleic acid classes mostly used in RNAi transfection experiments include 2-10 kilobase plasmid DNA or 19-25 base-composed short double-strand RNA (either in the form of siRNA or miRNA) [132]. When transfected to cells, two RNA molecule characteristics should be taken into account; 1) cell compartment where they are expected to function (cytoplasm or nuclei) and 2) their length (base or kilobase magnitude) [133]. Delivery methods of nucleic acids include a) non-pathological viruses which are mostly used for *in vivo* studies, b) viral-like particles and c) non-viral techniques [134]. Viruses are

specifically designed to include the nucleic acid sequence of interest into the viral genome and release their cargo into the cytoplasm of the infected cell. The viral genetic material then enters nucleus, integrates into the host genome and starts being expressed using the host transcriptional machinery [134]. Different viral types are currently used for this purpose including retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, parvoviruses and herpes simplex viruses. Eventhough displaying high transfection efficiency, usage of viral formulations show health concerns based on the host immune response induction and insertional mutagenesis. Other obstacles of using viral particles include challenges like complexity of their design, their packaging capabilities as well as the expensive procedures for their production [135]. Virus-like particles on the other hand, include recombinant viruses which except their capsid do not contain any other viral genetic information. The advantage of this technique is the easy packing possibility of the siRNA or plasmid DNA by spontaneous capsid self-assembly, via chemical dissociation and reassociation process. Most of these viruses are used as a vaccination tool, while several others are available in the market for research purpose such as: human polyoma JC virus, papillomavirus and polyomavirus [136].

1.2.2.2 RNAi transfection modalities

Cell membrane (a polar lipid bilayer) doesn't internalize negatively charged nucleic acid molecules spontaneously. This is the reason why many methods for delivering of nucleic acids to cells have been established. Non-viral nucleic acid delivery methods include 1) mechanical, 2) physical and 3) chemical methods. Mechanical method includes microinjection, particle bombardment and single-walled carbon nanotubes. By microinjection nucleic acid is directly delivered to a single cell using glass capillaries. Eventhough efficient, this is a difficult procedure for *in vivo* and cell culture application studies, since it includes large number of cells [137]. Particle bombardment on the other hand, is a delivery method by which gold or tungsten nanoparticles loaded with nucleic acids are given a high velocity in order to cross the cell membrane of many cells simultaneously. This method is mainly used for vaccination and plant studies [138]. Single walled carbon nanotubes are composed of one-dimensional layers of hexagonal carbon nanostructure. By addition of positively charged amine groups, they are made functional for carrying and transfecting the

nucleic acid to the host cell. This method has shown positive results of siRNA gene targeting even in *in vivo* studies [139]. Electroporation technique, as a means of physical transfection method, includes application of an electric field to cells. This treatment causes short-term depolarization and pore formation on the cell membrane, thus makes it possible for molecules like siRNA and plasmid DNA to be internalized into the cell. This technique is efficient and non-toxic, as far as multiple parameters are optimized and kept constant, such as: length of the pulse, number of pulses and temperature [140]. Permeabilization of cell membrane is also achieved by laser – beam-mediated-gene delivery (as a means of physical transfection method), which is performed by the application of a focused high-energy laser light. Eventhough showing very promising results on transfection efficiency and cell toxicity, laser-beam-mediated-gene delivery is quite challenging due to its manual application which makes it more appropriate for studies including limited cell number [141]. On the other hand, chemical method for nucleic acid delivery includes: 1) the widely used calcium phosphate method, 2) polymers, 3) PAMAM dendrimers and 4) lipid formulations. In the first technique, sodium phosphate and calcium chloride are mixed with nucleic acid molecule after which calcium phosphate crystals are formed. These crystals bind the nucleic acid and carry it to the cell. This method has a limited application because of being highly sensitive to pH changes and its cell type-dependent transfection efficiency [142]. Polymers used for transfection studies are mostly made up of synthetic polyethylenimines (PEIs). Composed of positive amine charges, PEIs formulations range from those carrying small oligonucleotides (such as siRNAs), to the ones carrying large plasmids; which are taken up by cells via endocytosis. Polymer efficiency and toxicity is based on the formulation size of the molecule [143]. Similar to PEIs, PAMAM dendrimers are polymers composed of amine groups, more appropriate for binding and transferring of plasmid DNA rather than short oligonucleotides. Several commercial PAMAM dendrimer formulations which have water solvable, biocompatible and non-immunogenic properties are now available for research purpose [144]. Lipid molecules on the other hand, are widely used for siRNA and plasmid DNA gene delivery purpose. They carry nucleic acids either by entrapping them inside the internal aqueous region, or by binding of nucleic acid on the lipid bilayer surface. Lipid designing and formation is a very flexible process specifically adapted to the purpose of usage by modifying their surface charge, size, composition and morphology. Another advantage of lipids as nucleic

acid carriers is their good stability due to being protected from the cell nucleases cleavage [145]. As a subclass of lipids, cationic lipids are widely used both in *in vitro* and *in vivo* studies of siRNA and plasmid transfection. Their overall positive charges make it possible for them to bind the cell membrane and induce membrane perturbation and fusion. Although showing no immunogenic responses and insertional mutagenesis in the host genome, these lipids don't show transfection efficiency as high as the viral particles. Moreover these lipids are inactivated in the presence of serum (both from *in vivo* and *in vitro* cell studies) [146]. Currently there are several cationic lipid formulations available in the market both for siRNA and plasmid DNA delivery, such as HiPerfect and Attractene Transfection Reagents, respectively. Specialized lipid-based formulations are elegant designs (mostly for *in vivo* gene delivery). By modifying their functional groups, such lipid formulations can be specialized for specific targeting and/or including pH-sensitive platforms for siRNA and plasmid-DNA drug designs [147].

1.2.3 RNAi machinery and cancer

1.2.3.1 Role of miRNAs in tumor progression and cancer treatment

To better understand the RNAi impact in human health, we should consider that human cells express approximately 100 types of endogenous miRNAs which are thought to regulate around 30% of the transcriptome. They regulate many cellular processes such as reproduction, wound healing, cardiovascular remodeling, pain, as well as show significant role in different pathologies such as epilepsy, cerebrovascular diseases, hypertension and diabetes [148, 149]. miRNAs are also key regulators of cancer initiation and progression, with important implications in metastasis process [150]. Extensive and increasing research on miRNA deregulation in cancer has revealed several of them as tumor suppressors, oncogenes or having both roles; depending on the cellular context and/or cancer type. Current studies also focus on exploiting miRNA signatures for their possible usage as prognostic, diagnostic, theranostic (treatment monitoring) markers and personalized therapy [151]. The fact that a single miRNA has multiple targets could be advantageous and indicates that modulating one miRNA's activity might open door to innovative therapies in breast

cancer. Targeting of miRNAs which show oncogenic activity could be an approach toward personalized therapy. This can be achieved either by natural/synthetic agents or oligonucleotides targeting overexpressed miRNAs; all of which have been experimentally demonstrated. Another benefit of targeting the endogenous RNAi machinery could be that of preventing future metastatic potential of cancer or treatment of an already developed metastasis [152]. In breast cancer, several miRNAs have been associated with cell cycle, cell morphology, cancer stem cell regulation and metastasis. Overexpression of miRNAs in breast cancer specimens is often an indication of genomic abnormalities. On the other hand, miRNA expression can be evaluated even from clinical specimens like fine-needle aspirates [153]. Accordingly, there has been intensive research conducted, focusing on the possibility of using circulating miRNA molecules for diagnosis and prognosis purpose. Few circulating protein molecules have already been used as biomarkers for metastatic breast cancer however they didn't show high sensitivity for diagnosis of primary breast cancers [154]. Using the circulatory miRNAs from microscale serum volumes of breast cancer patients has recently identified circulating miRNA signatures which might well sever as biomarkers for early breast cancer diagnosis [154].

1.2.3.2 Advances in research about endogenous siRNAs

Endogenous siRNAs (endo-siRNAs), are not as extensively investigated as the miRNA molecules. Endo-siRNAs share many similarities to the other small-RNA family members (miRNAs and piRNAs), which makes it difficult to separate and trace them specifically [155]. Most of the endo-siRNAs studies include their characterization in the invertebrate model organisms, while the vertebrate studies show not a clear mechanistic aspect and physiological role of endo-siRNAs, with discrepancies among several research groups [156]. In different organisms, pseudogenes-derived endo-siRNAs have been implicated to their ancestor-gene regulation [157]. In C.elegans studies, endo-siRNAs have shown role in chromosome organization and gene regulation [158, 159]. Drosophila-related studies have revealed endo-siRNAs role in mitochondrial metabolism, transposon regulation and ovarian cell gene regulation [160, 161]. Similarly, two nature papers in 2008 showed that endo-siRNAs regulate gene expression in mouse oocytes [162, 163] while other studies implicated endo-siRNAs in murine reproductive and nervous system [164, 165].

1.2.3.3 *In vitro* RNAi screening

Similar to chemical screening, high-throughput RNAi screening is now a well known method which serves to study large RNAi collections. Using this technique, RNAi is randomly introduced into cell either by transfection or viral transduction. New sophisticated instrumentation and software programs are now available for performing such studies and for analyzing their high-content data. Such methodology has shown to be a powerful tool to understand several gene functions which are now implicated with cancer, aging, infection, obesity, etc [166]. Currently RNAi method is intensely used as a way to better understand the biology of cancer, resistance to anti-cancer drugs and several other cellular functions. Moreover, *in vitro* cell studies and *in vivo* loss-of-function screening via RNAi have helped for phenotype characterization, in a cell, tissue or stage-specific way [131].

RNAi screening technology has a crucial role also in identifying novel targets and biomarkers as means of personalized medicine, a field of rapid development. Due to its ability to modulate gene expression, RNAi can be used as an efficient therapeutic means of treating several diseases, including cancer [167]. Several clinical trials have reported promising results using RNAi machinery to treat hypercholesterolemia, genetic diseases, skin disorders and amyloidosis [168].

1.2.3.4 *In vitro* RNAi studies in breast cancer

Numerous *in vitro* cell studies used siRNA-based gene silencing approach for finding potential targets for cancer therapy. For instance, silencing of CXCR4 in MDA-MB-231 breast cancer cell line decreased breast cancer metastasis as well as showed the requirement of CXCR4 for cell metastases [169]. A study conducted a decade later used a HER2-peptide fusion protein as a carrier of CXCR4-siRNA in BT-474 cells resulting in a potential, non-toxic targeted therapy for HER-positive breast cancers [170]. Similarly, in a study performed in MDA-MB-231 cells, siRNA against survivin (BIRC5) was delivered with PEI, which resulted in loss of cell viability, induction of apoptosis and increase in sensitivity toward several chemotherapeutic drugs [171]. Likewise, a combinatorial treatment of BIRC5-siRNA and two chemotherapeutic drugs in MCF7 cells increased the therapeutic efficiencies of these

anticancer agents [172]. In two other studies Wnt-1 and WT-1 gene downregulation in MCF7 cell line showed role of these genes in maintenance of cell proliferation [173, 174]. siRNA silencing of MDR gene expression rendered drug resistant MCF7 cells more sensitive to doxorubicin treatment [175]. In a screen of siRNAs against 28 kinase function bearing proteins, silencing of PLK kinase in several breast cancer cell lines resulted in elimination of tumor-initiating cells. In addition, simultaneous downregulation of PLK and treatment with severeal anticancer drugs, decreased the population of tumor initiating cells; implying the possibility that PLK knockdown in chemotherapeutic regiments could prevent tumor relapse, especially in TNBC cancers [102]. In several RNAi screens, targeting of several genes resulted in pro-proliferative or anti-mitotic phenotypes, which suggested the investigated genes as promising proliferation markers (i.e., CHRNA5 RNAi negatively affecting cell proliferation) [176-178].

There have been attempts also in using siRNA molecules for long-term silencing of target protein. For instance, Narz et al. (2005) have published a protocol of MAPK1 siRNA silencing (5nM) in MCF7 cells with a transfection time of more than 2 weeks [179]. MAPK1 gene expression was highly downregulated in cells treansfected after each split, without any effect of HiPerfect Transfection Reagent on cell viability [179].

1.2.4 New advances in siRNA research and human trials

1.2.4.1 Advances in siRNA developing methods

Most therapeutic applications of RNAi suggest using siRNAs as a mode of gene knockdown; however siRNAs have low stability, possible off-targets and immunostimulatory effects [180]. To prevent such side-effects of siRNA usage (especially for *in vivo* studies) one possibility is introducing a chemical modification to siRNA molecule [181], while another choice would be the usage of promoter expressed shRNAs/ miRNAs with their advantage of inducing a long-term gene silencing by only a single application [182]. In order to increase efficacy of siRNAs and prevent nuclease cleavage, several parameters need to be considered during siRNA design such as: the thermodynamic stability of 5’ end, GC content, selective

addition or substitution of nucleotide derivatives, binding of siRNAs to intramolecular structures or regulatory regions of the target [183]. In addition to the above mentioned modifications, conjugation to another molecule (like cholesterol derivatives and lauric acid) is performed in order to facilitate cellular uptake of siRNAs [184]. There are now several new and wise designs of siRNAs which show promises in facilitating cancer treatment via using RNAi machinery, one of which is the splice switching oligonucleotides (SSOs) [185]. Such siRNAs bind specifically to a pre-mRNA splice site and interfere with the splicing machinery by competing with the splicing factors and directing them to the other splice sites. Thus, SSOs don't shutdown the transcript translation but can: 1) block expression of a cancer-associated gene isoform, 2) upregulate alternative splice variants and/or 3) generate novel splice variants which might have therapeutic role for cancer [186]. This targeting method best serves for the alternatively spliced cancer-associated genes which express an isoform protein with anti-apoptotic function and another having pro-apoptotic role, like Bcl-x [187].

1.2.4.2 *In vivo* RNAi screening

In vivo RNAi studies initially started on *C.elegans*. Continuous research in this model organism made it possible to better understand several biological and biomedical dilemmas, mainly those related to aging and obesity [188]. On the other hand, RNAi studies in Drosophila have been generally performed for tissue and stage-specific gene expression analysis. Genome-wide RNAi screens in Drosophila have also contributed vastly to the medically-related topics such as gut infection, neurological diseases, etc [189-191]. Zebrafish siRNA-related research is more focused on loss-of-function studies using injected siRNA (termed as "morpholinos") agains specific genes [192]. Studying of developmental stages and miRNA profiling have been the focus of Zebrafish-RNAi [192], with attempts to make RNAi machinery a heritable and gene-specific system [193]. RNAi application in mice is mainly performed by inducible and tissue specific RNAi using viral vector expressing shRNA [194-196]. Studies related to siRNA application in murine model generally include novel designs of efficient and safe siRNA carriers using different molecules such as synthetic polymers, nanoparticles or stimulus-sensitive carrier systems [197, 198]. For instance, a study conducted by Juan et al. (2014) introduced

four siRNAs targeting different oncogenes, loaded in the same nanoparticle which significantly inhibited the target proteins as well as inhibited tumor growth in mice. Same study showed that lowering dosage of siRNAs was correlated to their lower target-off effect as well as revealed the finding that for successful gene delivery *in vivo*, very potent siRNAs are required; the ones which are able to induce knockdown of target gene, even at very low concentrations [199]. Such mammalian studies provide the shift from using RNAi machinery as a way of understanding biological background of several pathways and diseases, to the usage of RNAi as a therapeutic drug in clinical medicine.

1.2.4.3 siRNA-based human clinical trials

For around one decade substantial research has been performed to unravel the potentiality of siRNA as a therapeutic tool. Systemic administration has been of major interest regarding usage of siRNA for treatment of both local and metastatic tumors. Several human phase I studies of systemic siRNA administration as anti-cancer therapy have been already completed. These studies included patients of different solid tumors which showed no response to conventional therapies. The siRNAs administered in these clinical trials were targeting the already known cancer-related genes and showed the advantage of undergoing several cycles of target transcript-binding and cleavage [200].

Human clinical trials initially included the local administration of siRNAs in the eye [201]. Later on, the first human study introduced the systemic application of liposomal siRNA to a chronic myeloid leukaemia (CML) patient who showed no response to chemotherapy. No clinical adverse effect was observed after treatment. In addition, the overexpressed bcr-abl gene was downregulated as well as apoptosis was induced in the CML cells. This was the first human trial using safe, synthetic, systemic administered and non-viral RNAi-liposomal complex [202]. Subsequent human trials included siRNA complexes of improved delivery made possible by using different encapsulating molecules. A phase I clinical trial conducted in 2013 included 32 Transthyretin Amyloidosis patients and 17 health volunteers which were subjected to transthyretin-siRNA using two different lipid nanoparticle formulations [203]. Results showed decrease in the serum transthyretin protein level both of its

mutant and non-mutant forms. Subsequent clinical trials followed this study, which made it the most advanced investigation of siRNA-based therapeutics [204]. There are currently around 30 clinical trials of different phases for treatment of several pathologies, including cancer [205]. A promising outcome from all these studies is that there has been no reports on serious accumulation of siRNA formulations in liver, which has been the case in the shRNA-based *in vivo* treatments. However, different strategies might improve the siRNA-based therapeutic approach: 1) designing of multifunctional siRNAs; 2) multiple siRNAs-carrying formulations (simultanously targeting different pathways); and/or 3) usage of biodegradable lipid carriers for improving safety. A very important issue is also pre-screening of cancer patients recruited in the trials, so that only those having tumor-driving mutations could be treated by siRNA delivery [200].

1.3 Nicotinic Acetylcholine Receptors and CHRNA5

1.3.1 Acetylcholine

1.3.1.1 Neuronal acetylcholine synthesis and release

Acetylcholine (ACh) was initially isolated in 1914 from *Claviceps purpurea* fungi and afterwards showed to have stimulatory effects on intestinal smooth muscles and an inhibitory effect on heart functions. 15 years later, ACh was found to be expressed also in the spleen of mammals and subsequently it was determined as a transmitter of nerve impulses in heart after which it was found to be a neurotransmitter of nervous system [206]. ACh is now accepted as a major neurotransmitter of peripheral nervous system and one of the main modulators of central nervous system. ACh is produced by cholinergic neurons; including projection neurons and local interneurons [207]. Choline acetyltransferase (ChAT) synthesizes ACh form choline and acetyl coenzyme A (themselves synthesized from lipid metabolism and turnover). ChAT enzyme is thus considered as a key regulator of central and peripheral nervous system. The regulation of ACh synthesis is not very clear but there is evidence that phosphorylation of ChAT by protein kinase C (PKC) increases the catalytic activity of ChAT [208]. Other factors which are correlated with increase of ChAT activity

(thus ACh production) are nerve growth factor, leukemia inhibitory factor and cilliary neutrophic factors, which are also important to maintain the cholinergic phenotypes of ACh producing neurons [209]. Apart from ACh production, another factor that influences overall ACh activity is the storage and release of this neurotransmitter by synaptic vesicles after being loaded by the vesicular acetylcholine transporters (VAChT) [210]. These transporters (positioned mainly at the nerve terminals) are also regulated by phosphorylation mainly via Protein Kinase C (PKC) anzyme, indicating the immense role of PKC in regulation of ACh neurotransmission at several level [211]. The driving force that induces ACh concentration at the synaptic vesicles and load to VAChT is thought mainly to be the electrochemical potential generated from H^+ ATPases [212]. After being loaded to vesicles, ACh is released from the vesicles to the synaptic cleft by calcium-dependent fusion of the vesicle to the cell membrane. ACh then binds and activates the acetylcholine receptors (AChR) in the post-synaptic cells [213]. In the case of other neurotransmitters (i.e., GABA, serotonin), their action in the synaptic cleft is terminated by transporter removal however, ACh function is terminated via its hydrolysis by acetylcholinesterase (AChE) enzyme, after which choline is transported to the presynaptic neuron by special transporters. The produced choline is again used to synthesize more of ACh neurotransmitters [214].

1.3.1.2 Acetylcholine role in CNS

The nervous system development begins with the induction of neural plate formation and neural commitment of cells regulated by several genes (i.e., mash-1, neurogenin). The downstream targets (e.g., neuroD) are found to be responsible for cells to undergo a differentiation program [215, 216]. Together with the epigenetic factors, other molecules such as growth factors and neurotransmitters also play roles in keeping the committed state of neurons via regulation of neuron-specific genes as well as providing cell survival, proliferation and fiber elongation. Neurotransmitter expression is also known to start in early neurogenesis, before formation of synaptic contacts [217]. ACh is considered one of the major players among expressed neurotransmitters. By affecting the intracellular fluctuations of Ca2+, ACh has a role mainly in the regulation of cell proliferation, as well as the cell signaling transmission between the neuronal and non-neuronal cells [218].

As a neuromodulator, ACh is neither an excitatory nor an inhibitory neurotransmitter. Thus it functions in regulating the release of other neurotransmitters and regulation of neuronal networks upon internal or external stimuli. ACh's role depends on several factors such as: the site of release, receptors they bind to, the neuronal type they target, etc. Accordingly, the classical roles of ACh in the brain include: 1) regulation of synaptic transmission, 2) neuronal excitability, 3) synaptic plasticity, and 4) firing of neurons in a coordinated pattern [219]. The fact that ACh modulates neuronal networks on several brain areas (via both ionotropic and metabotropic receptors) makes it a major player in regulating behavioral and cognitive processes [220]. Some of the specific functions of ACh are: regulation of GABA-mediated neuronal response, drug reward, attention, learning, memory, food intake and stress [221].

1.3.1.3 Non-neuronal acetylcholine

ACh is considered as a fast-acting neurotransmitter, mainly functioning at the neuromuscular junction and autonomic ganglia, where it regulates various biological processes. In addition to its expression and function in neuronal cells, ACh is also detected in other cells like vascular endothelial cells and lymphocytes [222]. There are studies showing the circulation of low (but detectable) ACh levels in blood and even more interesting studies which show expression of AChE by other non-neuronal cells such as: $CD4^+$ T cells, trophoblasts in placenta, renal tubuli, cardiac myocytes and epithelial cells of airway [223]. The evidence of endogenous expression of ACh by these specific cells is also the detection of AChE protein in these cells [224]. Non-neuronal functions of ACh are divided according to their target receptor; the ionic events (if it binds to the nicotinic acetylcholine receptors (nAChR)) or the metabolic events (if it binds to the G protein-coupled muscarinic receptors (mAChR)). nAChR-based functions in human epithelial cells is mainly related to their cell cycle regulation and differentiation process; based on intracellular pathway signaling triggered from nAChR subtypes [222]. mAChR which are coupled to G-proteins, link the ACh activity to various cellular signaling cascades. One example of muscarinic receptor-based function of ACh includes airway smooth muscle inflammation and remodeling [225]. In immune cells (which express both nAChR

and mAChR), ACh stimulation positively regulates cell proliferation as well as increases the intracellular calcium (Ca^{2+}) concentration [226].

1.3.2 Cholinergic receptors

1.3.2.1 Cell membrane receptors

Lipid bilayer of the cell membrane is a barrier for the free passage of most polar molecules. Thus cells use transporters and channels for molecule-specific and -nonspecific transportation, respectively [227]. Ion concentration differences between the external and internal cell compartments generate potential energy for other molecule transportation. In contrast to transporters (which include coupled transporters, ATP-driven and light-driven pumps), ion channels mediate the transport of molecules only passively [227]. In addition ion channels provide movement of much more ions and at a greater rate. They are composed of narrow and hollow pores which open and close rapidly for transport of inorganic ions (mainly Na+, Ca2+, K+ or Cl-) down their electrochemical gradient [228]. Most of the ion channels open and close in response to a stimulus which can be 1) a change in voltage across membrane, 2) mechanical stress, or 3) binding of a ligand which induce opening of the ion channels. Ligand-gated ion channels in particular are either stimulated by an extracellular molecule (like a neurotransmitter), or an intracellular molecule (such as an ion or nucleotide). Compared to other ions, Na+ is the one which makes the highest contribution to the inward current, whereas Ca2+ has only a minor effect due to its low extracellular concentration [228].

1.3.2.2 Role of ion channels in cancer

Ion channels regulate physiological processes mainly through calcium (as a second messenger), which is considered as the key regulatory ion in the cell [229]. Cellular Ca^{2+} concentration itself depends on the electrical gradient developed from ion channels [230]. It is now a known fact that in cancer cells, the channels regulating Ca^{2+} concentration are modulated, which (in addition to other pathologies) implicate them with carcinogenesis [231]. Targeting of cancer cells by immune cells also

depends on calcium signaling [232], all of which implicate Ca^{2+} with cancer at several levels. These findings make ion channels be considered as a potential tool to target cancer [233]. Dysfunction of ion channels (thus alterations in Ca^{2+} concentration) contributes to neoplastic transformation, with roles in 1) sustained growth, 2) resistivity to anti-growth signals, 3) angiogenesis, 4) invasion, and 5) metastasis. Contribution of the ion channels to the cancer hallmarks are found to be cancer cell type-dependent [233]. For instance, one of the well-studied ion channels is CRAC/ORAI, which by controlling Ca^{2+} influx), regulate recognition and adherence of immune cells to cancer cells, as well as [234] cell proliferation in various cancers [235]. ORAI1 expression is shown to increase during lactation, while ORAI1 regulators (STIM1 and STIM2) were significantly modulated in 295 basal type (which belong to poorest prognosis) breast cancer patients [236].

1.3.2.3 Expression of cholinergic receptors

NAChRs, similar to other pentameric ligand-gated ion-channels (pLGICs), are composed of five homolog subunits coming together to form either a homomeric or heteromeric functional and symmetric channel which become permeable to K^+, Na^+ and Ca^{2+} upon stimulation.These subunits show multiple conserved sequences which makes them be included in a superfamily [237]. When divided into protein domains based on their topology, pLGIC subunits are composed of a signal sequence, an extracellular ligand-binding terminal domain, four transmembrane domains (forming the channel pore) and a considerably big cytoplasmic loop (between the third and fourth transmembrane domains). Among these compartments, transmembrane domains (composed of alpha helices) show the most conserved sequences [238]. The ligand-binding domain has high affinity for neurotransmitters, which (upon binding), induce allosteric changes propagating to transmembrane domains, resulting in opening of the pore. These internal domains serve at the same time as channel gates and as a filtering system for ion selection [239]. Human nAChR are encoded by 16 genes; 10 alpha and 6 non-alpha subunits. Muscle and neuronal nAChRs include different subunit compositions, whih non-neuronal nAChRs also differing among themselves based on the cell and tissue they are expressed in. Thus they show some pharmacological diversity [240]. The subunit types of nAChRs include α, β, γ and δ. Non-alpha subunits expressed in vertebrate central nervous system have been

assigned as β2- β4 while those in neuromuscular junctions are termed β1, γ, δ and ε commonly referred as 'neuronal' and 'muscle-type', respectively [241]. nAChRs are composed of 16 different subunits (γ, δ, ε, α1- α10, β1- β4). Among this subunits, only few (like α7) are able to form a functional homomeric receptors, while others do so only when co-assembled with other subunits [242] (Figure 1.4). Although the 16 functional nAChR subunits (in mammals) can potentially give rise to a considerable number of combinations, evidence shows that their assembly is restricted to limited combination possibilities. α4β2 is the most abundant heteromeric nAChR in mammalian brain while α1β1γδ and α1β1δε are the predominant receptor isotypes in muscular nAChRs [241].

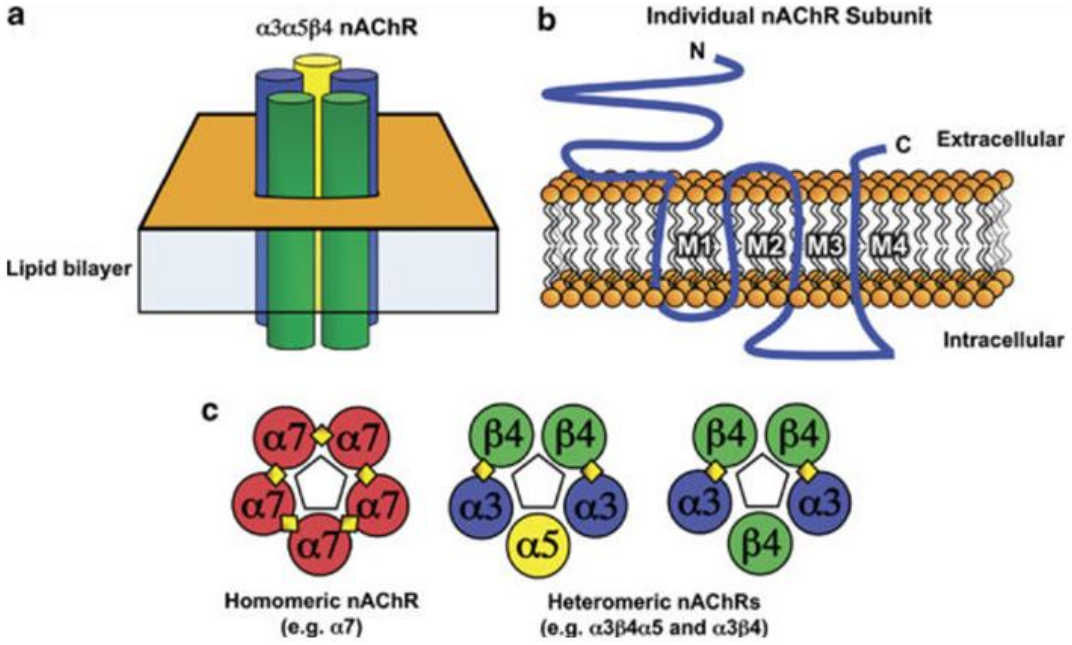

Figure 1.4: Structure of nAChRs.

Schematic representation of nAChRs based on their **A)** assembly, **B)** conserved domains and **C)** different combinations of their subunits (represented by circles) as well as positions of their ligand bindng domains (represented by yellow diamonds). This figure was reprinted with permission from Nature Publishing Group. Improgo et al., From smoking to lung cancer: the CHRNA5/A3/B4 connection, (2010) Oncogene.

1.3.2.4 Cholinergic receptors in CNS

nAChRs provide the influx of ions and facilitate depolarization of cell membrane, resulting in neuronal excitability. Internalization of Ca^{2+} in particular triggers several signaling cascades [243]. Taking into account the mentioned functions of ACh in CNS, AChRs (as targets of ACh) are involved in similar processes such as: cell

sensitivity and neurotransmitter release (at the cellular level), nervous system development and neuronal differentiation (as biological functions), as well as behavioral and cognitive roles insleep, stress and pain (as physiological functions). Other physiological pathways regulated by nAChRs depend on different ligands to which they might bind [244]. The role of nAChR in neuronal synaptic density and morphology is thought to activate continuous rearrangements of neuronal connections. Accordingly, *in vivo* knockdown experiments of AChRs showed impairment of memory and learning abilities in animals [245]. In addition, imbalance in AChR system contributes to several neurodegenerative diseases such as Alzheimer's, schizophrenia and visual hallucinations [246].

Nicotine is the primary addictive compound in tobacco, which, as an exogenous substance, binds to nAChRs and activates them. In the long-term it also upregulates the expression of nAChRs (α4 and β2 subunits), alters their assembly in the plasma membrane, affect the distribution of intracellular receptor proteins, all of which are shown to be associated with chronic nicotine exposure and nicotine addiction [247].

1.3.2.5 Cholinergic receptors at the neuromuscular junction

Skeletal muscle contractile activity is regulated by CNS via transmission of action potential from the motor neuron to the muscle cells. This impulse is transferred in a specialized region between the two cell compartments, named as 'neuromuscular junction synapse' [248]. Due to this synapse presence, cell membrane of skeletal muscle cells is composed of a very dense population of AChR. When the presynaptic nerve impulse reaches the nerve terminal, it induces depolarization of the plasma membrane and release of ACh in the synaptic cleft [228]. AChRs in the neuromuscular junction (NMJ) are mainly composed of α2βεδ [249]. Expression of the subunit genes is affected by the impulse characteristics (thus synapse-specifically) while AChR themselves affect the postsynaptic cell differentiation [250]. Exactly two ACh molecules bind to the AChRs which makes the receptors take an open conformation of the channel, until ACh is hydrolyzed by AChE [251]. Inward current of positive charged ions (mostly Na^{+}) activates voltage gated ion channels in sarcoplasmic reticulum to release Ca^{2+} into the cytosol, which then induces myofibrils and muscle cell to contract as a whole [248]. AChR deficiency in

NMJ (due to gene mutations or AChR-specific autoantibody production), causes congenital myasthenic syndrome or myasthenia gravis (associated to skeletal muscle weakness), respectively [252].

1.3.2.6 Cholinergic receptors in non-excitable tissues

An important clue of possible ACh role other than their synaptic activity, was the finding that nAChRs are expressed also in the non-synaptic region of neurons [253]. The detection of neuronal type-like nAChR in several non-excitable cells has been very interesting. This indicated a possibility for ACh to work as a local hormone or 'cytotransmitter', able to regulate several cell functions that related to fast adaptation to certain conditions [253]. Research groups have investigated and approved the presence of nAChR (mainly composed of α3, α5, α7, β2, β4) in tagumental cells which line the internal and external body surfaces, such as: skin keratinocytes, bronchial epithelial cells and blood vessel endothelial cells. These cells are also sensitive to nicotine, which raises the possibility that nAChRs mediate the nicotine-induced toxicity [254]. Moreover, expression of nAChR in bronchial epithelial cells was found to include neuronal-specific subunits such as: α3, α5, β2, β4, indicating a very similar subunit composition of nAChR between bronchial and neuronal cells [255]. Using intact trachea tissues, same research group verified that α3 and α5 are present on the functional nAChR cell membrane surface, with implications in cell shape and motility [256]. Several studies have found that expression of α3 is usually accompanied by the expression of α5 subunit, especially in the functional neuronal-like nicotinic receptors in human keratinocytes [257, 258]. Another study (blocking α3 by antagonists) showed that expression of α3 subunit was positively correlated to increased cell-cell and cell-substrate adherence (as well as more differentiated state of keratinocytes) and negatively correlated to their motility and migration [259]. The finding that nicotine (which activates nAChRs) increases Ca^{2+} uptake of keratinocytes in a dose-dependent manner implied that these receptors probably are able to transport Ca^{2+}, by which they affect the cellular motility and viability [260]. Similarly, presence of functional nAChRs was found also in human vascular endothelial cells. Similar to neurons in CNS, these cells expressed α3, α5, β2 and β4 subunits. It was proposed that the ligand of these receptors could be either ACh circulating in the blood, the endogenous ACh or both [261]. Both studies suggested

that these tagumental cells expressed functional neuronal-like nAChRs, which were proposed to be involved in the maintenance of a continuous lining of the tissue and the flat shape of the cells. In addition, studies on nAChRs role in endothelial cells have shown implications of these receptors in survival, migration and angiogenesis (mainly through α7 subunit) [262, 263].

Considerable amount of nicotine is detected from the blood of smokers. The fact that functional nAChRs are expressed in epithelial and endothelial cells makes it possible to think that tobacco-induced toxicity in the respiratory and cardiovascular system is induced by the direct contact of nicotine to these tissues, via binding to nicotinic receptors [264]. Nicotine is found to be full agonist (similar to ACh) in the α3 cholinergic receptors containing α5 subunit, however it is only partially behaving as an agonist (inducing less response and desensitization) in those cells which do not have α5 subunit in their nAChRs [265]. Moreover, evidence from *in vivo* experiments show that incorporation of α5 in the α4β2 receptor subtype reduces also the nicotine-dependent upregulation of this receptor subtype [266]. This indicates a very important role of α5 subunit in the nicotine-induced cellular response.

1.3.2.7 Cholinergic receptors and cancer

The finding that nAChRs were expressed in lung cancer cells and that they controlled several processes such as cell proliferation and apoptosis has been revolutionary for the cancer field, heavily impacted by the effects of smoking. Eventhough three out of four lung cancer subtypes develop in the non-smoker population, smoking still is a major factor increasing the risk of lung cancer development, which is though to be mediated through nAChR [267]. In addition to nicotine, several other agonists and antagonist agents regulate central cellular pathways by mediating the release of growth, neurogenic and angiogenic factors in the cell interior or exterior environment [268]. Genome-wide association studies in addition have shown that allelic variations in the α3-α5-β4 gene cluster is associated with the risk of lung cancer. In particular a non-synonymous variant in CHRNA5 (α5) subunit increases the susceptibility of lung cancer by 30% [269]. Subsequent studies on lung cancer showed that CHRNA3 expression negatively affected cell survival by regulating apoptotic pathway and was also suggested to have some negative correlation with the expression of CHRNA5

and CHRNA7 [270]. Another study including 28 small cell carcinoma samples showed that CHRNA5 and CHRNB3 upregulation in tumors was accompanied by changes in ACh and AChE expression [271]. Nicotine related studies showed that binding of nicotine to CHRNA7 in lung cancer cells triggered internalization of Ca^{2+} ions after which voltage-gated ion channels were activated with subsequent activation of MAPK. This caused increase in cell proliferation and downregulation of apoptosis. [272]. However function of CHRNA7 seems to be cell-dependent; inhibiting proliferation in well-differentiated NSCLC while promoting the proliferative effect of nicotine on cell division in poorly-differentiated NSCLC [273]. Homomeric CHRNA7 receptors together with heteromeric α5- β2-β4 isotypes were found to stimulate lung tumor progression by regulating cell proliferation and invasion [273].

Another cancer type affected by nicotine action is pancreatic cancer, where the role of nAChRs was investigated too. Nicotine was found to induce de-differentiation of the cells mainly by activation of α7 subunit, which was followed by positive regulation of AKT and MAPK pathway [274]. Interestingly, it was also found that nicotine induced the release of catecholamines via the activity of α3, α5 and α7 subunits and it inhibited GABA synthesis [275]. Nicotine also was found to increase cell proliferation and metastasis in several pancreatic cancer cell lines mediated by nAChRs activation and subsequent phosphorylation of ERK1/2 [274]. Similarly, in gastric cancer nicotine-related studies have shown the role of nicotine in tumor progression via activating nAChRs [276]. In another study of oral and lung cancer cell lines it was found that nicotine triggers cell survival mechanisms via cooperation of cell membrane nAChRs with EGF and VEGF receptors (by α7, β2 subunits), while it activates anti-apoptotic mechanisms of mitochondrial nAChRs via their association with PI3K and Src (by α7, β4 subunits); showing a dual role of nicotine as a positive and negative regulator of cell division [277]. The positive regulation of cell proliferation by nicotine was also suggested to be via induction of a resistance mechanism for protecting the tumor cells from radiotherapy and chemotherapy. In addition, nicotine was also found to act as a metastatic and angiogenic factor in lung cancer [278, 279].

Breast cancer cells MCF10a and MCF7 were found to express α3, α5, α7, α9, α10, β1, β2, γ, ε and δ subunits of nChR. Malignancy of breast cancer cells was associated

mostly with the expression of α1, α4, β4 and overexpression of α7 and α9 in MCF7 cell line [280]. In a study of 276 breast tumors (versus normal tissue samples), α9 subunit was found to be the highest in expression. Its downregulation via RNAi machinery in MDA-MB-231 decreased cell proliferation and tumor mass in murine model whereas its overexpression in MCF-10A rendered these cells carcinogenic and increased tumor growth when injected in mice [281]. Upregulation of α9 and increased proliferation in MDA-MB-231 upon nicotine treatment related breast cancerogenesis to another factor which is smoking behavior [282]. In addition, expression of α9 was found to be higher in ER+ breast cancer tissues (compared to ER- ones) and showed to increase via estrogen exposure; all of which demonstrate the regulation of nAChRs both by hormones and smoking; the well-known factors involved in breast cancerogenesis [283]. In another study, exposure of MCF7 cells to nicotine increased the population of ALDH-positive cancer stem cells while inhibition of α7 subunit blocked this effect of nicotine [284]. Survival analysis of 208 lymph-node negative breast cancer patients revealed that for the ER-negative patients, cholinergic pathway was significantly associated with the non-recurrence or cancer [285].

1.3.2.8 Cholinergic receptors as drug targets

Taking in consideration 1) the broad expression of nAChR (in central nervous system, peripheral nervous system, non-neuronal cells) and 2) various pathways these receptors are involved in and the variety of receptor isotypes combinations present, nAChRs offer very promising possibilities of treating several disorders in a unique way [286]. So far, only two drug-targets of nAChRs were put in market; Mecamylamine and Varenicline (for hypertension treatment and smoking cessation, respectively) [287, 288]. However, because of low efficacy as well as adverse effects in central nervous system and gastrointestinal system, these drugs didn't show successful therapeutic results. Substantial efforts are now given for new drug design, development and trial studies which have resulted in very promising treatment agents for several other pathologies and diseases, i.e., pain, inflammation, myasthenia, depression, addiction, epilepsy, schizophrenia, Alzheimer's and Parkinson's disease [286]. Except from the agonist binding site in the extracellular compartment of nAChR, there is also evidence for additional allosteric binding sites present on

nAChR extracellular compartment, which makes this receptor a good target for developing novel drugs either as agonists, antagonists or allosteric agents [289].

1.3.3 CHRNA5

CHRNA5 gene (encoding α5 subunit of nAChRs) is located on chromosome 15, locus 15q24, and forms a gene cluster with other two subunits of nAChRs, CHRNA3 (α3) and CHRNB4 (β4), which in immunoprecipitation studies, precipitate together (Figure 1.5). Unlike other subunits, α5 doesn't have the tyrosine residue thus has no ligand-binding domain [290].

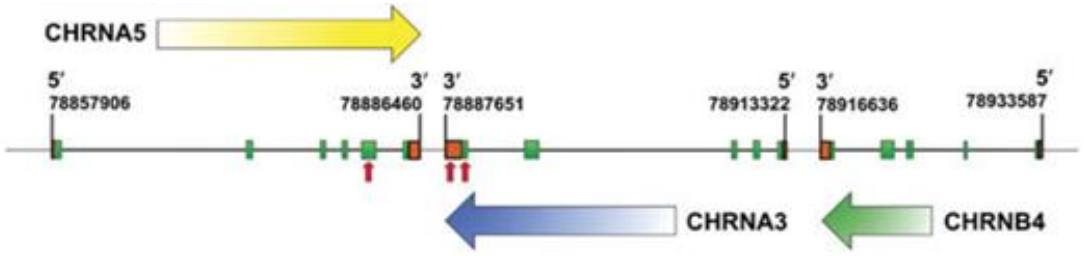

Figure 1.5: α3/α5/β4 gene cluster.

Schematic representation of α5 containing gene cluster. Horizontal arrows are used for indicating the direction of transcription, green boxes for exons, red boxes for untranslated regions, black lines in between for introns and grey ones for intergenic regions. This figure was reprinted with permission from Nature Publishing Group. Improgo et al., From smoking to lung cancer: the CHRNA5/A3/B4 connection, (2010) Oncogene.

1.3.3.1 CHRNA5 role in CNS

Recombinant expression of α3β4 receptors in neurons has shown that α5 subunit changes the functional properties of the nAChRs [291]. It increases the ACh sensitivity, desensitization and the Ca^{2+} permeability of neuronal α3β2 receptors showing lesser effect on α3β4 complexes. Interestingly CHRNA5 shows a dual role in nicotine treatment; increases sensitivity of α3β2 receptors while decreasing sensitivity in α3β4 receptors. Moreover it is suggested that α3β4 receptors (in contrast to the other two subunits) has only one copy of α5 [292, 293]. Knock-out mice studies have revealed that absence of α5 subunit in nervous system increases the nicotine intake which once more demonstrates the role of CHRNA5 in the nicotine addiction and reward system. More specifically this study presents

CHRNA5 as an inhibitory motivational signal which serves to limit the nicotine intake [294]. Another study conducted in 7500 smokers from different European populations has found a common haplotype in CHRNA3(α3): CHRNA5(α5): CHRNB4(β4) gene cluster which predisposes smokers to nicotine dependence [295]. In addition, an SNP in CHRNA5 locus has come up as a protective element for cocaine dependence (whereas it was previously found to be a risk factor of nicotine dependence), showing a possible bidirectional complex mechanism by which CHRNA5 modulates the drug-related reward pathways [296].

1.3.3.2 CHRNA5 role in cancer

There have been many publications based on the association between smoking and cancer regulated by CHRNA5. Same variants which in different genome wide-studies (GWAS) previously associated with lung cancer risk, have been found to have implications also in smoking behavior, or vice versa [297, 298]. A study conducted in lung cancer cell lines has demonstrated that downregulation of CHRNA5 by siRNA resulted in nicotine-like effect; increasing cell motility, invasiveness and Ca2+ influx [299]. Moreover CHRNA5 depletion (in the same study) resulted in downregulation of cell adhesion genes as well as that of p63 (a candidate oncogene), suggesting an important function of CHRNA5 gene also in cell proliferation, however these effects were blocked with downregulation of α7 (the subunit of homopentameric receptors). Another study conducted in A549 lung cell lines showed that CHRNA5 was activated by nicotine and its downregulation blocked nicotine-induced migration/invasion, suggesting nicotine regulation of lung cancer metastasis via CHRNA5 activity [300]. It was also found that nicotine induced cell proliferation via activation of CHRNA5 in gastric cell lines whereas downregulation of α5 via siRNA hindered the nicotine-dependent effects on cisplatin induced apoptosis [301]. Expression of CHRNA5 in HEK-293 cells was correlated to decreased levels in cell calcium response toward nicotine treatment [302]. When α5-siRNA was accompanied by PI3K/AKT pathway inhibition, an increase in apoptosis was observed, suggesting a role of CHRNA5 in nicotine-induced resistivity of cancer cells to chemotherapeutic treatment [301]. Another study conducted by Falvella et al. (2013) on lung cancer identified four exonV-alternatively splice variants of CHRNA5 and one unspliced transcript (suggested to be the only functional, cell

membrane spanning isoform) [303] (Figure 1.6). Moreover comparison of CHRNA5 mRNA expression between the normal lung tissue and lung adenocarcinoma (in the same study) showed upregulation of CHRNA5 in adenocarcinomas suggesting an involvement of this gene in lung carcinogenesis. A very recent study on gastric cancer similarly found that CHRNA5 expression was higher in gastric cancer tissues compared to the para-carcinoma ones [301]. In another study, tumors derived from mouse xenografts of CHRNA5-depleted small cell lung cell carcinoma (SCLC) cell line exhibited lower size and weight, compared to their controls [304].

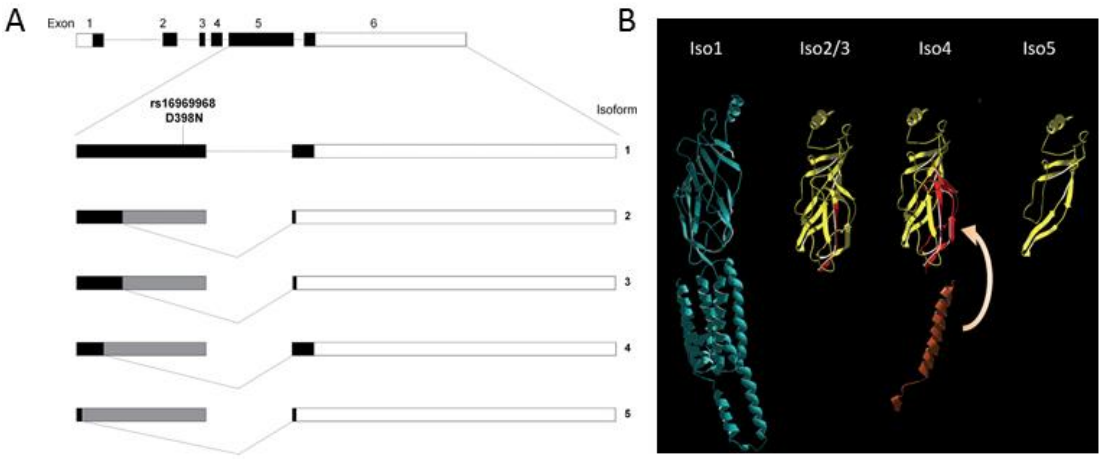

Figure 1.6: CHRNA5 isoforms.

Schematic representation of **A)** unspliced and exonV-spliced CHRNA5 variants in normal lung tissues and **B)** their correponding models, as introduced by Falvella et al., Multiple isoforms and differential allelic expression of CHRNA5 in lung tissue and lung adenocarcinoma, Carcinogenesis, 2013, 34, 6, 1281–1285, by permission of Oxford University Press.

1.4 Genes investigated in the context of CHRNA5 depletion in this study

Literature shows implication of CHRNA5 with cell proliferation (i.e., p63 regualation), motility and adhesion (i.e., ZO-1 regulation). Further investigation of its role in breast cancer requires usage of marker proteins regulating cytoskeletal arrangement, p53 and cell cycle pathways, which could test the role of CHRNA5 in breast tumorigenesis. The following are details on selected genes on which the effects of CHRNA5 RNAi has been tested. Identification of these genes is based on the microarray data analysis described in the methods and results section of this thesis.

1.4.1 MAP1B

Microtubule Associated Protein 1B (MAP1B) is predominantly expressed in neurons where it is found to regulate microtubule assembly (nucleation and stabilization). This protein contains both an actin-binding and a microtubule binding domain and its precursor protein undergoes proteolysis after which a heavy and a light chain is produced [305]. *In vivo* mice studies show that MAP1B is important in adult nervous system and critical in nervous system development (regulating axonal guidance, elongation and migration), mainly by regulating the expression of Rac1 and RhoA-GTP [306]. In cancer-related studies it is found that MAP1B interacts with p53 [307], it regulates invasion [308] and autophagy [309] in neuroblastoma, gastric cancer cells and melanoma cells, respectively. However, its role in cholinergic signaling and in particular breast cancer is not known.

1.4.2 CLDN1

Claudin 1 (CLDN1) is the major component of tight junction complexes. It regulates the permeability of small molecules and ions in epithelial cells via interacting with other family members and provides the barrier for water loss in epidermis [310]. CLDN1 loss is considered as a hallmark of carcinomas in general, with substantial loss observed especially in glioblastoma cells [311]. In addition downregulation of CLDN1 is linked to colorectal cell invasiveness [312] and breast carcinogenesis [313]. However its expression is found to be increased with cervical cancer progression (where CLDN1 regulates EMT transition via interaction with SNAI protein) [314] and in gastric tumors compared to normal controls [315]. Its association with cholinergic signals in breast remains to be studied.

1.4.3 GJA1

Gap Junction Protein Alpha 1 (GJA1, CX43) belongs to the connexin protein family. As a gap junction component, it has a role in forming the intercellular channels facilitating the diffusion of low molecular weight molecules (e.g., glucose, IP3, cAMP, K^+, Ca^{2+}) from one cell to the neighboring one, thus regulating cell growth, differentiation and providing tissue homeostasis [316]. It has a crucial role in heart

contractility and skeletal growth as well as shows involvement in hearing process and bladder functioning [317]. GJA1 is found to be upregulated in chronic wounds and regulate cell migration [318]. It is found to be upregulated also in myoepithelial cells during mammary gland development and pregnancy as well as have function in coordinating the cell contractility during milk ejection [319]. GJA1 is considered as a potential tumor suppressor protein whose expression is altered in primary breast cancers and downregulated during metastasis [320]. Its expression is also shown to correlate with glioma progression [321]. Nevertheless, its connection with CHRNA5 depletion induced cellular changes has not been addressed yet.

1.4.4 GADD45A

Growth Arrest and DNA-damage Inducible Protein GADD45 Alpha (GADD45A) protein expression is correlated with environmental stress conditions such as growth arrest and DNA damage treatments. Its expression is induced either by p53-dependent or p53-indendent mechanisms. Thus, it participates in pathways like cell cycle regulation, apoptosis, senescence, DNA repair, maintenance of genomic stability [322]. GADD45A is negatively correlated with cell proliferation and is considered as a player in the survival mechanism for cancer cells to evade apoptosis, provided by the physical interaction with proteins like p21, p38 and JNK [323]. GADD45A mutations have not been yet identified in cancer patients however, common mutations in TP53 gene are accompanied by upregulation in its protein level in several cancer cell lines [324]. In different leukemias it shows dual role as a promoter or inhibitor of tumorigenesis, in a cell type-dependent manner [325]. Considering CHRNA5 has previously been associated with reduced cell proliferation it is important to identify whether GADD45A has any role in breast cancer growth.

1.4.5 GPNMB

Glycoprotein non-metastatic B (GPNMB) is a type I trasmembrane glycoprotein with an integrin binding motif in the extracellular domain, which promotes the adhesive properties of GPNMB. Its function is not yet fully characterized however it is found to be involved in the regulation of inflammation and autophagy [326]. GPNMB is also considered as a potential marker for kidney injuries [327]. It is highly expressed

on the surface of cancer cells (glioma, melanoma, breast cancer) while in normal cells it is mostly located in cytoplasm, which makes it a candidate oncogene and a promising target for cancer therapies [326]. GPNMB is found to induce invasion in several malignancies, such as, oral cell carcinomas, glioma cells and prostate cancer cells [328, 329]. In breast cancer specifically, GPNMB expression is found to be a pro-proliferative and pro-metastatic factor via binding to integrins, interacting with HER2 pathway, regulating metalloproteinase gene expression and increasing cell adhesion to fibronectin [328-331]. Studies show that p53 binds to GPNMB promoter and regulates its expression. Despite the contradictory reports on the role of GPNMB in tumorigenesis, in breast cancer cell lines and gastric cancer cells it is observed to have tumor suppressive role [332, 333].

1.4.6 CDKN1A

Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) (p21, WAF1) protein expression is regulated by TP53 following DNA damage-inducing treatments. By inhibiting the activities of CDK2 and CDK4, CDKN1A induces cell cycle arrest (at G1 phase) and apoptosis. CDKN1A also regulates the activity of RB protein via inhibiting its phosphorylation [334]. In addition to its cell cycle-related function, CDKN1A is also involved in the regulation of transcription, DNA repair, cell differentiation, cell motility and senescence [335]. Apart from its accepted role as a tumor suppressor protein, CDKN1A can also function as an oncogene, mainly depending on the cell type and its intracellular localization. When it is localized in nucleus, this protein shows its canonical functions as a negative regulator of cell cycle however, when in cytoplasm, CDKN1A promotes tumorigenesis by regulating apoptosis and migration [336]. In melanoma cells p21 deregulation by MAPK signaling was found to be a key factor in maintaining cell proliferation [337]. However in a study of prostate cancer, deletion of CDKN1A in mice rendered cancer cells less tumorigenic [338]. Downregulation of this protein in MCF7 breast cancer cell line had an anti-angiogenic effect [339], while in a study of 65 primary breast tumor samples, CDKN1A expression was associated with poor prognosis and adverse pathologies; these show its potential role as a breast cancer biomarker [340]. Moreover in another study, ER alpha was found to physically interact with CDKN1A (in the absence of estrogen).

1.4.7 ANLN

Anillin Actin Binding Protein (ANLN) by binding and regulating the actin dynamics is involved in cell proliferation (specifically cytokinesis) and migration (especially in glomerulus cells) (http://www.genecards.org). ANLN expression in 156 urinary tract carcinomas was linked to different clinical outcomes, which was suggested to be associated with its subcellular localization [341]. Using RNAi machinery against ANLN in non-small lung cancer cell lines resulted in growth suppression, large cell morphology and multiple nuclei, while overexpression of this protein increased migration of cells and induced stress fibers [342]. Similarly, ANLN expression was correlated to colorectal cancer and gastric tumor progression [343, 344]. Two recent studies have validated its role in regulating proliferation rate and cell cycle in breast cancer cell lines and its high expression in poor survival-showing breast cancer patients, suggesting ANLN as a promising biomarker in breast cancer [345, 346]. However its modulation of expression in CHRNA5 induced changes in cell proliferation is not well known.

1.4.8 BIRC5

Baculoviral IAP Repeat Containing 5 (BIRC5) (Survivin) is mainly known for its involvement in inhibition of apoptosis (by deregulating CASP3 and CASP7), however it can also promote cell proliferation (BIRC5 isoform-specific functions) [347]. During mitosis BIRC5 functions in regulating the spindle organization whereas its own activity is controlled via phosphorylation of different residues by kinases like CDK1 and Aurora B. BIRC5 expression is high during fetal development but low in adult tissues. BIRC5 is negatively regulated by TP53, PTEN and BRCA1-SIRT1 [348]. It is a crucial factor for protecting stem cells against apoptosis and shows similar importance in keeping the cell malignancy in several tumors. BIRC5 expression was found to control cancer cell aggressiveness by providing protection from apoptosis, increasing drug resistance, maintaining the stemness of cancer stem cells and inducing metastasis; all of which have introduced survivin as a poor prognostic gene [349]. BIRC5 is found to correlate with the metastatic disease in several cancer types such as: colorectal [350], gastric [351] and non-small cell lung cancer [352]. Overexpression of BIRC5 in MCF7 cells was correlated with high proliferation rate [353], lymph node

metastasis, stage of breast cancer, risk of recurrence and overall survival [354]. BIRC5 has been associated with nicotine induced signaling. In Myasthenia Gravis patients, alpha subunit of AChRs was found to bind to ANLN-positive peripheral blood mononuclear cells (PBMC) [355].

1.4.9 WDHD1

WD Repeat and HMG-Box DNA Binding Protein 1 (WDHD1) protein, due to its domains, is involved in several pathways like: cytoskeletal and chromatin assembly, DNA repair, pre-mRNA processing, signal transduction, transcription and DNA replication. Its well known role is as an initiation factor during replication process where WDHD1 brings together DNA polymerase complex and MCM proteins (http://www.genecards.org). During replication stress, WDHD1 is found to be phosphorylated by ATR and subsequently activate CHK1, which results in recovery of DNA stalled forks [356]. Depletion of WDHD1 in cancer cells has been associated with increased DNA damage (due to aberrant double strand DNA repair mechanisms), cell cycle arrest at S phase and increase in apoptosis [357]. There are limited numbers of studies focusing on the role of WDHD1 in cancer. For instance, one research group found WDHD1 to be a poor prognostic marker for non-small cell lung and esophageal cancer. Moreover, downregulation of this gene resulted in decreased cell growth while its overexpression induced increase in cell proliferation [358]. The effects of cholinergic signaling on the expression of WDHD1 in breast cancer is not known.

1.5 Microarray Technology in Cancer

1.5.1 Microarray technology

1.5.1.1 Microarray platforms

Sequencing of human genome had substantial impact in the research field by making it possible to analyze the entire human genome (~30,000 genes) at once. Microarray technology has come up as a powerful tool to analyze gene expression profiles, either by custom-made or commercial platforms. Microarray platforms are composed of

immobilized probes either in a nylon membrane or glass slide [359]. Nowadays microarrays help in many different analyses such as analysis of gene expression, polymorphisms, pathogen detection, and biomarker discovery [360]. Mostly due to their ease of use, microarray technology is now the most widely used technology to analyze gene expression. DNA microarrays are divided mainly on the bases of their probe lengths, i.e., short oligonucleotide (~27 bases), long oligonucleotide (~65 bases) and cDNA (variable in length) microarrays [361]. Microarray probes target and hybridize with the labeled cDNA strands which are amplified from RNA by reverse transcription. To increase the specificity of probes to their target sequence, either the probe should have a minimum length of 50 nucleotides, or several probes of each gene have to be combined for analysis [360].

1.5.1.2 Affymetrix microarrays and data processing

Affymetrix GeneChip is the most widely used platform for microarray gene expression analysis, which originates from 1996 [362]. Affymetrix microarrays are composed of short oligonucleotide probes which are synthesized *in situ* using the photolithographic method. Each perfect match probe in Affymetrix is coupled with a mismatch probe (including a mismatch base in the middle of the sequence) which serves as an internal control for measuring non-specific sequence hybridization [363]. However, many normalization methods rely only on the perfect matched probes. Affymetrix oligonucleotide chips contain 11-20 oligonucleotide pairs for each transcript. In addition to these, Affymetrix microarrays also use little amount of RNA and generate less variable results (high reproducibility), all of which makes them advantageous among the other platforms [364]. Within the Affymetrix genome arrays there are several platforms in use, each with specific characteristics, such as: Affymetrix Human Genome U133A Array/GPL96 platform (introduced in 2001, including oligonucleotides of more than 33,000 human genes) and Affymetrix Human Genome U133 Plus 2.0 Array/GPL570 platform (created in 2003, covering 9,921 new probes of approximately 6,500 new genes) (https://www.ncbi.nlm.nih.gov). Since first introduction of Affymetrix arrays, there have been many advances also in production of new Affymetrix platform types, such as the development of Exon arrays (analyzing different exons of same gene) and Tilling arrays (analyzing genomic regions with unknown functions) [360].

1.5.2 Affymetrix GeneChip data analysis

1.5.2.1 Affymetrix GeneChip data processing

The microarray probes hybridize to a single sample after which absolute expression values are obtained from a fluorescent scanning image stored as DAT file. These probe intensities are then converted to CEL files (by GCOS: GeneChip Operating Software) which are subsequently used for statistical analysis [365].

1.5.2.2 Microarray data analysis standardization

Repetition of microarray experiments might produce data with significant variations; mainly due to different array platforms, experimental protocols (treatment procedure, data analysis means) and biological noise (certain genes' expressions might be sensitive to minor changes in experimental conditions). These factors might be avoided with standardization of experimental protocols and data analysis [366]. In addition to possible variability between microarray studies, data complexity and volume of Affymetrix GeneChip results have raised the necessity of evaluating the quality of generated microarray information [365]. Before hybridization procedure, RNA integrity measurement is a highly recommended step which can be performed by electrophoresis record of the extracted RNA sample [367]. Moreover there is an increasing necessity of standardizing microarray control procedures for the meta-analysis studies [368]. Since now there is a plausible standardization for Affymetrix GeneChip procedures and data processing, variability among experiments is thought to come mainly from the biological inconsistencies [369]. Preprocessing of microarray data is a necessary step for transforming the fluorescent signals to gene-expression measurements; this involves: 1) background adjustment (dividing the hybridization signals to background), 2) summarization (combining the probe-level intensities to expression units) and 3) data normalization (removing non-biological differences between arrays) [370]. RMA has been introduced as one of the most precise methods for preprocessing of microarray data [371]. In order to standardize thousands of microarrays performed worldwide, there have been attempts to publish some guidelines, an example of which is MIAME (Minimum Information About a Microarray Experiment) published in 2001 [372]. This guideline describes the

minimal information required to interpret a microarray result so that it can be verified independently and compared with microarrays of other research groups, which include experimental design, sample selection, and array design [372]. An important factor in microarray data analysis is how effectively and easily data can be analyzed. BRB-ArrayTools is a comprehensive, Excel-based statistical analysis system [373]. Being a widely used package, in an increasing number of publications and institutions worldwide, has made BRB-ArrayTool a powerful tool for microarray studies [374].

In addition to standardization of the microarray analysis, a critical step in Affymetrix data processing is to remove the probesets which appear as having redundancy and/or poor quality (due to concerns regarding probe design parameters as well as probe complementarity to its target and off-targets), a process defined as 'identifier filtering' [375]. There have been presented several identifier filtering methods (e.g, GeneAnnot, PlandbAffy, Jetset) which used different criteria of filtering such as, probe sensitivity, probe specificity, and protein coding potential [376]. Jetset Bioconductor package as an example, looks at features such as specificity, coverage, nucleotide alignment to reference genome, probe robustness, and accordingly selects the best probeset with the highest scores [377].

1.5.2.3 Statistical analysis of microarray data

After normalization of microarray data, one of several statistical tests can be applied to assess the genes differentially expressed among different samples (such as between treated cells versus their untreated control or between disease versus healthy cases) [378]. Examples of the widely used statistical tests for microarray analysis are ANOVA, t-test, F-statistic or the non-parametric tests such as Kruskal-Williams rank analysis and Mann-Whitney test [378].

1.5.2.4 Biological interpretation of microarray data

After analyzing microarray gene expression data, a large number of genes might pass the significance threshold and it becomes a challenge to interpret the results as well as to define the most interesting genes to be studied further. For this purpose there

are several databases which give a biological interpretation of the results [379]. Gene ontology softwares are programs which use the gene list one provides and compares the results to the literature in order to find potential pathways affected in the microarray data [380]. One example is DAVID (Database for Annotation, Visualization and Integrated Discovery) (http://www.david.niaid.nih.gov). This program includes four components: 1) the annotation tool (annotation of gene lists from public databases), 2) GoCharts (functional categorization of genes), 3) KeggCharts (classification of genes according to their biochemical pathways and metabolic processes), and 4) DomainCharts (classification of genes according to their protein domains) [381]. GSEA software is also one of the widely used databases to perform the gene enrichment analysis using the MSigDB (The Molecular Signatures Database) collection of annotated gene sets [382]. As advantages with respect to other resources, MSigDB includes: 1) a wide range of gene sources extracted from signatures of well known databases like GO and KEGG, 2) gene sets which are acquired from manual and automatic computational methods, and 3) the largest number of gene sets among all other gene collections [383]. Currently there are 18,026 gene sets in MSigDB which are included in 8 major collections and few other subcollections (http://software.broadinstitute.org). STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (http://string-db.org) database on the other hand offers the assessment of protein-protein interactions by covering both functional (indirect) and physical (direct) interactions between proteins as well as introduces both the known and predicted interactions (using a number of algorithms) in form of networks [384]. Protein interactions in STRING are given a confidence score and 3D structures of proteins, and their domains are also made available [385]. STRING currently covers protein information of more than 2000 organisms and it groups proteins in families. Connecting to other databases, STRING also annotates proteins based on their tissue and disease entities [386]. In addition to the above mentioned gene ontology softwares, gene lists retrieved from microarray studies can also be used to predict potential upstream gene regulators which might have mediated the treatment effects, one example of which is miRNET (http://www.mirnet.ca). This web-based tool, developed from high-throughput experimental approaches on miRNAs and mostly focusing on finding miRNA targets (forward mapping), also accepts microarray gene lists in its Data Input Processing module to map the associated miRNAs from their targets (termed as ‘reverse

mapping') [387]. Among the novelties of miRNET is the development of a network visualization method (taking into account also the synergistic/combinatorial relationship between different miRNAs) and performing of the functional enrichment analysis based on databases like KEGG and GO [387].

Microarray analysis of gene expression profiles provides the screening of the entire cell transcriptome and makes it possible to find the associations between a cell treatment and its corresponding effects. The exponential growth in microarray data has made a challenge for extracting the most useful information out of them [388]. Comparing a list of genes between different datasets can make it possible to identify common signatures between independent studies and to address some puzzling questions. The importance of microarray data usage in research is shown by the current contribution of meta-analysis studies and by the requirement that many journals have for microarray data publication to one of the available repositories like ArrayExpress or Gene expression omnibus (GEO) [389].

1.5.3 Microarray studies in cancer

1.5.3.1 Microarray technology impact in cancer field

Gene expression profiling has been an important tool in revealing the molecular mechanism and regulatory circuits behind a certain stimuli to cells as well as finding differences in gene expression between certain tissues and cell types [390]. Microarray impact especially in cancer field has been immense due to identification of gene markers (regulating cancer progression) and providing the biological background behind several clinical aspects of cancer. Expression profiling of cancer cells has contributed in prognosis, classification of cancer subtypes, defining the most appropriate therapy for treatment as well as predicting the response to therapy [390]. One example of the role of microarray r data in cancer cell classification has been to differentiate between two different leukemia cell types (AML and ALL) based on their expression signature. These two leukemia cell morphology sometimes appear very similar; yet microarray profiling has been the only means of diagnosis [391]. Similarly, molecular subtypes of breast cancer depends on classification of a large cohort of breast tumors using microarray data and revolutionarized the breast

cancer diagnosis (see the section below). However, the association of CHRNA5 depletion with microarray profiles of TOPO inhibitors, although all being shown to be cell cycle inhibitory agents, have not been investigated in the literature.

1.5.3.2 Impact of microarray technology in breast cancer treatment and diagnosis

In case of breast cancer, patients are assigned for chemotherapy according to prognostic and predictive markers, such as, age, hormonal status, tumor size, histological grade, menopausal and lymph node status; however (eventhough these method work well for group statistics) this is a poor predictive tool for individual patients [392]. The first study using microarray technology for breast cancer subtype classification was the one conducted by Perou et al. (2000). In this study, 8102 genes (of cDNA microarray) were used to classify 65 breast tumors into four subtypes, two of which were identified for the first time [393]. Another study of 81 breast cancer carcinomas (focusing on 4720 genome wide SNPs), found the candidate genes for defining breast cancer susceptibility [394]. Following research focused also on the retrospective analysis for discovering gene sets which could serve as predictors of survival. One of them could successfully predict prognosis of around 80 breast cancer patients and associated poor prognosis mainly to basal subtype [395]. Subsequent studies were focused on defining the best gene signatures which could predict metastasis and survival of patients [396, 397]. Other studies focused on predicting response to several chemotherapeutic treatments, such as, paclitaxel, fluorouracil, doxorubicin and tamoxifen, by determining certain genes as markers of response [398, 399]. The impact of microarray techonology on breast cancer field could be assessed by the commercial platforms which are now used to predict recurrence such as MammaPrint, PAM50 ROR, and GGI. These technologies have come up as being superior to the traditional histopathological examinations. [400].

1.5.3.3 Microarray studies in breast cancer research

Several other studies focus on functional characterization of single genes in breast cancer by downregulating their expression via RNAi methods and subsequently analyzing the expression profiles using the microarray technology. For instance a

study performed by Achari et al. (2015) in basal like breast cancer cell lines, downregulated PKCδ gene by siRNA in order to identify novel apoptotic regulators (by means of microarray technology), one of which came out to be claudin-like (CLDND1) gene [401]. Afterwards CLDND1 downregulation in several breast cancer cell lines resulted in induction of apoptosis thus validating the microarray studies and implying that CLDND1 is an important player in the maintenance of breast cancer cell proliferation [401]. Similarly, in another study conducted in the same year, downregulation of STC1 gene via shRNA in MDA-MB-231 and in murine mammary tumor cells revealed an important role for this gene in tumor growth and metastasis [402]. Subsequent microarray analysis following STC1 downregulation identified several genes regulated by STC1, the majority of which were already hallmarks of carcinogenesis [402].

Similar to RNAi exposure-based microarray analysis, chemotherapeutic drug treatments are frequently followed by microarray studies to identify the biological pathways and molecular mechanisms associated with their anticancer effects [403]. For instance in a study conducted by Iorio et al. (2010) eight chemotherapeutic drugs were used for treatment of mammary and ovary cell lines, MCF7 and A2780, respectively [404]. Microarrays were performed to analyze each drug-induced expression profile, which data were further used for bioinformatics studies. Together with repositories of other drug data, in this study they 1) analyzed transcriptional profiles of same drugs among different cell lines and drug concentrations, 2) compiled a ranked list of general drug effects, reducing dosage, cell line-dependent and toxicity effects, 3) quantified the distance between different compounds using GSEA enrichment scores, 4) set up communities of drugs based on their clustering and dense interconnections, 5) found similarities in the mode of action of different drugs, and finally they were able to 6) classify previously uncharacterized drugs based on their gene expression signatures [404]. Based on their analysis, TOPO inhibitors and cyclin-dependent kinase (CDK) inhibitors came up to be closest neighbors in drug networks, which was experimentally found to be due to p21-dependent inhibition of CDKs induced by SN38 (camptothecin derivative) and doxorubicin [404].

1.6 Aims and Rationale

Nicotinic acetylcholine receptors (nAChR) are very important regulators of central nervous system development and differentiation. They control neurotransmitter release and hence several behavioral and cognitive processes of CNS. The finding that nAChRs (including α5 subunit) were expressed and functional also in other tissues, such as tumors, was very important. Additional research showed the association of these receptors with cancer formation/progression and risk for lung cancer. Following publications focused on the molecular mechanisms by which CHRNA5 regulated lung cancer. Previous studies from our lab (performed by Sıla Özdemir, MS Thesis, September 2014) have shown the expression CHRNA5 isoforms (Azer A. Acıkgöz, MS Thesis, August 2013) in breast cancer cell lines; and that they have increased in breast tumors, compared to normals (as in the case of lung cancer; Sıla Özdemir, MS Thesis, September 2014). In addition CHRNA5 expression was found as a potential marker for separation of breast cancer stage and ER status (Sıla Özdemir, MS Thesis, September 2014). These findings inspired us to analyze and identify the functional role of CHRNA5 in breast cancer cell proliferation using RNAi, microarray analyses and qRT-PCR expression studies, assuming that it can be a potential oncogenic marker and its absence could affect proliferation status of the cells.

There was only one study defining the nAChR subunits expressed in MCF10A and MCF7 cell lines (including CHRNA5) [280], while another publication introduced 5 alternatively spliced isoforms of α5 subunit expressed in lung cancer cells [303]. My first aim in this thesis, was to sequence CHRNA5 isoforms and downregulate them using RNAi, taking the MCF7 as a model cell line. Recombinant expression of α3β4 receptors in neurons showed that α5 subunit changed the functional properties of the nAChRs. In addition, few studies in lung cancer and one study in gastric cancer used RNAi machinery to functionally characterize CHRNA5. Therefore, I have aimed to use multiple siRNA molecules against CHRNA5 to obtain CHRNA5-specific downregulation and examine related effects in breast cancer cell lines.

Literature shows that CHRNA5 has an important role in controlling cell proliferation (regulating p63 expression in lung cancer cell lines). My second aim was to study CHRNA5's role in breast cancer cell proliferation (in one TP53 wild type and two

TP53 mutant cell lines). In addition I wanted to examine the CHRNA5-specific role in cell cycle and define potential cell death mechanisms, if any exists.

Lung cancer studies have revealed modulation of cytoskeleton and cell adhesion molecules (via CHRNA5 downregulation) as well as changes in lung cancer cell motility. My third aim was to characterize such changes in MCF7 breast cancer cells using microarray analysis, RT-qPCR validations, phalloidin staining of actin fibers and the scratch assay.

Lung and gastric cancer studies have shown that nicotine activates CHRNA5 and it is via this subunit that nicotine induces its effects on cell proliferation. In addition, the gastric cancer study has shown implication of CHRNA5 in regulating cellular sensitivity to chemotherapeutic drugs induced by nicotine exposure. My forth aim was to better characterize the role of α5 subunit in drug sensitivity by hypothesizing that targeting of CHRNA5 via RNAi method could function synergistically with chemotherapeutic drugs and thus enhance breast cancer cell sensitivity.

My fifth aim was to identify genes/networks common to CHRNA5 RNAi and several drugs used in breast cancer treatment (TOPO and CDK inhibitors) based on comparisons of CHRNA5 RNAi microarray and public drug microarray datasets. Moreover, I have aimed to identify mRNA-miRNA networks common to TOPO inhibitors and CHRNA5 RNAi effects and select candidate microRNAs in connection with the CHRNA5 RNAi- microRNA microarray studies performed in Dr. Konu's Laboratory.

Co-delivery of chemotherapeutic drugs by different nanoparticle formulations is now applied both in *in vivo* studies and patient trials for clinical applications. There are currently several nanoparticle complexes used in their raw form for diagnostic purpose and others used (in combinations with drugs) in therapeutic regimens. My sixth aim was to help characterize a red emitting, pH-sensitive, non-toxic, CB7-capped oligomeric nanoparticle, developed by Dr. Tuncel's group at Bilkent University, for its usage as a drug carrier and imaging molecule. For such purpose I have aimed to characterize the effects nanoparticles on cell viability, alone or in combination with camptothecin, using two breast cancer cell lines, i.e., MCF7 and MDA-MB-231.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Laboratory reagents and kits used during experimental procedures

Laboratory reagents and kits including the products purchased from our projects as well as reagents and salts used in common from general laboratory stocks. Table 2.1 lists these items together with their catalog number and company information.

Table 2.1: Products used for experimental procedures

Product name	Catalog no.	Company (Country)
Water, molecular biology grade, nuclease free	SH30538.01	HyClone (USA)
Bacto-tryptone	1612	Conda (Spain)
Yeast Extract	1702	Conda (Spain)
Agar (microbiology grade)	05039	Sigma-Aldrich (Germany)
NaCl	31434	Sigma-Aldrich (Germany)
Pipes	A1079	Applichem (Germany)
Glucose	G7021	Sigma-Aldrich (Germany)
$CaCl_2$	A4689	Applichem (Germany)
KCl	12636	Sigma-Aldrich (Germany)
$MnCl_2{\cdot}2H_2O$	A146417	Merck (USA)
KOH	B534712	Merck (USA)
Dimethyl sulfoxide (DMSO)	A1584	Applichem (Germany)
EDTA	A3562	Applichem (Germany)
Glacial acetic acid	27225	Sigma-Aldrich (Germany)
Bromophenol blue	B5525	Prona (Spain)
Xylene cyanol	X4126	Sigma-Aldrich (Germany)
Glycerol	15524	Sigma-Aldrich (Germany)
Quick-Load Taq 2X Master Mix	M0271L	BioLabs (New England)
Agarose	BHE500	Prona (Spain)
Ethidium bromide	17898	Thermo Scientific (USA)
GeneRuler 100 bp DNA Ladder	SM0241	Thermo Scientific (USA)

Product name	Catalog no.	Company (Country)
GeneRuler 1 kb DNA Ladder	SM0312	Thermo Scientific (USA)
Agarose Gel DNA Extraction Kit	11696505001	Roche (Switzerland)
pGEM-T Easy Vector System I	A1360	Promega (USA)
$MgCl_2 \cdot 6H_2O$	A404233	Merck (USA)
$MgSO_4 \cdot H_2O$	M7634	Sigma-Aldrich (Germany)
Ampicillin	A0839	Applichem (Germany)
IPTG	R0392	Fermentas (USA)
x-Gal	R0401	Fermentas (USA)
PureLink Quick Plasmid Miniprep Kit	K210010	Invitrogen (USA)
RNeasy Mini Kit	74104	Qiagen (Germany)
2-mercaptoethanol	M3148	Sigma-Aldrich (Germany)
EtOH	B2221	Sigma-Aldrich (Germany)
RevertAid First Strand cDNA Synthesis Kit	K1622	Fermentas (Canada)
RNase-Free DNase Set (50)	79254	Qiagen (Germany)
LightCycler® 480 SYBR Green I Master	4887352001	Roche (Switzerland)
Vybrant MTT Cell Proliferation Assay Kit	V-13154	Invitrogen (USA)
SDS	71725	Sigma-Aldrich (Germany)
HCl	07102	Sigma-Aldrich (Germany)
NaOH	06203	Sigma-Aldrich (Germany)
S-(+)-Camptothecin (100mg)	C9911-100MG	Sigma-Aldrich (Germany)
Doxorubicin	5927S	Cell Signaling (USA)
5-bromo 2-deoxyuridine (BrdU)	B5002	Sigma-Aldrich (Germany)
MetOH	24229	Sigma-Aldrich (Germany)
Borax	B3545	Sigma-Aldrich (Germany)
BSA	A7906	Sigma-Aldrich (Germany)
Tween-20	822184	Merck (USA)
Na_2HPO_4	04272	Sigma-Aldrich (Germany)
KH_2PO_4	04243	Sigma-Aldrich (Germany)
DAPI	D9542	Sigma-Aldrich (Germany)
Propidium iodide solution	P4864	Sigma-Aldrich (Germany)
RNase A	34388	Serva (Germany)
Triton X-100	T8787	Sigma-Aldrich (Germany)
Cell Death Detection Elisa	11544675001	Roche (Switzerland)
PFA	76240	Fluka (USA)
Alexa Fluor® 488 Phalloidin	A12379	Life Technologies (USA)
UltraCruz Mounting Medium	sc-24941	Santa Cruz Biotechnology
Crystal violet	V5265	Sigma-Aldrich (Germany)

Product name	Catalog no.	Company (Country)
NaCl	K47011104609	Millipore (USA)
6-Aminocaproic acid	A2504	Sigma-Aldrich (Germany)
Trisma HCl	T3253	Sigma-Aldrich (Germany)
Trisma Base	T1503	Sigma-Aldrich (Germany)
Glycine	G8898	Sigma-Aldrich (Germany)
Ammonium persulfate	A3678	Sigma-Aldrich (Germany)
TEMED	A11480100	Applichem (Germany)
NP-40	A16940250	Applichem (Germany)
cOmplete, EDTA-free	11873580001	Roche (Switzerland)
Bio-Rad Acrylamide Kit	161-0183	Bio-Rad (USA)
PageRuler Prestained Protein Ladder	26616	Thermo Scientific (USA)
PVDF membrane	3010040	Roche (Switzerland)
ECL Plus Western Blotting Detection System	RPN 2232	GE Healthcare (UK)
MXBE-Film	771 0783	Carestream (USA)

2.1.2 Products and reagents used in cell culture procedures

Cell growth media, buffers, enzymes and reagents used for maintenance and treatment procedures of cultured cells. The products are listed in Table 2.2 together with their catalog number and company information.

Table 2.2: Products used for cell culture procedures

Product name	Catalog no.	Company (Country)
Dulbecco's Modified Eagles Medium (DMEM)	SH30021.01	HyClone (USA)
PBS without Ca++ Mg++ or phenol red	17-516F	Lonza (Switzerland)
Trypsin/EDTA	CC-5012	Lonza (Switzerland)
Dimethyl sulfoxide (DMSO)	A1584	Applichem (Germany)
L-glutamine	BE17-605E	Lonza (Switzerland)
Pen/Strep stock	DE17-602E	Lonza (Switzerland)
NEAA 100X	BE13-114E	Lonza (Switzerland)
HiPerFect transfection reagent	301704	Qiagen (Germany)
Tryptan blue	T8154	Sigma-Aldrich (Germany)

2.1.3 RNAi molecules used in experimental procedures

Nucleic acids used during RNAi procedures of this study, all of which were provided from Qiagen 'FlexiTube siRNA' products. Sequences as well as additional information about siRNAs are listed in Table 2.3.

Table 2.3: Nucleic acids used for RNAi studies

siRNA name	Annotation	Catalog no.	Target sequence
AllStars Negative Cnt siRNA	siRNA-CN	1027280	"Proprietary"
Hs_CHRNA5_5	siRNA-1	SI03051111	5'-ATGGATCACAGGTTGATATAA-3'
Hs_CHRNA_6	siRNA-3	SI03096940	5'-CTGGACTCCACCGGCAAACTA-3'

2.1.4 Antibodies used in Western blot and immunofluorescence studies

Primary and secondary antibodies used for Western blot (WB) and Immunofluorescence (IF) experimental procedures. The corresponding catalog number and company information are listed in Table 2.4.

Table 2.4: Antibodies used in WB and IF studies

Antibody name	Catalog no.	Company (Country)
Anti-CHRNA5	AAS67349E	Antibody Verify (USA)
Anti-CHRNA5 monoclonal	EPR5395	Abcam (UK)
Acetyl-CoA Carboxylase	3676	Cell Signaling (USA)
Anti-Calnexin	ab2301	Millipore (USA)
FAS	8023	Cell Signaling (USA)
BID	2002	Cell Signaling (USA)
GAPDH	25778	Santa Cruz (USA)
Anti-rabbit IgG, HRP-linked	7074	Cell Signaling (USA)
Anti-mouse IgG, HRP-linked	7076	Cell Signaling (USA)
BrdU (Bu20a)	5292	Cell Signaling (USA)
Anti-mouse Alexa Fluor-555	4409	Cell Signaling (USA)

2.1.5 Primers used in the study

Primer sequences used in the study for gene expression analysis by RT-qPCR (together with their working conditions) are listed in Table 2.5.

Table 2.5: Primer pairs used in RT-qPCR studies

Name	Primer sequence (5'-3')	Size of amplicon (bp)	Tm (ºC)	Amplification efficiency
CHRNA5_v1	F: AGATGGAACCCTGATGACTATGGT	104	79.06	1.87
	R:AAACGTCCATCTGCATTATCAAAC			
CHRNA5_v2	F:GGAAACTGAGAGTGGTAGTGGA	122	79.3	1.95
	R:CTTCAACAACCTCACGGACA			
CHRNA5_v3*	F:CATCAGGTGTTGAAGATTGGAAAT	101	78.29	1.92
	R:AAAAAGCCCAAGAGATCCAACAAT			
CHRNA5_iso2**	F:TGGAGAATGGGAGATTGTGAGTGCA	78	81.11	1.97
	R:CCAATCTTCAACAACCAGCAACAGC			
CHRNA5_iso3**	F:TGGAGAATGGGAGATTGTGAGTGCA	84	81.41	1.96
	R:CCAATCTTCAACAACGGATACCAGC			
TPT1	F:GATCGCGGACGGGTTGT R:TTCAGCGGAGGCATTTCC	100	85.2	1.95
SDHA	F:TGGGAACAAGAGGGCATCTG	86	78.24	2.01
	R:CCACCACTGCATCAAATTCATG			
MAP1B	F:GTTGGAAGGAAAGGCTCAGT	110	82.88	1.81
	R:CTTGCTGTTTCTCATGGGTC			
CLDN1	F:CTGTCATTGGGGGTGCGATA	118	80.18	1.88
	R:CTGGCATTGACTGGGGTCAT			
WDHD1	F: AGCAGCCAAGGACGAGTAAA	192	79.42	1.95
	R:CTTCGGCTTTGGAATCAGAG			
ANLN	F:TAAAGCAGGTGATTGTTCGG	180	78.13	1.97
	R:GTTCTTCATCAACACAGCAG			
BIRC5	F:GTTGCGCTTTCCTTTCTGTC	141	78.51	1.92
	R:TCTCCGCAGTTTCCTCAAAT			
CDKN1A	F:GTCACTGTCTTGTACCCTTGTG	129	86.36	1.82
	R:CGGCGTTTGGAGTGGTAGAA			
GPNMB	F:TGCTGACTGTGAGACGAACC	204	86.16	1.92
	R:ACACCAAGAGGGAGATCACAG			
GJA1	F: TCTGAGTGCCTGAACTTGCC	171	83.68	2
	R:CCCTCCAGCAGTTGAGTAGG			

Name	Primer sequence (5'-3')	Size of amplicon (bp)	Tm (ºC)	Amplification efficiency
GADD45A	F:TCTCGGCTGGAGAGCAGAAGAC	121	85.91	1.96
	R:AGCTTGGCCGCTTCGTACAC			
ACHE	F:TCTCGAAACTACACGGCAGA	164	88.78	NA
	R:CGCAGGTCCAGACTAACGTA			

[a] *Melting temperatures determined according to RT-qPCR results*

**(Warzecha et al. 2009) **(Falvella et al. 2013)*

2.1.6 Laboratory equipments used in the study

Machines and devices used to perform or analyze experimental results of the study. The assays where these instruments are used together with their company information are listed in Table 2.6.

Table 2.6: Laboratory instruments used for experimental procedures

Name of the instrument	Assay	Company (country)
PCR Thermal Cycler 2720	RT-PCR	Applied Biosystems (USA)
Thermal cycler TC-512	Gradient PCR	Techne (UK)
Midicell Primo EC-330	Gel electrophoresis	Thermo Scientific (USA)
LightCycler 480 Instrument	RT-qPCR	Roche (Sweitzerland)
Zeiss-Axioimager A1 Carl	IF-BrdU	Carl Zeiss (Germany)
ECLIPSE-TS100 microscope	Cell culture, scratch assay	Nikon (Netherlands)
Coolpix 4500 camera	Cell culture, scratch assay	Nikon (Netherlands)
Biofuge Pico centrifuge	All assays	Heraeus instruments (Germany)
Xl-90 Ultracentrifuge	Competent cell preparation	Beckman (USA)
Innova4300 incubator shaker	Cloning, competent cell prep.	New Brunswick Scientific (UK)
µQuant elisa reader	MTT	Biotek (USA)
BD Accuri C6	PI staining-FACS	BD Biosciences (USA)
DU640 spectrophotometer	SDS-PAGE, competent cell	Beckman (USA)
Hyperprocessor (x-ray)	Western blot	Amersham (Mexico)
NanoDrop ND-1000	RNA isolation, cloning	Thermo Scientific (USA)

2.2 Solutions and Media Preparations

Preparation of buffers, solutions and media which were used during experimental procedures. Preparation guidelines as well as the assays these solutions are used for, are listed in Table 2.7.

Table 2.7: Solutions and buffers used in the study

Solution name	Preparation	Assay	Total volume
10x PBS	80 g NaCl, 2 g KCl, 2 g KH2PO4, 25.6 g Na2 HPO4.7H_2O, ddH_2O	General	1 L
L-agar	10 g tryptone, 5 g yeast extract, 5g NaCl, 15 g agar, dH_2O	Cloning	1L
AIX agar	10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, 1 ml 100 mg/ml, ampicillin, 1ml 100 mM IPTG, 1ml 3% X-gal, dH_2O. pH 7	Cloning	1L
L-broth	10 g Tryptone, 5 g yeast extract, 5 g NaCl, dH_2O. pH 7	Competent cell preparation	1 L
LB-ampicillin	10 g Tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 100mg/L ampicillin, dH_2O. pH 7	Competent cell prep, Cloning	1 L
TB buffer	2 ml 0.5M pipes, 3 ml 0.5M $CaCl_2$, 25 ml 1M KCl, 5.5 ml 1M $MnCl_2$,25 ml 1M KOH, dH_2O. pH 6.7	Competent cell preparation	100 ml
50x TAE buffer	242 g Trisma base, 18.6 g EDTA, 57.1 ml glacial acetic acid, dH_2O	RT-PCR	1 L
1.5% Agarose gel	1.5 g agarose, TAE buffer, 3 µl 1mg/ml EtBr	RT-PCR	100 ml
6x Loading dye	12 x 10^{-5} g xylene cyanol, 12 x 10^{-5} g bromophenol blue, 80 µl 0.5M EDTA, 800 µl glycerol	RT-PCR	1 ml
SOC medium	20 g tryptone, 5 g yeast extract, 0.59 g NaCl, 0.19 g KCl, 2.03 g $MgCl_2$, 2.47 g $MgSO_4$, 2 ml 1M glucose, dH_2O	Cloning	1 L
SDS-HCL solution	1 g SDS, 0.01M HCl	MTT assay	10 ml
Borate buffer	3.8 g borax, dH_2O. pH 8.5	BrdU	100 ml
PI solution	5 µl 1mg/ml PI, 5 µl 100% Triton-X, 100 µl RNase A, NF-H_2O	PI staining	10 ml
PBX buffer	0.1 L Triton-X, 1x PBS	Phalloidin staining	100ml
BBX buffer	1g BSA, 0.1 L Triton-X, 1x PBS	Phalloidin staining	100ml
Cryastal violet	5% crystal violet, 25% MetOH, dH_2O	Scratch assay	100 ml

Solution name	Preparation	Assay	Total volume
solution			
RIPA buffer	75 µl 2M NaCl, 50 µl 1M Tris-HCl, 10 µl 10% SDS, 40 µl 25x proteinase inhibitor, 815 µl ddH_2O	SDS-PAGE	1 ml
10x Running buffer	30.3 g Trisma base, 144.1 glycine, 100 ml 10% SDS, ddH_2O	SDS-PAGE	1 L
Anode I buffer	36.3 g Trisma base, 20% methanol, ddH_2O	SDS-PAGE	1 L
Anode II buffer	3 g Trisma base, 20% methanol, ddH_2O	SDS-PAGE	1 L
Cathode buffer	5.24 g 40mM aminocaparoic acid, 20% methanol, ddH_2O	SDS-PAGE	1 L
10x TBS	12.19 g Trisma base, 87 g NaCl, ddH_2O. pH 7.6	Western blot	1 L
Blocking solution	5 g milk powder, 100 µl Tween-20, 1x TBS, ddH_2O	Western blot	100 ml

dH_2O: distilled water. ddH_2O: double distilled water. NF-H_2O: Nuclease Free water.

2.3 Methods

2.3.1 Maintenance and handling of cell lines

MCF7, BT-20 and MDA231 breast cancer cell lines were preserved in liquid nitrogen in aliquots of cryo-vials. On the day of usage cells were thawed in 37^oC water . Cells were subsequently diluted by Dulbecco's Low glucose Modified Eagles Medium (DMEM), centrifuged at room temperature to obtain pellet, removed from freezing medium and resuspended in fresh DMEM. Afterwards cells were transferred to T25 tissue culture flasks and maintained in 5% CO_2, 37^oC incubator. Next day cells were washed with PBS, trypsinized 5 min with 0.25% Trypsin/EDTA and centrifuged to obtain cell pellet. After being resuspended in 15ml DMEM cells were transferred to T75 tissue culture flasks and maintained in incubator. After at most 3 days of incubation, when reaching 70%-80% confluency, cells were split again 1:2 to 1:4, according to their growth rate. At the day of usage, cells were washed with PBS, trypsinized with 0.25% Trypsin/EDTA and pellet was subsequently resuspended in 10 ml fresh DMEM. Cells were counted using a glass haemocytometer and a coverslip. 10ul from evenly distributed cell mixture was taken twice and both chambers underneath the coverslip were filled with cell suspension. Using 10X objective of inverted microscope, cells within the middle square of area 1mm^2 were

counted and the average cell number 'C' from both chambers was used to calculate volume (ml) of cell suspension to be used for experimental purpose 'v' which includes the number of cells required for experimental purpose 'E', by taking into account also the haemocytometer constant '10^4', as given in Eq (1).

$$v = \frac{\mathrm{E}}{\mathrm{C}x\ 10^4} \qquad \text{Eq (1)}$$

Accordingly, 2000 or 200 000 cells were seeded in each well of 96 or 6-well plates respectively. Cells were used at maximum 5th passage after thawing. On the day of freezing, cells were washed with PBS, trypsinized with 0.25% Trypsin/EDTA, centrifuged and split in 4 to 5 cryo-vials after being diluted in freshly prepared freezing medium containing 90% sterile filtered FBS and 10% DMSO. Cryo-vials were incubated at -20^{o}C for 1 hour followed by overnight incubation in -80^{o}C freezer. Next day vials were transferred to liquid nitrogen and preserved in nitrogen tanks. All mentioned procedures were performed under sterile conditions.

2.3.2 Competent *E.coli* cell preparation from DH5α strain

Single DH5α colonies were picked from agar plate into 15ml LB and grown overnight at 37^{o}C with 225rpm agitation. Overnight culture was added onto 200ml fresh LB until optical density (OD_{600}) reached 0.2. The mixture was then incubated at 25^{o}C with shaking until OD_{600} reached 0.6. LB mixture was transferred into 465ml precooled ultracentrifuge tubes and centrifuged at 4300 rpm for 10min at +4^{o}C. Supernatant was discarded and cells were resuspended in 64ml ice-cold TB. Bacterial mixtures were then transferred into precooled 30ml ultracentrifuge tubes and left on ice for 10min, after which they were centrifuged under the same conditions as before. Supernatant was again discarded and cells were resuspended in 8ml ice-cold TB containing 7% DMSO. Aliquots of competent cells were put directly into liquid nitrogen and preserved at -80^{o}C freezer until the day of usage.

2.3.3 RT-PCR

Reverse Transcriptase-PCR (RT-PCR) was performed as the first step of cloning CHRNA5 isoforms. Multiple isoforms amplifying primer set (Forward 5'-

GGAGATACCCTGATGATGACTTAA-3', reverse 5'-AAAAAGAAGCCCAGAAACAATTCC-3') [405] was used with cDNA of MCF7 cells. The following PCR mixtures of 50µl total volume were prepared: 20µl H_2O, 25µl Quick-Load Taq 2X Master Mix, 1µl forward primer (10pmol), 1µl reverse primer (10pmol),3µl cDNA. A negative control reaction with no cDNA was included to detect any possible contamination of reagents. Reaction mixtures were incubated in PCR machine with 40 cycles of 95ºC, 58ºC and 72ºC each lasting 30 seconds. Product mixtures and 5µl of DNA ladder were run in 1.5% agarose gel containing EtBr (Figure 3.1). TPT1 was used as a housekeeping gene in RT-PCR studies.

2.3.4 Cloning of CHRNA5 isoforms

PCR products were visualized under UV light. ExonV-unspliced (CHRNA_v2) and the shortest known, exonV spliced (CHRNA_v3) amplicon' bands (Figure 3.1) were cut out with a blade from 1.5% agarose gel and transferred to eppendorf tubes. Nucleic acids were extracted from the gel using Agarose Gel Extraction Kit (Roche) according to the kit protocol. Pure PCR products were cloned into pGEM-T Easy vector (Promega) according to kit instructions, including positive and negative control reactions. Mixtures were incubated 1 hour at RT for ligation to take place. Afterwards ligated constructs were transformed into *E.coli* competent cells. In brief 150ul of competent cells were added to ligation mixtures and heat shocked for 50 seconds in water bath at exactly 42ºC, followed by addition of SOC medium. After incubating mixtures at 37ºC for 1.5 hours with 225rpm agitation, transformed bacteria were spread into 100µg/ml Ampicilline, 100mM IPTG, 3% X-Gal (AIX) plates. Plates were incubated overnight in 37ºC incubator. Next day blue-white selection was used to distinguish between negative and positive transformants respectively. Single white colonies were picked and grown overnight in 3ml LB-ampicillin, at 37ºC with agitation at 225rpm. Plasmids of bacterial cultures were isolated using PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to manufacturer's protocol. Plasmid concentrations were determined with NanoDrop ND-1000 spectrophotometer.

2.3.5 Isoform sequencing and isoform specific primer design

Plasmids were sent for sequencing to RefGen (Ankara). After sequencing with SP6 promoter-specific primer, resulting sequences were extracted from Chromas Lite (2.01) and aligned to full CHRNA5 mRNA sequence obtained from NCBI (NM_000745.3), using Blast (http://www.ncbi.nlm.nih.gov). Accordingly, two forward primers were specifically designed corresponding to the two isoforms (CHRNA5_v2, CHRNA5_v3; Table 2.5) including the two specific splice sites for CHRNA5_v3 in the forward primer. In addition, another primer pair (CHRNA5_v1) was used to amplify multiple isoforms, together with two other published primer pairs amplifying exonV-spliced isoforms (Iso2 and Iso3; Table 2.5) [303]. A graphical representation of the primer sequences on exon structure of CHRNA5 was obtained using ApE 8.5.2.0 software (Figure 3.3).

2.3.6 RNA extraction and cDNA synthesis

RNA was extracted from frozen cell line pellets obtained from 6-well plates by using RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. As a last step, 35µl nuclease free water was added to the extracted RNA pellets. RNA concentrations were measured with NanoDrop ND-1000 spectrophotometer by using 1µl of total RNA. RNA quality of microarray samples was determined by RIN values using Agilent 2100 Bioanalyzer.

Corresponding volumes of 1µg total RNA was used for reverse transcription with oligo-dT primers using RevertAid first strand cDNA synthesis kit (Fermentas). The following suggested reaction mixtures of 20µl total volume were prepared: 1µg total RNA template, 1µl oligo-dT primer, up to 12µl using nuclease free water, 4µl 5x Reaction buffer, 1µl RiboLock RNase inhibitor, 2µl 10nM dNTP mix, 1µl RevertAid M-MuLV reverse transcriptase. Mixtures were incubated for 60 minutes at 42^{o}C and reaction was terminated by heating at 70^{o}C for 5 minutes. cDNA samples were maintained at -20^{o}C for further use.

2.3.7 siRNA transfection

24 hours before treatment, 2 x 10^5 of MCF7 cells were seeded in 6-well plates. Transfection was performed using 10nM of a siRNA targeting several CHRNA5 isoforms (SI03051111, Qiagen) and same concentration of a negative control, using HiPerFect transfection reagent (Qiagen). An untreated control group 'siRNA-CN' was used to detect possible transcriptome changes induced by the transfection reagent as well as the nucleic acid treatment. Reaction tubes containing respective volume from each of siRNAs/ siRNA-CN and 12µl of transfection reagent were incubated 10 minutes at RT for complexation to take place. Transfected cells were incubated at 37ºC for 72h (or 120h when required) using at least two biological replicas. For experiments with a total of 120 hour treatment, an additional transfection was performed 72 hours after first one. Cell pellets were kept at -80ºC until the RNA isolation procedure was performed. siRNA-1 validation experiments were repeated with another siRNA molecule (FlexiTube, SI03096940, Qiagen; siRNA-3) which (similar to siRNA-1), targeted exonV of CHRNA5 transcript. Higher doses (50nM) of siRNA-3 and an additional 48 h transfection period was needed for siRNA-3 to obtain significant downregulation of CHRNA5 mRNA level.

In BT-20 and MDA-MB-231 breast cancer cell lines, RNAi treatment against CHRNA5 splice variants was performed using 10nM and 50nM of siRNA-1 (SI03051111, Qiagen), respectively.

2.3.8 Microarray and data analysis

To analyze the expression profile of siRNA-1 versus siRNA-CN treated (10nM) MCF7 cells (using two biological replicas for each), Affymetrix HGU133plus2 platform was used. Data were accessed by GeneChip Operating Software (Affymetrix HGU133 plus 2). *AffyQCReport* and *simpleaffy* packages [406] were used to perform the quality control in R. Data were then RMA normalized and differential expression of genes was obtained by *limma* package (performed by Huma Shehwana) [407]. BRBarray tool was used for quality control of the microarray data as well as correlation analysis.

Enrichment of KEGG pathways was tested using DAVID (http://www.david.ncifcrf.gov) seperately for upregulated and downregulated probesets from limma. Similarly, probesets were tested using CGP analysis in MSigDB (http://www.broadinstitute.org) to pick up enriched datasets related to E2 and tamoxifen treatment in MCF7 cells.

2.3.9 Primer design and primer efficiency

Primer design for RT-qPCR studies was performed using 'Primer-BLAST' option from National Center for Biotechnology Information (NCBI) online software (http://www.ncbi.nlm.nih.gov). After several template-specific primers were introduced, the primer pair containing forward and reverse strand hitting exons with at least one long intron in between (or one of them spanning exon-exon junction) was selected. Primers were also chosen so that they had the most optimal qualities and working conditions. Primer strands were rechecked from 'In Silico PCR' in UCSC Genome Bioinformatics website (http://www.genome.ucsc.edu) to verify single gene hit and amplification of multiple isoform transcripts of the selected gene.

Primer efficiencies were determined as needed using serially diluted cDNA as either 5-fold or 2-fold (for low expressed genes). Log_{10} (cDNA dilution) versus related Ct values were used to plot a linear graph. The graph' slope was used to calculate average amplification efficiency (Eff) for each primer pair (Eq 2), as listed in Table 2.5. Sıla Özdemir and Ilgın Cağnan contributed in the primer efficiency studies.

$$Eff = 10^{-1/slope} \qquad \text{Eq (2)}$$

2.3.10 RT-qPCR and expression analysis

For all qPCR experiments, real-time RT-qPCR was performed with the SYBR Green mix (04707516001, Roche) using LightCycler® 480 Instrument. A total volume of 10µl was prepared for each reaction containing 1µl H_2O, 5µl 2X Master Mix, 1µl forward primer (10 pmol), 1µl reverse primer (10 pmol) and 2µl cDNA mixture. Reaction conditions were adjusted as in Table 2.8.

Table 2.8: Reaction conditions of RT-qPCR

Cycle step	Hold temperature (^{O}C)	Hold time (seconds)	No. of cycles
Pre-incubation	95	5	1
Denaturation	95	10	
Annealing	58 / 60	20	40
Extension	72	20	
	95	5	
Acquisition	55	60	1
	95	Continuous acquisition	
Final extension	40	30	1

Samples were run in duplicates which Ct (cycle threshold) values were averaged and used for further analysis. SDHA was used as reference gene in RT-qPCR studies (Table 2.5). Relative expression value (ΔΔCt) for each sample was calculated using the difference in Ct values between control and treatment of target gene normalized to that of reference gene, using the equation as given in Eq (3). Corresponding primer efficiencies were used as in Table 2.5. Statistical analysis and representation of siRNA-1 expression profile was performed using $\log_2$ of efficiency corrected ΔΔCt values. Microarray calculations were performed subtracting average logFC values of siRNA-CN controls from each of siRNA-1 treatments.

$$Relative\ expression\ (\Delta\Delta Ct) = \frac{Eff_{target}{}^{\Delta CP_{target}(control-treatment)}}{Eff_{ref}{}^{\Delta CP_{ref}(control-treatment)}} \qquad \text{Eq (3)}$$

2.3.11 siRNA and drug toxicity using MTT assay

24 hours prior to treatment, $2x10^3$ MCF7 cells were seeded in each well of 96-well plates, at least in three replicas. After 72 or 120 hours of treated cells' incubation, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed using Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) and solutions from general laboratory use. In brief, treated MCF7 cells were replenished with growth media, were given MTT (4h, 37ºC) and SDS-HCL solution (18h, 37ºC), after which color absorbance was determined using Microplate Spectrophotometer (μQuant, Biotek) at a wavelength of 570nm. Results were normalized against the measurements from at least three replicas exposed to no treatment.

For siRNA experiments in MCF7 cells, an additional 48h siRNA treatment on top of the 72h exposure was performed (total 5 day treatment) prior to MTT treatment, using siRNA-1 together with another siRNA against CHRNA5 (siRNA-3) with an initial cell seeding of $0.8x10^3$. 72h and 120h exposure MTT results were analyzed separately. MTT assay was perfomed also in BT-20 and MDA-MB-231 cell lines after 72h siRNA-1 exposure at different cell seeding densities, by keeping the treatments and protocol similar to the above mentioned one.

For experiments related to CPT and DOXO drug exposure, cells were treated with either siRNA (10nM), CPT/DOXO (2x dilution within 0.125 – 2μM range) or a combination of siRNA and drug. Experiments were performed with an incubation time of 72h. Prior to drug experimental procedures, a control treatment of DMSO (within a range of 0-0.04%) was applied, which resulted in no change of cell viability (data not shown). Untreated groups included at least eight replicas whereas treatments were performed in at least three replicas. Statistical analysis was performed between each drug concentration, the corresponding siRNA exposure (siRNA-1/siRNA-CN) and the group including the combination of both.

2.3.12 DNA replication using BrdU staining

MCF7 cells incubated 72h with siRNA-1 or siRNA-CN, were treated with 5-bromo 2-deoxyuridine (BrdU) for 2h, followed by fixation with cold ethanol. Cells were then exposed to anti-BrdU (1h) and anti-mouse AlexaFluor-555 (1h), after which counterstained with DAPI for 10min. Images of at least four areas from duplicates of each treatment were captured using fluorescent microscope (Zeiss-Axioimager A1 Carl). Cell proliferation was calculated by taking the ratio between BrdU- positive (new cells) and DAPI-positive (total cells) after manual counting.

2.3.13 Cell cycle progression using PI staining and FACS analysis

Cell seeding was performed using $2x10^5$ MCF7 cells in 6-well plates. Next day treatment was performed in triplicates using 10nM siRNA-1or corresponding siRNA-CN, after which plate was incubated at 37ºC for 72h. Prior to the staining procedure, PBS rinse and cell suspension were obtained for Propidium Iodide (PI)

exposure, together with the detached cells after trypsinization. PBS washing and fixation in 70% ethanol followed, after which cells were incubated at +4 ^{o}C for 72h. Cells were again washed in PBS twice and stained with PI solution (50ug/ml PI, 0.1mg/ml RNase A, 0.05% Triton X-100, in PBS) within an incubation step of 40min at 37^{o}C. MCF7 cells were then analyzed in BD Accuri C6, using Cell Cycle Analysis 1.8 Software.

2.3.14 Apoptosis detection using CDD assay

MCF7 cells were treated with 10nM siRNA-1 or siRNA-CN for 72 hours in 6-well plates, as described before. Experiment was performed using two biological replicas. Camptothecin treatment was used as positive control at a concentration of 0.5μM or 0.25μM. At the end of incubation period, cells were rinsed in PBS, trypsinized in 0.25% Trypsin/EDTA and counted. The following experimental steps were performed using Cell Death Detection Elisa (CDD) (Roche) kit contents and provided protocol. Accordingly, $7x10^{4}$ cells were used from each well, centrifuged at 1500g for 5 minutes after which supernatant was removed. Cells were then treated with Incubation Buffer, centrifuged 20 0000g for 10 minutes after which supernatant containing cytoplasmic fraction of cell lysate was used. Supernatant was diluted 1:10 with Incubation Buffer, mixed with Coating Solution and put into Elisa plates (duplicated for each reaction) provided by kit. Two negative control reactions exposed to no treatments were also included in this step and plate was incubated overnight at +4^{o}C. Next day Coating Solution was removed followed by addition of Sample Solution and 90 minutes of incubation. Cells were replenished with Conjugate Solution and reactions were terminated using Substrate Solution. Each step after addition of Coating Solution was followed by three washing steps using Washing Solution. Elisa plate was incubated on plate shaker with 250rpm agitation for 35 minutes until yellow color was developed. Color absorbance was measured using Elisa Reader (μQuant, Biotek) at a wavelength of 405nm and 490nm.

Apoptosis was quantified by mono- and oligo-nucleosomes released into cytoplasm detected by color change. Absorbance values were used to calculate ‘Enrichment Factor of CDD’ formula as instructed by kit protocol. In brief, double absorbance measurements were averaged; background values (490nM measurements) were

subtracted from each of the averages; and resultant values of treatments were divided by siRNA-CN samples' average.

2.3.15 Trypan blue excision assay of flowthrough

MCF7 cells were treated with 10nM siRNA-1or siRNA-CN in four replicas and corresponding controls, in 6-well plates. At the end of 72 hour treatment, cell suspension was removed from each well separately, centrifuged and resuspended in 50µl DMEM. Each cell suspension was mixed with same volume of trypan blue solution and 20µl of cell mixture was immediately analyzed on a haemocytometer. Blue-stained cells were counted as nonviable.

2.3.16 SDS-PAGE

Three day (72h) and five day (120h) treatments of siRNA-1 and corresponding siRNA-CN in MCF7 cells were performed in 6-well plates in triplicates, as previously described. Prior to protein isolation, cells were scraped in ice-cold PBS. RIPA buffer was used to extract total protein, which concentration was subsequently determined by Bradford reagent. Proteins were run in 12% SDS-PAGE (Bio-Rad Acrylamide Kit) using kit contents and protocol, as indicated in Table 2.1, Table 2.9.

Table 2.9: SDS-polyacrylamide gel preparation

Solutions	Resolver Gel	Stacker Gel
Solution A (ml)	4	2
Solution B (ml)	4	2
10% APS (µl)	40	20
TEMED (µl)	4	4

Gel was incubated 35 minutes at RT to solidify followed by gel run in 1x running buffer (Table 2.7) for around 50 minutes at 170mV, until loading dye reached the bottom of gel system.

2.3.17 Western blot

Proteins run in SDS-PAGE were transferred onto PVDF membrane by semi-dry transfer method using semi-dry transfer system and suggested buffers (Biorad). At the end of proteins transfer, membrane was blocked in 5% milk TBS-Tween (TBST), overnight at 4 ^{o}C. Next day, membranes were added either: anti-Cholinergic Receptor Nicotinic Alpha 5 (CHRNA5) (Antibody Verify (polyclonal), Abcam (monoclonal), anti-Acetyl-CoA Carboxylase (ACC) (Cell Signaling), anti-Calnexin (CANX) (Millipore), Fas cell surface death receptor (FAS) (Cell Signaling), anti-BH3 Interacting Domain Death Agonist (BID) (2002, Cell Signaling) at a dilution of 1:10,000 for (CANX) and 1:1000 for the rest of the primary antibodies at an overnight incubation of 4^{o}C. Anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (25778, Santa Cruz) was used as a loading control, at a dilution of 1:1000, overnight at 4^{o}C. After primary antibody incubation, membranes were exposed to 5:15:5:5 minutes washing steps with washing buffer and then incubated 1h at RT with either of the following secondary antibodies: anti-rabbit (Cell Signaling) or anti-mouse (Cell Signaling). At the end of a second round of similar washing steps, targeted protein bands were detected by ECL Plus Western Blotting Detection System (GE Healthcare) on MXBE-Film (Carestream). Corresponding protein expressions were analyzed using Image J software and normalized to the expression level of GAPDH. Resultant values were normalized according to the highest expression showing treatment (either siRNA-1 or siRNA-CN), indicated as '1'.

2.3.18 Cell morphology based on phalloidin staining

24h prior to treatment, 2x10^{5} MCF7 cells were seeded on 22 x 22 mm^{2} coverslips, previously placed in 6-well plates under sterile conditions. Transfection was performed using 10nM siRNA-1 or corresponding siRNA-CN exposures (as previously explained), in duplicates. Two wells exposed to no treatment were also included. After 72h or a total of 120h incubation, cells were washed in PBS, fixed in 4% paraformaldehyde (PFA), permeabilized in PBX and blocked in BBX solution (Table 2.7). Fixed cells were again washed with PBX and incubated with 1:100 diluted phalloidin (Molecular Probes) for 40min at RT. After two washing steps with same solution, cells were then incubated with DAPI for 10 minutes at RT, washed

again twice and mounted on slides using UltraCruz Mounting Medium. İmages from at least five different fields were captured using fluorescent microscope (Zeiss-Axioimager A1 Carl) with 20X, 40X and 100X objectives.

2.3.19 *In Vitro* wound healing assay

$4x10^5$ MCF7 cells were seeded in each well of two 6-well plates. Next day 10nM, 20nM treatments of siRNA-CN were performed once and 10nM, 20nM treatments of siRNA-1 were performed in replicas. Same treatments were performed in a control plate. After 48 hours incubation at 37ºC, two parallel scratches were made with 20µl pipette tips in each well. Immediately after, the control plate was fixed in ice-cold 100% methanol while experimental plate was replenished with 2% FBS-DMEM. Next day experimental plate was fixed too. After fixation step, cells were washed three times with tap water and stained with 0.2% crystal violet for 30 minutes at RT. Fixed cells were washed again three times and photographed at 10X magnification. Three different regions of each well were photographed from each well and three different regions were randomly selected from each image to quantify wound dimensions. Wound length was manually quantified by measuring the distance between cells of opposite wound sides and their averages were obtained to assess motility. % Closure was calculated by wound closure difference between 72 hours treated 'experimental' samples and corresponding 48 hours treated 'control's, as in Equation 4.

$$\%\ Closure = \frac{(\text{control} - \text{experimental})}{\text{control}} \text{ x } 100 \qquad \text{Eq (4)}$$

An additional scratch assay was conducted for assessing a shorter CHRNA5 RNAi exposure (48h) in MCF7 cells. In this experimental set a single siRNA-1(10nM) and corresponding siRNA-CN treatments were performed and no 'control' plate was included. 24h prior to transfection end time, a scratch was made and next day cells were fixed and stained (as explained above). Three images of different scratched areas were captured, from which three random wound distances were measured manually. Values of siRNA-1 and siRNA-CN wound lengths were statistically analyzed using One-Way Anova. To confirm the results, invasiveness of MCF7 cells was evaluated by using Image J software. Two parallel vertical lines were drawn

from two opposite sides of wound borders (at the same position in each image) and number of cells inside the depicted area was measured automatically by Image J. Three measurements coming from each sample were compared using One-Way Anova.

2.3.20 Comparative transcriptome analysis of CHRNA5 siRNA-1 and chemotherapeutic drugs

GSE18552 dataset was based on treatment of two cell lines, MCF7 and A2780, with 8 different drugs, in order to test a newly generated tool (MATRA, http://mantra.tigem.it) predicting the molecular effects and mode of action of drugs based on similarities in the gene expression profiles between an undefined compound and the well-known ones [404]. In GSE18552 MCF7 cells were treated 6h with either SN38 (0.165 μM), DOXO (1.5 μM), 17AAG (0.5 μM), NVP-AUY922 (0.07 μM), PHA-793887 (6.0 μM) or PHA-848125 (8.5 μM). The corresponding microarray datasets (performed by Affymetrix HG-U133 Plus 2 platforms) were retrieved from GEO database (accession number: GSE18552) [404] and corresponding .CEL files were normalized in R. CHRNA5 siRNA-1 raw data was processed as described before. Only probesets including Entrez gene ID and gene symbol (54636 out of 54675 probesets) were included in differential expression analysis using *limma* package [407] in R. Significant probesets with $p<0.05$ and logFC>0.5/logFC<-0.5 were considered for further functional annotation analysis. For network representation, the combination (including up-up, down-down, up-down, down-up genes) showing the most densely connected network (highest number of edges) between the siRNA-1 and drug expression profile were retrieved form STRING [386]. DAVID [379] was further used for functional annotation where enrichment tests were performed using KEGG analysis. The most affected pathways with $p<0.05$ were plotted accordingly.

2.3.21 Prediction of regulatory miRNA networks from CHRNA5 siRNA-1 and TOPO drugs microarray datasets

Microarray datasets of siRNA-1 and two TOPO inhibitors (SN38, DOXO) retrieved from GSE18552 were merged in R. Gene probesets significantly modulated in the

same direction (up/up/up or down/down/down) with logFC>0.5 or logFC<-0.5 were picked up. To determine the common regulatory miRNAs among the three treatments, indicated gene sets were used to retrieve miRNA networks from miRNET software [387]. Shortest path filter tool was applied to reduce the dense networks (http://www.mirnet.ca). The microRNA microarray data for CHRNA5 RNAi in MCF7 cells, which have been obtained based on 114S367 project and provided by Şahika Cıngır, were used to determine some of the candidate microRNAs, significantly modulated by CHRNA5 RNAi, and were reported herein. .

2.3.22 Nanoparticle-based MTT assay in breast cancer cell lines

MCF7 and MDA-MB-231 cell lines were seeded using 2×10^3 cells/well in 96-well plates. After 24 hours, cells were exposed (in four replicas) to either of the treatments: CPT, NP, CB, CB+NP, NP+CPT, CB+NP+CPT, DMSO. Cells were incubated 72h at 37 °C, after which MTT treatment was performed, as described in section 2.3.11. Absorbance measurements of each group were normalized according to the untreated controls.

2.3.23 Nanoparticle-based cell imaging in breast cancer cell lines

2×10^5 MCF7 and MDA-MB-231 cells were cultured on coverslips in each well of 6-well plates, under sterile condition. After 24h cells were added either 1μM NP or CB+NP at a ratio of 4 μM /1 μM and incubated 72h at 37 °C. A control group exposed to no treatment was also included for comparison. Cells were washed once in PBS, after which fixed and permeabilized in 4% PFA and 0.1% Triton-X (respectively). Fixed cells were incubated with 1:100 diluted phalloidin (Molecular Probes) for 40min at RT and added DAPI for 10min. Cells were then washed twice in PBS and mounted on slides. Images from different fields were captured by Axio Imager fluorescent microscope with 100X objective.

2.3.24 Statistical Analysis

Figures were drawn using Graphpad (Prism 6) [408]. Experimental results of RNAi-based studies were analysed in Matlab® (R2009a) using One-Way ANOVA and

multiple test comparisons (Tukey HSD test). RT-qPCR results of siRNA-1 and siRNA-3 (120h) including two replicas of siRNA and one of siRNA-CN, were analysed by one-sample t-test.

For nanoparticle-based studies, differences in MTT cell viability were analyzed separately for each treatment dose using One-Way ANOVA and multiple test comparison in Matlab®. IC50 values were calculated in GraphPad using four parameter polynomial analysis.

CHAPTER 3

RESULTS

3.1 CHRNA5 cloning and isoform identification

Cholinergic receptor nicotinic alpha 5 (CHRNA5) is known to have six exons with exonV being alternative spliced in three of the known isoforms (Ensembl, Version 88) (Figure 3.2A). No information has been available about sequences of spliced isoforms in any of biotechnology information softwares. In order to determine the sequences of expressed CHRNA5 variants in breast cancer, the following primer set amplifying all possible variants of spliced exonV was used: Forward 5'-GGAGATACCCTGATGATGACTTAA-3', Reverse 5'-AAAAAGAAGCCCAGAAACAATTCC-3') [405] (Figure 3.1). RT-PCR was performed using cDNA from MCF7 exposed to no treatment. At least five different isoforms were observed (Figure 3.1). Spliced isoforms corresponding to 1000bp (longest), 500bp and 200bp (shortest) were extracted from the agarose gel and sequenced. The resultant three sequences belonged to CHRNA5 mRNA (NM_000745.3, NCBI), the longest one corresponding to the unspliced transcript (Figure 3.2B) and other two ones to variants having spliced exonV (Figure 3.2C, 3.2D). To specifically amplify the alternatively spliced isoforms, primer pairs were manually designed so that forward primer contained two spliced sites. According to amplification efficiencies, only the primer pair amplifying the shortest variant 'V3' was suitable for further PCR reactions (Figure 3.3A, Table 2.5). Previously designed two other primer pairs which amplified all known and longest (exonV-unspliced) CHRNA5 transcript were also used (V1 and V2 respectively) (Figure 3.3A). In addition, primer pairs (Iso2 and Iso3) amplifying two alternatively spliced isoforms were included in our study after Falvella et al. (2013) publishing five (one full-length and four alternatively spliced) transcripts expressed in normal lung tissue [303] (Figure 3.3B).

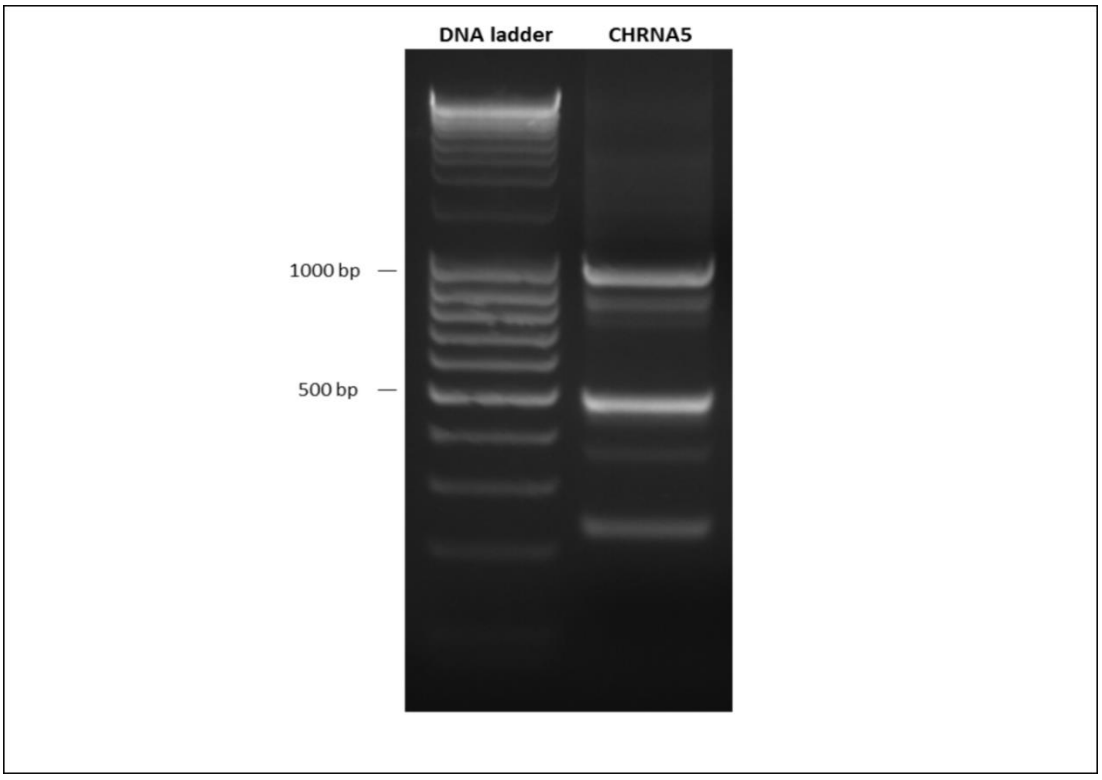

Figure 3.1: Agarose gel electrophoresis of RT-PCR amplified CHRNA5 variants.

Agarose gel representation of CHRNA5 variants after RT-PCR ,using primer pairs amplifying all exonV spliced/unspliced isoforms.

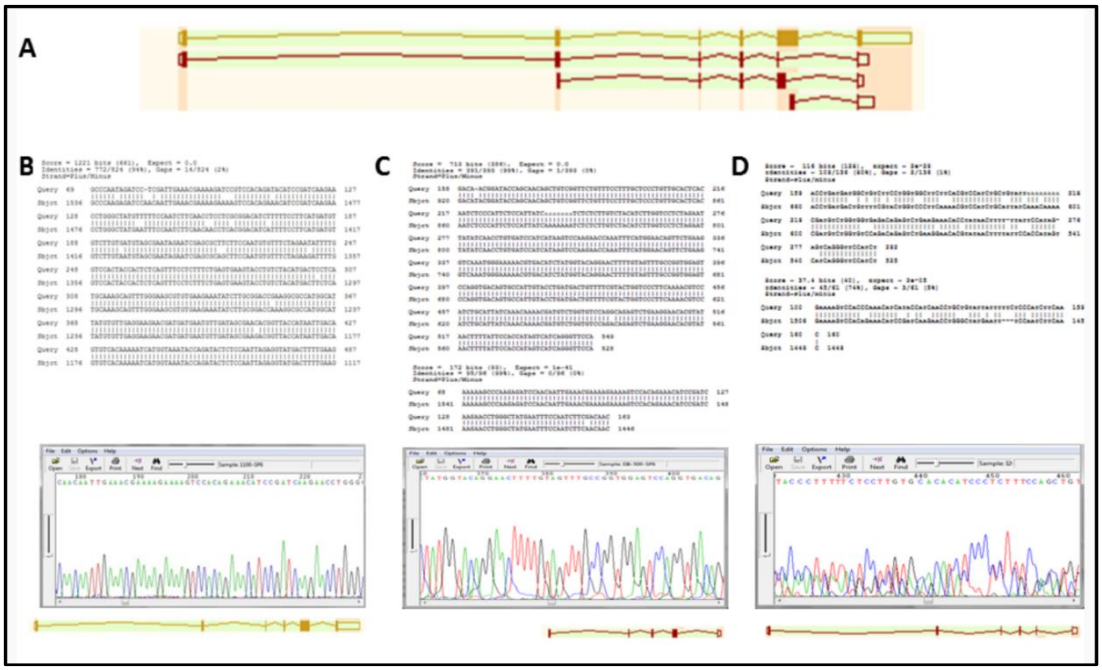

Figure 3.2: Cloning of CHRNA5 isoforms.

A) CHRNA5 isoform representation of one unspliced and three spliced isoforms from Ensembl. **B)** Sequencing results of cloned, exonV-unspliced CHRNA5 variant **C, D)** Sequencing results of exonV-spliced CHRNA5 isoforms. Corresponding Ensembl transcript illustrations are depicted at the bottom of each sequencing results.

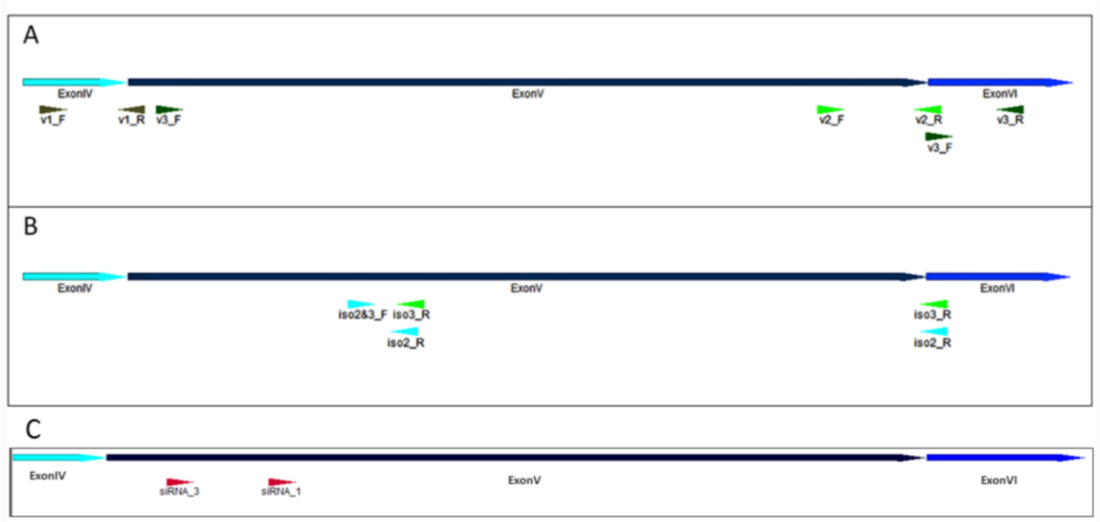

Figure 3.3: Graphical representation of CHRNA5 primer pairs and siRNA molecules used in the present study.

Location of primer pairs and siRNA targeting regions are represented on the exon structure of CHRNA5. **A)** CHRNA5_v1_F&R, which amplifies multiple isofoms, designed as spanning exon IV and ExonV, while forward (F) and reverse (R) primers for V2 and V3 are specific to the cloned products mentioned in Materials and Methods. **B)** Localization of previously reported (Falvella et al. 2013) CHRNA5 isoforms used in the experiments. (Only ~150bp of ExonVI was showed in this figure; 1446bp-1596bp). As illustrated by Sıla Özdemir. **C)** siRNA-1 and siRNA-3 targeting sites on CHRNA5 ExonV.

3.2 RNAi studies and microarray analysis

3.2.1 CHRNA5 siRNA treatment in breast cancer cell lines

In the present study, CHRNA5 was downregulated in three breast cancer cell lines: MCF7, BT-20 and MDA-MB-231 using siRNA as the targeting method. MCF7 was used as a representative cell line in this study for all assays while BT-20 and MDA-MB-231 were used in qPCR and MTT procedures for comparisons between cell lines with different ER and p53 status (MCF7: ER+, p53 wild type; BT-20, MDA-MB-231: ER-, p53 mutant (BT-20, c.394A>C, missense mutation; MDA-MB-231, c.8396G>A, missense mutation) [409]. Two different nucleic acid strands were used against CHRNA5 transcripts (Flexitube siRNA, Qiagen) (Table 2.3) transfected by 12µl HiPerfect transfection reagent. AllStars Negative control siRNA (Qiagen) was used as a control (Table 2.3). In several experimental methods, MCF7 with no treatment was included to exclude any effect arising from the use of transfection reagent or nucleic acid internalization into the cell. siRNA transfection was performed one day after addition of 200.000 cells per each well of 6-well tissue

culture plates at a concentration of 10nM in MCF7, BT-20 and 50nM in MDA-MB-231 with treatments lasting 72 hours (if not otherwise stated).

3.2.2 siRNA treatment optimization

To determine the most effective and minimal dose of siRNA, MCF7 cells were treated with 10nM and 50nM siRNA-1 (72h) together with corresponding highest dose of negative control siRNA 'siRNA-CN'. RT-PCR results showed similar downregulation at the transcript level between both concentrations of siRNA-1 (Figure 3.4A). In order to reduce the possible target-independent toxicity of siRNA-1 molecule [410], its lowest dose (10nM) was used for most of the following RNAi studies. To validate the previous results of CHRNA5 downregulation by 10nM siRNA-1, an additional 10nM transfected MCF7 experimental set was prepared with triplicates of siRNA-1 and corresponding controls. Successful silencing of CHRNA5 was observed in all biological replicas (Figure 3.4B). Accordingly, 10nM concentration of siRNA-1 was used for further experimental procedures in MCF7 cells.

Prior to RT-qPCR studies, primer efficiencies were determined as previously indicated (Table 2.5). Relative expression of all CHRNA5 isoforms at once (V1) and four known exonV-spliced variants (V2, V3, Iso 2, Iso 3) were determined in untreated MCF7 cells (Figure 3.5A). Similar expression of all spliced isoforms was observed in between. Downregulation of all known CHRNA5 variants by siRNA-1 was validated by RT-qPCR (Figure 3.5B) resulting in approximately 5-fold decrease in expression. Similar CHRNA5 silencing by siRNA-3 was observed using the CHRNA5_v1 primers tested (Figure 3.3C, 3.5C). CHRNA5 was successfully silenced with both siRNAs; therefore following experiments were mainly performed using one of the siRNAs, i.e., siRNA-1, as the representative one (Figure 3.3). CHRNA5 transcripts (amplified by CHRNA5_v1) were significantly downregulated also in BT-20 and MDA-MB-231 cell lines treated with siRNA-1 (Figure 3.5D). CHRNA5 downregulation in MDA-MB-231 approached significance at $p=0.056$.

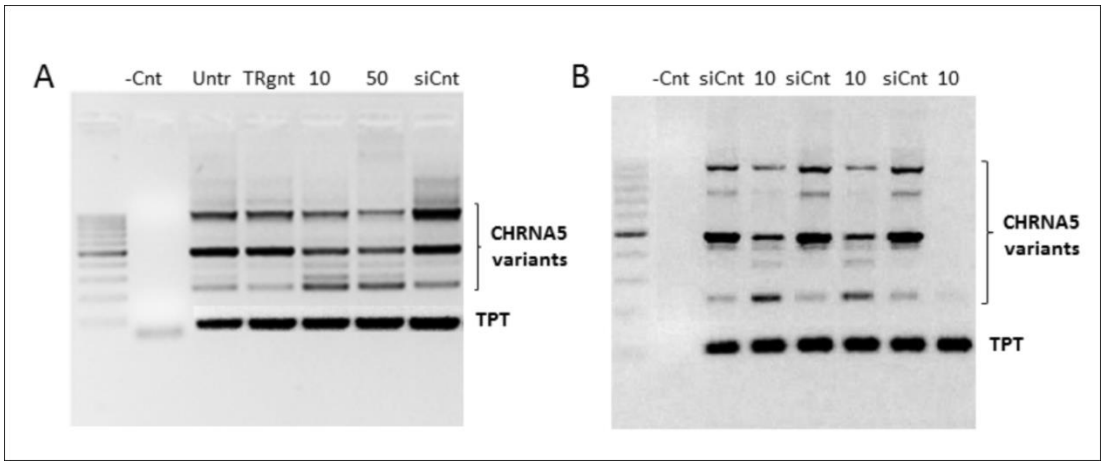

Figure 3.4: Optimization of CHRNA5 siRNA-1 doses in MCF7 cells.

A) RT-PCR results of siRNA-1 optimization study. Two concentrations (10nM and 50nM) of siRNA-1 molecule were used to target CHRNA5. Labeling on each well stands for: '-Cnt'= negative control; 'Untr'= Untreated; 'TRgnt'= Transfection Reagent-only; '10'= 10nM siRNA-1, '50'= 50nM siRNA-1, 'siCnt'= 50nM siRNA-CN. **B)** RT-PCR results of an additional experiment including three replicates of the lowest working siRNA-1 dose (10nM) and corresponding siRNA-CN controls, as defined from the previous experiment. Primer pairs amplifying exonV-spliced/uspliced transcripts were used. TPT1 was used as a loading control.

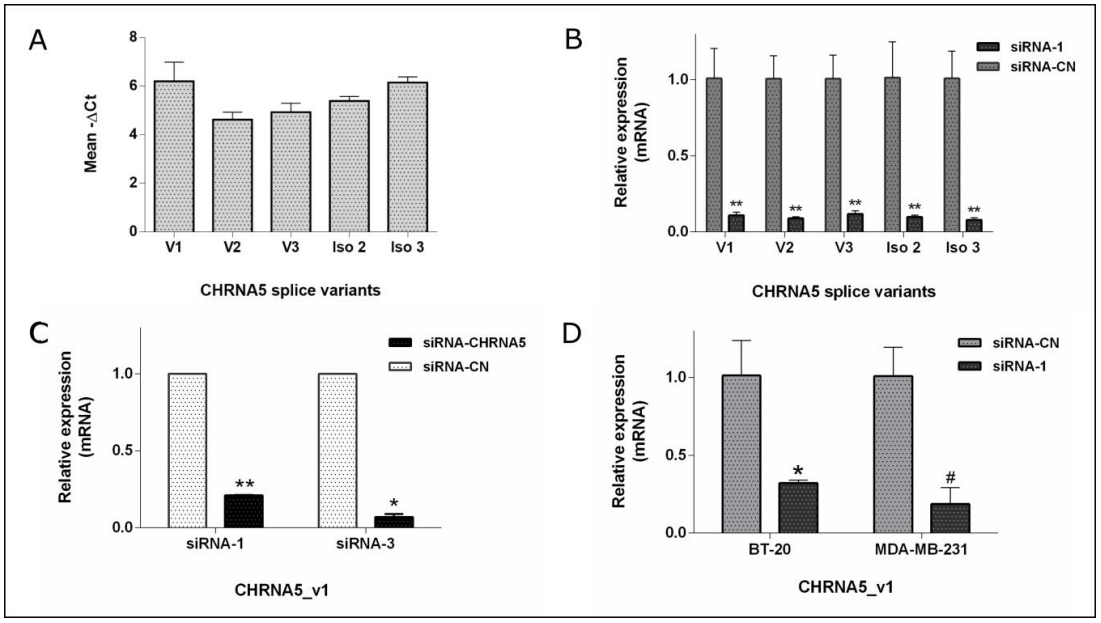

Figure 3.5: CHRNA5 isoform expression and downregulation using RNAi.

Differential expression of CHRNA5 isoforms in MCF7 cells grown under **A)** normal growth conditions for 72h, **B)** transient transfection with siRNA-1 for 72h, **C)** transfection with an additional siRNA molecule 'siRNA-3' (120h, 50nM), targeting nearby regions of CHRNA5 transcript, as well as 120h siRNA-1 treatment, **D)** 72h siRNA-1 exposure in BT-20 (10nM) and MDA-MB-231 (50nM). Expression of SDHA gene was used as reference. *p<0.05, **p<0.01, ***p<0.001, #p<0.1 (One-Way ANOVA).

3.2.3 RNAi validation studies

After optimizing the siRNA treatments, an independent set including triplicates of scrambled control (siRNA-CN) and siRNA-1 of 10nM treatment was applied for 72 hours in MCF7 cells. RNA was isolated and RIN values were determined using Agilent 2100 Bioanalyzer (Figure 3.6A), before microarray set up. Scatterplot analysis of microarray expression profiles revieled good quality data (Figure 3.6B). Same samples were reverse transcribed to cDNA and RT-qPCR was performed to validate the downregulation of CHRNA5 at the transcript level. RNAi was applied similarly in another experimental set to obtain total protein and verify downregulation of CHRNA5 at the protein level. Significant decreases in all transcripts were observed by RT-qPCR (Figure 3.6C). Western blot studies using CHRNA5 antibody (AAS67349E, Antibody Verify) showed a considerable decrease of the longest transcript (upper band) and even higher downregulation of spliced transcript(s) (lower band) [303] (Figure 3.6D). One of biological replicates of siRNA treatment 'si4', showed abnormal total protein loading as well as CHRNA5 expression.

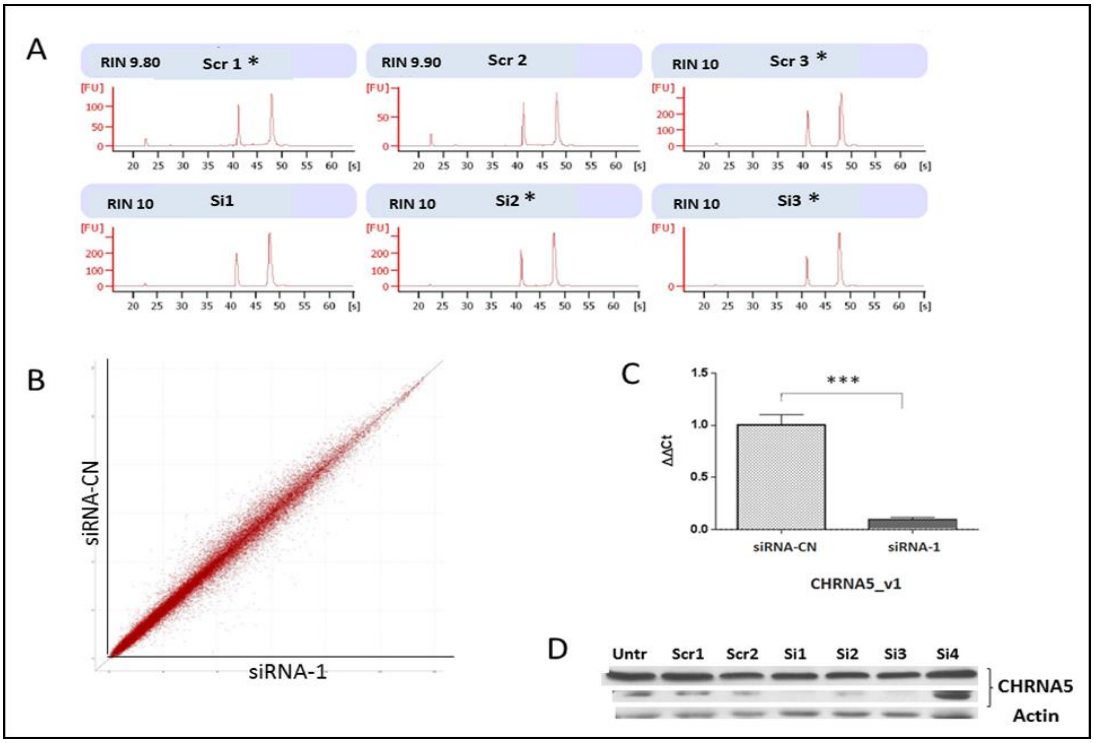

Figure 3.6: Microarray and related validation studies.

A) RNA integrity measurements after siRNA-1 (10nM, 72h) application in MCF7 cells. **B)** Scatterplot of microarray data from BRB-Array Tools Analysis **C)** RT-qPCR for CHRNA5 downregulation represented by V1 which amplifies all known CHRNA5 isoforms and **D)** Western blot validations of microarray results with independent siRNA-1 experiments.

3.2.4 Transcriptional modulations by CHRNA5 RNAi based on microarray analysis

Microarrays were performed using two scrambled controls (siRNA-CN) and two siRNA-1 treatments, as mentioned above (shown by asterisk in Figure 3.6A). After normalization and quality control of the microarray data, probe sets with significant change at $p<0.05$ (*limma*) were selected and tested using KEGG pathways from DAVID and CGP from MSigDB (as described in Materials and Methods). KEGG analysis of significantly affected pathways demonstrated that upregulated genes were enriched in p53 signaling, tight junctions as well as pathways involved in cancer while downregulated genes were enriched in cell cycle, DNA replication and DNA repair mechanisms (Figure 3.7A). After CGP analysis, expression signatures related to E2 and tamoxifen treatment in MCF7 cells were picked up. Results showed many expression overlaps between the siRNA and E2 profiles but in the opposite direction; genes upregulated in siRNA-1 data were downregulated in E2 data and vice versa (Figure 3.7B). Similarly, ESR1 negative MCF7 cells showed downregulation of genes which were upregulated in siRNA-1 case. On the other hand, around 23% of genes upregulated in tamoxifen resistant MCF7 cells showed same regulation pattern as in the CHRNA5 depleted MCF7 cells (Figure 3.7B).

A

Upregulated Gene List Results

KEGG Pathway Term	Gene Count	%
Ubiquitin mediated proteolysis	38	1.6
Pathways in cancer	70	3.0
Colorectal cancer	27	1.1
Tight junction	35	1.5
ErbB signaling pathway	26	1.1
Adherens junction	24	1.0
P53 signaling pathway	22	0.9
Wnt signaling pathway	37	1.6
Pancreatic cancer	22	0.9
Chronic myeloid leukemia	22	0.9

Downregulated Gene List Results

KEGG Pathway Term	Gene Count	%
DNA replication	30	1.2
Cell cycle	59	2.3
Mismatch repair	18	0.7
Homologous recombination	18	0.7
Oocyte meiosis	36	1.4
Base excision repair	17	0.7
Spliceosome	35	1.4
Pyrimidine metabolism	28	1.1
Progesterone-mediated oocyte maturation	24	0.9
RNA degradation	18	0.7

B

Upregulated Gene List Results

Description	Expression	No. of genes	Genes in overlap
MCF7, 24h estradiol treatment	Down	505	194
MCF7, ESR1 negative	Down	781	225
MCF7 tumors, resistant to tamoxifen	Up	578	170

Downregulated Gene List Results

Description	Expression	No. of genes	Genes in overlap
MCF7, 24h estradiol treatment	Up	324	244

Figure 3.7: Microarray data annotation.

A) Top 10 DAVID-KEGG pathways affected by knockdown of CHRNA5 in MCF7 cell line, with $p<0.05$. **B)** MSigDB-CGP expression signatures showing overlapping profiles with CHRNA5 siRNA-1 treated microarray dataset. Only E2 and tamoxifen related profiles were picked up, with $p<0.05$.

3.2.5 Microarray data verification by RT-qPCR

To verify consistency in CHRNA5 downregulation-dependent transcriptome changes, an siRNA-1 experimental set other than the one used in microarray studies was run. Three genes from some of the most affected pathways of microarray studies were picked up and RT-qPCR was performed as explained in Materials and Method part. Microarray data expressions of related genes were displayed as barplots nearby RT-qPCR results to compare expression from two siRNA-1 sets (Figure 3.8A). *MAP1B* (Microtubule Associated Protein) and two probe sets of cell-cell junction proteins, *GJA1* (gap junction protein) and *CLDN1* (tight junction constituent), were included as representatives of 'cytoskeletal' group. All three probe sets and

transcripts showed similar pattern of upregulation. *GADD45A, GPNMB* and *CDKN1,* negative regulators of cell cycle progression, were selected for 'p53 pathway'. All three transcripts were upregulated in the three experimental sets (i.e., microarray 72h and qPCR; 72h and 120h siRNA-1 transfection). *ANLN, BIRC5* and *WDHD1* were chosen as representatives of 'cell cycle' genes. Both ANLN and BIRC5 are positive regulators of cell cycle having role in cytokinesis while WDHD1, also a positive regulator of cell cycle, is a DNA replication initiation factor (http://www.uniprot.org). All three transcripts showed similar downregulation in the experimental sets. Overall these data implicated significant changes induced by CHRNA5 siRNA in cytoskeletal organization and cell cycle progression in MCF7.

To confirm CHRNA5-specific downregulation of siRNA-1, another siRNA (siRNA-3) was used to transfect MCF7 cells (as explained in Materials and Methods). Both siRNAs target nearby regions of fifth exon in CHRNA5 transcript (Figure 3.3C). The primer panel of Figure 3.8A was repeated for siRNA-3. Similar pattern of selected gene regulation was confirmed with this experimental set (Figure 3.8B).

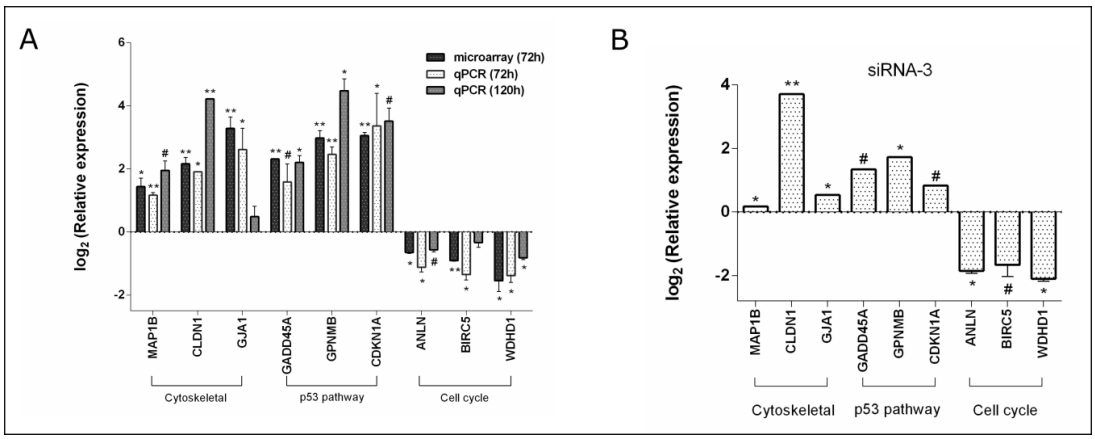

Figure 3.8: RT-qPCR confirmation of genes affected by CHRNA5 RNAi.

A) RT-qPCR studies (72h and 120h siRNA-1 exposure) of several genes together with corresponding micrarray expression. Microarray calculations were performed by subtracting average logFC values of siRNA-CN from each replica of siRNA -1 (72h) treatment**s. B)** RT-qPCR of the selected genes upon prolonged siRNA-3 exposure (120h transfection, n=2). SDHA was used as a reference gene. *p<0.05, **p<0.01, ***p<0.001, #p<0.1.

3.2.6 RT-qPCR analysis of BT-20 and MDA-MB-231 cell lines

siRNA-1 treatment in BT-20 and MDA-MB-231 resulted in downregulation of the known CHRNA5 isoforms (Figure 3.5D), represented by V1. To find whether

transcriptome changes of CHRNA5 depleted cells were similar as in MCF7 (TP53 wild type, ER+) in ER- and P53 mutant breast cancer cell lines [409, 411], all three representative genes of each pathway in Figure 3.8A were used to perform RT-qPCR of BT-20 and MDA231-MB-231. These three affected pathways in MCF7 were not as highly modulated by CHRNA5 depletion performed in BT-20 and MDA-MB-231 cell lines (Figure 3.9A-B). Moreover, some of them showed the opposite direction of expression; for instance, cell cycle genes showed a trend of increase in expression while GPNMB resulted in a decrease in the case of BT-20 (10nM siRNA-1) (Figure 3.9A). Similarly, CDKN1A expression in MDA-MB-231 (50nM siRNA-1), showed an opposite direction of expression (compared to MCF7 cells) approaching significance at p=0.07 (Figure 3.9B).

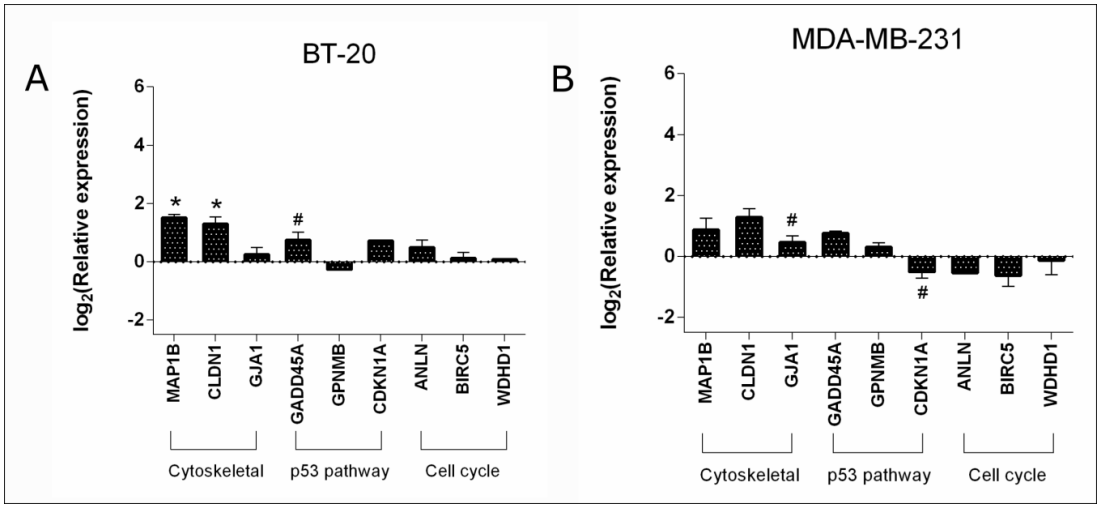

Figure 3.9: RT-qPCR expression analysis of selected genes upon siRNA-1 treatment in BT-20 and MDA-MB-231.

Gene expression analysis from three selected pathways after 72 hours siRNA-1 transfection in **A)** BT-20 (10nM) and **B)** MDA-MB-231 (50nM). SDHA was used as a reference gene. *p<0.05, **p<0.01, ***p<0.001. All statistical tests were performed using One-Way ANOVA.

3.3 Functional analysis of CHRNA5

3.3.1 Cell viability using MTT assay

I performed MTT assay to assess viability of asynchronous cells upon siRNA-1 treatment. There was a 30% reduction of viable cells after 72h of CHRNA5 downregulation (Figure 3.10A). However, siRNA-CN subjected cells also showed reduced cell viability compared to untreated control. Another MTT study was

performed including Transfection Reagent (TR)-only as control group, which was statistically not different from the untreated and siRNA-CN samples (Figure 3.10B). Further studies can help clarify the differential effect of TR from that of siRNA-CN, if any.

The MTT experiment was repeated using both siRNAs targeting CHRNA5, for a 72h and 120h treatment (at the optimized doses of application) (Figure 3.10C). Similar results were observed from siRNA-3 exposure, with more effect on cell viability with 120h RNAi application.

In contrast to the RT-qPCR studies, which showed no difference in expression of genes regulating cell cycle, cell proliferation status in BT-20 and MDA-MB-231 (measured by MTT assay) was significantly modulated upon siRNA-1 exposure, yet depending on the initial cell seeding density (Figure 3.11).

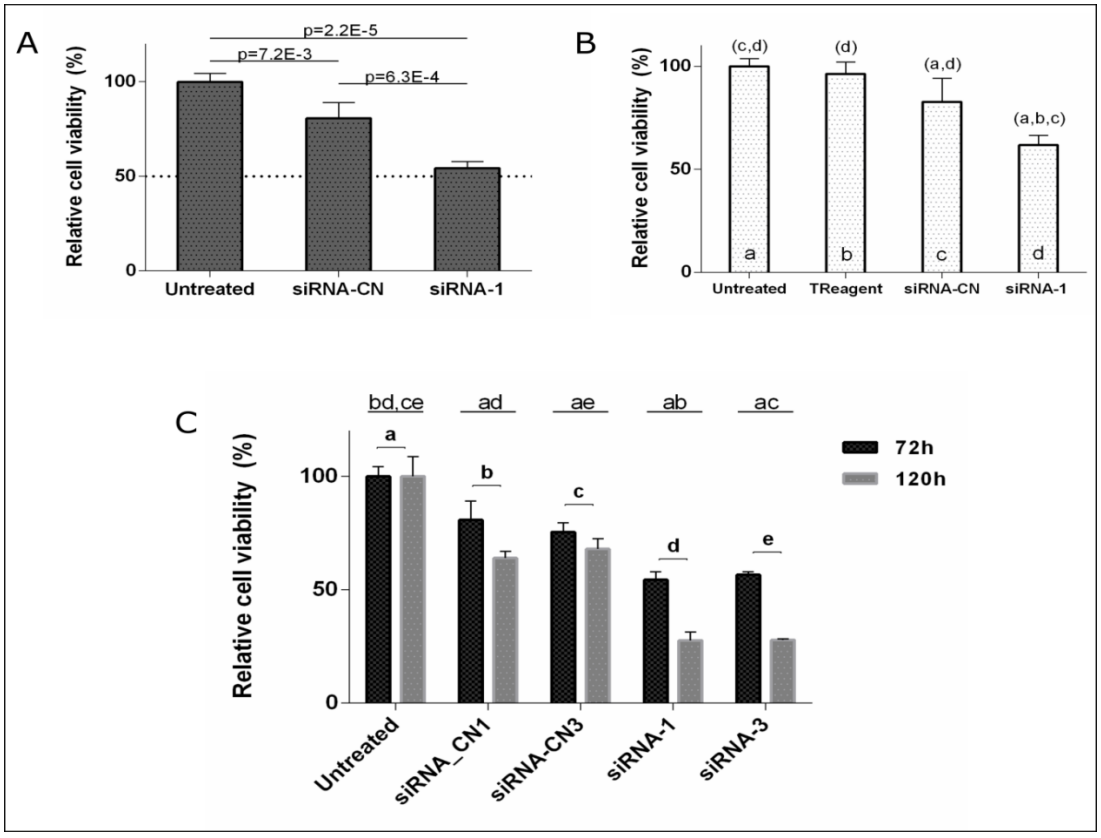

Figure 3.10: Effects of CHRNA5 depletion on MCF7 cell viability.

Relative cell viability of MCF7 cells (as measured by MTT assay), treated with **A)** 10nM siRNA-1 (72h) and corresponding controls, **B)** 10nM siRNA-1 (72h), including a Transfection Reagent-only (12µl) 'TReagent' control group, **C)** siRNA-1 and siRNA-3 of both 72 and 120 hours transfection period, at their most affective doses of application. Corresponding siRNA-CN control treatments (siRNA-CN1 for 10nM and siRNA-CN2 for 50nM concentrations) were included. Multiple pairwise test differences were performed to assess significance in viability difference, as indicated above each bar, at $p<0.05$.

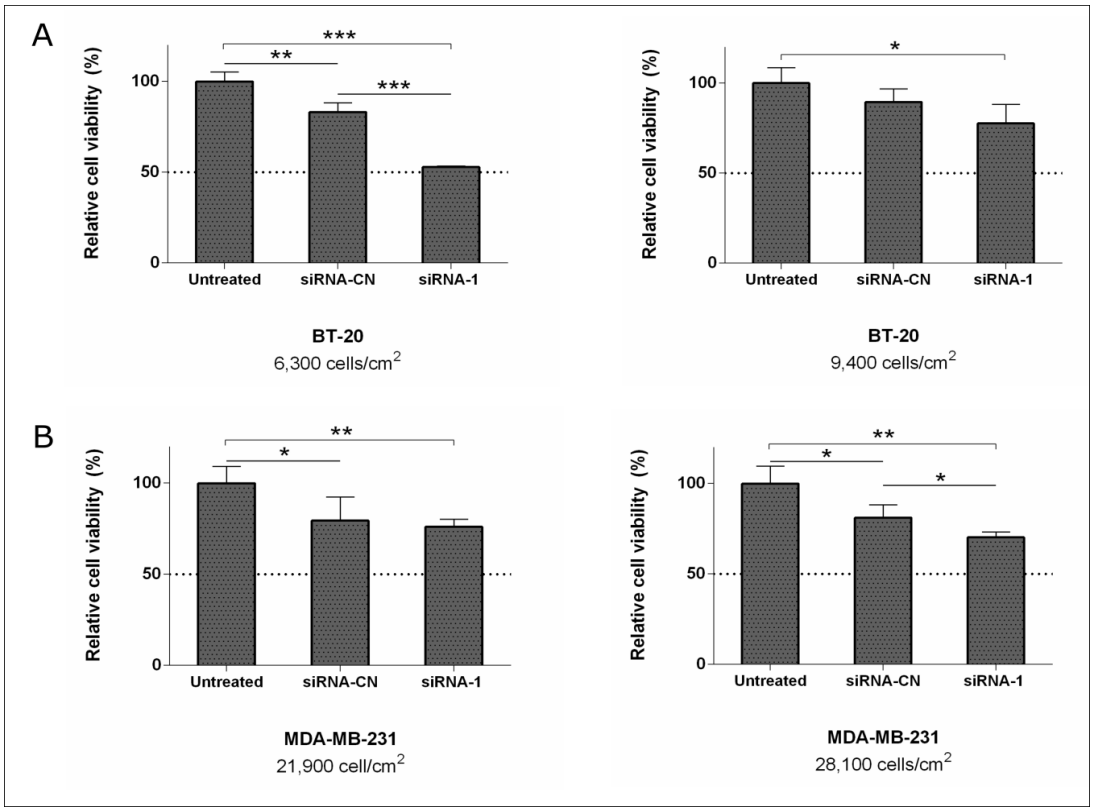

Figure 3.11: Cell viability of siRNA-1 treated BT-20 and MDA-MB-231 cell lines.

Cell viability measured by MTT assay under **A)** 10nM, 72h siRNA-1 treatment in BT-20 cells and **B)** 50nM, 72h siRNA-1 treatment in MDA-MB-231, at two different cell seeding densities. *p<0.05, **p<0.01, ***p<0.001.

3.3.2 Testing of DNA replication using BrdU staining

After MTT results, BrdU (5-Bromo-2'-deoxyuridine) incorporation assay was performed to test whether reduced DNA replication could be the cause of low cell viability. BrdU was added after 72 hours of siRNA-1 treatment and cells were fixed 2 hours later. BrdU incorporation was detected using immunostaining and quantification was manually performed as described in Materials and Methods. Results showed that 72 h siRNA-1 treated samples had lower percentage of BrdU-positive cells, indicating reduction in the rate of DNA synthesis (Figure 3.12).

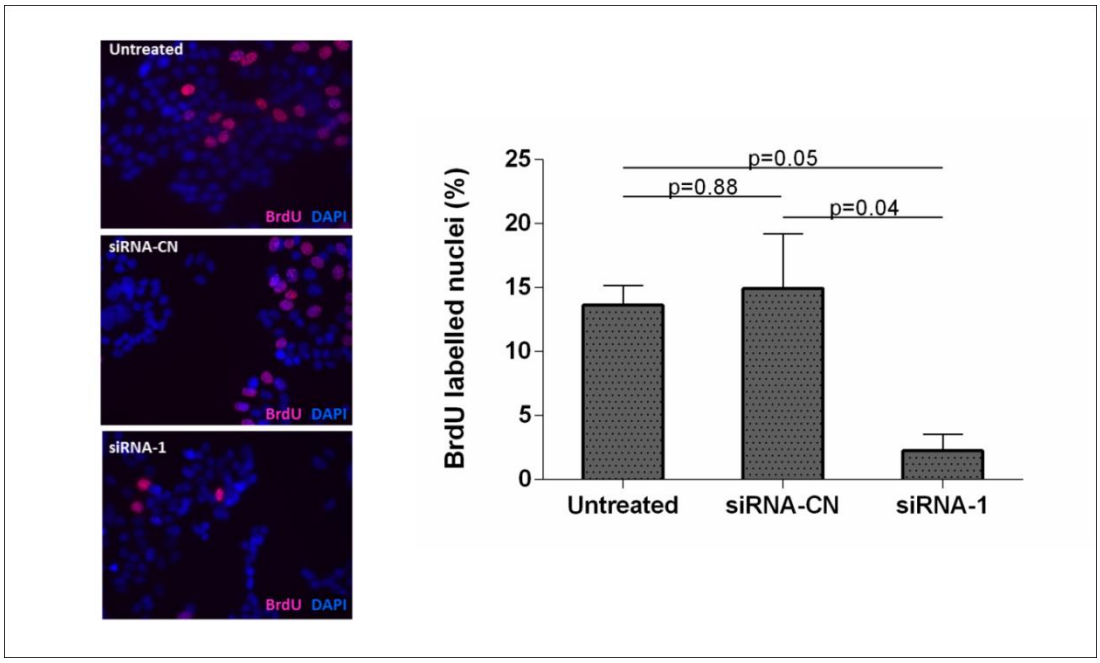

Figure 3.12: Effects of CHRNA5 RNAi on DNA synthesis.

DNA replication of 72h siRNA-1 treated MCF7 cells was measured after 2h of BrdU incorporation. Representative figures of each treatment group are shown in the left panel while statistical analysis of BrdU-positive to total cell number are shown in the right panel.

3.3.3 Cell cycle progression using PI staining and FACS analysis

BrdU results were further validated by propidium iodide (PI) staining of 72h siRNA-1 subjected MCF7 cells. siRNA treatment was performed in triplicates in a 6-well plate, as indicated in Materials and Methods. After PI staining and FACS analysis, cell cycle distribution of CHRNA5 depleted cells showed enrichment of treated cells at G1-phase with similar percentage in reduction of the G2-M phase population (Figure 3.13A). A significant increase in the subG1 cell percentage was also observed, which suggested a proportion of treated cell population might have undergone cell death. These results pointed to a G1 to S-phase cell cycle arrest upon CHRNA5 downregulation in MCF7 cells (Fig. 3.13A).

3.3.4 Apoptosis using CDD kit

After cell cycle analysis, next step was to understand whether cell cycle arrest resulted in programmed cell death (apoptosis). Cell Death Detection Elisa assay (Roche) was used to determine induction of apoptosis by measuring the mono and oligo-nucleosomes in the lysate of MCF7 cells. Two measurements from each

sample were obtained at 405nm wavelength and normalized to the corresponding 490nm values in order to calculate 'Enrichment Factor', according to kit instructions. Two camptothecin (CPT) concentrations were included as positive controls and one mixture having kit reagents but no cell lysate was included as a negative control. Results showed mild apoptotic enrichment in one of siRNA-1 samples (Figure 3.13B), with no significant differences from other groups. Future studies are needed to test the significance of these findings.

3.3.5 Trypan blue staining of cells in suspension

As a next step, trypan blue dye exclusion assay was performed using small aliquots of flowthrough from RNA-1 treated samples in 6-well plates, after 72h (3 day) treatment, as explained in Materials and Methods. The aim of this study was to understand whether treated MCF7 cells were more inclined to detach from tissue culture flask and be part of floating cell population. Indeed, results showed that the overall number of floating cells in siRNA-1 samples has increased (although not significantly), while dead cell fraction has decreased by 10% (Figure 3.13C). Results indicated that MCF7 cells in the siRNA-1 treated samples have the tendency to detach from the culture surface and survive in suspension; however more replicates are needed to conclude.

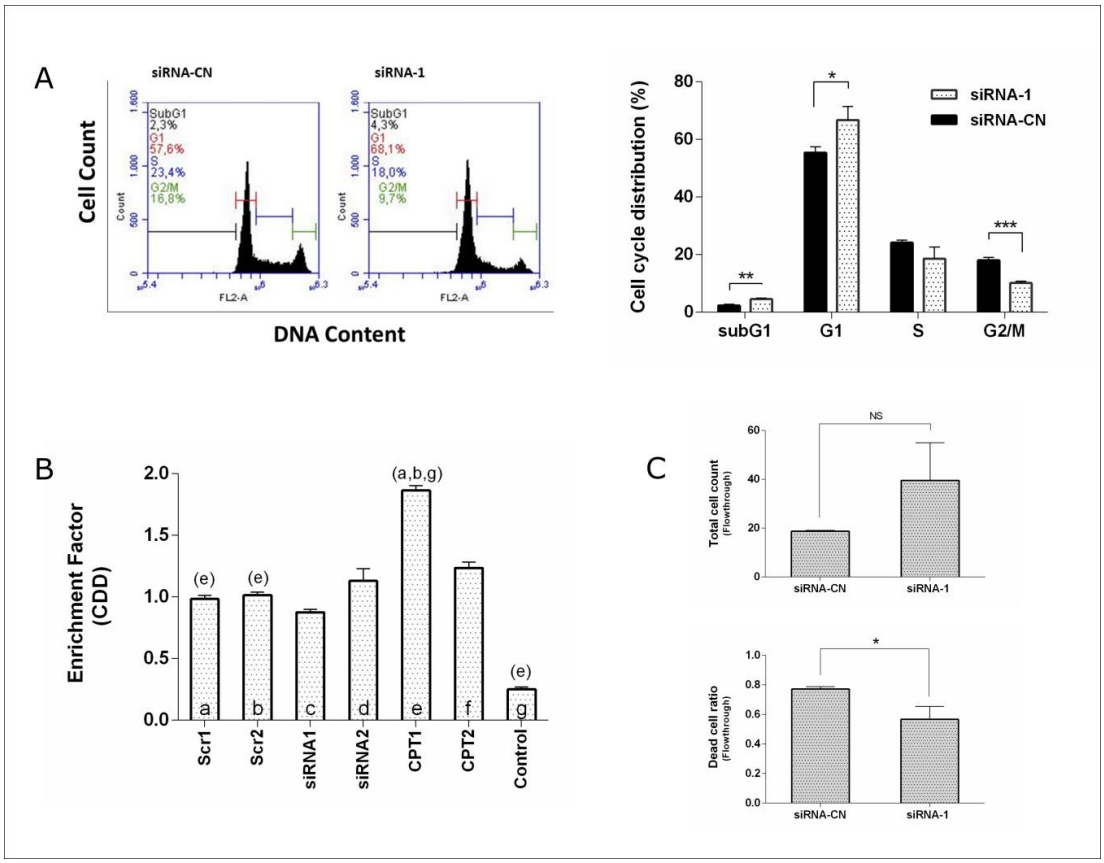

Figure 3.13: Effects of CHRNA5 RNAi on cell cycle, apoptosis and detachment of MCF7 cells.

Effects 72h siRNA-1 transfection in MCF7 cells were further determined by **A)** Cell cycle analysis performed using propopidium iodide (PI) staining after 72h siRNA-1 and analysed by flow cytometry. Data on the right represent percentage of cell population present in each phase of cell cycle (n=3). **B)** Apoptotic cell death evaluation using cell death detection ELISA. **C)** Tryptan blue viability assay of flowthrough cell population. *p<0.05, **p<0.01, ***p<0.001

3.3.6 Cell morphology using phalloidin staining

Short-term loss of CHRNA5 for 72h (3 days) and 120h (5 days) induced observable morphological changes in MCF7 cells. Cells became more elongated and seemed to lose their rounded appearance as well as their cell-cell contacts. To better characterize these observations and visualize the actin cytoskeleton, treated cells and corresponding controls were stained with phalloidin. RNAi treated cells stained with phalloidin showed changes in contours of cell junctions implying restructuring of MCF7 epithelial monolayer (Figure 3.14A, B). Additionally, treated cells showed differences in morphology and additional features not characteristic of MCF7 cells. RNAi subjected cells gained filopodia-like structures resembling to either motile-like (Figure 3.15A) or rigid-like (Figure 3.15B) protrusions. Predominant structures of

abnormal cell groupings existed such as tubule formation (Figure 3.15C) and grape-like nuclei (Figure 3.15D). Interestingly, cells in multiple sites formed actin foci (Figure 3.15E) and multilayer-like structures (Figure 3.15F). An additional 2-day exposure to RNAi showed even more pronounced changes in morphology (Figure 3.16). 120h CHRNA5 depleted cells exhibited rigid-like filopodia extensions (Figure 3.16B). Cell-cell borders seem to have been more disrupted resulting in higher proportion of cells growing with no cell-cell contacts, as shown in Figure 3.16. Long motile-like filopodia were predominantly observed in 72h siRNA-1 exposure while 120h siRNA-1 treated cells exhibited more cell polarization and increased accumulation of actin foci (Figure 3.16B).

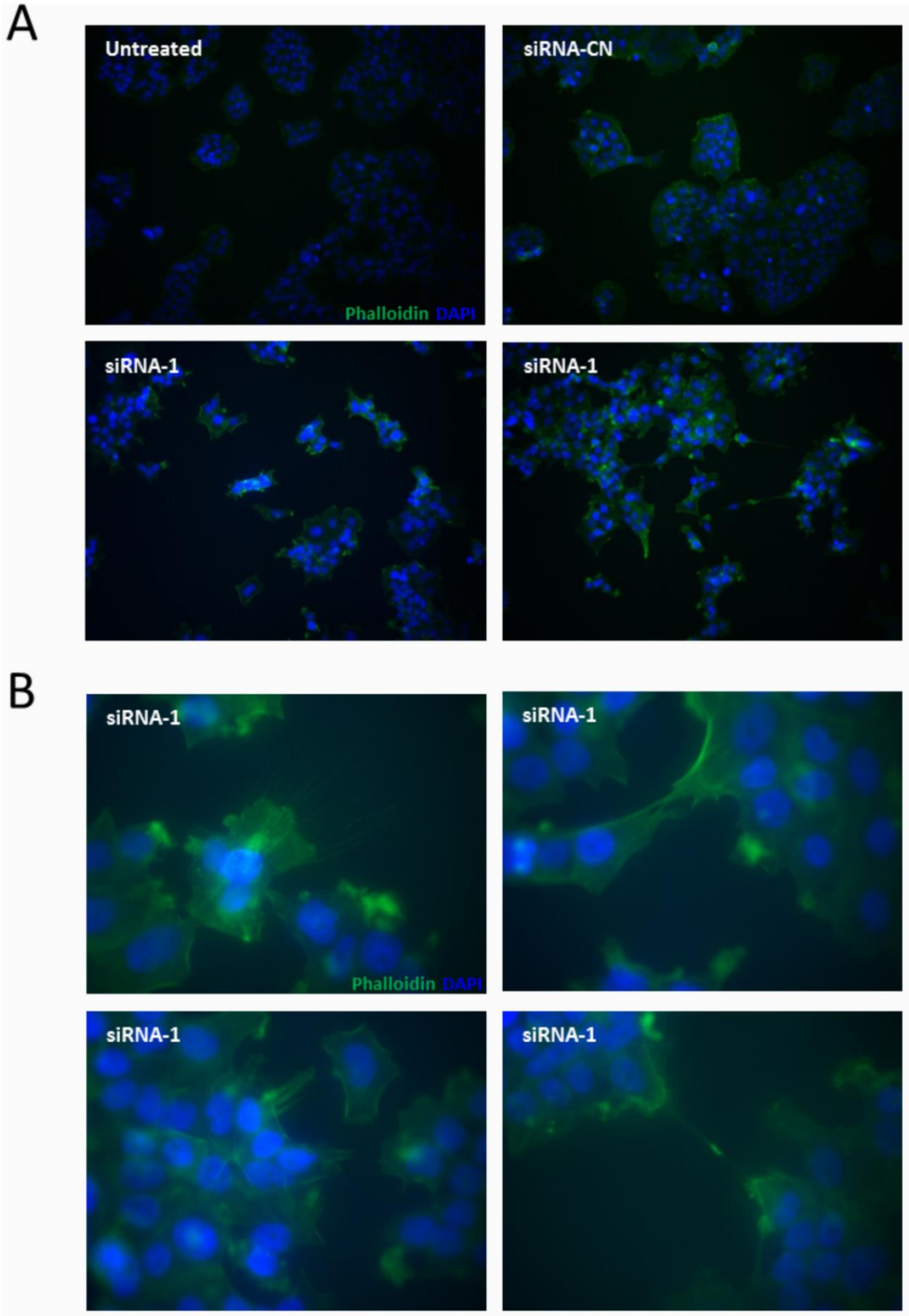

Figure 3.14: Effects of 72h CHRNA5 depletion on MCF7 cell morphology.

Images of F-actin stained (by phalloidin) MCF7 cells after 72h of siRNA-1 transfection, in comparison with siRNA-CN treated cells, at magnification of **A)** 20X and **B)** 100X using fluorescent microscope.

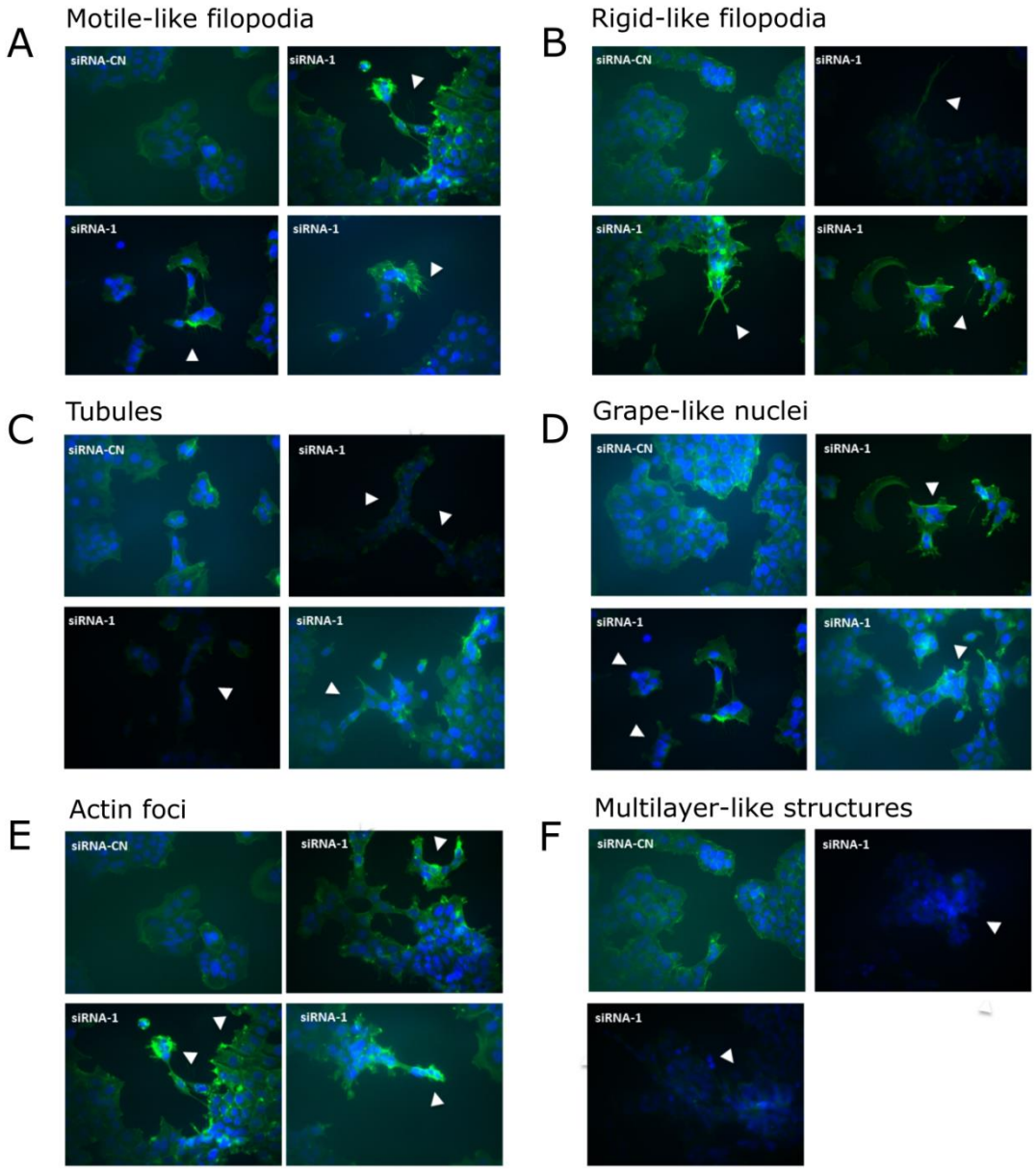

Figure 3.15: Effect of 72h CHRNA5 depletion on MCF7 cell morphology.

Images of F-actin stained (by phalloidin) MCF7 cells after 72h of siRNA-1 transfection, in comparison with siRNA-CN treated cells. The most predominant morphological features observed in treated cell populations are indicated at the top of images and shown by white arrowheads.

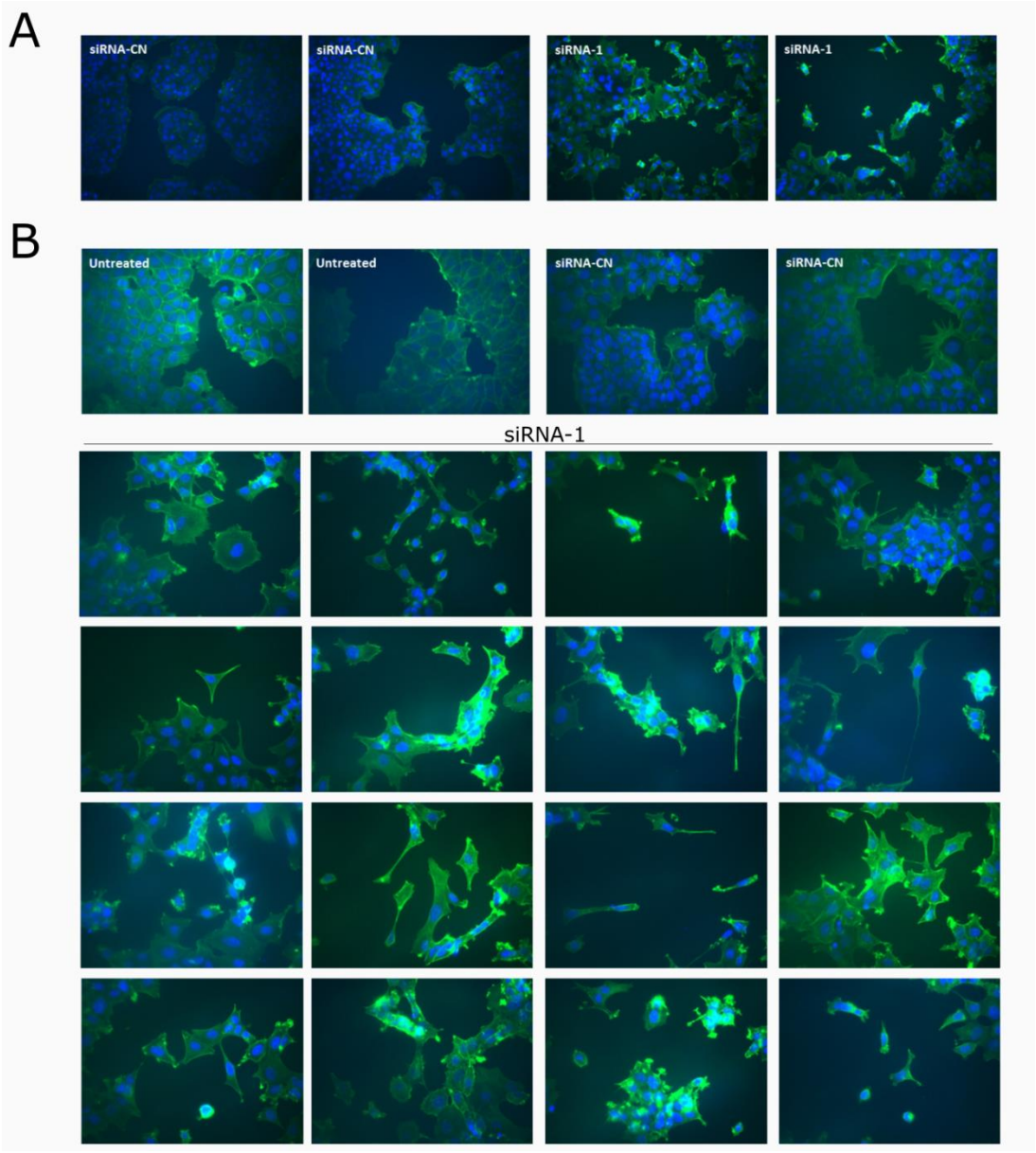

Figure 3.16: Effects of 120h CHRNA5 depletion on MCF7 cell morphology.

Images of phalloidin stained MCF7 cells after 120h of siRNA-1 transfection, in comparison with siRNA-CN treated cells, using **A)** 20X and **B)** 40X magnification in fluorescent microscope.

3.3.7 Western blot analysis

To understand the cell death mechanism of the RNAi subjected MCF7 cells, protein lysates of both 72h and 120h siRNA-1 treated cells were used to examine expressions of two pro-apoptotic marker gene proteins (FAS and BID) using Western blot method. The drastic increase in FAS protein was similar to the observations from the Affymetrix microarray data (Figure 3.17A, C). As a follow-up of potential apoptosis, I tested protein expression of BID and observed an increase in the full-

length BID although cleaved BID was absent (Figure 3.17A). The prolonged CHRNA5 knockdown by siRNA-1 (120h) showed higher levels of upregulation for both FAS and BID proteins (Figure 3.17A). At the protein level, I also tested several genes whose transcriptions were modulated significantly by RNAi at the mRNA level; CANX and ACC (Figure 3.17A, C).

To validate the previous Western blot results, depletion of CHRNA5 was quantified from protein lysates of an independent experiment. Same antibody as in microarray validation study (polyclonal, Antibody Verify) was used for detection of CHRNA5 longest transcript (upper band; corresponding to CHRNA5_v2) and potentially one or more spliced CHRNA5 isoforms (the lower bands) (Figure 3.17A). 72 h of siRNA-1 treatment showed reductions close to 20% for the CHRNA5_v2 isoform while no further decrease was detected at the 120h. However, 120 h of siRNA-1 treatment further reduced the expression of a shorter isoform (Figure 3.17A). Another antibody (monoclonal, Abcam) recognizing a common region of all known CHRNA5 variants, with the highest recognition of the exonV-unspliced (longest known) variant, V2, was further used (performed in collaboration with Sahika Cingir). Western blot results showed a decreasing trend for 72h siRNA-1 exposure and a statistically significant decrease of CHRNA5 protein at 120h treatment (Figure 3.17B). To further validate the microarray results and support that functions related with cholinergic signaling, such as acetylcholine metabolism, acetylcholinesterase (ACHE) expression was analyzed. CHRNA5 RNAi treated MCF7 cells showed increased ACHE (ACh degrading enzyme) mRNA levels, suggesting that acetylcholine signaling was diminished in response to reduced CHRNA5 levels (Figure 3.17D).

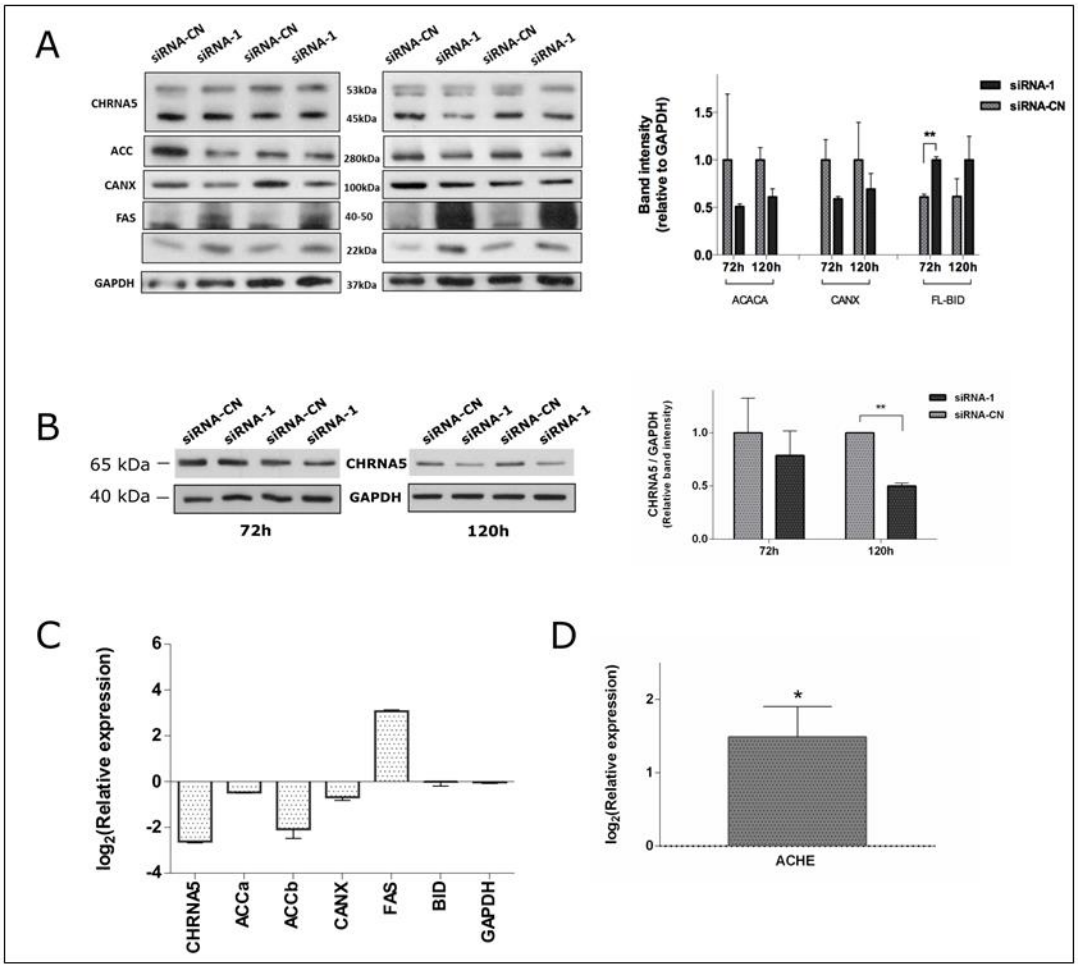

Figure 3.17: Validation of microarray data using RT-qPCR and Western blot analysis.

A) Western blot validations of CHRNA5 protein expression (using polyclonal antibody) and selected genes for 72h and 120h siRNA-1 treated MCF7 cells. Corresponding mage J quantifications are shown on the right. **B)** Western blot analysis of CHRNA5 protein expression of same protein lysates using a monoclonal antibody, together with Image J quantification on the right. GAPDH was used as loading control. **C)** Microarray expression corresponding to each protein studied in A and B part. **D)** RT-qPCR expression of ACHE. *p<0.05, **p<0.01, ***p<0.001. All statistical tests were performed using One-Way ANOVA.

3.3.8 *In vitro* would healing assay

Phalloidin staining of stress fibers after CHRNA5 downregulation showed substantial changes in MCF7 cell morphology, thus implying a possible role of CHRNA5 on motility and migration. Accordingly, scratch wound healing assay was performed to assess the migratory ability of siRNA-1 transfected MCF7 cells, initially optimized with short exposure time of siRNA-1 (48h). No significant change in cell migration was observed between the siRNA-1 treated and siRNA-CN sample (Figure 3.18A). The experiment was repeated for 10nM and 20nM siRNA-1 concentration for a longer exposure time (72h). Wound closure of 72h siRNA-1

treatment plate was calculated according to the corresponding control plate which wells were scratched and fixed the previous day. Only one replica of 10nM and 20nM siRNA-CN was included as control treatments. Results showed a trend of reduction in cell motility for 10nM siRNA-1 and even more pronounced decrease in migration for 20nM siRNA-1 treated MCF7 cells (no statistical analysis possible) (Figure 3.18B). Future studies are needed to better characterize the motility and migrative abilities of siRNA-1 treated MCF7 cells.

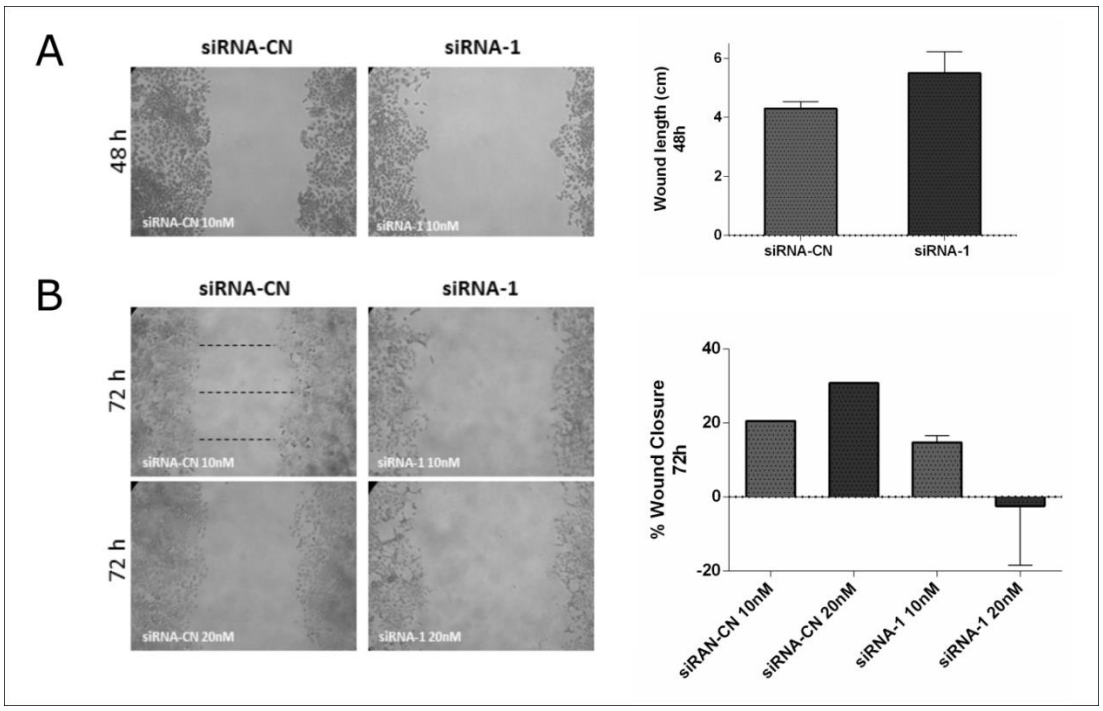

Figure 3.18: Effects of CHRNA5 RNAi on MCF7 cell motility.

Wound closure ability of siRNA-1 exposed MCF7 cells and corresponding controls after **A)** 48h and **B)** 72h treatment. No significance test was performed due to absence of replicates for each treatment; n=1 for siRNA-CN 10nM, n=1 for siRNA-CN 50nM, n=2 for siRNA-1 10nM, n=2 for siRNA-1 50nM. Analysis were performed after obtaining three measurements at identical positions from top to bottom of each image.

3.3.9 Microarray data expression analysis of actin-binding proteins

Out of 53 probe sets belonging to proteins/protein subunits which regulate actin assembly, dynamics or adhesions to ECM [412, 413], 20 of them showed differential expression in CHRNA5 depleted cells (Figure 3.19). The most upregulated one in the microarray data was gelsolin (GSN), a protein whose activity is shown to be regulated by Ca^{2+} in non-muscle cells [414, 415]. In the siRNA-1 dataset, members of RHO-family GTPases also were modulated. RHOA with roles in formation of

stress fibers and focal adhesions [416] and RHOC, similarly to RHOA regulating cell locomotion [417], showed opposite expression. ARP 2/3 subunit ARPC2, controlling nucleation of new actin filaments and directing the cell protrusions [413], was upregulated. Signal proteins of WASP family (WASF3, WASF2, WASF1) known to be regulators of ARP 2/3 [413, 416] showed difference in their direction of expression. There was no change on transcripts of ERM proteins, controlling control cell-cell and cell-matrix adhesion (by forming the bridge between actin cytoskeleton and plasma membrane [418, 419]), however ITGB1 was overexpressed. Vinculin, a focal adhesion protein involved in cell morphology and locomotion [420, 421], was one of the transcripts showing downregulation in the siRNA-1 dataset (Figure 3.19).

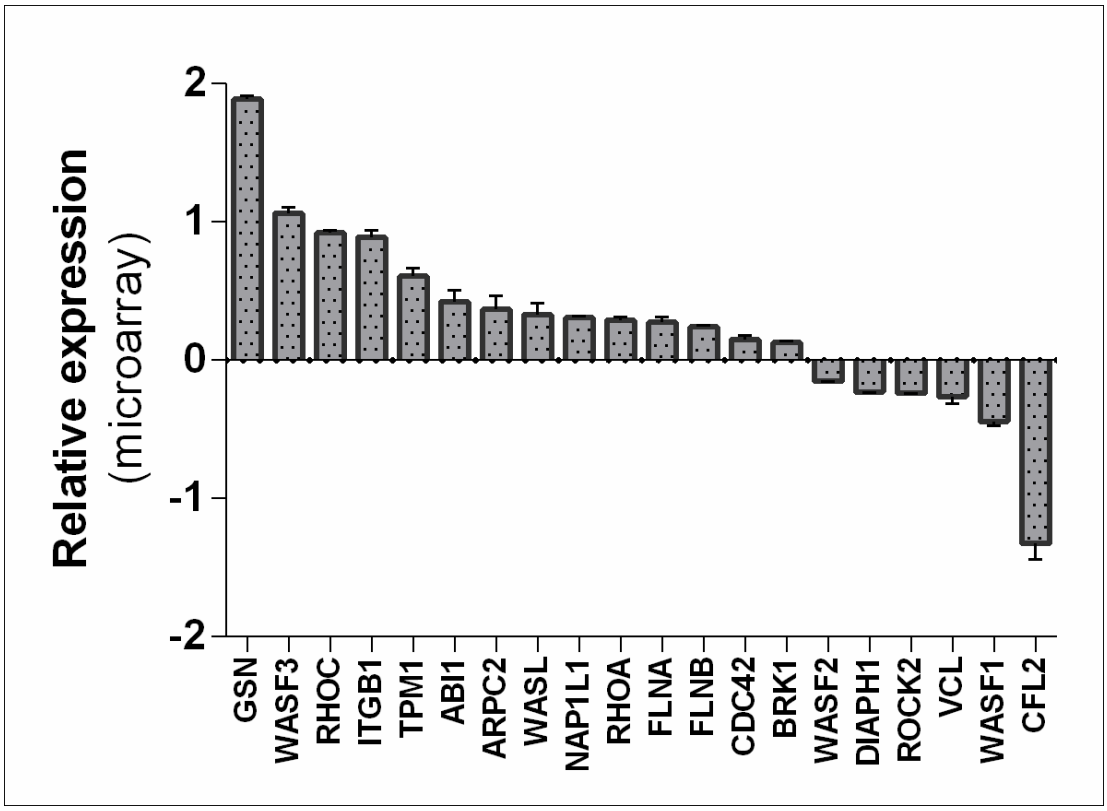

Figure 3.19: Effect of CHRNA5 RNAi on the expression of actin regulatory genes.

Microarray probe set expression of significantly affected actin binding genes/gene subunits (out of 53 probe sets) by siRNA-1 treatment in MCF7 cells.

3.4 Sensitivity of CHRNA5 depleted MCF7 cells to TOPO inhibitors

Drug resistance is a major obstacle in cancer chemotherapy field. Certain *in vitro* methods are used to increase the sensitivity of cancer cells toward chemotherapeutic agents, one of which includes RNA interference studies [175]. Camptothecin (CPT)

and Doxorubicicin (DOXO) are topoisomerase inhibitors widely used as anti-cancer agents due to their role in terminating the DNA replication process via stabilization of the enzyme-DNA complex [44]. It is important to test whether CHRNA5 depletion interacts with drugs commonly used in breast cancer.

Treatment of CPT/DOXO-alone or in combination with siRNA-1/siRNA-CN in MCF7 cells were tested for their effect on cell viability using MTT assay. Results showed that lower CHRNA5 enhanced the cellular sensitivity to CPT exposure (Figure 3.20A) and to DOXO (although with a lesser effect) (Figure 3.20B) in MCF7, a cell line known to be drug resistant [175].

Considering the above findings on CHRNA5 relation to anticancer drug sensitivity, I proposed that there might be an effect of drug treatment on CHRNA5 expression level, suggesting the model that anticancer drugs might block cell proliferation via CHRNA5 downregulation. To elucidate this hypothesis, a 72h single treatment of either 3mM camptothecin, 5 mM doxorubicin or highest corresponding dose of DMSO were performed in MCF7 cells. Western blot results showed considerable downregulation of CHRNA5 in the treated samples relative to DMSO control (Figure 3.21). Future studies are needed to test significance of this finding.

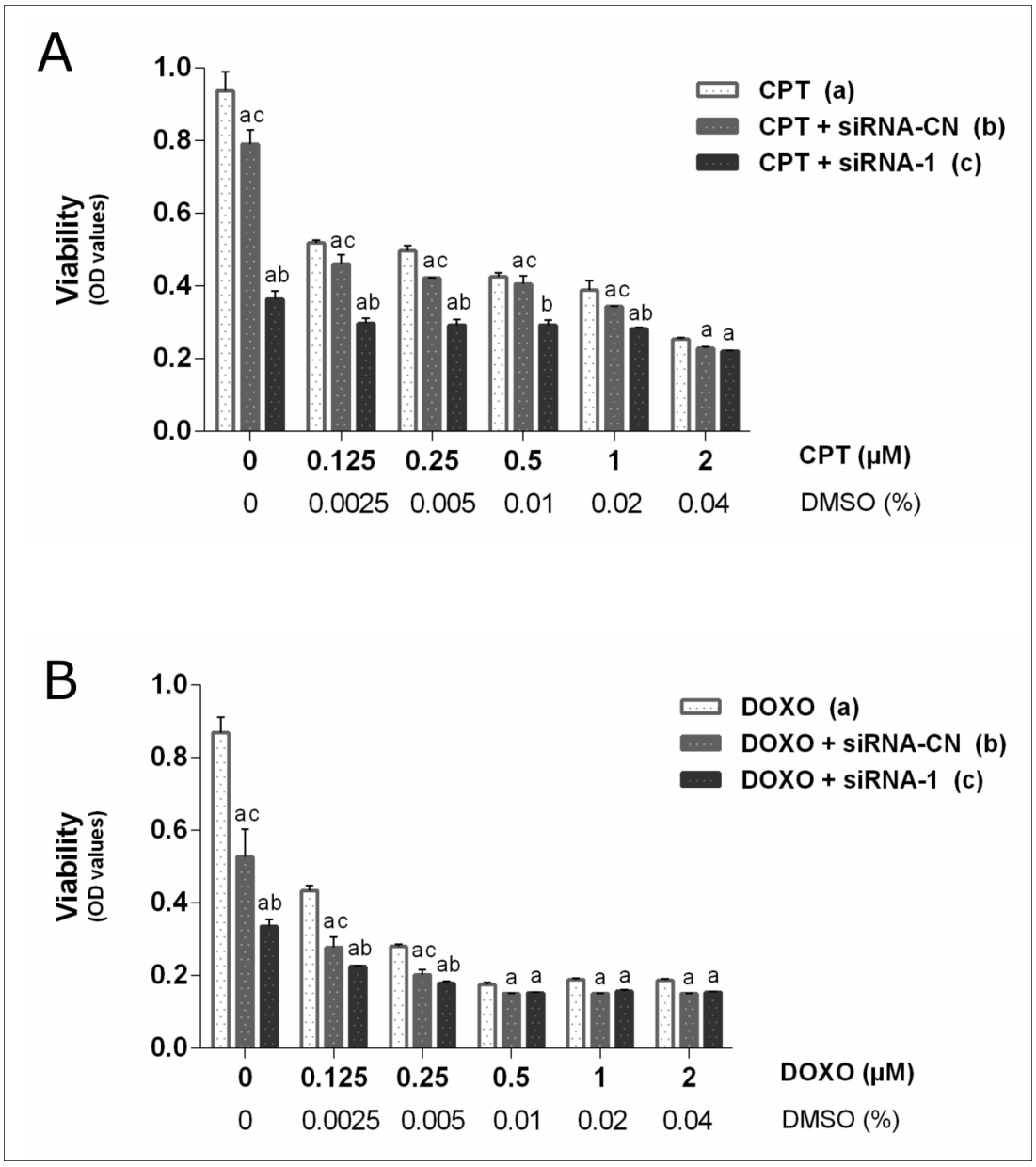

Figure 3.20: Effects of CHRNA5 depletion on the sensitivity of MCF7 cells to TOPO inhibitors.

MCF7 cells were treated with serial dilutions (within the range of 0-2µM) of **A)** CPT, **B)** DOXO or a combination of drug and siRNA molecules. X-axis indicates the drug concentration (first raw) and corresponding DMSO percentage present in each treatment (second raw). Multiple pairwise test differences are indicated above each bar by treatment groups labeled either as 'a' (drug-only), 'b' (drug + siRNA-CN) or 'c' (drug + siRNA-1).

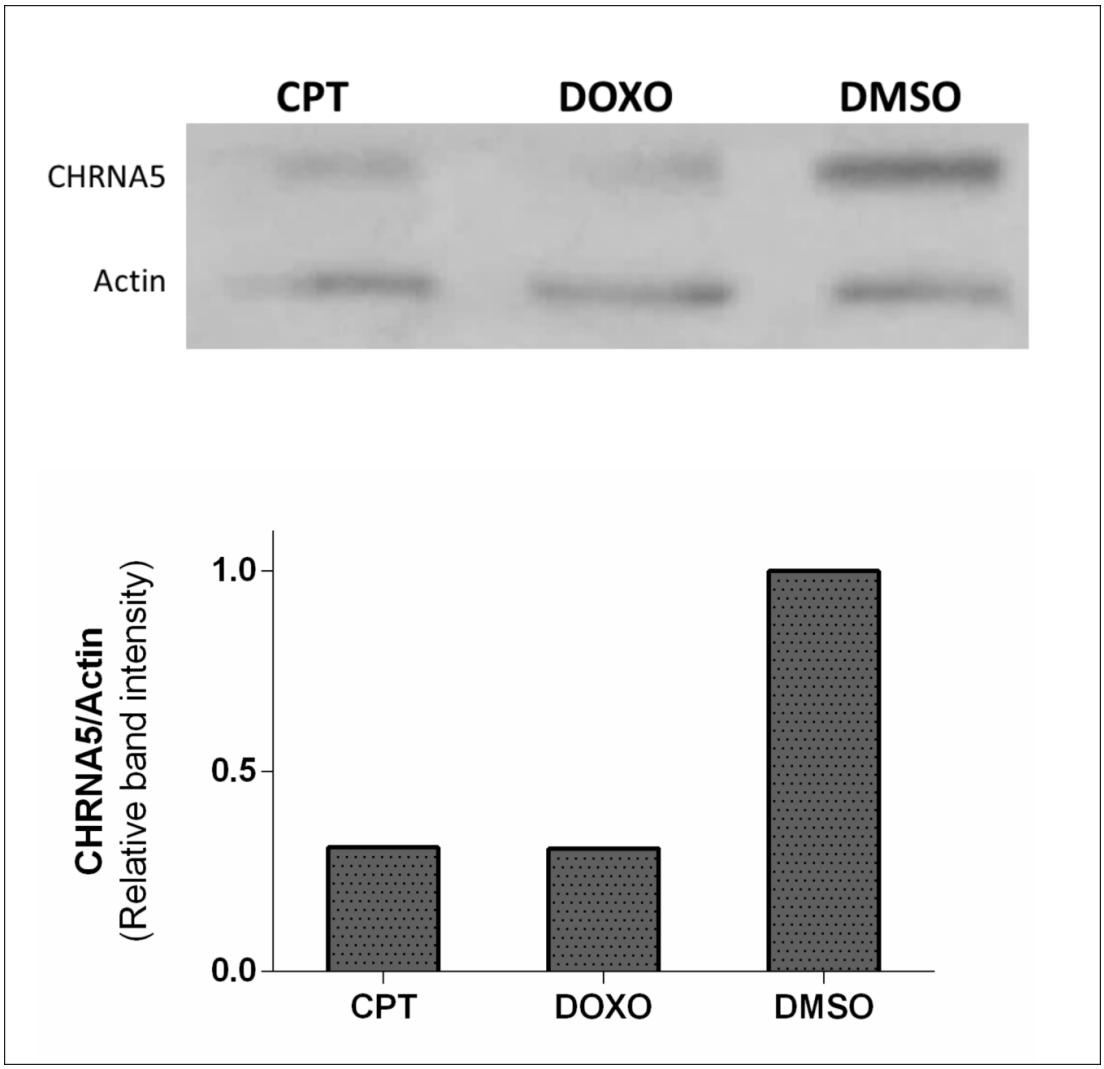

Figure 3.21: CHRNA5 expression in TOPO drug treatments.

CHRNA5 protein expression (using polyclonal antibody) under 72h exposure of MCF7 cells to either 3mM CPT or 5mM DOXO. DMSO was used as negative control group while actin antibody as loading control.

3.5 mRNA-miRNA networks of CHRNA5 depletion upon filtering with drug expression profiles

3.5.1 Extraction of common signatures of CHRNA5 RNAi and drug expression profiles and STRING analysis

Comparing a list of genes between different datasets makes it possible to identify common signatures between independent treatments/studies; such an appraoch serves to better characterize the complexity of the less known compound and to address further questions [389]. Microarray data analysis of CHRNA5 RNAi has shown that

multiple cellular pathways were affected (i.e., cell cycle, DNA repair mechanisms); many of these pathways were also known to be modulated upon chemotherapeutic drug treatments. In addition, previous MTT-drug study has shown that CHRNA5 expression affects viability as well as the sensitivity of MCF7 cell line to chemotherapeutic drugs like CPT and DOXO. To compare the expression profiles and to find possible similarities in the mode of action between siRNA-1 and different anticancer agents in MCF7 cells, I retrieved public microarray datasets of two representatives from each of the following chemotherapeutic agent groups: 1) TOPO inhibitors: SN38, DOXO 2) HSP90 inhibitors: Tanespimycin (17AAG), Luminespib (NVP-AUY922) and 3) CDK inhibitors: PHA-793887, PHA-848125 from GEO (accession number: GSE18552) [404]. Highly connected networks were found between siRNA-1 expression profile and that of topoisomerase inhibitors, when analyzing their common differentially expressed downregulated gene groups (STRING analysis) (Figure 3.22A). Similarly, genes deregulated in both CDK inhibitors and siRNA-1 treatment profiles, came up as densely connected nodes (Figure 3.22B, Table 3.1), with common KEGG pathways including DNA replication, cell-cycle and DNA repair mechanisms (Table 3.2). On the other hand, MCF7 cells treated with proteasome inhibitors shared less differentially expressed genes with siRNA-1 microarray profile and resulted in comparatively less densely interconnected networks in STRING (Figure 3.22C, Table 3.1-3.2).

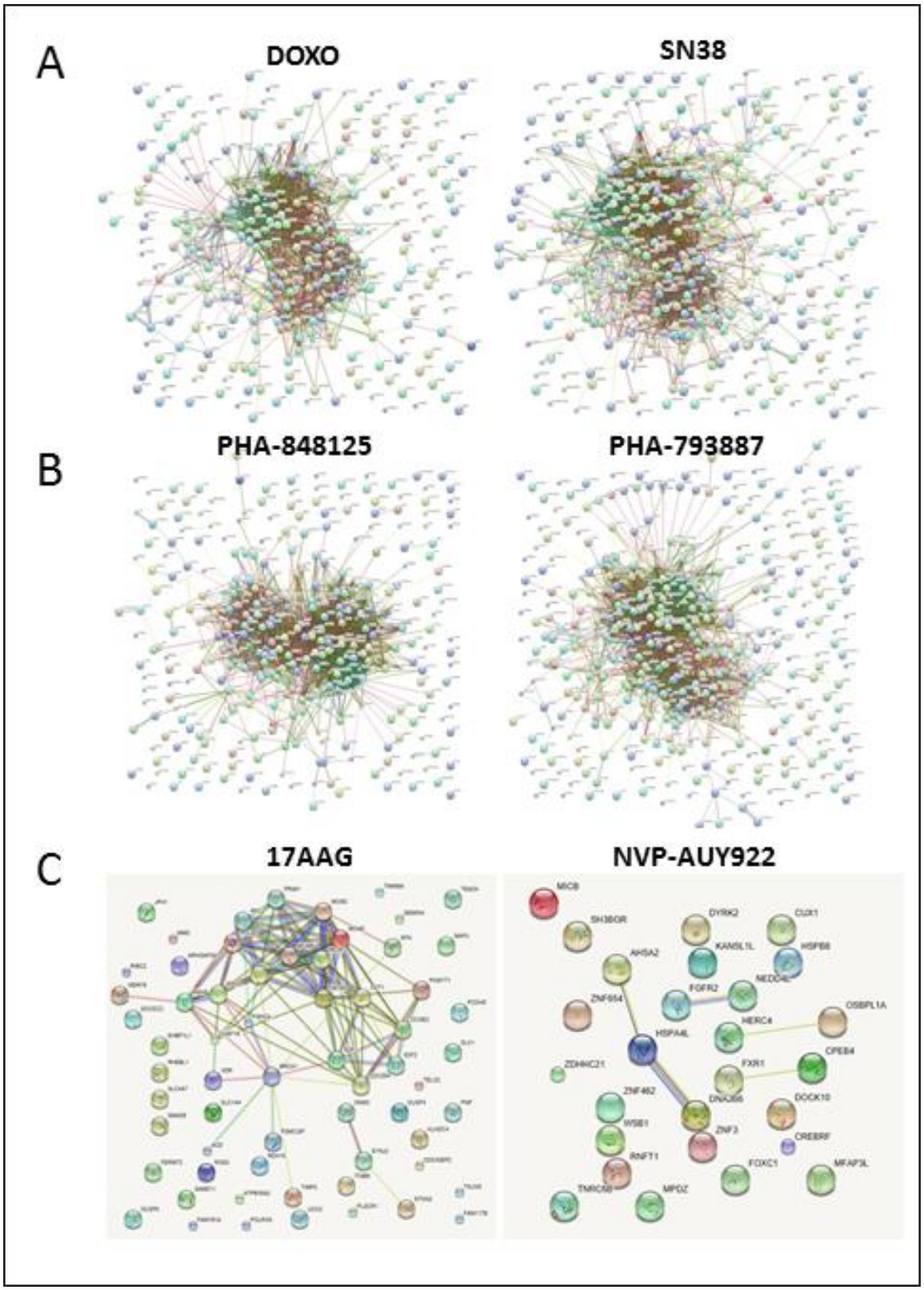

Figure 3.22: Microarray comparisons between CHRNA5 siRNA-1 and chemotherapeutic drug profiles.

The most highly connected networks (from up-up, up-down, down-down, down-up genes) between siRNA-1 and individual drugs were picked up for **A)** topoisomerase inhibitors (represented by down-down genes), **B)** CDK inhibitors (represented by down-down genes) and **C)** HSP90 inhibitors: 17AAG (Tanespymicin) represented by down-down and NVP-AUY922 represented by up-up genes, as retrieved from STRING network analysis.

Table 3.1: STRING network statistics of expression profile comparison between siRNA-1 and individual drug treatments in MCF7 cells

Drug name/symbol	No. of nodes	No. of edges	Avg. node degree	Avg. clustering coefficient	Expected no. of edges	PPI enrichment p-value
DOXO	281	1358	9.67	0.423	298	0
SN38	360	2094	11.6	0.416	562	0
CDK-inhibitor 125	327	2009	12.3	0.46	411	0
CDK-inhibitor 887	387	2255	11.7	0.43	512	0
17AAG	63	107	3.4	0.307	18	0
AUY922	27	5	0.37	0.296	1	0.00607

Table 3.2: Significant KEGG pathways showing common regulation between siRNA-1 and individual drug expression profiles

DOXO (down-down)	#	SN38 (down-down)	#	CDK-inhibitor 125 (down-down)	#	CDK-inhibitor 887 (down-down)	#	17AAG (down-down)	#	AUY-922 (up-up)
Cell cycle	21	Cell cycle	20	Cell cycle	23	Cell cycle	25	DNA replication	9	NA
DNA replication	7	Fanconi anemia pathway	11	Fanconi anemia pathway	10	Fanconi anemia pathway	11	Cell cycle	11	
Mismatch repair	6	Oocyte meiosis	12	DNA replication	8	DNA replication	8	Mismatch repair	4	
Fanconi anemia pathway	7	DNA replication	7	Oocyte meiosis	11	Mismatch repair	6	MicroRNAs in cancer	6	
Oocyte meiosis	9	Homologous recombination	6	Mismatch repair	6	MicroRNAs in cancer	12	Pyrimidine metabolism	5	
Homologous recombination	5	Mismatch repair	5	Homologous recombination	6	Oocyte meiosis	9	Nucleotide excision repair	3	
Viral carcinogenesis	10	Progresterone-mediated oocyte meiosis	8	Base excision repair	5	Base excision repair	5	Fanconi anemia pathway	3	
MicroRNAs in cancer	8	Colorectal cancer	6	Progresterone-mediated oocyte meiosis	7	Progresterone-mediated oocyte meiosis	7			
Base excision repair	4	P53 signaling pathway	6	Nucleotide excision repair	5	Nucleotide excision repair	5			
						Homologous recombination	4			
						P53 signaling pathway	6			

3.5.2 Constructing mRNA-miRNA networks of common expression profiles obtained from CHRNA5 RNAi and TOPO inhibitor studies

In order to identify a common mRNA-miRNA network for CHRNA5 depletion and exposure to TOPO inhibitors I have used mirnet.ca. Common UP-UP and common DOWN-DOWN gene lists were uploaded to miRNET and for each a network was obtained (Figure 23A,B). The characteristics of the network were given in the Table 3.3. Shortest path analysis revealed several microRNAs that can be further leads to study in connection with drug sensitivity and CHRNA5 RNAi (Figure 3.23C,D). Selected candidate microRNA expression modulations from microRNA arrays performed in the context of 114S367 project (Cingir et al. manuscript in prep) were indicated (Table 3.4). Accordingly, miRNET analysis based on the commonly regulated gene sets between siRNA-1, SN38 and DOXO revealed a set of miRNAs some of which can be further studied as common regulatory factors of agents affecting the interaction between genotypes and drugs (Figure 3.23, Table 3.4).

Table 3.3: miRNET statistics of common differentially expressed genes in siRNA-1 and drug treatments (DOXO, SN38) in MCF7 cells

	Upregulated gene list	**Downregulated gene list**
No. of genes	181	265
No. of miRNAs	1755	1790

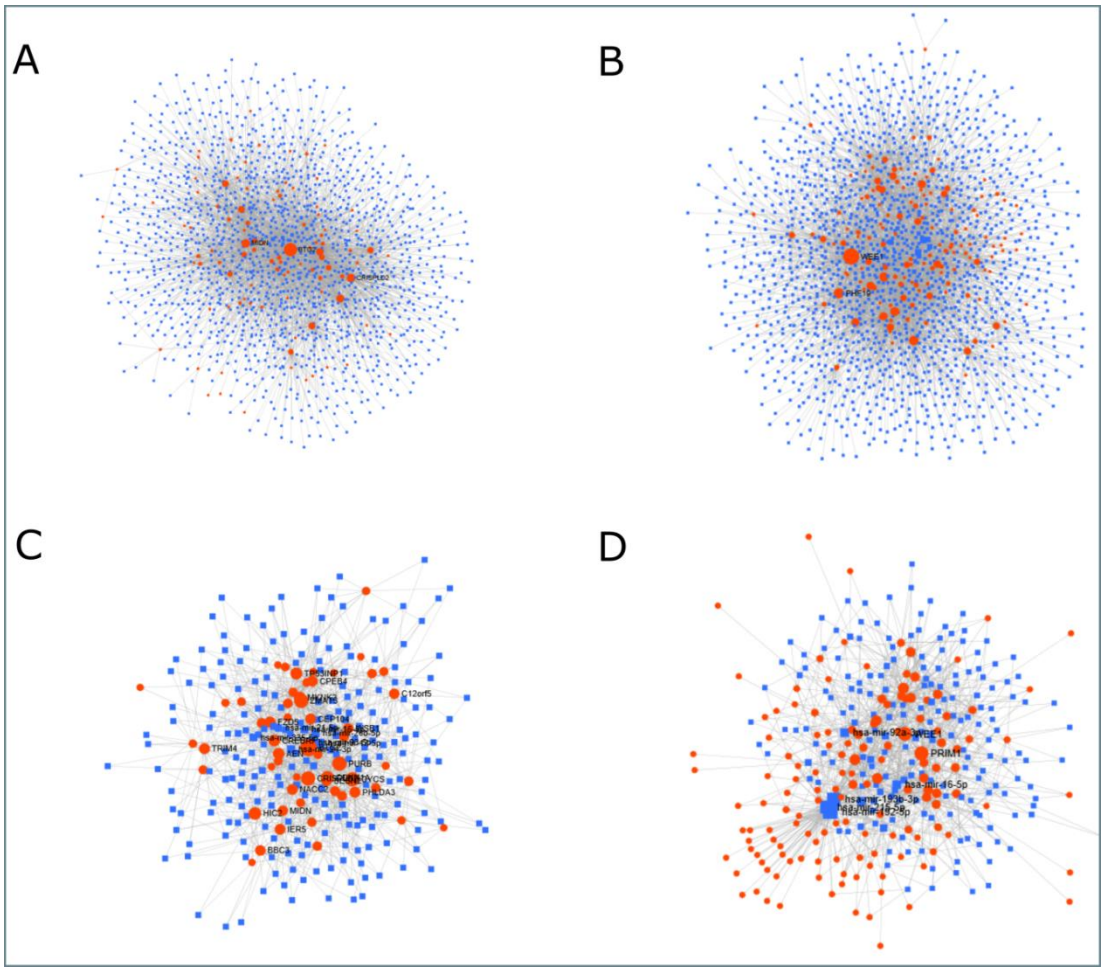

Figure 3.23: Common signatures between topoisomerase inhibitors and siRNA-1 treatment.

A) Common miRNA network of the upregulated probesets (p<0.05, logFC>0.5) and **B)** downregulated probesets (p<0.05, logFC<-0.5) between SN38, DOXO and siRNA-1 miroarray expression profiles**; C)** Shortest path filtering of upregulated probesets; **D)** Shortest path filtering of downregulated probesets.

Table 3.4: Candidate miRNA molecules suggested to affect the functional properties of CHRNA5 and TOPO drugs

miRNA ID	Gene list	miRNA array p-value	miRNA array logFC
hsa-mir-34a-5p	Up, down	0.0017	0.78
hsa-mir-21-3p	Up, down	0.007	-0.82
hsa-mir-10b-5p	Up, down	0.0038	-0.70
hsa-miR-125b-5p	Up, down	0.0154	0.51
hsa-miR-326	Up, down	0.0005	-1.04
hsa-miR-195-5p	Up, down	0.03	-0.384

3.6 Red conjugate oligomer nanoparticle (Red-CON) characterization

3.6.1 Cytotoxicity study of Red-CON

Different formulations of nanoparticles are now available for delivery of chemotherapeutic molecules, a candidate of which is a CB7-capped, red-emitting conjugated oligomer nanoparticle (CON) [94]. MCF7 and MDA-MB-231 adenocarcinoma cell lines were included in the study, as breast cancer representatives, to evaluate the effect on cell viability of the abovementioned CON. Compared to MCF7 cell line, MDA-MB-231 cell line was found to be more resistant to the nanoparticle-induced cytotoxicity, for the first two applied concentrations (0.125−0.5 μM) (Figure 3.24A). Nanoparticle in its un-encapsulated form (NP) showed higher toxicity than its encapsulated complex (NP+CB). CB7 and NP+CB7 formulation (similar to the CB7-only treatment) had comparable, non-toxic effects on both cell lines (Figure 3.24A).

3.6.2 Red-CON usage as a drug delivery agent

In order to study the effectivity of the above mentioned CON as a drug loading and releasing agent, camptothecine (CPT) was used as a model chemotherapeutic drug in a dose-dependent treatment. When comparing the treatment groups, NP+CPT and NP+CB+CPT showed similar and significant reductions on cell proliferation (Figure 3.24B). Both drug loading nanoparticle formulations (CPT+NP and CPT+NP+CB) showed differences in cell viability compared to the respective CPT-only concentrations (One-way ANOVA, multiple test comparison) (Figure 3.24B).

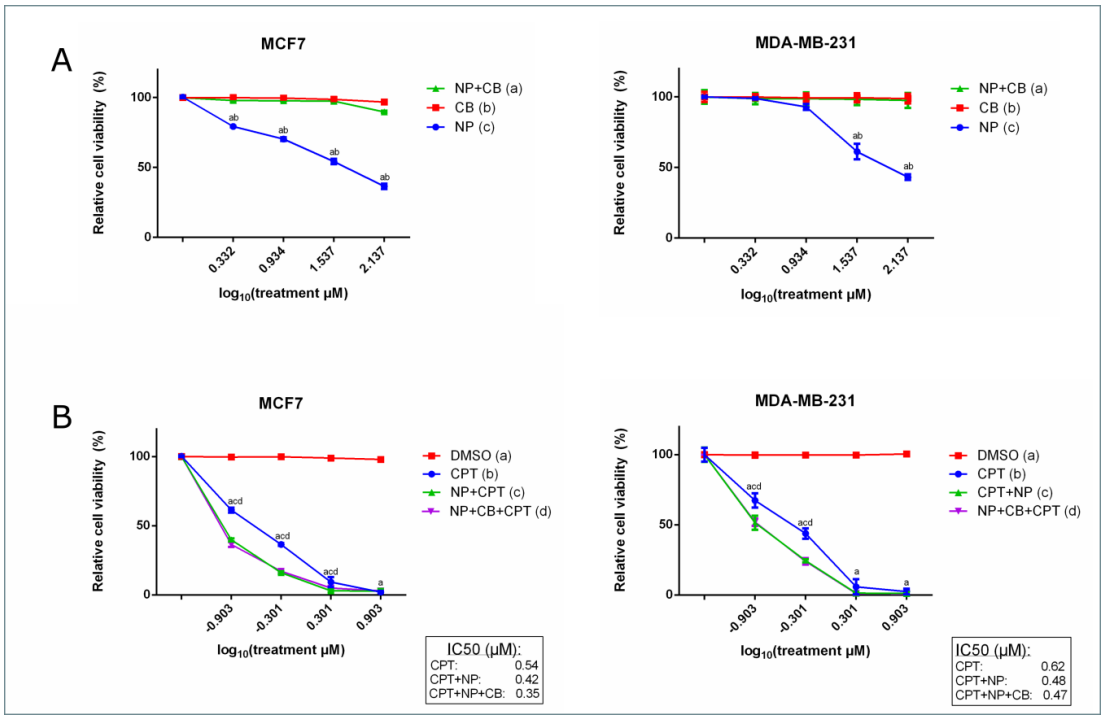

Figure 3.24: Effects of red-emitting nanoparticle on breast cancer cell viability.

Relative cell viability results after MTT assay at indicated concentrations (log10) and treatment groups. **A)** NP+CB, CB, NP and **B)** CPT, NP+CPT, DMSO, NP+CB+CPT together with IC50 values calculated using four parametric polynomial group in MATLAB. Data were normalized according to the untreated controls, in both breast cancer cell lines (MCF7 and MDA-MB-231). Treatment groups with significant multiple pairwise differences were indicated on each concentration of CPT viability curve. This figure was reprinted with permission from ACS publications. Copyright (2014) American Chemical Society. Pennakalathil et al., Red Emitting, Cucurbituril-Capped, pH-Responsive Conjugated Oligomer-Based Nanoparticles for Drug Delivery and Cellular Imaging, (2014) Biomacromolecules.

3.6.3 Red-CON usage as an imaging agent

Fluorescent microscopy is an important technique for describing and predicting the localization of nanoparticle after its cellular uptake. II used phalloidin staining of actin cytoskeleton counterstained with DAPI and took overlaying images from both cell lines treated either with non-encapsulated NP or CB7-encapsulated complex (NP+CB). Fluorescent images of NP-treated MCF7 and MDA-MB-231 cell lines revealed the presence of nanoparticles in perinuclear regions, which indicated the possibility that they might be enriched in endosomes and/or lysosomes (Figure 3.25). CB7 encapsulation on the other hand could affect the photo-stability of nanoparticle and/or its light emitting properties.

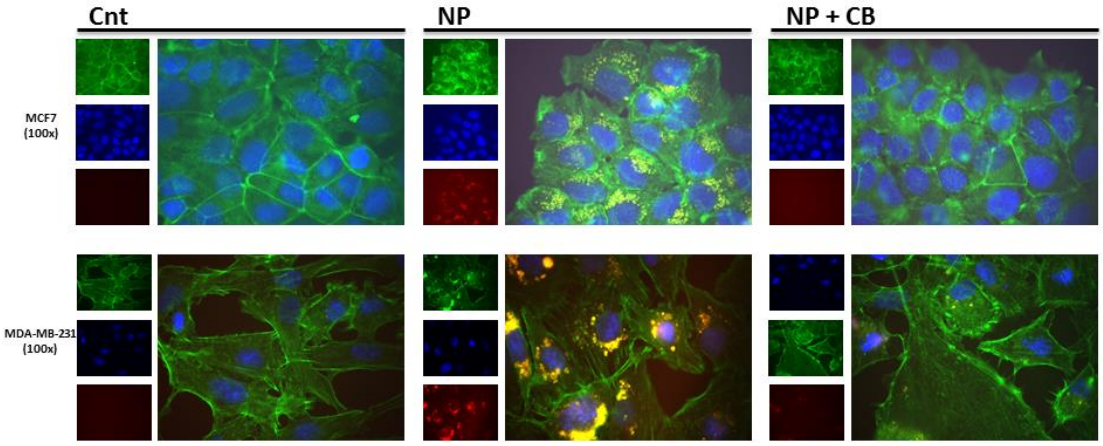

Figure 3.25: Characterization of red-emitting NP compartmentalization and light emission in breast cancer cells.

Emission from nanoparticle (yellow) internalized in MCF7 and MDA-MB-231 cell lines both in the non-encapsulated (NP) and encapsulated formulation (NP+CB), in comparison to the untreated control groups (Cnt). Nuclei (blue) and F-actin (green) were stained with DAPI and phalloidin, respectively and images were obtained using 100x magnification. This figure was reprinted with permission from ACS publications. Copyright (2014) American Chemical Society. Pennakalathil et al., Red Emitting, Cucurbituril-Capped, pH-Responsive Conjugated Oligomer-Based Nanoparticles for Drug Delivery and Cellular Imaging, (2014) Biomacromolecules.

Chapter 4

Discussion and rationale

4.1 CHRNA5 in breast cancer cells

4.1.1 CHRNA5 targeting via siRNA

In this study I have incorporated two different siRNA molecules targeting nearby regions of *CHRNA5* transcript which resulted in similar effects, revealing the specificity of the RNAi treatment. The mild toxicity of siRNA-CN on cell viability (MTT assay) might have arisen due to target-independent effects of RNAi [410].

High decreases in CHRNA5 transcript levels were observed eventhough reduction at the protein level was comparatively lower. The Western blot observation of CHRNA5 downregulation could partly be explained by the lengthy turnover rate of CHRNA5. Upregulation of nAChR subunits induced by nicotine treatment is known to be associated with regulation in proteasome activity [422]. Literature evidence shows that the rate of protein turnover might also depend on several other factors (i.e., multi-subunit protein turnover might happen in sub-complexes at different rates for some of subunits [423]). Other factors include subcellular location and functional constraints; half-life of cell surface proteins is known to be relatively longer due to slower degradation [423, 424]. In addition, there is evidence that long-lived proteins, when exposed to growth arrest-inducing drugs (like camptothecin), become longer lived [425]. Accordingly CHRNA5_v2 a part of a heteromeric pentameric complex that spans the cell membrane [303], might have longer half-life than the other forms in siRNA treatment.

4.1.2 CHRNA5 RNAi role in cell proliferation

Several of the experimental results presented herein similarly demonstrated an antiproliferative effect of CHRNA5 downregulation. Decrease in the DNA synthesis rate displayed by BrdU data, reduction of cell cycle gene representatives, *BIRC5*, *ANLN* and *WDHD1,* and increases in the subG1 cell population suggested a role for

CHRNA5 in regulating cell cycle and cell death, at least in the case of MCF7 cell line (p53 wt, ER+).

CHRNA5 RNAi was successfully applied to two additional breast cancer cell lines, BT-20 and MDA-MB-231 (TP53 mutant, ER-) [409]. In contrast to the strong TP53 response induced by CHRNA5 depletion in MCF7, TP53 induced genes as well as the cell cycle-related genes were not significantly regulated in BT-20 and MDA-MB-231; however proliferation of both cell lines was affected by siRNA-1 in a cell seeding density manner (as evaluated by MTT assay). These data suggested differential effects of CHRNA5 silencing on cell proliferation, potentially based on the TP53 status of cell lines. A high 6-well seeding density of BT-20, i.e., 21,100 cells/cm^2) that was used for gene expression studies may not directly correspond to the seeding densities used in MTT analyses; thus the two different datasets (expression and MTT) may conflict each other. One study on transient gene silencing in smooth muscle cells (96-well plates) has shown that treatment effectiveness depends on the initial cell seeding density [426]. Same study also showed that high seeding densities might have masked treatment-dependent changes in cell viability, highlighting the importance of cell seeding counts on RNAi treatments. These suggest caution is needed when interpreting expression studies and functional tests dealing with the gene knockdown-overexpression studies.

4.1.3 CHRNA5 RNAi role in MCF7 morphology and motility

MAP1B, *CLDN1* and *GJA1* are implicated with cell motility, migration and carcinogenesis in several cancer types. Interestingly *GJA1* is also upregulated during mammary gland development, pregnancy and milk ejection [427]. Microarray- and qPCR-measured upregulation of these genes indicated a role for CHRNA5 depletion in modulation of cellular morphotype as well as implication with cell motility and cancer progression.

In the present study, siRNA subjected cells showed both biological and structural alterations with diverse morphological changes observed by stress fibers staining. Interestingly, at 120h of siRNA-1 treatment, there was absence of few structures which had developed in 72h siRNA-1 subjected cells such as motile-like filopodia

and multilayer cell aggregates. Cell extensions are mandatory structures for cells to migrate. Extracellular signals cause cells to initially acquire a polarized morphology by actin assembly and to extend its protrusions by forming flattened structures called 'lamellipodia' and fingerlike ones called 'filopodia' [413]. Cell then forms adhesive anchoring on the surrounding by linking cytoskeletal actin of lamellipodium to the extracellular matrix (ECM); thus forms protrusive structures and move forward [413]. Each step in cell migration is regulated by different actin-binding proteins which control actin assembly, stability and organization. The regulation of actin-binding protein expression (by CHRNA5 depletion) might be explained by a possible change in cytoplasmic Ca^{2+} upon CHRNA5 downregulation [299].

Out of 20 transcripts belonging to actin-regulating proteins which were significantly regulated in siRNA-1 microarray dataset, gelsolin (GSN) was the most upregulated one. GSN protein has a dual function of actin capping and severing, resulting in actin network remodeling [415]. Expression of GSN is found to affect motility [428] and migration [429] *in vitro* as well as induce metastasis *in vivo* [430, 431]. Older studies considered gelsolin also as a regulator and effector of apoptosis [432] after being activated by caspase-3 cleavage, prior to cell death in MCF7 cells [433]. Recent data also show that caspase-3, stimulated by FAS, cleaves GSN in functionally active fragments, which further cleave actin filaments [434]. However, absence of CASP3 in MCF7 cells indicates that either gelsolin is not activated (eventhough upregulated in the microarray dataset) or that it might have been activated by other factors.

Out of 7 integrin subunits, only ITGB1 shows upregulation in siRNA-1 expression profile. ITGB1 is a part of focal adhesion which constitutes the major part of trans-membrane receptors that provide interaction between actin cytoskeleton and extracellular matrix [435]. It regulates many cellular processes such as cell proliferation, differentiation, polarity, angiogenesis, migration and has a direct effect on mammary tumor development [436]. There is evidence that integrin-β_1 is also responsible for detachment of cells from culture flask [437]. This could be the reason why I observed an increase of living cell number in flow-through cell population in siRNA treated samples (tryptan blue assay). Viability of detached MCF7 cells in treated samples also implies that somehow these cells can survive independent of cell-cell and cell-matrix interactions. Krais et al. have reported decreases in two cell adhesion molecules (P120 and ZO-1) upon CHRNA5 downregulation, while in this

MCF7 CHRNA5 RNAi study ZO-1 (TJP1) was upregulated at the mRNA level (microarray data for TJP1: 202011_at: logFC= 0.65171, p=8.69E-05; P120=no data available). Such variability among different cancer cell lines implies the possibility for CHRNA5 action to be cell-type dependent.

F-actin staining of CHRNA5 depleted cells has revealed structural changes implying a tendency of treated cells to overcome the characteristic monolayer growth pattern toward a 3-dimensional like growth, shown by tubule formation, multilayer structures and grape-like cell groupings. Comparable results have been obtained from MCF7 cells grown in scaffold-free 3D cultures with non-adhesive hydrogel [438]. In contrast to 2D growth systems, cells in 3D cultures form microtissues with luminal spaces and expressed breast specific markers [438]. Another study shows that MCF7 cells grown in 2D cultures exposed to microgravity also result in production of 3D cell aggregates like spheroid cells as well as forming glandular structures which resemble the *in vivo* cancer tissue composition [437]. Both real space mission and induced microgravity in MCF7 cell line has shown alterations in cytoskeletal structure [439, 440]. Similarly, the siRNA-treated MCF7 cells might have the tendency of differentiating toward their normal progenitor cell profile.

Vinculin is another downregulated actin-regulating gene by CHRNA5 RNAi. In a recent study, its knockdown showed cell polarity disruption with reversal of migration direction as well as reduced speed in MDA-MB-231 cells [441]. Vinculin is one of the transcripts showing downregulation by CHRNA5 RNAi. As a focal adhesion protein, it is involved in cell morphology and locomotion [420, 421]. Vinculin has been downregulated in the study of simulated zero gravity in MCF7 [439]. Moreover, in a recent study, its knockdown shows cell polarity disruption with reversal of migration direction as well as reduced speed in MDA-MB-231 cells [441]. Overall, the microarray data and phalloidin results of CHRNA5 depleted MCF7 cells imply a gross change in actin cytoskeleton organization and dynamics leading to changes in morphology.

The wound healing data have shown no significant change in motility of siRNA-1 treated MCF7 cells. CHRNA5 depletion treatment might have rendered MCF7 cells less motile (although with no significance). Additionally, the migratory ability of MCF7 cells could be proportional to the level of CHRNA5 downregulation. Yet I have

observed no gross change in motility; and it is not clear how much the loss in cell proliferation might have affected the migratory profile of siRNA-1 treated MCF7 cells (i.e., less cells, less motility observed). Number of stress fibers, lamellipodia, filopodia and cell-matrix adhesions are not always directly proportional to rate of migration. For example, fibroblasts have many such structures but slow moving rates, whereas keratinocytes (with only a single protruding lamelipodium, no stress fibers and small focal adhesions), move much faster [413]. As a result, more than the number of cell protrusions and changes in trancriptome, knowing exact mechanism of regulation by actin reorganization could be more be more conclusive in the present study.

4.1.4 CHRNA5 RNAi as an inducer of cell death

Experimental results also have suggested that CHRNA5 depletion is probably not associated with apoptosis. Although upregulated FAS at the mRNA and protein levels indicates induction of a possible FAS-induced apoptotic pathway in siRNA transfected MCF7 cells, no increases in its ligand (FASL) at the transcript level (microarray data for FASL: 210865_at adj p=0.776) has been detected. For apoptosis to occur, a strict regulation of FASL expression and further activation of FAS is required [442]. To make sure that neither extrinsic nor intrinsic apoptotic pathways is the cause of reduced cell proliferation in the siRNA treated cells [443], I further have looked at BID protein cleavage profiles in siRNA-1 treated cells. WB analysis has shown upregulation of full length BID (FL-BID) but absence of its truncated form (tBID) in siRNA-1 treated cells. Even though cleavage of BID is not necessary for apoptosis a in every cell line [443], in MCF7 cells this is reported as being so [30, 31, 32]. The microarray data analysis also has pointed out pathways involved in DNA damage response, DNA replication stress and cell cycle arrest that might occur in RNAi with no apparent apoptosis. Several studies show that BID in its uncleaved form has a role in replicative stress response by binding to RPA protein, facilitating binding of other proteins and inducing cell cycle arrest [444, 445]. Upregulated FL-BID protein in the present study might be the subsequent response to the cell stress after CHRNA5 downregulation. Drastically upregulated FAS at the mRNA and protein levels, yet absence of truncated BID (tBID) in siRNA-1 treated MCF7, as well as no detection of apoptosis in CDD assay, implicate that apoptosis may not be complete or has never initiated.

One of the hallmarks of apoptotic cell death is nuclear and DNA fragmantation via DNA Fragmentation factor subunit alpha (DFFA/ICAD) (the major apoptotic nuclease) whose enzymatic activity makes the histones (bound to DNA fragments) to be released into the cytoplasm. Activation of ICAD is performed by the cleavage of its inactive form ICAD-DM via caspase-3 (CASP3) (with apoptosis progression) [446]. MCF7, a CASP3-deficient cell line (due to a deletion mutation in CASP3 gene), has failed to undergo such morphological changes during apoptosis [447]. However apoptotic cell death could be observed under certain stress conditions in MCF7 cell line [448], probably since CASP3 (although needed in most cases of apoptosis) is not a necessary requirement for apoptosis pathway to occur [447]. The principle of CDD kit (Roche), used in this study to detect apoptosis, is via binding of specific antibodies to the histone proteins of cytoplasmic DNA fragments released in cytoplasm. The probable absence of such proteins in treated MCF7 cytoplasm [447] could be the reason I have detected no apoptosis in siRNA-1 treated MCF7 cells.

4.1.5 CHRNA5's role in cholinergic signaling

Literature evidence shows that cholinergic signaling regulation in SCC tumors is not limited only to cholinergic receptors subunits, but also includes changes in ACh synthesis and degradation, reported as: higher a5, β3, ACh, ChAT and lower ACHE expression (compared to normals) [271]. In the microarray study, CHRNA5 depletion has been accompanied by an increase in ACHE suggesting CHRNA5 might be an essential factor orchestrating the cholinergic circuit and could even be the inducer of tumorigenic events. Upregulation of ACHE in the present study also implies the possibility that CHRNA5 might regulate cell cycle via ACh (known to promote cell proliferation [222]), or through ACHE via the non-cholinergic functions for controlling cell survival [449].

4.1.6 CHRNA5 role in drug sensitivity

In a study performed in 2002 [450], around 75% of studied tumors and corresponding normal tissues were CASP3 deficient (while CASP8 and CASP9 levels were normal), which was considered as a mechanism responsible for survival and drug resistivity in tumors [450]. CASP3-deficient MCF7 cells have shown

resistivity to several apoptotic agents but were quite sensitive toward inhibitos of cyclin-dependent kinases (CDKs), due to the intact p53 protein they harbor [451]. However, reconstitution of CASP3 in MCF7 rendered cells sensitive to several chemotherapeutic drugs, including DOXO [452]. CHRNA5 downregulation in the present study has increased the sensitivity of the drug-resistant MCF7 cells toward CPT and DOXO, two topoisomerase inhibitors, known to induce double-strand breaks [453]. Hence, the present thesis introduces the possibility that CHRNA5 targeting might be beneficial in reducing the therapeutic doses of agents like CPT, DOXO and their clinical analogs, in p53-wild type, ER+ (and possibly CASP3-deficient) breast tumors. Advances in nanotechnology field have opened avenues for recently designed nanocarriers which carry both drugs and siRNA molecules [94, 454]. Moreover, recent products include degradable implants which carry siRNA based drugs administered to human patients (in clinical trials) in combination to the classical chemotherapeutic regimens [455, 456]. Therefore using TOPO inhibitors in breast carcinomas expressing lower CHRNA5 levels could be helpful. Moreover, the combinatorial strategy of CHRNA5 RNAi and topoisomerase targeting in CHRNA5-high breast tumors might be a therapeutic lead that has come out of the present study. In addition, increase in sensitivity of MCF7 to apoptotic inducers such as CPT and DOXO indicates a possible regulation of cell death via CHRNA5 deregulation in a CASP3-independent manner.

The comparative transcriptomics studies of TOPO and CDK inhibitors with CHRNA5 RNAi expression profiles have led me to identify a series of genes similary up- or down-regulated, for the first time in literature. Common downregulated pathways by both treatments included mainly cell-cycle related pathways and DNA repair mechanisms, suggesting that CHRNA5 depletion has similar effects on MCF7 cells as topoisomerase and CDK inhibitors. Topoisomerase inhibitors were found to be close neighbors of CDK inhibitors (with similar responses) according to the MATRA website tool [404]. This finding strongly confirms the similarities based on commonly modulated genes I observed between each of the drug families (represented by SN38 and DOXO) and CHRNA5 siRNA-1 datasets. In addition, MANTRA study has identified p21/CDKN1A upregulation as a common event induced both by topoisomerase and CDK inhibitors, which (according to the RT-qPCR results in this thesis) might be an important intersection/overlapping

point also for the antiproliferative effect of CHRNA5 downregulation. These results offer a potential usage of CHRNA5-targeting agents to decrease resistivity and increase therapeutic potential of not only topoisomerase but also CDK inhibitors. Very interestingly, the preliminary Western blot study using one CPT and one DOXO sample indicated that apoptosis inducers could apply their effect via downregulation of CHRNA5; this needs to be further validated.

4.1.7 Common miRNA networks of CHRNA5 and TOPO drugs

smiRNET nalysis of commonly regulated gene sets between siRNA-1 profile and profiles of public SN38, DOXO datasets revealed some promising miRNA molecules with important implications in cancer progression. For instance, hsa-mir-34a-5p (which was upregulated in siRNA-1 treatment), is a key player of p53 pathway. Its regulation is shown to be linked with tumor progression, yet in a cell context manner [457]. Synthetic mimics of this molecule are being studied in phase I clinical trials for treatment of several cancer types [458]. On the other hand, hsa-mir-21-3p (downregulated in siRNA-1) is considered as an ocogenic miRNA molecules due to its upregulation in several cancer types [459]. It is implicated with overian cancer initiation and resistance to chemotherapeutic drugs [460]. Similarly, hsa-mir-10b-5p (downregulated in siRNA-1) is considered as an oncogenic miRNA [461]. In a study of 770 breast cancer patients, hsa-mir-10b-5p was associated with poor prognosis [462]. Another miRNA, hsa-miR-125b-5p (upregulated in siRNA-1), is reported to have tumor suppressor activity in several cancers [463], including breast, where it is found to target MUC1 transcript [464]. Its expression in breast cancer is also correlated with increase in resistance to chemotherapy [465]. On the contrary, hsa-miR-326 (downregulated in siRNA-1) is found to increase sensitivity of breast cancer cell lines to chemotherapeutic drugs, while its deregulation was observed in aggressive breast cancer tissues [466]. Expression of another miRNA molecule, hsa-miR-195 (downregulated in siRNA-1), was similarly found by multiple studies to regulate fatty acid synthesis in breast cancer cell lines as well as reduce angiogenesis, cell proliferation and migration [467, 468]. Overall, downregulation of miRNAs (in the siRNA-1 profile) showing oncogenic activity and upregulation of those having tumor suppressive role, strongly suggest implication of miRNA networks in CHRNA5 induced effects.

4.2 Red-CON as a drug delivery and imaging agent

In the recent years nanoparticles have evolved as 1) promising drug carriers for therapy of several pathologies (especially cancer), 2) agents of photothermal therapy, and 3) live-cell imaging [95]. Nanoparticles in general and positively charged nanoparticles in particular, have been shown to own ability of accumulating in tumor sites and being used as RNAi carriers, respectively [469]. Characterized by their well-defined size and easier metabolism, CONs (compared to conjugate polymeric nanoparticles; CPNs) show promises in being used for theranostic applications where they can be utilized as both therapeutic and diagnostic agents [94]. I further investigated the potential of using the red light emitting, pH-responsive conjugate oligomer nanoparticle developed by Pennakalathil et al. (2014) for its probable usage as an imaging and drug delivery molecule in breast cancer. Previous data have shown good stability of the Red-CON in different media (BSA, milk cell growth media), while in human blood serum it is non-precipitative only when encapsulated with cucurbituril (CB7) macrocycle. In addition, Red-CON has exhibited good entrapment and drug (CPT) loading efficiencies, as well as high drug release in acetate buffer (pH 5.0). In the biological studies of Red-CON presented in this thesis, the toxicity range of this nanoparticle was tested and its encapsulation by CB7 molecule, rendered this nanoparticle less toxic to MCF7 and MDA-MB-231 cells. This could be due to shielding of outer Red-CON positive charges, which are known to be responsible for toxicity of NPs [470]. In its encapsulated form (NP+CB7), this formulation showed promising results of drug loading and delivery potential (with high effectiveness and low toxicity), which can be further investigated with other drugs and cell lines so that it is utilized not solely as a cancer- and drug-type specific delivery agent. In its un-encapsulated form this nanoparticle can also be used as an imaging agent (at low concentrations). Thus, the oligomer nanoparticle complex introduce in this study has the potential to be used for future *in vivo* and clinical studies of breast cancer.

Chapter 5

Future Perspectives

- Future studies should focus on testing changes in the storage, compartmentalization and endocytosis of CHRNA5 isoforms to explain the disconcordance in levels of reduction and the lag period observed in reduction between mRNA and protein levels of CHRNA5 upon RNAi application.
- Effects of CHRNA5 RNAi on DNA damage/repair mechanisms (based on the microarray data) should be further characterized experimentally.
- Further Western blot experiments should characterize CHRNA5 reduction in the two triple negative cell lines, BT-20 and MDA-MB-231.
- CHRNA5 silencing in BT-20 and MDA-MB-231 cell lines showed discordant effects on cell cycle based on the differences between the gene expression analysis and MTT assay. Future studies should investigate the proliferative effects of CHRNA5 downregulation on differing cell densities in breast cancer cell lines.
- Future studies should focus on simultaneous treatments of CHRNA5 and TP53 downregulation (i.e., using siRNA molecule) as well as treatment with CHRNA5 RNAi in CASP3-reconstituted MCF7 cells.
- BrdU and further cell proliferation experiments should be applied in the other breast cancer cell lines to better understand, and generalize (if possible) the role of CHRNA5 in breast cancer carcinogenesis.
- Very new sofisticated 3D cell migration assessment platforms are now commercially available with *in vivo*-like ECM proteins [471]. These products could help to better characterize the role of CHRNA5 in breast cancer cell motility and migration (if any).
- Additional experiments are needed to further characterize the flowthrough (living) cell population in treated samples. This cells can be passaged and observed for any possible re-attachment to the flask as well as their proliferation status should be determined.

- Future studies should focus on using other markers of apoptosis, such as phospatidylserines, via Annexin A5-specific antibody (with the additional advantage of detecting an early event of apoptosis [472]), as a possible mechanistic effect of CHRNA5 depletion on cell death.
- The drug sensitivity experiments need to be validated with several other replicates and anticancer drugs, including CDK inhibitory agents. Additionally, simultaneous drug and CHRNA5 overexpression treatments would indicate further the importance of CHRNA5 in regulating apoptosis.
- Predicted miRNAs in connection with CHRNA5 RNAi and TOPO inhibitory action need to be validated experimentally.
- CHRNA5 also comes as a candidate regulator of cholinergic pathway, which requires more supportive data; i.e., measuring ACh level as well as expression of cholinergic signaling regulators in CHRNA5 depleted cells.
- Further studies would help in finding the mechanisms of CHRNA5 action as well as the therapeutic potential of RNAi in CHRNA5-high breast cancer patients.
- For the Red-CON study, additional experiments might be needed to further validate the drug loading and delivery abilities of this nanoparticle with additional drugs and other cell lines.
- The positively charged, pH-responsive red-emitting CON has potential in being used as an antisense-oligonucleotide molecule carrier and tumor targeting nanoparticle; however, this might require further chemical modifications.
- Future studies might evaluate the possibility that the herein presented biologically active CON might be used as a theranostic (multifunctional) molecule based on its hydrophilicity, cell-imaging and drug delivery abilities.

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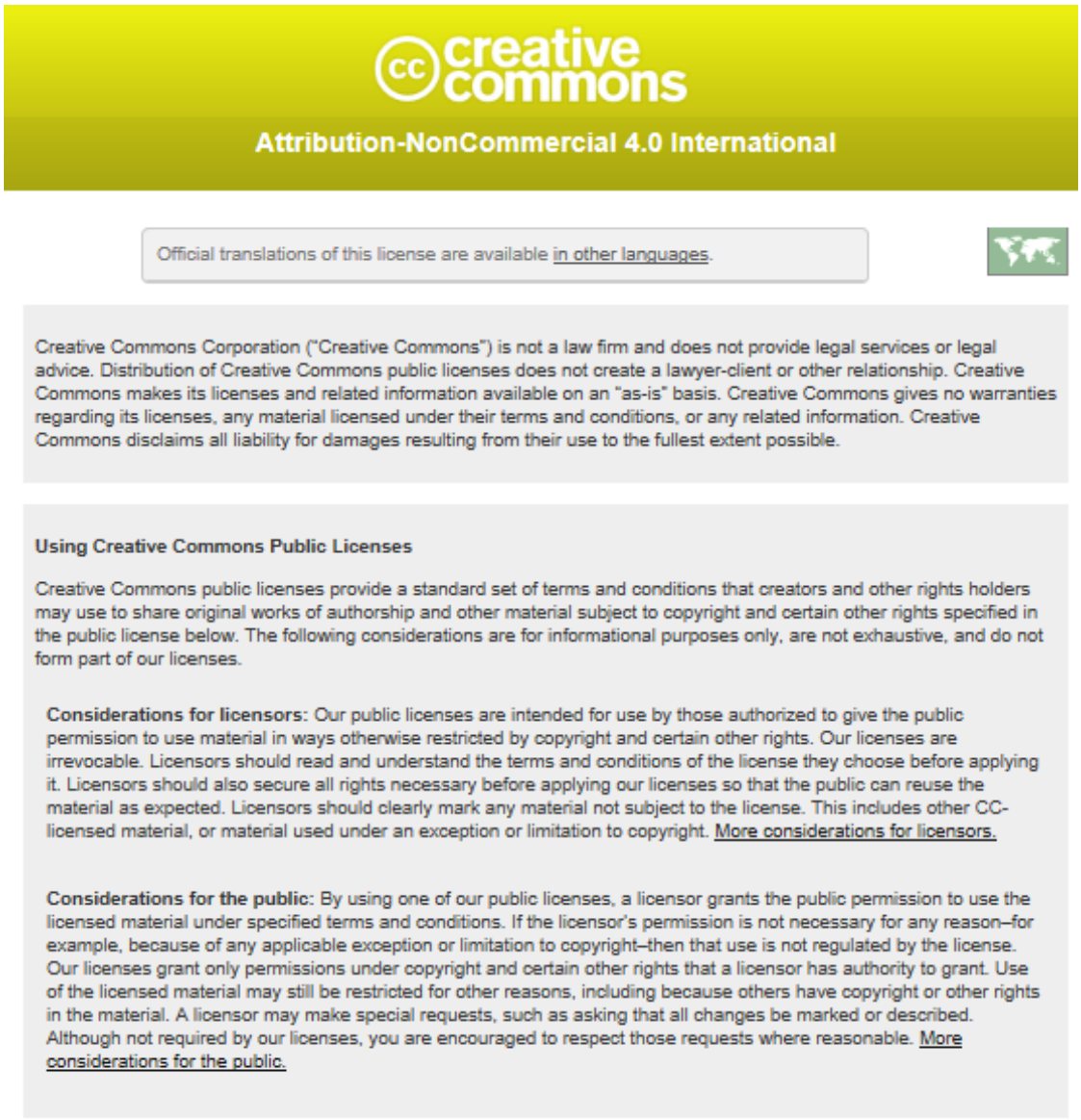

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