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Development of the system for functional analysis of BRCA1 mutations in breast cancer cell lines

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LIST OF ABBREVIATIONS:

53BP1: p53-Binding Protein 1

ATM: Ataxia-Teleangiectasia Mutated

ATR: ATM and Rad3-Related

BACH1: BRCA1-Associated C-terminal Helicase 1 (alternative name: BRIP1)

BARD1: BRCA1-Associated Ring Domain 1

BRCA1: Breast Cancer 1

BRCT: BRCA1 Carboxyl-Terminal

BRIP1: BRCA1-Interacting Protein 1 (alternative name: BACH1)

CHK: Cell Cycle Checkpoint Kinase

CtIP: CtBP-Interacting Protein

DSB: Double-Stranded DNA Break

GADD: Growth Arrest- and DNA Damage-Inducible Gene

HR: Homologous Recombination

IR: Ionizing Radiation

MDC1: Mediator of DNA Damage Checkpoint Protein 1

MRE11: *S. Cerevisiae* Meiotic Recombination 11 Gene Homolog

MRN: MRE11-RAD50-NBS1 complex

NBS1: Nijmegen Breakage Syndrome Gene 1 (alternative name: Nibrin/p95)

NHEJ: Non-Homologous End-Joining

PCNA: Proliferating Cell Nuclear Antigen

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

RAD50: *E. coli* RecA Protein Homolog

RAD51: *S. Cerevisiae* Rad50 Gene Homolog

RB: Retinoblastoma protein

RING: Really Interesting New Gene

shRNA: Small hairpin RNA

Ub: Ubiquitin

Due to space limitations, only abbreviations used in more than one chapter are listed here. Remaining abbreviations are included in the main text.

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1. INTRODUCTION

BREAST CANCER 1 gene (BRCA1; OMIM # 113705) was originally cloned as a gene that confers strong genetic predisposition to early-onset breast and ovarian cancers [1]. The second major breast/ovarian cancer susceptibility gene – BRCA2 – was cloned a year later, in 1995 [2]. Both BRCA1 and BRCA2 proteins are ubiquitously expressed in normal, non-malignant cells and share many biological functions. Despite their similar names, the structure of BRCA1 and BRCA2 proteins is unrelated. In my thesis I will focus on the *BRCA1* gene and its functions.

2. STRUCTURE OF BREAST CANCER 1 (BRCA1)

2.1. STRUCTURE OF BRCA1 GENE

BRCA1 gene is located on chromosome 17q21 [3, 4]. The gene is spanning ~ 110 kb and consists of 23 exons out of which 22 are coding ones [5]. The gene's structure is unique since it contains unusually long (~ 3.5 kb) exon 11, which is coding for more than 60% of BRCA1 protein (Fig. 2.2). Notably, there is a little confusion in the numbering of *BRCA1* exons. Coding exons are numbered 2-24, although there are only 22 of them. The "missing" one is the exon 4, which was originally annotated in one of the clones isolated from placental cDNA library, but is not present in mature BRCA1 mRNA [1].

The 5' end of *BRCA1* gene lies within a duplicated region of chromosome band 17q21 ([6]; Fig. 2.1) head-to-head with the 5' end of *NBR2* (Neighbor of BRCA1 gene) gene with a physical distance of 218 bp between their transcription start sites [7, 8]. *BRCA1* gene is localized centromeric to the *NBR2* gene and both

genes are transcribed in opposite directions from the bi-directional promoter embedded in a large CpG island [9, 10].

As a result of duplication, a *BRCA1* pseudogene, *ΨBRCA1*, lies ~ 30 kb upstream of *BRCA1* gene. The duplicated region contains related, albeit degenerated, *ΨBRCA1* exons 1A, 1B and 2 and *NBR1* exons 1 and 2 and their surrounding introns. The *NBR1* gene is located head-to-head with the *ΨBRCA1* pseudogene (similarly as *BRCA1* and *NBR2* genes), separated by the promoter region paralogous to that between *NBR2* and *BRCA1* genes ([8] and Figure 2.1).

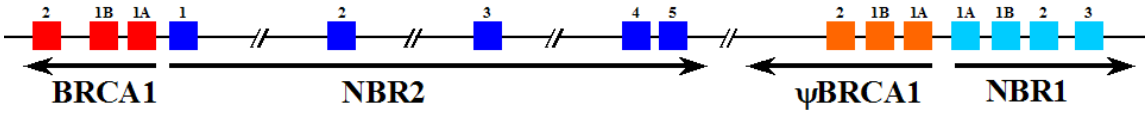

Figure 2.1. Human *BRCA1*/*NBR2*/*NBR1* genomic locus. Schematic representation of the duplicated region on chromosome 17q21 showing relative position and homology between *BRCA1* (red), *ΨBRCA1* (orange), *NBR1* (cyan) and *NBR2* (blue) genes. Boxes represent exons. Arrows indicate direction of transcription (centromere is to the left). The schema is not drawn to scale. NBR: Neighbour of BRCA1 gene.

The predominant product of *BRCA1* gene is mRNA ~7.8 kb in length. However, many BRCA1 alternative splicing variants were described [11-13]. The variability of BRCA1 mRNA begins with the first exon of the gene coding for 5' UTR [14]. The usage of different promoters is responsible for generation of two main BRCA1 mRNA variants with unchanged coding potential encompassing exons 1a or 1b, respectively [15]. Both forms are expressed differentially in various tissues, including testes and thymus. Interestingly, only the variant 1a is detected in mammary gland, whereas the form 1b is unique for placenta. Different 5'-UTR results in different efficiencies of translation initiation, probably as a result of the

presence of upstream ORFs in the exon 1b [15]. However, the functional significance of the existence of different 5'-UTR in BRCA1 mRNA is unknown.

Despite the existence of various BRCA1 splicing variants, only several of them were analyzed in more details (for review see [11]). The alternative splicing of BRCA1 was studied mainly in tumour samples and the results are somehow conflicting. The conflicting results may be explained in part either by different methodologies used or by the heterogeneity of tumour samples used in the studies. It can be speculated that alternative splicing of BRCA1 plays an important role in certain cellular functions and in tumour suppression, possibly in tissue-dependent manner. However, the exact function of splice variants and the extent they contribute to overall BRCA1 function remains elusive.

Recently, the BRCA1-IRIS splicing form was described [16]. BRCA1-IRIS is a 1,399-amino-acid *BRCA1* gene product encoded by an uninterrupted open reading frame that extends from codon 1 of the known BRCA1 open reading frame to a termination point 34 triplets into the intron 11. Whether the expression of BRCA1-IRIS is driven by a specific promoter or a promoter used by other BRCA1 exon 1a-containing transcripts is unknown [17]. BRCA1-IRIS is over-expressed in multiple sporadic human breast and ovarian cancer cell lines including "BRCA1-negative" ones, HCC1937 and SNU251 [18]. BRCA1-IRIS is functionally different from BRCA1 (p220); it is exclusively chromatin-associated with unique nuclear immunostaining and fails to interact with BARD1, a major BRCA1 (p220)-interacting protein. BRCA1-IRIS interacts with replication-licensing proteins and inhibits geminin-negative functions at DNA replication origins [16]. BRCA1-IRIS was also implicated in JNK/c-Jun/AP1-mediated, ERα-independent up-regulation of the cyclin D1 expression in breast cancer cells [18]. Thus, BRCA1-IRIS, unlike BRCA1 (p220), may have oncogenic-like properties due to promoting cell proliferation during S phase.

2.2. STRUCTURE OF BRCA1 PROTEIN

The BRCA1 is a 1,863 amino acids long protein containing two conserved protein-protein interaction domains: the N-terminal RING finger domain and the tandem of two acidic C-terminal repeats, termed the BRCT domains (Fig. 2.2). The N-terminal RING domain (amino acids 24-65) possesses an E3 ubiquitin ligase activity when complexed with another RING domain-containing protein, BARD1 [19]. C-terminally located BRCT domains (BRCT1: amino acids 1,642-1,735; BRCT2: amino acids 1,755-1,855) are mediating phosphopeptide-specific binding to other targets [20-22]. BRCA1 contains also so called Ser-Gln (SQ) cluster domain, a region harboring clusters of serine and glutamine residues within consensus sequence feasible for ATM- and ATR-mediated phosphorylation. Phosphorylation within this domain appears to be functionally important because a mutated BRCA1 protein lacking two phosphorylation sites within SQ domain failed to rescue the radiation hypersensitivity of a BRCA1-deficient cell line [23].

BRCA1 is a nuclear-cytoplasmic shuttling protein, however is mainly localized and functions in the nucleus [24]. BRCA1 contains at least two nuclear export signals (NES; amino acids 22-30 [25] and amino acids 81-99 [26]). The export of BRCA1 from the nucleus to cytoplasm is dependent on CMR1/exportin pathway [26] as well as functional p53 protein [27].

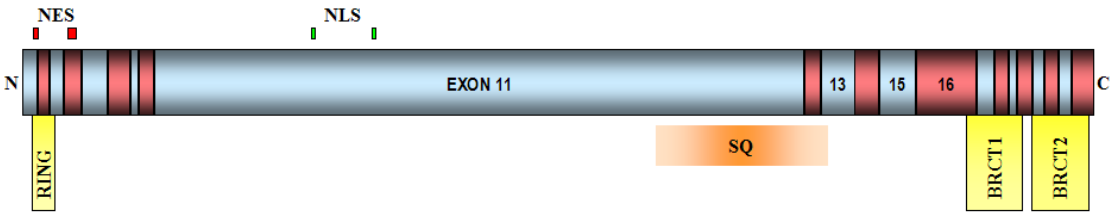

Figure 2.2. Human BRCA1 protein structure. Schematic representation of BRCA1 protein and a layout of corresponding coding exons are shown. The schema is drawn to scale. RING: Ring Finger Domain; BRCT: BRCA1 C-Terminal Domain; SQ: SQ domain; NES: Nuclear Export Signal; NLS: Nuclear Localization Signal.

BRCA1 contains two nuclear localization signals (NLS, amino acids 503-508 and 606-615) which facilitate nuclear import by binding to importin α/β receptor complex [28]. However, nuclear localization of BRCA1 protein lacking these NLS sequences was reported [29, 30] indicating the existence of other mechanisms. Fabbro *et al.* [31] found that nuclear import of BRCA1 is stimulated by its binding to BARD1. BARD1 binds to the extreme N-terminus of BRCA1 (amino acids 1-107) and acts as a chaperone. Moreover, binding of BARD1 masks NES signals in BRCA1 protein, thus anchoring BRCA1 in the nucleus [31]. Subcellular localization is also regulated by BRAP2 (BRCA1-Associated Protein) protein. BRAP2 binds to BRCA1 and masks NLS signal(s) thus retaining BRCA1 in the cytoplasm [32]. It can be speculated that other proteins binding to the same region(s) of BRCA1 may compete for interaction between BRCA1 and the nuclear import receptor, importin α, similarly as BRAP2 does (Fig. 3).

Accurate localization of nuclear-cytoplasmic shuttling proteins is critical for their function and several tumour suppressor genes, including BRCA1, are regulated as regards their localization [33]. BRCA1 nuclear localization and transcriptional activity is enhanced by heregulin β_1-induced PI-3-K/Akt-mediated phosphorylation on Thr^{509} ([34]; Tab. 2.2). Although little is known about signaling pathways regulating BRCA1 localization, cytoplasmic mislocalization of BRCA1 is frequently found in tumours and may have direct impact on cancer development [29, 35-37].

BRCA1 is a phosphoprotein which is predominantly phosphorylated on Ser (S) compared to Thr (T) or Tyr (Y) residues [38]. The exact biological function(s) and kinase(s) responsible for phosphorylation on each particular residue are still mostly unknown (Tab. 2.2). However, phosphorylation is an important regulator of BRCA1 function. BRCA1 can be phosphorylated in a response to extracellular signals by PI3K/Akt pathway [34]. BRCA1 is phosphorylated on several residues by ATM and ATR [23, 39], DNA-PKcs and Chk2 kinases during DNA damage repair and after cell cycle checkpoints activation [40]. Cell cycle-dependent phosphorylation of BRCA1 is mediated by Chk2, Cdk2 (Cyclin-Dependent Kinase) and Aurora A kinases (for review see [41]). BRCA1 phosphorylation is also important for apoptosis, since BRCA1 deficient cell lines HCC1937 and SNU251 are

resistant to caspase-3 cleavage and UV-induced apoptosis [42]. Despite the precise dynamics of BRCA1 phosphorylation on particular residues is not known, it is generally accepted that BRCA1 phosphorylation differentially influences its functions.

Amino Acid	Cdk	Akt	ATM	ATR	DNA-PKcs	Chk2	Aurora-A	Biological consequences
Ser^{308}							+	G_2 → M transition Mitotic entry
Thr^{509}		+						Regulation of BRCA1 localization
Ser^{988}						+		Dissociation from Chk2 DNA repair
Ser^{1148}				+				?
Ser^{1189}			+					?
Ser^{1239}			+	+				?
Ser^{1280}				+				?
Ser^{1298}			+					?
Ser^{1330}			+	+				?
Ser^{1387}			+	+	+			S phase checkpoint
Ser^{1423}			+					G_2/M checkpoint Caspase 3 activation
Ser^{1457}			+					?
Ser^{1466}			+					?
Ser^{1497}	+							?
Ser^{1524}			+					Caspase 3 activation
Ser^{1542}			+					?
Thr^{1720}			+	+				?

Table 2.2. Phosphorylation sites in BRCA1 protein. Phosphorylation residues in BRCA1 tumour suppressor, the potential kinases responsible for phosphorylation and biological consequences of such modification are listed. Cdk: cyclin-dependent kinase; Akt: v-akt Murine Thymoma Viral Oncogene Homolog; ATM: Ataxia-Teleangiectasia Mutated; ATR: ATM and Rad3-Related; Chk2: Checkpoint Kinase 2; DNA-PKcs: catalytic subunit of DNA-Dependent Protein Kinase; Aurora-A: Aurora Kinase A (also known as Serine/Threonine Protein Kinase 15, STK15).

3. FUNCTION OF BREAST CANCER 1 (BRCA1)

BRCA1 is a multifunctional protein. Some of the diverse functions associated with BRCA1 are mediated through interactions with specific partner proteins (Fig. 3). There are more than 130 functional interactions involving BRCA1 described in literature [43]. The main interacting proteins and functions of BRCA1 will be discussed in separate chapters, here I mention some of "less characterized" interactions. However, I would like to stress out that all functions of BRCA1 are interconnected and cannot be viewed independently without considering all other BRCA1 functions and interacting partners.

BRCA1 interacts with PABP1 [Poly(A)-Binding Protein], a highly conserved protein involved in mRNA stabilization and protein translation [44]. Interaction between the BRCT domain of BRCA1 and the N-terminus of PABP1 occurs in cytoplasm. This interaction seems to be physiologically relevant since depletion of BRCA1 by siRNA decrease protein synthesis and disease-associated BRCA1 mutations abolish interaction with PABP1 [44]. BRCA1 modulates protein translation independently of its other functions and may exert some of its tumour suppressor functions by this way.

BRCA1 was shown to interact with ACCA (Acetyl Coenzyme A Carboxylase α) [45, 46]. ACCA is a rate-limiting enzyme catalyzing *de novo* fatty acids biogenesis and is an essential gene for breast cancer cell survival. Inhibition of ACCA in human breast cancer cell lines leads to depletion of the cellular pool of palmitic acid and subsequent induction of apoptosis, formation of reactive oxygen species and mitochondrial impairment [47]. The ACCA-BRCA1 interaction is mediated by BRCA1 C-terminal BRCT domains which recognize ACCA phosphorylated on Ser^{1263} [46]. Phosphorylated form of ACCA is enzymatically inactive and the interaction with BRCA1 prevents its dephosphorylation. The regulation of fatty acid metabolism by modulation of ACCA activity may contribute to tumour suppressor functions of BRCA1 [45].

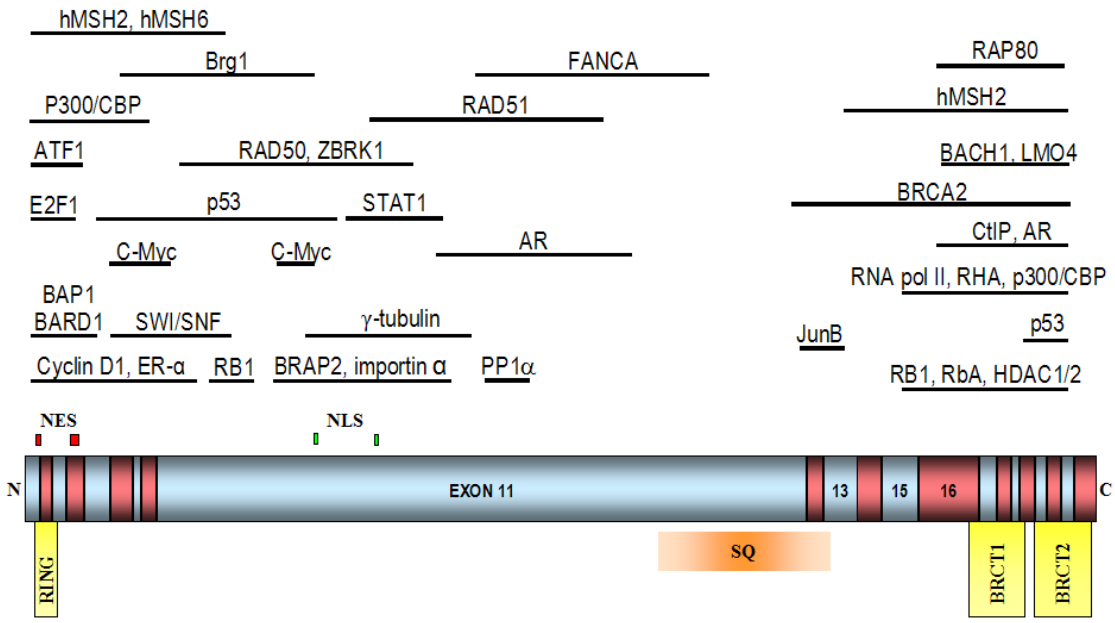

Figure 3. BRCA1 binding partners. Schematic representation of BRCA1 protein structure and approximate localizations of major BRCA1-binding partners are shown. RING: Ring Finger Domain; BRCT: BRCA1 C-Terminal Domain; NES: Nuclear Export Signal; NLS: Nuclear Localization Signal. For full proteins' names and their function with relation to BRCA1 see the main text.

The central part of BRCA1 encoded by the exon 11 was shown to interact with all three isoforms of PP1 (Protein Phosphatase 1) [48, 49]. Moreover, the expression of PP1 isoforms was altered in sporadic breast cancer tumour samples and low levels of PP1 were associated with negative ER status [49]. PP1α interacts with ^{898}KVTF^{901} motif in BRCA1 and dephosphorylates ATM, ATR and Chk2 phosphorylation sites in BRCA1. PP1 may play role in development of breast cancer through its association with BRCA1 and (de)regulation of the balance between kinase and phosphatase activities at the sites of DNA damage.

BRCA1 was implicated in the maintenance of intact telomere by several mechanisms. BRCA1 co-localizes with telomere-binding proteins TRF1 (Telomeric Repeat-Binding Factor) and TRF2 [50]. BRCA1 influences telomere length through

the direct regulation of telomerase activity [51, 52] and inhibits myc-induced expression of telomerase reverse transcriptase gene. Moreover, BRCA1 protects telomere against the formation of anaphase bridges [53]. Thus, disruption of BRCA1-mediated regulation of telomere status may contribute to the telomere-mediated type of genomic instability found in sporadic and hereditary breast cancers [54, 55].

3.1. BRCA1 AND UBIQUITINATION

Ubiquitination is s stepwise process by which a target protein is modified by covalent attachment of mono- or poly-ubiquitin (Ub) chains. The process is initiated by ATP-dependent activation of Ub by Ub-activating enzyme (E1). Second step involves transfer of activated Ub^* from E1 enzyme to Ub-conjugating enzyme (E2). Finally, Ub-ligase (E3) catalyzes transfer of Ub^* from E2 to the target protein. Proteins modified by poly-Ub chains are often destined for degradation by proteasome, whereas mono-Ub modification has regulatory purposes [56-61].

BRCA1 posses E3 Ub-ligase activity through its N-terminally located RING finger domain [62]. E3 Ub-ligase activity is stimulated when BRCA1 forms heterodimer with BARD1 [63] and requires UbcH5 as an E2 [64]. Relevance of BRCA1/BARD1-mediated ubiqutination *in vivo* was supported by purification of stable complex called BRCC (BRCA1-BRCA2-Rad51-Containing Complex) possessing E3 Ub-ligase activity. The BRCC complex contains BRCA1 binding partners BARD1, BRCA2, Rad51, BRCC45 and BRCC36 [65]. The E3 Ub-ligase activity is impaired by cancer-predisposing mutations in RING domain of BRCA1 and BARD1 [66]. Moreover, the physiological importance of BRCA1-BARD1 interaction is emphasis by other common features: **(a)** embryonic lethality of respective knockout mice, **(b)** induction of genetic instability when depleted from cells and **(c)** stabilization of both proteins upon interaction, as the respective monomers are unstable [67, 68].

BRCA1 interacts with a de-ubiquitinating enzyme (DUB) BAP1 (BRCA1-Associated Ubiquitin Protease) [69]. DUBs are enzymes specifically cleaving Ub molecules from substrate proteins and antagonizing the function of E3 Ub-ligases. Moreover, BAP1 interacts with the RING domain of BRCA1, thus may, at least theoretically, compete with BARD1 for BRCA1 binding [70]. Interestingly, BRCC36, a component of a BRCC complex [65] bears homology to the JAMM-domain family of DUBs and may function as regular DUB [71].

Target proteins for BRCA1-mediated ubiquitination are not well understood [70]. BRCA1/BARD1 complex catalyzes the formation of multiple poly-Ub chains on itself. This auto-ubiquitination was reported to be mediated through less conventional Lys^6 residue in the Ub peptide and increases ~ 20-fold the E3 Ub-ligase activity of BRCA1/BARD1 complex [72-75]. However, the importance of Lys^6 residue in Ub for linkage remains controversial [76].

Another targets for BRCA1-mediated ubiquitination are histone H2A and its subtype H2AX [66, 75]. This links BRCA1-mediated ubiquitination to DNA damage repair, a process highly regulated by ubiquitination ([77]; see Chapter 3.6.). Morris and Solomon [74] detected BRCA1-mediated ubiquitination at stalled replication forks in S-phase following hydroxyurea treatment as well as at sites of DSB repair following exposure to IR. Recently, Zhao *et al.* [78] described critical role for the E2 enzyme Ubc13 in initiating HR response and recruitment and activation of the E3 Ub-ligase activity of BRCA1 at sited of DSBs. However, how exactly is BRCA1 enzymatic activity activated following DNA damage and the identity of ubiquitinated proteins at sites of DNA damage remains to be elucidated.

BRCA1 potentially ubiquitinates Npb1/nucleophosmin/B23 [79], γ-tubulin [80, 81] and HMMR (Hyaluronan-Mediated Motility Receptor; [43]) proteins, all present and function at centrosomes. Despite the exact functional significance of their ubiquitination remains unknown, it may be one of the ways in which BRCA1 influence cell cycle and cell division (for details see Chapter 3.4). Besides its potential role at centrosome, BRCA1/BARD1 heterodimer is capable to ubiquitinate several cell cycle proteins *in vitro* and *in vivo* and target them for proteasomal

degradation. Such activity may be linked to the regulation of cell cycle checkpoints following DNA damage and may be controlled by BRCA1 phosphorylation [82].

Potential role of BRCA1-mediated ubiquitination of RNA polymerase II and topoisomerase IIα are discussed in Chapters 3.2. and 3.3., respectively.

Ubiquitination is important for breast cancer tumorigenesis [83]. Recently, estrogen receptor α (ERα) was described as a putative substrate for BRCA1/BARD1-mediated ubiquitination [84]. Ubiquitination of ERα may represent the regulatory mechanisms for repression of ERα transcriptional activation by BRCA1. Regulation of ERα activity by BRCA1 could have significant implications in controlling breast tissue proliferation and may provide the link between BRCA1 and tissue-specific tumorigenesis [85].

BRCA1 ubiqutinates CtIP (CtBP-Interacting Protein) [86]. CtIP is a candidate tumour suppressor gene originally isolated as a component of transcriptional repressor CtBP (C-terminal region of adenovirus E1A Binding Protein) [87]. CtIP binds to tandem BRCT domains of BRCA1 in a phosphorylation-dependent manner [88, 89]. Ubiqutinated CtIP associates with chromatin following DNA damage and participates in G_2/M checkpoint control [86]. Thus, CtIP may represent a new group of proteins which function is regulated in a phosphorylation-dependent manner by BRCA1-mediated ubiquitination through non-proteasomal pathways not involving substrate degradation [90].

Recently, Christensen *et al.* [91] reported six new E2 partners for BRCA1/BARD1 heterodimer and defined structural determinants for their binding to BRCA1. Four of these E2s, UbcH6, Ube2E2, UbcM2 and Ube2w, direct monoubiquitination of BRCA1, while Ubc13-Mms2 complex and Ube2k direct the synthesis of Lys^{63}- or Lys^{48}-linked poly-Ub chains, respectively. Thus, single E3 (in this case the BRCA1-BARD1 hetodimer) can promote different Ub conjugation reactions depending on its E2 partner. The ability to synthesize different types of ubiquitination products implies BRCA1 in targeting individual substrates for different fates. For example, Ubc13 E2 was shown to play critical role in HR [78] and Ube2k promotes poly-Ub of RNA Polymerase II [92].

Taken together, BRCA1's E3 Ub-ligase activity influences possible all of BRCA1 functions [19]. Ubiqutination not only marks target proteins for proteosomal degradation, but also modifies its function. BRCA1 interacts with more than 100 proteins, frequently in a phosphorylation-dependent manner through its BRCT domains. We can speculate that ubiquitination, and phosphorylation, dynamically regulate complex BRCA1 pathway(s) and help to switch between diverse BRCA1 executory functions.

3.2. BRCA1 AND TRANSCRIPTION

BRCA1 protein contains transactivation domain (TAD) at its C-terminus [93, 94]. BRCA1 TAD (amino acids 1,293-1,863) was demonstrated to recruit RNA polymerase II (RNAPII) to synthetic reporters and stimulate transcription [95-97]. However, direct evidence for BRCA1 binding to promoter regions of genes is lacking, albeit BRCA1 is capable to bind DNA directly [98-100]. BRCA1 DNA-binding activity is stimulated by heterodimerization with BARD1 in both ubiquitination-dependent and independent ways [101].

The majority of BRCA1 is unphosphorylated or hypophosphorylated in undamaged cells and cells in $G_{0/1}$ phase [102] and associates with transcriptional complex of RNAPII holoenzyme [93, 103-105]. This interaction seems to involve some proteins associated with the core RNAPII complex, namely RHA (RNA Helicase A) [104, 106], hRPB2 (RNA Polymerase II Subunit B2), hRPB10α [107] and transcriptional enhancers NUFIP (Nuclear Fragile X Mental Retardation Protein-Interacting Protein) and pTEF-b (Positive Transcription Elongation Factor B) [108]. BRCA1 C-terminal TAD domain is primary important for interaction with RNAPII, however other regions of BRCA1 may contribute to this interaction as well [100, 109, 110]. BRCA1 protein is bound to RNAPII as a heterodimer with BARD1; this complex acts as a fully active E3-ubiquitin ligase [109]. Recently, RNAPII subunits RPB1 [92, 111] and RPB8 [112] were identified as ubiquitination targets of BRCA1/BARD1 complex. BRCA1 and BARD1 are both necessary for

ubiquitination and consequent proteasomal degradation of RNAPIIO, the elongating form of RNAPII, in a response to UV-induced stalled replication [113]. Specificity for RNAPII ubiquitination is determined by phosphorylation of YSPTSPS heptapeptide repeat motif in the carboxy terminal domain (CTD) of RNAPII. Only RNAPII hyperphosphorylated on Ser^5 within heptapeptide repeat is ubiquitinated by BRCA1/BARD1 [111]. Degradation of RNAPII inhibits transcription-coupled RNA processing and facilitates DNA repair [92]. This is in agreement with the role of BRCA1 in polyadenylation [114, 115]. BRCA1/BARD1 complex binds through BARD1 to CstF-50 (Cleavage Stimulation Factor) component of the polyadenylation complex and inhibits its function by sequestration of CstF-50.

BRCA1 regulates transcription of several stress-response genes including $p21^{waf1/cip1}$ [116, 117], $p27^{kip1}$ [118, 119], GADD45 [120], estrogen receptor regulated genes [121], VEGF (Vascular Endothelial Growth Factor) [122], IGF-I (Insulin-like Growth Factor), GADD153, cyclins and cyclin-dependent kinases [123], PCNA, and many others [124-127]. BRCA1 can modulate transcription of target genes through protein-protein interactions with other transcription factors, activators and/or repressors including NELF-B/COBRA1 (Negative Elongation Factor B/Cofactor of BRCA1), CtIP, HIF-1α (Hypoxia-Inducible Factor), p53, Rb, c-Myc, p65/RelA subunit of NF-κB, estrogen receptor, histone acetyltransferase p300/CBP and histone deacetylases HDAC1-3 [128, 129]. BRCA1-mediated transcriptional regulation is complex and the overall effect depends on the interplay with other transcription factors. An example may be the GADD45, a tumour suppressor gene playing an important role in the control of cell cycle checkpoints, DNA repair and signal transduction [130]. GADD45 regulation by BRCA1 is indirect and depends on p53 protein [120]. BRCA1 inhibits GADD45 transcription through interaction with the KRAB domain of transcription factor ZBRK1 (Zinc Finger and BRCA1-Interacting Protein with KRAB Domain) [131]. This interaction is relieved after phosphorylation of BRCA1 by ATM kinase after DNA damage. On the other hand, BRCA1 is capable to activate GADD45 transcription through interaction with Oct-1 and NF-Yα transcription factors.

Taken together, BRCA1 plays important role in transcription regulation either by direct binding to transcription factors and RNAPII or indirectly via chromatin remodeling (for details see Chapter 3.3). BRCA1 may connect transcription with transcription-coupled DNA damage repair [132-135]. Under normal conditions, BRCA1 is predominantly hypophosphorylated and interacts with highly processive elongating form of RNAPII, RNAIIPO. Following genotoxic insult, BRCA1 becomes phosphorylated by ATM and/or ATR kinases, dissociates from RNAPII transcriptional complex and subsequently associates with sites of DNA repair. So, BRCA1 and associated DNA-damage surveillance factors may be connected with the RNAPII and monitor elongation success. Once transcription halted (due to DNA lesion) or is otherwise disrupted, BRCA1 becomes phosphorylated, relocates to sites of DNA damage, recruit DNA-repair proteins and activates cell cycle checkpoints (for details see Chapter 3.5.).

3.3. BRCA1 AND CHROMATIN MODIFICATION

BRCA1 plays a role in X-chromosome inactivation [136]. Equality of X-linked genes dosage between males (XY) and females (XX) is in mammals achieved by inactivation of one of the two X chromosomes (Xi) in each somatic female cell. The process of X chromosome inactivation takes place early in the developing embryo and is relatively temporary restricted [137]. XIST (X Inactivation-Specific Transcript) RNA is critical for this process, but the exact mechanism of its action is unknown [138-140]. BRCA1 and its heterodimeric partner, BARD1, were shown to interact with XIST RNA and intact function of BRCA1 was needed for proper XIST staining of Xi [136]. BRCA1 contributes to association of Xi with molecules (e.g. histone macroH2A1 variant) that play role in the genesis of Xi heterochromatin in early embryonic cells. BRCA1 dysfunction increases the risk of failure of the maintenance of X chromosome inactivation and results in deregulation of expression of X-linked genes. There is further evidence for role of Xi heterochromatization in female-specific breast and ovarian cancers. The detectable Xi heterochromatin

(Barr's body) was absent in a subset of highly aggressive breast and ovarian cancers and BRCA1-deficient ovarian cancers over-express a set of X-linked genes which are normally silenced [141-143]. Abnormal Xi inactivation was present in the majority of breast cancers with basal-like phenotype, a hallmark of BRCA1 defect [144, 145].

However, direct interaction between BRCA1 and XIST RNA was recently questioned [146-148] and novel, more general role of BRCA1 in maintaining heterochromatin structure and regulating replication-linked maintenance of centric and pericentric heterochromatin was suggested [143, 149, 150]. Such a broad effect of BRCA1 on chromatin structure may impact XIST RNA metabolism and the Xi heterochromatin formation and maintenance. Thus, BRCA1 loss and subsequent deregulation of heterochromatin maintenance may contribute to genomic instability and cancer. On the other hand, further evidence for direct interplay between BRCA1 and XIST RNA in Xi heterochromatin regulation was published recently [151].

BRCA1 plays also important role in DNA decatenation. BRCA1 directly interacts during the S-phase with phosphorylated topoisomerase IIα through its BRCT domains and regulates topoisomerase activity and distribution through ubiquitination [150, 152]. Topoisomerase IIα is essential for chromosome decatenation after DNA replication and its inhibition results in a defect in chromosome segregation. Similar defects are apparent after loss of BRCA1 implicating BRCA1 in the regulation of topoisomerase IIα activity. Chromatin remodeling surrounding the sites of DSBs mediated by histone acetyltransferases and other chromatin remodeling factors participates in DNA repair by dissolving higher order chromatin structure otherwise interfering with recruitment of DNA repair proteins to DSB sites. Thus, BRCA1 may participate in DNA repair not only as a scaffold protein by orchestrating DNA repair proteins interactions but also by direct regulation of chromatin structure and its accessibility do DNA repair [153].

BRCA1 interacts with large SWI/SNF-related chromatin remodeling complex through binding to bromo-domain containing protein BRG1 (BRM/SWI2-Related Gene) [154]. BRCA1 also interacts with histone deacetylase complex, paralogous histone acetyltransferases CBP and p300 and pRB-associated proteins RbAp46 and RbAp48 [155-157]. So, BRCA1 is able to alter histone modifications and resulting

higher-order chromatin structure. Regulation of local chromatin structure may be important during BRCA1-mediated transcriptional regulation (see Chapter 3.2.) as well as DNA damage repair (see Chapter 3.6).

Taken together, heterochromatin maintenance including X chromosome inactivation and the epigenetic regulation of gene expression plays an important role in breast and ovarian tumorigenesis. BRCA1 participates in the regulation of these processes; however the exact mechanism and timing of its action await complete understanding.

3.4. BRCA1 AND CELL CYCLE CONTROL

Cell cycle checkpoint is a multi-layered network of interacting pathways coordinating cell cycle progression with DNA repair, chromatin remodeling, transcriptional programs and metabolism. Cell cycle checkpoints are activated as a response to damaged or structurally abnormal DNA, e.g. after ionizing or UV radiation or replication errors (for review see [158-162]). Key components of checkpoint pathways are checkpoint kinases (ATM, ATR, Chk1, Chk2), which are activated by DNA damage and phosphorylate many down-stream targets, thus amplifying, coordinating and spreading the DNA damage-induced response (Fig. 3.6.3). Phosphorylation, and possibly other post-translation modifications, may affect DNA damage-induced checkpoint activation through modification of specific down-stream targets. BRCA1 is a well-known target for all these checkpoint kinases (Tab. 2.2). BRCA1-deficient cells exhibit progressive impediment of cell proliferation and spontaneous chromosomal instability, similarly as do ATM- or ATR-deficient cells. BRCA1 participates in the cell cycle control during all phases of cell cycle, which is complement to its role in DNA damage repair process, allowing adequate time for DNA repair to occur ([135, 163], see Chapter 3.6.). BRCA1 function in cell cycle control is also intimately connected to its role in transcription (see Chapter 3.2.).

A key down-stream target of BRCA1 in the regulation of G_1/S checkpoint is $p21^{cip1/WAF1}$ [116]. Direct $p21^{cip1/WAF1}$ activation requires BRCA1 association with

CtIP and CtBP and is dependent on BRCA1 phosphorylation [32]. Moreover, BRCA1 was shown to regulate $p21^{cip1/WAF1}$ in an indirect manner through p53. BRCA1/BARD1 heterodimer is required for DNA damage-induced phosphorylation of p53 on Ser^{15} and subsequent G_1/S arrest following IR-induced DNA damage [164]. BRCA1 participates in G_1/S checkpoint also via retinoblastoma protein pathway. BRCA1 directly interacts with hypophosphorylated form of pRB, which binds to and inactivates E2F transcription factors [165]. Binding of BRCA1 keeps pRB in the hypophosphorylated state and achieving growth arrest. BRCA1 binds through BRCT domains to two pRB-interacting proteins RbAp46 and RbAp48 and to histone deacetylases HDAC1 and HDAC2 [155]. The pRB-HDAC complex suppresses transcription of E2F-responsive genes, which are necessary for cell cycle progression into S-phase.

The intra-S checkpoint primarily represents an inhibition of DNA replication initiation upon DNA damage. A lack of IR-induced S-phase checkpoint results in persistent (radioresistant) DNA synthesis. The role of BRCA1 in intra-S checkpoint is less well understood. BRCA1 deficient HCC1937 breast cancer cells were reported to be defective in S-phase checkpoint. IR-induced DNA damage and subsequent ATM-mediated phosphorylation of BRCA1 on Ser^{1387} was shown to be required for intra-S checkpoint [166]. Similarly, ATR-mediated phosphorylation of BRCA1 on Ser^{1423} is required for S-phase checkpoint activated by stalled replication forks. [167]. Thus, ATR and ATM kinases activate intra-S checkpoint in analogous ways. BRCA1 also interacts with several other proteins implicated in S-phase checkpoint regulation. These include MDC1 [168], 53BP1 and MRN complex [169]. It is also possible that BRCA1 regulate S-phase progression through transcriptional up-regulation of $p21^{cip1/WAF1}$ or $p27^{kip1}$ [118].

BRCA1 transcriptionally regulates several proteins associated with G_2/M checkpoint. BRCA1 regulates the expression, phosphorylation and cellular localization of Chk1 kinase, a known component of the G_2/M checkpoint [170]. BRCA1 represses cyclin B that is responsible for activation of cdc2 kinase and mitotic entry [171]. BRCA1 regulates chaperone protein 14-3-3σ, which targets cdc25C phosphatase following DNA damage, sequesters it in the cytoplasm and

prevents it from activating cyclin B-cdc2 kinase complex [170]. Moreover, BRCA1 stimulates transcription of wee-1 tyrosine kinase that is necessary for inhibitory phosphorylation of cyclin B-cdc2 complex [170]. BRCA1 also inhibits PLK1 (Polo-like Kinase 1), a kinase required for G_2 to M transition in response to IR [172]. One of the most important targets is GADD45, a protein inhibiting the kinase activity of cyclin B-cdc2 complex by sequestering cdc2 [173]. BRCA1-mediated control of G_2/M checkpoint after irradiation is regulated by ATM-mediated phosphorylation on Ser^{1423} [174] and requires ERK1/2 (Extracellular Signal-Regulated Protein Kinase) activity [175]. The G_2/M-phase checkpoint defects were also reported in the absence of BRIT1 (BRCT-Repeat Inhibitor of hTERT Expression) protein. The checkpoint defects in the absence of BRIT1 are likely to result from deregulation of BRCA1, NBS1 and Chk1, since BRIT1 is required for their proper expression [176].

BRCA1 may also regulate the spindle checkpoint since it sensitizes breast cancer cells to the spindle poisons paclitaxel and vinorelbine [177, 178]. The exact mechanism how BRCA1 control spindle checkpoint is not known, however the role of activation of MEKK3 (Mitogen-Activated Protein Kinase Kinase Kinase 3; [179]), GADD45 [177] and MAD2 (Mitotic Arrest-Deficient) [180] was suggested. MAD2 is the most important target since it is a key component of the spindle assembly checkpoint controlling the activity of cdc20/Anaphase Promoting Complex (APC/C). BRCA1 transcriptionally controls other genes involved in spindle checkpoint, e.g. Bub1 and BubR1 [163, 181].

Taken together, BRCA1 participates in the control of checkpoints in all cell cycle phases. It coordinates cell cycle progression with sensing of DNA damage and fidelity of DNA replication. Defects in BRCA1 function may lead to errors in cell division and ultimately to genomic instability and cancer development. However, complete checkpoints' composition and exact role of BRCA1 are under investigation.

3.4.1. BRCA1 AND CENTROSOME DYNAMICS

The centrosome functions as the primary microtubule-organizing centre in animal cells, and so regulates cell motility and adhesion in interphase, and facilitates the organization of the spindle poles during mitosis. Centrosomes undergo duplication during S-phase once every cell cycle so that their number remains stable, like the genetic material of a cell [182]. Abnormalities in the spindle pole-organization function of centrosomes occur in many cancers and are associated with genomic instability. Extra and often irregular centrosomes may give rise to aberrant cell division. Centrosomes were also reported to be a part of a signalling network connecting cell cycle arrest and repair signals in response to DNA damage [183, 184].

The first link between BRCA1 and centrosomes came from the observation that BRCA1 localizes to this organelle during mitosis [185, 186] as well as interphase [81, 187]. Despite the localization of BRCA1 to centrosomes was questioned, mainly due to non-specific binding of some anti-BRCA1 antibodies [188], BRCA1 is an integral part of centrosomes during whole cell cycle [189]. There is evidence that BRCA1 may control centrosome amplification in breast cells probably by preventing centrosome reduplication [190]. The HCC1937 breast cancer cells lack functional BRCA1 and have amplified centrosomes [106]. Inhibition of BRCA1 causes centrosome amplification in breast cell-specific manner [80]. BRCA1 localizes to centrosome as a heterodimer with BARD1 and resulting E3 Ub-ligase activity is necessary for controlling centrosome function [80]. The γ-tubulin, the key component of γ-TuRC (γ-Tubulin Ring Complex) complex that nucleates microtubule polymeration, was identified as an important target for BRCA1/BARD1 E3 Ub-ligase activity (see Chapter 3.1.). Moreover, BRCA1 is able to directly bind γ-tubulin through a domain comprising residues 504-803 [191]. Recently, BRCA1/BARD1 heterodimer and its E3 Ub-ligase activity was reported to be required for proper organization of microtubules within centrosomes through targeting the protein TPX2 to spindle poles [192, 193]. Pujana *et al.* [43] linked BRCA1 down-regulation to centrosome amplification/hypertrophy via

BRCA1/BARD1-mediated ubiquitination of HMMR (Hyaluronan-Mediated Motility Receptor) protein. HMMR interacts with BRCA1 and together control centrosome number in breast tumor- and mammary epithelium-derived cells. HMMR over-expression is a risk factor for breast tumorigenesis thus directly linking breast cancer susceptibility to centrosome dysfunction.

Taken together, defects in spindle pole integrity and centrosome function may lead to chromosome segregation defects and aneuploidity, abnormalities that are characteristic for BRCA1-deficicent cells and many tumours. The BRCA1/BARD1-mediated control of centrosome function via ubiqutination may represent another mechanisms by which BRCA1 maintain genomic stability and exerts its tumour-suppressor function.

3.5. BRCA1 AND APOPTOSIS

Role of BRCA1 in apoptosis is intimately connected with its role in cell cycle regulation and DNA damage (see Chapters 3.4. and 3.6.) since apoptosis is a final outcome of prolonged cell cycle arrest as well as excessive DNA damage. However, BRCA1 regulates apoptosis also independently on DNA damage response. There are several points of evidence that BRCA1 is involved in apoptosis both as an inducer and a suppressor.

It has been shown that BRCA1 sensitizes breast cancer cell lines to INF-γ-mediated apoptosis. BRCA1 induced a subset of interferon-inducible genes when co-expressed with INF-γ, but not INF-α or INF-β [194]. BRCA1 binds and functions as a co-activator of STAT1 (Signal Transducer and Activator of Transcription), the major effector of INF-γ signaling pathway [195, 196] and regulates INF-γ signaling through mechanism involving type I interferons [197]. Mutation or loss of BRCA1 may result in attenuated induction of INF-γ target genes and therefore a decrease ability of INF-γ to suppress tumour cell growth. Thus, BRCA1 may function also as an important mediator of immuno-surveillance and innate anti-tumour activity.

Exogenous over-expression of BRCA1 induced apoptosis through JNK/SAPK (c-Jun N-terminal Kinase/Stress-Activated Protein Kinase) pathway in correlation with the induction of GADD45 [120]. Subsequently, it was demonstrated, that BRCA1 modulates stress-induced apoptosis through H-ras/MAPKs/JNK pathway including FAS (Tumor Necrosis Factor Receptor Superfamily) antigen/FAS ligand and caspase-9 activation [198]. BRCA1 is necessary for hypoxia-mediated apoptosis in breast cancer cell lines [199]. Hypoxia increases cell surface expression of TRAIL (TNF-Related Apoptosis-Inducing Ligand) and subsequent TRAIL-dependent increase in BRCA1 nuclear localization and apoptosis.

BRCA1 functions also as a modulator of chemotherapy-induced apoptosis. It mediates resistance to a vide range of DNA-damaging agents including etoposide and cisplatin while sensitizing breast cancer cells to apoptosis induced by anti-microtubule agents paclitaxel and vinorelbine [178]. BRCA1 induced G_2/M cell cycle checkpoint after exposure to all DNA-damaging and anti-microtubule agents. The exact mechanism responsible for differential regulation of apoptosis is not known at present but it may be connected to p53 pathway since BRCA1 modulates apoptosis via p53-dependent [200-202] and independent pathways [31, 178].

Together, BRCA1 appears to regulate apoptosis in response to diverse stress signals including several DNA-damaging chemoterapeutic agents. In contrast, BRCA1 could mediate anti-apoptotic signals after DNA damage and in general confers an anti-apoptotic resistant phenotype [120, 178].

3.6. BRCA1 AND DNA DAMAGE

The DNA is subject to continuous damage and cell has an arsenal of ways of responding to such injury (for reviews see [203-205]). Multiple distinct mechanisms for repairing damaged bases exist: replication by-pass (translesion DNA synthesis) [206], nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR). Both BER and NER use somehow different mechanisms depending

on whether the DNA damage is located in regions undergoing active gene expression (transcription-coupled repair) or are transcriptionally silent (global genomic repair).

Besides various excision repairs coping with damaged bases (BER, NER) or mistakes during replication (MMR), cells frequently suffer breakage of one or both chains of DNA duplex. The DNA double-strand break (DSB) is probably the most cytotoxic cellular lesion, since as little as one unrepaired DSB is capable to cause cell cycle arrest and trigger apoptosis [207]. DSBs are cytotoxic lesions generated by ionizing radiation (IR) and radiomimetic chemicals, are caused by mechanical stress on chromosomes, and arise when DNA replication forks encounter other lesions such as DNA single-stranded breaks [208, 209]. Cells use several strategies to couple with DSBs: essentially error-free homologous recombination (HR) which depends on the existence of homologous sequences (chromosomes) [210-212] and error-prone single stranded annealing (SSA) and non-homologous end-joining (NHEJ) capable of joining any broken DNA ends [213, 214]. While its ability to ligate essentially any two DNA ends makes NHEJ a very effective pathway of DSB repair, some end-processing is normally required before ligation, making NHEJ an intrinsically mutagenic repair mechanism.

3.6.1. HOMOLOGOUS RECOMBINATION

Homologous recombination (HR) act predominantly in G_2 and S phases of cell cycle when sister chromatids are present. HR can be conservative (in the form of gene conversion; further referred as HR in a strict sense) or non-conservative (in the form of single strand annealing; SSA). Gene conversion uses identical sequence to copy and replace damaged DNA, namely the sister chromatids, in an error-free manner. During SSA, homologous sequences on either side of DSB are aligned followed by the deletion of the intermediate non-complementary sequence, thus being potentially error-prone [215].

One of the first steps during HR is recognition of DSBs by a protein complex comprising MRE11, RAD50 and NBS1 (MRN complex). The MRN complex is

proposed to perform multiple structural and enzymatic functions in DNA end processing and alignment [212, 216, 217]. During the central step in HR, RAD51 forms a nucleoprotein filament with the 3' overhanging ssDNA of the resected DSB and catalyzes homologous pairing and strand exchange. The role of RAD51 is supported and activated by its cofactors such as RAD52, RAD54 and RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3. RAD51 paralogs facilitate RAD51-mediated strand exchange within heterotetrameric RAD51B/C/D/XRCC2 and hetorodimeric RAD51D/XRCC2, RAD51B/C and RAD51C/XRCC3 complexes [216-219].

BRCA1 is necessary for efficient HR [127, 215, 220-223]. Cells lacking BRCA1 are inefficient in the repair of DSBs by HR [224]. BRCA1 protein is rapidly phosphorylated after DNA damage at different sites by at least three protein kinases: ATM, ATR and Chk2. ATM and ATR are key kinases regulating extensive protein networks in a response to DNA damage [39]. ATM is activated by DSB-induced specific alterations in the higher order nuclear chromatin structure [225]. Active ATM phosphorylates various DNA repair factors, including BRCA1, as well as the downstream signalling kinases, including Chk2 and c-Abl [219, 226-228]. The histone variant γ-H2AX is rapidly phosphorylated upon DSB and facilitates the focal assembly of many proteins at the region of DSB. The adaptor proteins 53BP1, BRCA1 and MDC1 further expand assembly of DNA repair proteins nucleated at the sites of DSB marked by γ-H2AX. Importantly, BRCA1, together with NSB1, are necessary for full activation of ATM and its recruitment at sites of DSBs. At sites of DSBs, ATM phosphorylates its substrates and orchestrates DNA repair and checkpoint responses [225, 227, 229].

Upon irradiation, BRCA1 was detected in the nucleus in discrete foci at sites of DNA damage, where it interacts with many proteins involved in HR, including e.g. MRN complex, BARD1, ATM, RecQ helicase BLM (Bloom Syndrome), MSH2/MSH6 (MutS Homologue 2/6), MLH1 (MutL Homologue 1), RFC (DNA Replication Factor C), RAD51/BRCA2, FANCD2, 53BP1, MDC1, SMC1 (Structural Maintenance of Chromosomes 1) and phosphorylated γ-H2AX histone variant [219, 230, 231]. Formation of irradiation-induced foci positive for BRCA1,

RAD50, MRE11 or NBS1 was shown to be dramatically reduced in breast cancer cells carrying a homozygous mutation in BRCA1 but was restored by transfection of wild-type BRCA1 [232]. The BRCT domain, a phosphoprotein interacting motif that has been identified in several other proteins involved in cell-cycle regulation, seems to be important in assembly of multiprotein complexes in response to DNA damage [20, 21, 233-235].

After irradiation, BRCA1 was shown to form stable heteromeric complex called BRCC (BRCA1-BRCA2-Rad51-Containing Complex) with its binding partners BRCA2, RAD51, BARD1, BRCC45 and BRCC36 [65]. BRCC36 and BRCC45 have sequence homology to a subunit of the signalosome and proteasome complexes. Reconstituted four-subunit complex containing BRCA1, BARD1, BRCC45 and BRCC36 revealed an enhanced E3 Ub-ligase activity compared to that of BRCA1/BARD1 heterodimer and ubiqutinated p53 *in vitro*. Thus, BRCC complex functions as an E3 Ub-ligase that enhances cellular survival following DNA damage [65].

Recently, another stable complex including BRCA1 was identified at DSBs sites [236-238]. BRCA1 BRCT repeats directly bind protein Abraxas in a phosphorylation-dependent manner. Abraxas binds BRCA1 to the mutual exlusion of BRIP1/BACH1 and CtIP, forming a third type of BRCA1 complex. Abraxas recruits Ub-binding protein RAP80 (Receptor-Associated Protein) to BRCA1. Moreover, BARD1 (E3 Ub-ligase) and BRCC36 (potential DUB) were detected in RAP80 complexes as well. Both Abraxas and RAP80 are substrates for ATM/ATR kinases and are phosphorylated after DNA damage. The RAP80-Abraxas complex lies upstream of BRCA1 and may serve as a adaptor protein to recruit BRCA1/BARD1 E3 Ub-ligase to sites of DNA damage in a Ub-dependent manner, thus controlling BRCA1-mediated regulation of DNA repair and cell cycle checkpoints [236-239].

Taken together, BRCA1 functions as a scaffold platform for the assembly of the HR machinery as well as recruitment of checkpoint factors (see Chapter 3.4.) thus linking DNA damage sensing to biological responses through distinct protein-protein interaction.

3.6.2. NON-HOMOLOGOUS END-JOINING

Compared to relatively well-defined role of BRCA1 in HR, the role of BRCA1 in NHEJ is far less clear and often conflicting [224, 240-247]. The main evidence for BRCA1 role in NHEJ comes from its interaction with MRN complex, which is known to play a role in both HR and NHEJ [248]. There is also evidence that HNEJ pathway is impaired in $BRCA1^{-/-}$ mouse embryonic fibroblast [243, 244] and BRCA1-defective HCC1937 human breast cancer cell line [242]. However, recent evidence suggest more prominent role of MRN complex in HR compared to NHEJ. Possible existence of HNEJ sub-pathways was suggested [249]. BRCA1 may play role only in particular NHEJ sub-pathway, which repairs DNA damage with higher fidelity comparable to HR. Recently, Zhuang *et al.* [250] reported that BRCA1 promotes error-free NHEJ while suppressing microhomology-mediated error-prone NHEJ and restricts sequence deletion at the break junction during repair. The promotion of precise DBS end-joining by HNEJ was dependent on phosphorylation by Chk2 kinase [250, 251].

Based on published results, a hypothetical model was proposed where BRCA1 acts upstream in the DNA damage response pathway [250]. BRCA1 may help to determine whether error-free HR or error-prone NHEJ repairs a DSB. In addition, BRCA1 may modulate NHEJ process to increase its fidelity and restrict sequence alterations. The function of BRCA1 and determination of the exact repair mechanism used for DSB repair is regulated by upstream kinases, like Chk2 and ATM/ATR [251]. Taking together, by promoting HR and increasing the fidelity of NHEJ, BRCA1 may exert its tumour suppressor activity.

3.6.3. FANCONI ANEMIA

Fanconi anemia (FA) is a rare, genetically heterogenous autosomal recessive or X-linked disorder (Tab. 3.6.3) characterized by congenital abnormalities (short statue, microcephaly, heart, renal and gastrointestinal defects, mental retardation),

progressive bone marrow failure (aplastic anemia, pancytopenia, myelodysplastic syndrome with progression to acute myelogenous leukemia) and increased cancer susceptibility (squamous cell carcinoma of head, neck and esophagus, gynaecological cancers in women including breast cancer) [252-256].

FA Group	Gene name	Approximate frequency in FA patients [%]	Chromosomal localization
A	FANCA	60 %	16q24.3
B	FANCB (FAAP95)	Rare	Xp22.31
C	FANCC	15 %	9q22.3
D1	FANCD1 (BRCA2)	5 %	13q12.3
D2	FANCD2	5 %	3p25.3
E	FANCE	Rare	6p21.3
F	FANCF	Rare	11p15
G	FANCG (XRCC9)	10 %	9p13
I	FANCI (KIAA1794)	Rare	15q25-q26
J	FANCJ (BRIP1; BACH1)	Rare	17q22
L	FANCL (FAAP43)	Rare	2p16.1
M	FANCM (FAAP250)	Rare	14q21.3
N	FANCN (PALB2)	Rare	16p12
	FAAP100	?	17q25.3
	FAAP24	?	19q13.11
	H2AX	?	11q23.2-q23.3

Table 3.6.3. Fanconi anemia genes. A list of Fanconi anemia (FA) complementation groups and corresponding components of FA complex is presented. Note that FAAP100, FAAP24 and H2AX are components of FA complex, but their mutations were not detected in FA patients and thus specific complementation groups were not assigned to them. FANC-: Fanconi Anemia Complementation Group; FAAP: Fanconi Anemia-Associated Polypeptide; XRCC: X-Ray Repair, complementing defective; BRIP: BRCA1-Interacting Protein; BACH: BRCA1-Associated C-terminal Helicase; PALB: Partner and Localizer of BRCA2

Cells isolated from FA patients demonstrate chromosomal instability and increased sensitivity to DNA cross-linking agents, such as mitomycin C [257] and cisplatin [258], features that are used in diagnostic process.

FA pathway is activated in S-phase of cell cycle in a response to stalled replication. FA pathway consists of two main complexes: core complex exhibiting E3 mono-Ub ligase activity and chromatin-associated FANCD2/FANCI/BRCA2 DNA repair complex. The core complex consists of at least 10 cloned FA proteins: FANCA, B, C, E, F, G, L and M, FAAP100 [259, 260] and FAAP24 [261]. FANCL is the putative catalytic element, bearing E3 Ub-ligase activity [262-264]. The core complex is required for monoubiquitination of both FANCD2 (FANCD2-Ub) on Lys^{561} and its paralogue FANCI [265] on Lys^{253} during S-phase of the normal cell cycle as well as in response to DNA damaging agents, UV-C or IR. FANCD2-Ub/FANCI-Ub complex (so called ID complex) is targeted into chromatin-associated foci where it co-localizes with other proteins playing role in DNA damage regulation, e.g. γ-H2AX [266], BRCA1, RAD51, BRCA2, PCNA and NBS1 (for review see [267-270]). However, the exact role of ID complex in DNA damage repair is currently unknown.

FANCD2 protein also plays role in cell cycle arrest. FANCD2 is phosphorylated by ATM kinase on several residues, including Ser^{222}, following IR [271] and by ATR kinase in response to UV-C or DNA cross-linking agents [272]. Phosphorylation of FANCD2 on Ser^{222} residue is required for intra-S checkpoint activation. Phosphorylation and monoubiquitination of FANCD2 are probably independent events [273] and FANCD2 thus seems to function at the intersection of two signaling pathways. Similarly as FANCD2, FANCI protein participates in the control of G_2/M and intra-S checkpoints [265].

There are several lines of evidence that BRCA1 is functionally connected with FA pathway. BRCA1 is not only co-localized with FANCD2/FANCI/BRCA2 DNA repair complex in the chromatin-associated foci, but also interacts with FA core complex through binding to FANCA protein [274].

BRCA1 directly binds to BRIP1/BACH1, a member of the DEAH-box helicase family, through its BRCT domains [275]. BRIP1/BACH1 is necessary for efficient double-strand break repair in a manner that depends on its association with BRCA1. BRIP1/BACH1 is both a DNA-dependent ATPase and a 5'-to-3' DNA helicase [276, 277]. BRIP1/BACH1 participates in the DNA damage response and

supports BRCA1 localization at site of DSBs marked by histone γ-H2AX [278]. BRIP1/BACH1 was recently reported to be defective in FA complementation group J (Table 3.6.3) [279-282]. However, BRCA1-independent function of BRIP1/BACH1 helicase in FA pathway was suggested as well [283].

BRCA1- and ATR kinase-mediated activation of FA pathway is required for G_2/M checkpoint activation and DNA damage repair in response to endogenous re-replication [284] and for DNA crosslink-induced S-phase checkpoint [285]. BRCA1 is necessary, together with phosphorylated form of histone γ-H2AX, for recruitment of FANCD2-Ub to damaged DNA loci [266]. Given that loss of BRCA1 severely affects chromatin foci formation of several DNA-repair factors in response to diverse DNA-damage inducing agents, BRCA1 may function as an interacting platform regulating and coordinating multiple DNA-repair processes. Despite the exact role of BRCA1 in FA pathway is currently unknown, it was proposed that BRCA and FA proteins forms integrated network which biological function is to overcome blocks to DNA replication (Fig. 3.6.3; [259, 269, 270, 286]).

Identification of BRCA2 as the FANCD1 protein was the first direct evidence of connection between FA pathway and breast cancer [287]. Interestingly, besides high-penetrance BRCA1 and BRCA2, mutations in other two FA proteins, namely BRIP1/BACH1/FANCJ [288] and PALB2/FANCN [289], predispose to breast cancer. Notably, all breast cancer predisposing genes are down-stream of the core complex in the FA pathway. Why biallelic mutations in BRIP1, PALB2 and BRCA2 genes predispose to FA (where the incidence of breast cancer is actually very rare) whereas monoallelic defects predispose to breast cancer is not understood. It is probable that consequences of mutant alleles depend not only on the character of mutation *per se*, but also on the context of the development and function of complex tissues. The important role of BRCA/FA pathway in tumorigenesis is supported by observed alterations of BRCA/FA pathway sporadic breast cancer (for review see [290]).

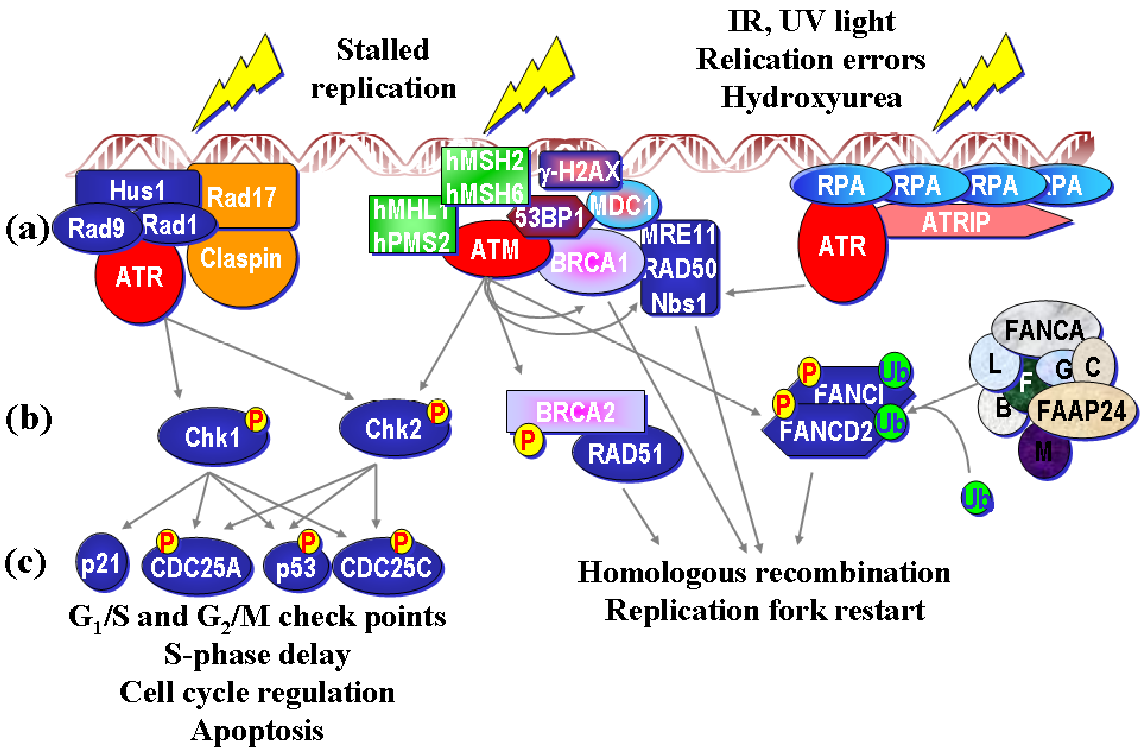

Figure 3.6.3. The BRCA-FA protein network in a response to DNA damage. A speculative and simplified model is presented. Several genes (*ATM*, *CHEK2*, *BRCA1*, *BRCA2*, *FANCJ*) whose inactivation predisposes people to breast and other cancers participate in the error-free repair of breaks in double-stranded DNA by homologous recombination. Response to genomic DNA damage (induced by UV light, IR, chemotherapeutics or replication errors) can be divided into three levels: **(a)** sensors and mediators of DNA damage, **(b)** signal transducers and **(c)** effectors. Many proteins were implicated DNA sensing process; e.g. Hus1/Rad9/Rad1 complex together with Rad17, replication protein A/ATR/ATRIP complex or histone γ-H2AX. These proteins signal to and activate ATM and ATR protein kinases that are key components of DNA damage-induce response. ATM and ATR phosphorylate diverse proteins allowing them to assemble into large multiprotein complexes at the sites of DNA damage (so called "foci"). These foci contain BRCA1, BRCA2/RAD51 complex, MRE11/RAD50/Nbs1 (MRN) complex, hMSH2/hMSH6 and hMHL1/hPMS2 complexes, PCNA, 53BP1, ATM and ATR kinases by themselves and many other proteins. Interaction of ATM and ATR kinases with these proteins is necessary for their full activation. Fully active ATM and ATR then phosphorylate and activate other signal transducers, mainly Chk1 and Chk2 protein kinases. Chk1 and Chk2 subsequently activate variety of effector proteins participating in different functions like cell cycle regulation, checkpoints activation, homologous recombination and ultimately apoptosis.
FA core complex is activated by DNA damage by yet unknown mechanism. Activation of the FA core complex triggers mono-ubiquitination of FANCD2 and

FANCI through the FANCL component of core complex. This results to activation and translocation of FANCD2/FANCI complex to sites of DNA damage ("foci").
The model predicts that BRCA1, BRCA2 and different FA proteins will have distinct functions within the network of processes that respond to DNA cross-links or replication blocks. In turn, these functional differences could engender differences in clinical syndromes, cancer susceptibility or therapeutic responses that are associated with mutations in the different proteins.

4. BREAST CANCER

Breast cancer (OMIM #114480) is the most frequent cancer among women in the Czech Republic as well as Western world and its incidence is steadily rising in these societies [291]. Breast cancer is a hormone-sensitive cancer. Its pathogenesis is determined by mammary gland architecture and its changes during menstrual cycle [292].

As other solid tumours, breast cancer is a histopathologically and etiologically heterogeneous disease. Several prognostic factors, including histological type and grade of the tumour, tumour size, lymph-node involvement, estrogen receptor (ER) and HER2/Neu oncogene status are used for prediction of patient's outcome [293]. However, the heterogeneity of the disease is responsible for different clinical course of patients with clinically and pathologically similar tumours [294]. The heterogeneity of breast and colorectal cancers was confirmed by Sjöblom *et al.* [295]. An average of 90 mutated genes was found to be present in individual tumours, but probably only a subset of them contributes to the neoplastic process.

Besides molecular heterogeneity, there are two fundamental types of breast cancer: hereditary form, responsible for ~ 5-10% of all breast cancer cases and sporadic form. Both forms will be discussed separately and the potential role of BRCA1 will be outlined.

4.1. HEREDITARY FORM

Understanding of pathogenesis of hereditary forms of cancer was based on the original "two-hit" hypothesis suggested by Alfred Knudson for retinoblastoma [296]. Individuals with hereditary form of cancer have inherited mutation in one allele of particular disease-causing gene (tumour suppressor) that is present in all cells within their body. Inactivation of the second allele, the so-called "second hit" is sufficient for the development of cancer. The "second hit" occurs somatically and usually involves either mutation or loss of chromosome part containing the functional allele (LOH; Loss of Heterozygosity). However, recent evidence suggests that the situation is not such a simple. Some tumours may require more than two mutations and some may occur even without the "second hit" [297]. Hereditary cancer, like sporadic one, may arise by a variety of molecular mechanisms, with loss of both alleles of a particular tumour suppressor gene being a frequent, but not invariably necessary or sufficient event.

Mutations in at least 10 genes were implicated in the pathogenesis of hereditary breast cancer [298, 299]. Mutations in *BRCA1* and *BRCA2* genes are quite frequent and confer very high risk of breast and ovarian cancer. Mutations in *TP53* and *PTEN* (Phosphatase and Tensin Homologue) genes lead to very high breast cancer risk, but are associated with rare cancer syndromes: Li-Fraumeni syndrome and Cowden disease, respectively. Mutations in remaining genes are associated only with a moderate increase in breast cancer risk. These genes include *LKB1/STK11* (Serine/Threonine Protein Kinase 11; mutated in Peutz-Jegher syndrome), *CHEK2*, *ATM*, *NBS1*, *RAD50*, *BRIP1/BACH1/FANCJ*, *PALB2/FANCN*, *TGFB1* (Transforming Growth Factor β_1) and *CASP8* (Caspase 8) [298, 299]. Despite many genes implicated in the pathogenesis of hereditary breast cancer, roughly ~ 50% of familiar cases remains unresolved by any of these genes.

Germline mutations in *BRCA1* gene (an up-to-date list of known mutations can be found in the Breast Information Core database [300]; http://research.nhgri.nih.gov/bic/) confer very high risk of hereditary breast and/or ovarian cancers. The risk of developing cancer at age 80 years was estimated to be ~

90% and ~ 25% for breast cancer and ovarian cancer, respectively, with some differences among particular studies [301]. The particular study design, number and heterogeneity of patients involved as well as the statistical method of analysis influence the risk estimation. Moreover, it is highly probable that other factors influence the penetrance of BRCA1 mutations [302-304].

4.2. SPORADIC FORM

Sporadic breast cancer is a "common" form of breast cancer occurring among women (and also men, albeit in much lower prevalence) population.

Breast cancer is usually classified based on its histopathological properties. However, such classification only partially corresponds to prognosis and treatment sensitivity [294]. Recently, comprehensive analysis of gene expression patterns in breast cancer using microarray technology was performed [294, 305-310]. Based on microarray results, following subtypes of breast cancer were proposed: **(a**) luminal-like, expressing "luminal" cytokeratins 8 and 18; **(b)** basal-like, characterized by the expression of "basal" cytokeratins 5 and 17; **(c)** HER2/Neu-positive, expressing higher amount of HER2/Neu receptor and **(d)** normal-like. Additional clinically relevant subgroups were predicted within the luminal category. Some evidence suggests that all categories may have ER-positive and ER-negative subsets, based on the presence of estrogen receptor. Breast cancers bearing BRCA1 mutations were classified as ER-negative and HER2/Neu-negative with higher amount of lymphocytic infiltrate and were classified as "basal-like" [308, 311, 312]. It was reported that basal-like cancers are more likely to be BRCA1-defficient [313] and share some defects with BRCA1-deficient cells, like Xi inactivation and sensitivity to chemotherapy (see Chapters 3.3. and 3.5. for details). However, BRCA1-deficiency is not a general characteristic of basal-like tumours [314, 315].

Somatic BRCA1 mutations are reported rarely in sporadic breast cancers, but BRCA1 expression is often reduced [316]. Therefore, other mechanisms are suggested to down-regulate BRCA1 expression in sporadic breast tumours. These

include methylation of BRCA1 promoter [317-319] and LOH of BRCA1 locus occurring in 7-31% and 15-45% of breast cancers, respectively [320]. Other potential mechanisms influencing BRCA1 expression include dysregulation of transcription factors (HMGA1, Ets-2) involved in the regulation of BRCA1 expression and dysregulation of other proteins functioning in BRCA1 pathway(s). BRCA1 expression is also negatively regulated by extracellular matrix; HER2/Neu activation by heregulin induced PI3K/Akt mediated phosphorylation of BRCA1 C-terminus and its down-regulation [321].

4.3. IMPACT OF BRCA1 DEFICIENCY ON BREAST CANCER PROGNOSIS

Determination of BRCA1 status may have also profound clinical consequences. The exact classification, based on microarray gene expression profile may be important for prognosis [310]; e.g. basal-like breast cancers have poor prognosis compared to luminal-like ones. Breast cancer molecular subtypes have also differential response to chemotherapy. Most importantly, basal-like subtype, which is often BRCA1-deficient, may be sensitive to chemotherapy regiments which are not used as the first-line therapy [314, 322-325]. BRCA1 deficient cells were shown to be sensitive to cisplatin and other drugs causing DSBs, but resistant to paclitaxel and vinorelbine [178].

Recently, it was shown that BRCA1-deficient and BRCA2-deficient cells are selectively killed by PARP-1 [Poly(ADP-Ribose) Polymerase] inhibitors *in vitro* [326-329]. PARP-1 is a key enzyme participating in base excision repair and is predominantly involved in the recognition of single-stranded DNA breaks. BRCA1 and BRCA2 dysfunction, and resulting defect in HR, profoundly sensitizes cells to the inhibition of PARP-1 enzymatic activity. It seems that inhibition of PARP-1 leads to the persistence of DNA lesions that are normally repaired predominantly by HR. However, several reports did not confirm these results *in vivo* [330] and detected BRCA1-independent inhibition of breast cancer cells. But the exact cause of "resistance" to PARP-1 inhibitors remains elusive [331].

Theoretically, based on the exact molecular classification of breast cancer, it may be possible to apply cancer-specific chemotherapy with the best curable potential [294]. The PARP-1 inhibitors may serve as an approach for the prevention of BRCA-related breast cancer and may be used in combination with other chemotherapeutic agents in the treatment of breast cancer [332, 333].

4.4. FUNCTIONAL ANALYSIS OF BRCA1 MUTATIONS IN BREAST CANCER

Besides many disease-causing mutations in *BRCA1* gene, numerous unclassified sequence variants, splicing variants and gene polymorphisms were reported. Different mutations and/or splicing variants of BRCA1 gene account for different phenotypic manifestations of breast and ovarian cancer [334]. On the molecular level, particular mutation could influence defined subset of multiple signalling pathways orchestrated by BRCA1. This contributes to different biological behaviour of tumours arising from the affected cell population. BRCA1 mutation may affect cell function by several mechanisms: **(a)** haploinsufficiency of BRCA1 may be sufficient to increase breast cancer risk and tumorigenesis [335]; **(b)** mutated BRCA1 protein interferes with wtBRCA1 function, e.g. by sequestrating endogenous BRCA1-binding partners, like BARD1 [68]; and **(c)** mutated BRCA1 protein gains new dominant-negative function(s) [336]. Despite intensive research on this field, the exact mechanism by which BRCA1 inactivation may lead to malignant transformation of cells remains unknown [334, 337].

What is the exact significance of each particular alteration for breast cancer development? The pathogenicity of particular BRCA1 mutation is mostly determined by segregation studies in affected families or by *in silico* prediction algorithms [338-340]. Up to now, limited functional studies determining disease causativeness of BRCA1 mutation have been published [127, 341-343]. Universal *in vivo* functional test exactly correlating any mutation with a corresponding risk of breast/ovarian cancer development is currently unavailable. Such *in vivo* system would be a valuable tool for clinicians and could be used in following situations:

- Better prediction of relative risk of breast and/or ovarian cancer development in patients bearing germinal BRCA1 mutation depending on the type of mutation in *BRCA1* gene. Answering patient's question like: Confers my mutation high or low risk of cancer development?
- Better prediction of chemosensitivity/chemoresitance of cancer cells to mostly used chemotherapeutics, which will allow optimal selection of chemotherapeutic regimens based on results of *in vivo* analysis. Answering physician's question like: Which regimen is optimal for this particular patient bearing this particular BRCA1 mutation?
- Better follow-up intervals and preventive care prediction for patients in risk of breast and ovarian cancer development according to the type of BRCA1 germ-line mutation. Answering patient's question like: Will I need some sort of preventive therapy including surgery? Answering physician's question like: What is the optimal interval for follow-up controls?

All these points are critical in prognosis and clinical follow-up of patients with BRCA1 mutations and are not clear enough in present days [344, 345].

5. AIMS

The major aim of this study was to develop the system enables the analysis of BRCA1 mutations found during screening of women with familiar breast and/or ovarian cancer syndrome in the Czech Republic. The system was used for characterization of selected alterations in BRCA1 gene. The issue was approached through the following stepwise goals:

- To set up methods needed for functional characterization of BRCA1 mutations

- To down-regulate the expression of endogenous wild-type BRCA1 in model breast cancer cell lines

- To reconstitute the expression of selected mutated forms of BRCA1 in cell lines depleted in endogenous wild-type BRCA1

- To determine the impact of wild-type BRCA1 down-regulation and mutated BRCA1 reconstitution on growth properties of breast cancer cell lines.

6. MATERIAL AND METHODS

6.1. CELL LINES AND CELL CULTURE

Epithelial breast adenocarcinoma cell line MCF-7 (#HTB-22) was purchased from American Type Culture Collection (ATCC, USA). MCF-7 cells express endogenous estrogen receptor and wilt-type (wt) p53 tumour suppressor gene.

MDA-MB-231 (#HTB-26, ATCC) epithelial breast adenocarcinoma cells lack endogenous estrogen receptor and express mutated form of p53 tumour suppressor containing mis-sense G>A mutation in exon 8 (pR280K) (IARC TP53 Mutation Database; http://www-p53.iacr.fr).

HCC1937 (#CRL-2336, ATCC) ductal breast adenocarcinoma cells are considered to be BRCA1 negative (contain homozygous c.5266dupC mutation leading to the production of unstable premature-terminated BRCA1 protein), do not express endogenous estrogen and progesterone receptors and express mutated form of p53 tumour suppressor containing non-sense C>T mutation in exon 8 (pR306X) (IARC TP53 Mutation Database).

HeLa cervical carcinoma cells and NIH3T3 cells were kindly provided by P. Johnson (LPDS, NCI-Frederick).

Cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich) in 5% CO_2 at 37 °C. Culture medium for MCF-7 cells was further supplemented by 0.01 mg/ml bovine insulin (Gibco). For some experiments MDA-MB-231 cells were cultured in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine and 10% FBS at 37 °C in atmosphere air, without CO_2 supplementation. NIH3T3 cells were cultured in DMEM + 10% Calf Serum (Colorado Serum Company).

6.2. TRANSIENT TRANSFECTIONS

Cells were seeded in 6-well plates at density $1.5x10^5$ (MCF-7, MDA-MB-231 and EM-G3) or $1x10^5$ (HeLa) cells per well 24 h before the transfection. A 1 μg portion of plasmid DNA was transfected using FuGENE 6 (Roche), Lipofectamine 2000 (Invitrogen) or Metafectene (Cambio) transfection reagents according to manufactures' protocols. Where appropriate, pBluescript plasmid DNA was added to equal total amount of DNA per well. Media were changed 24 h after the transfection and cells were collected and analyzed 48 h post-transfection.

6.3. INFECTION

Infection of human cell lines was performed using Phoenix amphotropic packaging cell line (kindly provided by P. Johnson, NCI-Frederick; http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). Briefly, Phoenix cells were transfected with retroviruses containing shRNA or BRCA1 sequences using standard calcium phosphate method. Transfection was stopped after 12 h by changing fresh media to Phoenix cells. At 24–72 h after transfection, viral supernatants from Phoenix cells were collected every 12 h, pooled, filtered through 0.45 μm membrane (Millipore), supplemented with 8 μg/ml Polybrene (Sigma-Aldrich) and used to infect target cells in the logarithmic phase of growth. Four infections were performed in total. Selection by puromycin (Sigma-Aldrich), blasticidin (Sigma-Aldrich) or hygromycine (Invitrogen) was started 24 h after the last infection. Media with selection antibiotic were changed every 48 h if necessary. After completed selection, infected cells were passaged and plated for experiments. Multiple genes were introduced by sequential infection and drug selection.

6.4. GROWTH CURVES AND COLONY ASSAYS

For growth curves analysis, infected cells after selection were seeded in 24-wells plates at density $2x10^4$ (MCF-7, MDA-MB-231 and EM-G3) or $1.2x10^4$ (HeLa) cells per well. Cells were cultured in media containing selection antibiotic(s) and the culture media were changed at the day 3. Cell staining was performed at days 0, 2, 4 and 6 (day 0 is the first day after plating the cells). Cells were washed twice in PBS and fixed in 10% formaldehyde for 30 minutes. After rinsing with water cells were stained by 0.1% crystal violet (Sigma-Aldrich) solution in 10% ethanol for at least 30 minutes. Stained cells were extensively rinsed with water and plates were dried. The dye was extracted with 10% acetic acid and an absorbance of solution was measured at λ = 590 nm; "staining" an empty well without cells was used as a control to zero the instrument. If needed samples were diluted with water to obtain absorbance < 1. Data were plot as relative absorbance ratio to day 0.

For colony assays, cells were plated in 10 cm Petri dish (the same amount as for one well in growth curve experiment). Cells were cultured in media containing selection antibiotic(s) and the culture media were changed every 3 days. Cells were washed twice in PBS and fixed in 10% acetic acid for 30 minutes. After washing in water cells were stained by 0.4% crystal violet (Sigma-Aldrich) solution in 10% ethanol for at least 30 minutes. Stained cells were extensively washed in water and plates were dried.

6.5. PLASMIDS

6.5.1. PLASMIDS EXPRESSING BRCA1 VARIANTS

The Rc_BRCA1 plasmid [178] coding for wtBRCA1 was kindly provided by Paul D. Harkin (Department of Oncology, Cancer Research Centre, Queen's University Belfast, Northern Ireland). BRCA1 c.1866A>T, c.3819_3823del5 and

c.5285insC mutations were constructed using PCR. Briefly, BRCA1 sequence was amplified by PCR using common forward primer (introducing Hind III restriction site) BRCA_Fwd: 5'- TTCTgATCAAgCTTCAgAAAgAAATggATTTATCTgCTCTTCgC and mutation-specific reverse primers (introducing Xho I restriction site):

1866A>T_Rev: 5'- gCTTATACTgCTCgAgTTAggTTCAgCTTACgTTTTgAAAgCAgATTC

3819del5_Rev: 5'-CATAACTACTCgAgTTAACTAgTAgACTgAgAAggTATATTgTTTACCAAATAACAAgTgTTg

5385dupC_Rev: 5'- gTAATAACTACTCgAgTTACTCACACATCTgCCCAATTgCATg

PCR was performed using high fidelity DNA Polymerase (Takara). Specific PCR products were gel-purified (DNA Gel Recovery Kit, ZymoResearch) and cloned into pcDNA3.1(+)_Hygro plasmid (Invitrogen). Final constructs were verified by sequencing (ABI Prism 310, Applied Biosystems).

Mutation c.300T>G was constructed in wtBRCA1-contaning pcDNA3.1(+)_Hygro plasmid using Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. Clones were screened by Ava II restriction and finally verified by sequencing.

For retroviral infection, BRCA1 inserts were transferred from pcDNA3.1(+)_Hygro plasmid into pWZL_Hygro or pWZL_Blast MMLV-derived retroviral plasmids (kindly obtained from P. Johnson, NCI-Frederick) bearing hygromycine or blasticidin resistance, respectively.

6.5.2. PLASMIDS USED FOR RNA INTERFERENCE

ShRNA targeting 3'-UTR of BRCA1 mRNA were constructed in pSUPER.retro.puro plasmid ([346]; OligoEngine; www.oligoengine.com) expressing shRNAs under the control of human H1 promoter. For each shRNA, two complementary oligonucleotides were synthesized (sequences of sense oligonucleotides are listed in Table 6.4.2.1). These oligonucleotides consist of (for sense oligonucleotide, from 5' to 3' end): 5' overhang complementary to Bgl II restriction site, sense (passenger) shRNA strand, loop sequence, antisense shRNA strand ("mature" shRNA), stop signal and 3' overhang complementary to Xho I restriction site. Corresponding sense and anti-sense oligonucleotides were annealed

and cloned into pSUPER.retro.puro plasmid according to the manufacturer's protocol. Positive clones were checked by sequencing in both directions, using following primers: pSUPER_Fwd: 5'-CATCGTGACCTGGGAAGCCTTG and pSUPER_Rev: 5'-GACGTCAGCGTTCGAATTCTACC.

shRNA	Sequence 5'→ 3'
shRNA_5890	GATCCCCCTACTGTCCTGGCTACTAATTCAAGAGA**TTAGTAGCCAGGACAGTAG**TTTTTGGAAC
shRNA_6069	GATCCCCGCAAGATGCTGATTCATTATTCAAGAGA**TAATGAATCAGCATCTTGC**TTTTTGGAAC
shRNA_6073	GATCCCCGATGCTGATTCATTATTTATTCAAGAGA**TAAATAATGAATCAGCATC**TTTTTGGAAC
shRNA_6095	GATCCCCGCCCTATTCTTTCTATTCATTCAAGAGA**TGAATAGAAAGAATAGGGC**TTTTTGGAAC
shRNA_6252	GATCCCCGGATCGATTATGTGACTTATTCAAGAGA**TAAGTCACATAATCGATCC**TTTTTGGAAC
shRNA_6965	GATCCCCCATACAGCTTCATAAATAATTCAAGAGA**TTATTTATGAAGCTGTATG**TTTTTGGAAC

Table 6.4.2.1. Sequences of oligonucleotides synthetized for each shRNA cloned into pSUPER.retro.puro plasmid. Predicted mature shRNA sequences are highlighted in blue. Number indicates the position of shRNA in BRCA1 reference mRNA sequence (NCBI; NM_007294).

Another subset of shRNA sequences targeting 3'-UTR of BRCA1 mRNA was constructed in miR-30-based LMP retroviral plasmid ([347, 348]; OpenBiosystems; www.openbiosystems.com) expressing shRNAs under the control of CMV promoter. In this case, 97-bp oligonucleotide was synthesized for each shRNA (sequences are listed in Table 6.4.2.2.) and used as a template in PCR reaction with following priers: miR-30-Fwd: 5'-CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG and miR-30_Rev: 5'- TGCCTACTGCCTCGGAATTCAAGGGGCTACTTTAG. Specific 138-bp PCR product was gel-purified, digested with Xho I/EcoR I and cloned into LMP plasmid. Sequencing using pLMP_Fwd primer (5'-GAATCGTTGCCTGCACATCTTGG) was used for screening.

All shRNAs are numbered according to the position of the first nucleotide of shRNA's target sequence in BRCA1 reference mRNA (GenBank accession number NM_007294.2).

shRNA	Sequence 5'→ 3'
shRNA_6335	TGCTGTTGACAGTGAGCGAAGGCAGGTATTAGAAATGAAATAGTGAAGCCACAGATGT ATTTCATTTCTAATACCTGCCTCTGCCTACTGCCTCGGA
shRNA_6867	TGCTGTTGACAGTGAGCGACATGAATATTTCATATCTATATAGTGAAGCCACAGATGT ATATAGATATGAAATATTCATGCTGCCTACTGCCTCGGA
shRNA_6965	TGCTGTTGACAGTGAGCGCCCATACAGCTTCATAAATAATTAGTGAAGCCACAGATGT AATTATTTATGAAGCTGTATGGTTGCCTACTGCCTCGGA

Table 6.4.2.2. Sequences of oligonucleotides synthesized for each shRNA cloned into LMP plasmid. Predicted mature shRNA sequences are highlighted in blue. Number indicates the position of shRNA in BRCA1 reference mRNA sequence (NCBI; NM_007294). All sequences are based on the RNAi Codex database (OpenBiosystems; http://codex.cshl.edu/scripts/newmain.pl).

6.6. BACTERIAL ARTIFICIAL CHROMOSOME (BAC)

Selected mutations were engineered in Bacterial Artificial Chromosome (BAC) carrying BRCA1 gene by the „hit & fix" method [349]. This method of BAC DNA modification is based on bacteriophage λ Red recombination system [350] and uses oligonucleotides as targeting vectors. In the first step, about 6-20 nucleotides are changed, including nucleotide(s) that are due to be mutated. In the second step, the modified bases generated in the first step are restored to original sequence except for the insertion of the desire mutation. Since several nucleotides are changed in each step, the recombinant BACs can be easily screened by PCR using a primer specific to the modified bases, by restriction analysis (restriction sites are included in the nucleotides inserted in the first step) or by hybridization with specific probe.

Briefly, two sets of pair of 100-mer oligonucleotide probes with 20-bp overlapping region were synthesized (Invitrogen) for each mutation to be constructed: c.300T>G, c.1866A>T, c.3819_3823del5 and c.5285insC. In the set used for the first recombination step, the overlapping region was changed to sequence: 5'-GGATCCTAGAATTCCTCGAG. A 180-bp targeting vector was generated by PCR using a pair of 100-bp oligonucleotides as a template. Specific PCR product was gel-purified and denaturized to obtain single-stranded DNA. A 300

ng portion of denaturized targeting vector was electroporated into bacterial cells containing λ prophage and HB1-812 BAC (kind gift of S.K. Sharan, MCGP, NCI-Frederick). Cells were diluted and grown for 24 h at 32 °C on LB agar containing 20 μg/ml chloramphenicol. Positive colonies were screened by hybridization with ^{32}P-labeled probe identical to 20-bp overlapping region of a corresponding pair of oligonucleotides used for PCR. Final constructs were verified by direct sequencing in both directions.

6.7. REPORTER ASSAY

pGL4.10-SV40_3UTR reporter plasmid containing human BRCA1 3'-UTR sequence was constructed in two steps. First, SV40 promoter from phRL-SV40 plasmid (Promega) was transferred into promoter-less pGL4.10[Luc2] plasmid (Promega). Next, BRCA1 3'-UTR (NM_007294; region 5820-7102) was PCR-amplified from HeLa cells' genomic DNA using following primers: 3UTR_Fwd: 5'-GCAGACTCTAGAGCCCAGGACCCCAAGAATGAG and 3UTR_Rev: 5'-CTGATGTCTAGAGTCTTCACTGCCCTTGCACACTGG. Specific PCR product was gel-purified and cloned into Xba I site of pGL4.10-SV40. Final construct was verified by sequencing in both directions using the same primers as for the initial PCR.

NIH3T3 cells were plated 16 h prior to the transfection ($1.5x10^5$ cells per well in 6-well plates). A 2 ng portion of pGL4.10-SV40_3UTR reporter plasmid was co-transfected with 50 ng – 1.5 μg of particular shRNA-expressing plasmid using FuGENE 6 (Roche). Where appropriate, pBluescript plasmid DNA was added to equal total amount of DNA per well. pGL4.10-SV40 reporter plasmid without BRCA1 3'-UTR and irrelevant shRNAs targeting mouse CCAAT/Enhancer Binding Protein γ gene were used as negative controls. Culture media were changed 24 h after transfection. At 48 h after transfection, the cells were lysed in Passive lysis buffer (Promega) and analyzed using the Luciferase assay system (Promega). Luciferase values were normalized to protein levels (Bio-Rad Protein Assay).

6.8. WESTERN BLOTTING

Whole-cells lysates were prepared in RIPA lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5mM EDTA; pH 7.2) supplemented with protease inhibitors (Calbiochem). Samples were cleared by high-speed centrifugation and supernatants were frozen and stored at -80 °C.

Nuclear extracts were prepared by detergent lysis procedure. Briefly, cell were washed once with PBS and scraped into hypotonic lysis buffer (buffer A: 20 mM HEPES pH 7.9, 1 mM EDTA, 10 mM NaCl, 1 mM DTT, 0.25% Nodinet P-40) supplemented with protease inhibitors and incubated on ice for 10 minutes. Nuclei were pelleted by centrifugation at 3,000 g for 10 minutes. Proteins were extracted from nuclei by incubation with buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM DTT, 25% glycerol) supplemented with protease inhibitors at 4 °C for 20 minutes with vigorous shaking. Nuclear debris was pelleted by centrifugation at 14,000 g for 5 minutes and supernatant was collected and stored at -80 °C.

A 20-50 μg portion of nuclear extract or 50-100 μg of whole cell lysate was resolved by 8% SDS-PAGE. After the SDS-PAGE, gels were equilibrated in transfer buffer (12.5 mM Tris-HCl, 96 mM glycine, 10% methanol) and blotted on the PVDF membranes (Immobilon-P, Millipore) using Criterion blotter apparatus (Bio-Rad) at constant voltage (100 V, 105 min). Membranes were blocked overnight in the TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.02% Tween 20 and 5% non-fat dry milk powder. Immunostaining was performed using following primary antibodies: anti-BRCA1 (K-18, H-100, I-20 and D-20; Santa Cruz), anti-BRCA1 (#KAP-ST020, StressGen Biotechnologies), anti-β-actin (AbCam), anti-p53 (Ab-2; Calbiochem). Secondary antibodies conjugated to horseradish peroxidase (Promega) were used to detect antigen-antibody complexes. Protein bands were visualized by chemiluminiscence (SuperSignal West Pico Detection System; Pierce).

6.9. FLOW CYTOMETRY

For BRCA1 expression analysis, cells were harvested by Trypsin/EDTA, washed with PBS and re-suspended in PBS to the final concentration 10^6 cells per ml. Cells were fixed in 1% formaldehyde for 15 minutes, and the fixation was stopped by adding glycine to the final concentration 125 mM. After washing with PBS, cells were fixed in 70% cold ethanol. Cells were stained with anti-BRCA1 antibody (D-20; Santa-Cruz) for 1 h at room temperature followed by anti-rabbit AlexaFluor 488-conjugated secondary antibody (Molecular Dynamics) for 1 h at room temperature. All antibodies were diluted in PBS containing 1% BSA and 0.1% Triton X-100. In controls, the primary antibody was omitted or replaced with an unspecific IgG or pre-incubated with specific blocking peptide (sc-641P; Santa-Cruz). After staining, cells were washed with PBS and analyzed by FACSort flow cytometer (Becton–Dickinson). Collected data were processed using the WinMDI 2.8 software.

For cell cycle and apoptosis analysis, formaldehyde fixation step was omitted. Fixed cells were washed 3 times in PBS and finally resuspended in 500 μl PBS containing 20 μg/ml RNase A (Roche) and 50 μg/ml propidium iodide (Sigma-Aldrich). Cells were incubated 15 minutes at room temperature in the dark and analyzed on FACSort flow cytometer. Collected data were processed using the WinMDI 2.8 and Cylchred software.

6.10. RNA ISOLATION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated by RNA Blue kit (Top-Bio). Reverse transcription was performed by SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. A 1 μl aliquot of prepared cDNA was used as a template for quantitative real-time PCR (qRT-PCR). qRT-PCR was performed on LightCycler 2.0 System (Roche) using Light Cycler Fast Start DNA Master SYBR Green I kit

(Roche). BRCA1-specific primers F1 (5’-AGAGTGTCCCATCTGTCTGGAGTTG) and R1 (5’-GGACACTGTGAAGGCCCTTTCTTC) targeting BRCA1 coding sequence (mRNA: 185-304 bp) were used for qRT-PCR. Reactions were cycled 50 times at 95°C for 10 s, 70°C for 10 s and 72°C for 10 s. Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD) were analyzed from the same cDNA at amplification conditions identical to those described for BRCA1. The following primers were used: GAPDH sense primer 5’-GGTGAAGGTCGGAGTCAACGG, GAPDH antisense primer 5’-CGCTCCTGGAAGATGGTGATGG, PBGD sense primer 5’-ATGTCTGGTAACGGCAATGCGG and PBGD antisense primer 5’-TGTCCCCTGTGGTGGACATAGC. qRT-PCR results were analyzed by LightCycler software (Roche) and values of crossing points (CPs) and amplification efficiencies were evaluated for each reaction. Statistical significance of changes in BRCA1 mRNA levels relative to housekeeping genes was calculated by pair wise fixed reallocation randomization test using the REST-2005 software [351].

7. RESULTS AND DISCUSSION

7.1. BRCA1 MUTATIONS

For the functional analysis we chose *BRCA1* variants c.300T>G, c.1866A>T, c.3819_3823del5 and c.5385dupC which were found during the screening of *BRCA1* gene variations in probands from high-risk families and patients with early onset breast or ovarian cancer in the Czech population [352, 353] (Fig. 7.1). The c.5385dupC (p.Gln1756fsX1829) mutation in the *BRCA1* gene is the most frequent one and may be the dominant founder mutation in the Czech Republic. The c.1866A>T (p.Lys583X) mutation has not been reported previously and may also be characteristic founder mutation in the Czech population. Mutations c.3819_3823del5 (p.Leu1252fsX1241) and c.300T>G (p.Cys61Gly) are frequently detected among screened population (Fig. 7.1).

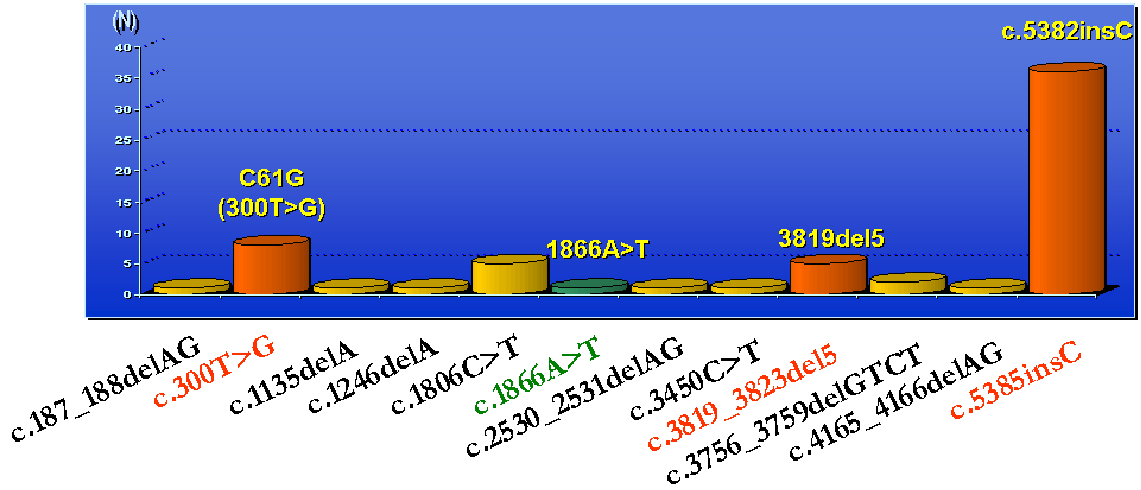

Figure 7.1. Spectrum and frequencies of mutations in *BRCA1* gene. Results of screening of *BRCA1* mutations in breast/ovarian cancers performed in patients from high-risk families and patients with early onset breast or ovarian cancer in the Czech Republic population [352, 353]. Traditional nomenclature of BRCA1 mutations is used.

7.2. DOWN-REGULATION OF ENDOGENOUS WILD-TYPE BRCA1 BY RNA INTERFERENCE

7.2.1. RNA INTERFERENCE

RNA interference (RNAi) is a phylogenetically conserved mechanism of double-stranded RNA-mediated mRNA silencing [354]. RNAi can be triggered either by small interfering RNAs (siRNAs) which are produced from exogenous double-stranded RNA (dsRNA) or by endogenously produced ~ 21-22 bp long non-coding RNAs, called microRNAs (miRNAs). Detailed mechanisms of miRNA/siRNA biogenesis, RISC (RNA-Induced Silencing Complex) assembly and mechanisms of miRNA/siRNA function were currently reviewed [354-357].

For experimental purposes, RNAi can be triggered in mammalian cells either by exogenous application of siRNAs or by intracellular expression of small hairpin RNAs (shRNAs) from transfected plasmids. SiRNAs are 19-bp long, synthetic dsRNA molecules bearing 2-bp 3‘ overhangs. These are transiently transfected to target cells and enter RNAi machinery at the stage of RISC complex formation. Effect of transiently transfected siRNAs is short, lasting only a couple of days in proliferating cells, generally not longer than a week [358, 359]. On the contrary, shRNAs are expressed from plasmids as precursor molecules similar to endogenous pre-miRNAs. These precursors enter the endogenous processing pathway leading to the production of mature shRNA similar in structure and function to siRNA/miRNA. The expression of shRNA can be driven by RNA polymerase II-based [348], RNA polymerase III-based [346] or RNA polymerase I-based promoters [360]. ShRNA expression cassettes cloned into retroviruses enable infection of hard-to-transfect cell lines and primary cultures [361-363]. The effect of intracellularly expressed shRNAs is long lasting and is not influenced by cell proliferation as is the case for transiently transfected siRNAs. ShRNAs were used through this study for induction of RNAi response.

7.2.2. DESIGN OF SHRNAS

All shRNAs used in this study were designed to target 3'-UTR of human BRCA1 mRNA. Although variety of siRNAs/shRNAs targeting the coding region of BRCA1 mRNA were published, targeting the 3'-UTR region of BRCA1 mRNA circumvents the need to engineer RNAi-resistant construct for each shRNA tested for control purposes. For all 3'-UTR-directed shRNAs, wtBRCA1 open-reading frame sequence can be used as a "general" RNAi-resistant control. Moreover, 3'-UTR directed shRNAs target all BRCA1 mRNA variants present in cells, including sequence variants and alternatively spliced mRNAs and are indifferent to potential mutations/SNPs in BRCA1 coding region.

Sh_5890, sh_6073 and sh_6095 were designed manually according to accepted rules [364-366]. Sh_6069 and sh_6252 were predicted by BIOPREDsi siRNA-predicting algorithm ([367]; http://www.biopredsi.org). All these shRNAs were cloned into pSUPER.retro.puro retroviral plasmid [346], where the expression is under the control of RNA polymerase III-driven human H1 promoter.

During the progression of this study, RNA polymerase II-driven shRNA expression plasmids based on endogenous miR-30 were described [347]. We constructed additional sh_6335, sh_6867 and sh_6965 in miR-30-based LMP retroviral plasmid. All three shRNA sequences used are listed in the RNAi Codex database which was design specifically for polymerase II-driven expression of shRNA [368]. Because of the sh_6965 was also predicted by BIOPREDsi as the best potential target, we cloned the sh_6965 in pSUPER.retro.puro plasmid as well to have the same shRNA sequence in both types of plasmids.

Sequences sh_6069, sh_6252, sh_6335 and sh_6965 were also predicted by recently published siExplorer algorithm implementing some new rules [369]. Although, the rules for siRNA/shRNA prediction are far from to be definitive and 100% effective [365, 366, 370], majority of our shRNAs fulfil recently accepted guidelines.

7.2.3. SHRNA ARE CAPABLE TO DOWN-REGULATE BRCA1 EXPRESSION IN REPORTER SYSTEM

To test shRNAs function, we used luciferase-based reporter system in transiently transfected murine NIH3T3 cells. We co-transfect pGL4.10-SV40_3UTR reporter plasmid together with shRNA-expressing plasmids and monitored the effect of human BRCA1 3'-UTR on luciferase activity. The luciferase expression was inhibited by all shRNA constructs tested (Fig. 7.2.3) in a concentration-dependent manner (data not shown) when BRCA1 3'UTR sequence was present in the reporter plasmid. Inhibition > 90% was observed at concentrations 50 ng and 1.5 μg for pSUPER.retro.puro and LMP plasmid, respectively (data not shown). Observed shRNA-mediated inhibition of luciferase signal was specific for BRCA1 3'-UTR, since luciferase signal from pGL4.10-SV40 reporter plasmid was not affected (data not shown). Irrelevant shRNA constructs targeting mouse CCAAT/Enhancer Binding Protein γ were used as negative controls (Fig. 7.2.3). At concentrations higher than 200 ng, luciferase activity was also inhibited by these control shRNAs, but in much less extend (~10%, ~25% and ~40% inhibition at 200 ng, 500 ng and 1.5 μg, respectively; data not shown). We expect this inhibition to be mediated by a non-specific, miRNA-like translation inhibition rather than expected siRNA-like mRNA cleavage mechanism [357].

The performance of shRNAs cloned into pSUPER.retro.puro plasmid was overall better than those cloned into LMP plasmid (Fig. 7.2.3). The difference between plasmids was still apparent at 1.5 μg, the highest concentration tested, where LMP plasmids inhibited luciferase activity by ~90% (data not shown). We expect the majority of luciferase activity detected in the assay originate from the beginning of the experiment before shRNAs are produced and luciferase mRNA is inhibited by RNAi. This means that the action of LMP-derived shRNAs is delayed compared to pSUPER-derived shRNAs. This variation may be due to different levels of shRNAs expression from polymerase III-driven (human H1; [346]) and polymerase II-driven (viral LTR; [347]) promoters used in pSUPER.retro.puro and

LMP plasmid, respectively, or by differences in the efficiency of shRNA-precursors' processing and loading into the RISC complex.

We conclude that all shRNAs are proficient in down-regulating reporter luciferase expression in BRCA1 3'-UTR-dependent manner and that pSUPER.retro.puro plasmids are more potent than LMP plasmids in this assay system probably due to faster and higher expression levels achieved from H1 promoter.

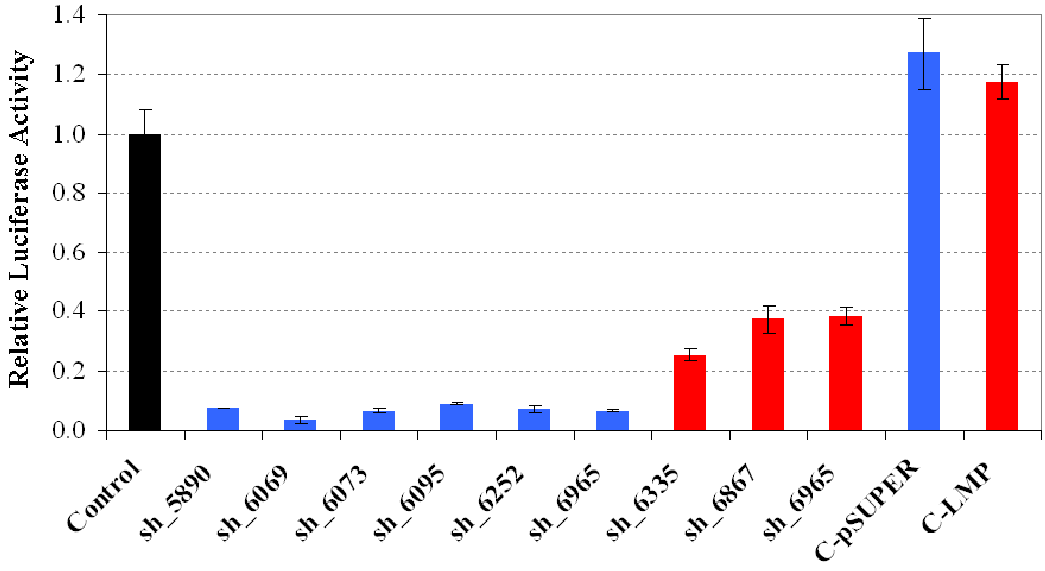

Figure 7.2.3. Luciferase Reporter Assay. NIH3T3 cells were transiently transfected with 2 ng of pGL4.10-SV40_3UTR reporter construct either alone or together with 50 ng shRNA-expressing plasmids. Reporter activity was normalized to protein levels and the value for reporter construct alone was set to 1. Data are plotted as relative activity ± SEM (average of 3 independent transfections) and represent the typical experiment. Black bar: control (transfection of reporter plasmid alone); blue bars: pSUPER.retro.puro plasmid; red bars: LMP plasmid. C-pSUPER and C-LMP are irrelevant control shRNAs targeting mouse CCAAT/Enhancer Binding Protein γ expressed from pSUPER.retro.puro and LMP plasmid, respectively.

7.2.4. TRANSIENT SHRNA-MEDIATED BRCA1 DOWN-REGULATION

To verify shRNA function on the protein level in more physiological settings, we transiently transfected HeLa cells with shRNA constructs and looked at

endogenous BRCA1 protein levels using flow cytometry. The BRCA1 protein levels were down-regulated by all shRNAs except sh_6095 (Fig. 7.2.4 and data not shown). We also tested co-transfection of two shRNA-expressing plasmids, but the resulting down-regulations were not superior compared to individual shRNAs (data not shown). This is consistent with published observations that the effect of perfectly complementary siRNAs is not additive, whereas miRNAs can function in a combinatorial way [371].

Observed down-regulation was only moderate compared to results obtained in reporter system. One reason for just moderate BRCA1 down-regulation can be low transfection efficiency in HeLa cells. To confirm this, we transfected HeLa cells by pEGFP-C1 plasmid and monitor EGFP expression by flow cytometry. Typically, less than 25% of HeLa cells were EGFP-positive independently of transfection reagent used (data not shown). Low transfection efficiency was also confirmed by the selection of transiently transfected cells by puromycin. Thus, overall moderate BRCA1 protein down-regulation observed in HeLa cells is in part due to low percentage of shRNA-expressing cells.

Another possibility for moderate BRCA1 down-regulation may be that shRNAs are not expressed sufficiently at the time of analysis, i.e. 48 h post-transfection. This is, however, not probable since shRNAs were working well in the reporter system at the same time point (Fig. 7.2.3). Moderate down-regulation can be seen also in the cases, when the target protein has long half-life or exists in specific compartments (e.g. preferential nuclear localization of BRCA1 compared to cytoplasmic action of RNAi). Here, sufficiently long time is necessary for depleting the protein from all stores. However, longer post-transfection intervals were not tested in our assay system because the levels of gene expression induced by transient transfection are decreasing rapidly from 48 h post-transfection as assessed by EGFP expression analysis (data not shown).

We conclude that all shRNAs except sh_6095 are able to down-regulate endogenous BRCA1 expression in transiently transfected HeLa cells, despite with different potency. However, to achieve a complete BRCA1 down-regulation, long-lasting shRNA expression may be needed.

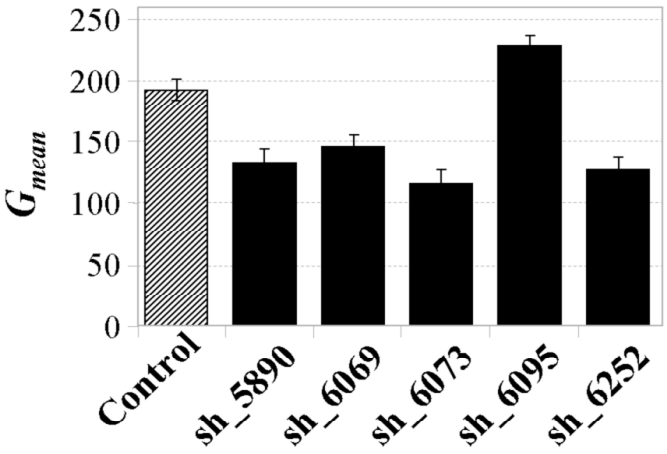

Figure 7.2.4. BRCA1 down-regulation in HeLa cells. HeLa cells were transiently transfected with shRNA-expressing pSUPER.retro.puro plasmids. BRCA1 expression was measured by flow cytometry 48 h post transfection. Intensity is expressed as G_{mean} ± CV (coefficient of variation). Data from a representative experiment are shown

7.2.5. LONG-TERM BRCA1 DOWN-REGULATION BY SHRNAS

To establish long-lasting BRCA1 down-regulation *in vivo*, we advantaged of viral infections, which provide more uniform expression in target cells compared to transient transfections. We infected human breast cancer cell line MCF-7 at a low MOI (Multiple of Infection) to accomplish even more uniform shRNA expression (theoretically at low MOI each cell is infected only by single retroviral particle). After selection, population of surviving cells was analysed for changes in BRCA1 mRNA levels by qRT-PCR and changes in BRCA1 protein levels by western blotting. The levels of BRCA1 mRNA were significantly down regulated by all shRNAs tested (Fig. 7.2.5.1) by the factor of ~ 2 ($p<0.001$). Correspondingly, BRCA1 protein expression was decreased in MCF-7 cells infected by shRNAs (Fig. 7.2.5.2). BRCA1 down-regulation (both on mRNA as well as protein levels) was cell line-specific, since no consistent effect of shRNAs was present in MDA-MB-231 or HeLa cells (data not shown). Interestingly, sh_6095 showed significant BRCA1 mRNA up-regulation in MDA-MB-231 and HeLa cells (data not shown). This effect was confirmed by qRT-PCR from independently prepared cDNAs. Although, siRNAs/shRNAs are supposed to silence homologous sequences, Li *et al.* [372] observed long-lasting, sequence-specific induction of target genes by siRNAs directed to promoters of E-cadherin, $p21^{WAF1/CIP1}$ and VEGF. Argonaute 2 (Ago2)

protein and the 5' end of siRNA ("seed" sequence) were critical for observed activation [372], which is reminiscent of microRNA action [354, 357] and siRNA-mediated transcriptional silencing [373, 374]. Moreover, this effect was siRNA- and cell type-specific similarly as in our case. Recently, Vasudevan and Steitz [375] observed activation of TNFα mRNA translation in serum starved HEK293 and monocytic THP-1 cells mediated by AU-rich sequence in 3'-UTR of TNFα mRNA and absolutely dependent on Ago2 and FXR1 (Fragile-X-Mental-Retardation) proteins [375]. It is not known whether observed Ago2-mediated translation activation was dependent on miRNA(s) or not. However, it can be speculated that such context-dependent activation effect is more general. Exact conditions that may be responsible for observed cell line-specific "stimulatory" effect in our system are under investigation.

We conclude that shRNAs delivered to target cells via infection are able to down-regulate endogenous BRCA1 mRNA and protein levels in a cell type-specific manner.

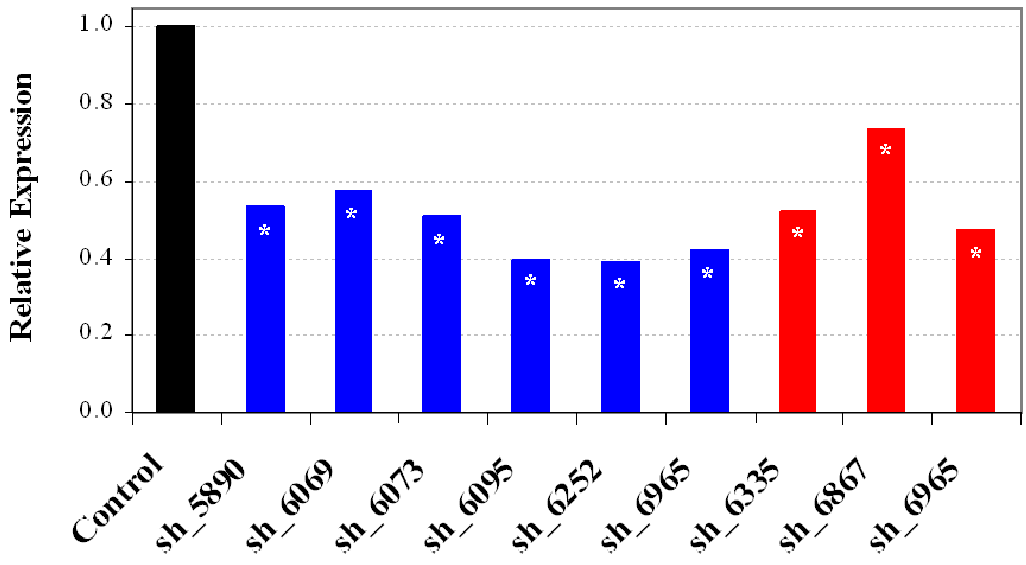

Figure 7.2.5.1. Down-regulation of BRCA1 mRNA levels in MCF-7 cells. Quantitative real-time PCR (qRT-PCR) analysis of BRCA1 mRNA expression in MCF-7 cells infected by shRNA-expressing plasmids. Housekeeping genes

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD) were used as internal controls. qRT-PCR results were evaluated by REST-2005 software and changes in BRCA1 mRNA expression levels relative to housekeeping genes were calculated based on the efficiencies of PCR reactions. BRCA1 relative expression in control MCF-7 cells (treated with empty pSUPER.retro.puro or LMP plasmid) is equal to 1. Statistical significance of changes in BRCA1 mRNA levels was calculated by pair wise fixed reallocation randomization test using the REST-2005 software and p values (marked by *) are p=0.001. Black bar: control (infection of empty plasmid); blue bars: pSUPER.retrop.puro plasmid; red bars: LMP plasmid. Data from a typical experiment are presented.

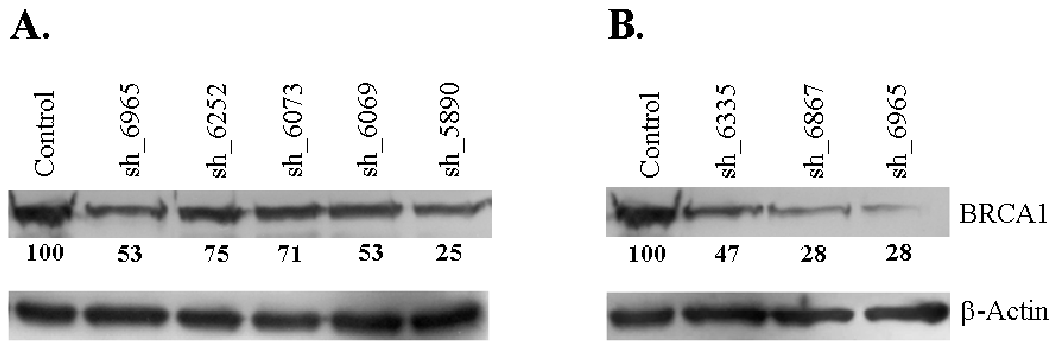

Figure 7.2.5.2. Decrease of BRCA1 protein expression in shRNA-infected MCF-7 cells. Western blotting analysis of BRCA1 protein expression in MCF-7 cells infected by shRNA-expressing **(A)** pSUPER.retro.puro or **(B)** LMP plasmids. β-actin expression was used as loading control. Numbers under BRCA1 bands indicate relative band intensity after normalization to corresponding β-actin band intensity. Intensity of BRCA1 band in control MCF-7 cells (infected with empty retroviral plasmid) is set to 100. Control: MCF-7 cells infected with empty retroviral plasmid. Data from a typical experiment are presented.

7.2.6. FUNCTIONAL EFFECT OF LONG-TERM BRCA1 DOWN-REGULATION

We next investigated the influence of BRCA1 down-regulation on proliferation of MCF-7 cells. Majority of shRNAs tested reduced the proliferation rate of MCF-7 cells (Fig. 7.2.6.1). The sh_6252 and sh_6069 were the most potent shRNAs expressed from pSUPER.retro.puro plasmid (~ 40-50% growth inhibition; p

= 0.001; Student's T-test); sh_5890 and sh_6095 had an intermediate effect (~ 20-30% growth inhibition; $p < 0.01$); the effect of sh_6073 and sh_6965 was only marginal and not statistically significant. The effect of shRNAs expressed from LMP plasmid was overall better compared to the expression from pSUPER.retro.puro plasmid; all shRNAs inhibited the proliferation of MCF-7 cells by ~ 60-80 % ($p < 0.001$).

ShRNA-mediated inhibition of cell proliferation was cell line specific, since the proliferation rate was reduced only marginally in MDA-MB-231 cells (statistically not significant; data not shown). The growth-inhibitory effect in HeLa cells was comparable to that in MCF-7 cells, but was less reproducible. The decrease of cell proliferation correlated with the magnitude of BRCA1 mRNA as well as protein down-regulation in each particular experiment (data not shown). BRCA1 regulates cell cycle through mediating the effects of checkpoint kinases (ATM, ATR, Chk1, Chk2) and was implicated in the regulation of S, G_1 as well as G_2/M checkpoints [159]. BRCA1 down-regulation may attenuate correct checkpoint function and together with delay in DNA damage repair may slow-down cell cycle progression. Cell cycle analysis revealed 5-8% decrease of cells in S-phase with corresponding increase of cells in $G_{0/1}$ phase in MCF-7 cells (Fig. 7.2.6.2). Although the effect is not huge, even such moderate changes in cell cycle may cause differences in proliferation rate over the period of 6 days as we assayed. Moreover, the changes in cell cycle distribution corresponded to the results of growth curve experiments (Fig. 7.2.6.1) and were higher in cells infected with LMP plasmid compared to pSUPER.retro.puro-infected cells (Fig. 7.2.6.2). There were no reproducible changes in cell cycle in MDA-MB-231 or HeLa cells. No indication of apoptosis was detected in any cell line (data not shown).

What is the basis of cell line-specific effect? One possibility is that the inhibitory effect of shRNAs on cell proliferation corresponds to the magnitude of BRCA1 mRNA down-regulation. MCF-7 cells express relatively high levels of endogenous BRCA1 compared to other cells lines [336] indicating potential important role of BRCA1 in this cell line. Expression levels of BRCA1 and its functional importance may prerequisite the final shRNA's action.

Another possibility is that cell type-specific effect on proliferation rate may be due to intrinsic differences between cell lines used. MCF-7 and MDA-MB-231 cells are derived from breast adenocarcinomas, whereas HeLa cells are derived from cervical adenocarcinoma. Mutations in BRCA1 are known to predispose to breast and ovarian cancers but having no effect on cervical cancer [302]. Thus, the lack of shRNAs action in HeLa cells may be related to tissue-specific functions of BRCA1. As opposed to MCF-7 cells, MDA-MB-231 cells lack endogenous estrogen receptor and express non-functional, mutated form of p53 tumour suppressor containing missense G>A mutation in exon 8 (pR280K). The role of hormonal exposure and especially estrogen receptors was anticipated in tissue-specific action of BRCA1 [376]. The tumour suppressor p53 plays a key role in coordinating responses to stress factors including DNA damage [377, 378] where BRCA1 plays an important regulatory role [379]. Thus, inhibition of BRCA1 expression by shRNAs may delay DNA damage repair and this may signal to activate p53 followed by cell cycle arrest or ultimately by apoptosis. Such an effect may be more apparent in cells expressing wt p53 with preserved checkpoints regulation (e.g. MCF-7 cells). It will be interesting to follow up cells with down-regulated BRCA1 expression for more passages and monitor the accumulation of DNA defects.

Finally, the possibility of negative selection against cells with highly down-regulated BRCA1 expression cannot be ruled out. Such a negative selection may act in cell type-specific and/or p53-specific manner. If negative pressure toward BRCA1 expression is considered, only cells with low BRCA1 down-regulation (due to e.g. epigenetic silencing of inserted retrovirus) and thus mitigate effect on proliferation rate will preferentially survive the selection. To rule out possibility of negative selection, conditional expression of shRNAs should be implemented [347].

Nevertheless, majority of our shRNAs are able to down-regulate BRCA1 expression (Fig. 7.2.6.1). This down-regulation has cell type-specific functional consequences *in vivo*. Exact mechanisms involved in cell specificity are under investigation.

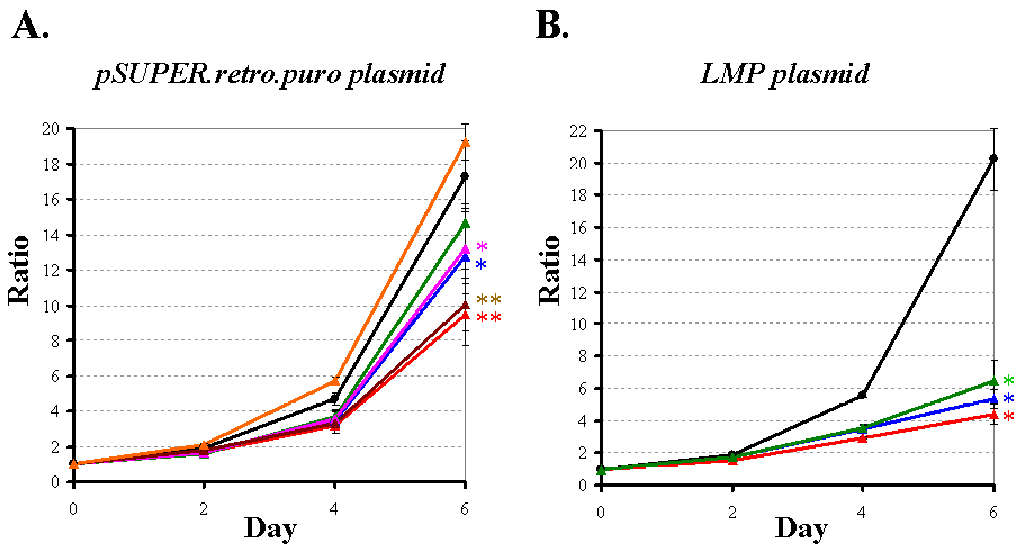

Figure 7.2.6.1. Functional effect of BRCA1 down-regulation on proliferation of MCF-7 cells. MCF-7 cells were infected with control (empty) or shRNA-expressing retroviruses, drug selected and used for growth assays. Cell proliferation was monitored over a 6-day period. Each value was normalized to the cell number at day 0. Data are expressed as average ± SEM from at least two independent experiments performed in triplicates. **[A]** Infection with pSUPER.retro.puro plasmid. Black line: control; blue line: sh_5890; red line: sh_6069; green line: sh_6073; magenta line: sh_6095; brown line: sh_6252; orange line: sh_6965. **[B]** Infection from LMP plasmid. Black line: control; blue linee: sh_6335; green line: sh_6867; red line: sh_6965. P-values are indicated in colour corresponding to particular growth curves, as follows: * p = 0.05; ** p = 0.001 (paired, two-tailed Student's T-test).

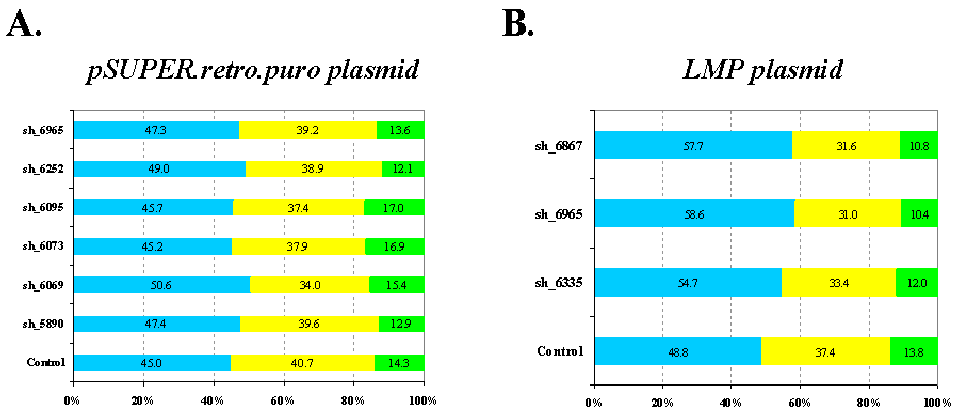

Figure 7.2.6.2. Effect of BRCA1 down-regulation on cell cycle distribution in MCF-7 cells. MCF-7 cells were infected with control (empty retrovirus) or shRNA-expressing retroviruses pSUPER.retro.puro **[A]** or LMP **[B]**, respectively, drug selected and plated at equal density. Cell cycle distribution was evaluated by propidium iodide staining. Data were processed by WinMDI and Cylchred software. Blue bars: G_1 phase; yellow bars: S phase; green bars: G_2/M phase.

7.3. Over-Expression of BRCA1 Variants

7.3.1. Over-Expression of BRCA1 Variants Using Conventional Plasmids

To overexpress mutated BRCA1 variants, we transfected MCF-7 and MDA-MB-231 breast cancer cell lines by pcDNA3.1(+)_Hygro plasmids containing appropriate BRCA1 variants. Permanent clones were selected by hygromycine. Unfortunately, we were not able to get any BRCA1-positive colony. qRT-PCR analysis showed that all surviving hygromycine-resistant cells did not express corresponding BRCA1 variants (data not shown). The reason for unsuccessful BRCA1 expression was low transfection efficiency as monitored by flow cytometry after transfection of EGFP (data not shown). Transfection efficiency was improved neither using transfection reagents from various suppliers (data not shown) nor using "easy-to-transfect" HeLa cells (data not shown).

7.3.2. Over-Expression of BRCA1 Variants Using Retroviral Infections

To circumvent problems with low transfection efficiency, we used retroviral infections to over-express mutated BRCA1 variants. We infected MCF-7 and MDA-MB-231 cells with pWZL_Hygro retroviral plasmids expressing wtBRCA1 and mutated variants c.300T>G, c.1866A>T, c.3819_3823del5 and c.5385dupC. We observed very low efficiency infecting both MCF-7 and MDA-MB-231 cells by retroviral plasmids expressing BRCA1 variants. The most probable reason for low infection efficiency was the large size of BRCA1 insert (~ 5.5 kb) bringing total plasmid size up to ~ 12 kb which is at the upper limit for efficient plasmid packaging and infection of target cells. This is supported by the observation of higher infection

efficiency obtained with empty pWZL_Hygro plasmids and to a lesser extend also with pWZL_Hygro plasmids containing BRCA1 c.1866A>T variant, the shortest one used (data not shown). Thus, we tried to subclone BRCA1 variant to pWZL_Blast plasmid that is ~ 1 kb shorter than pWZL_Hygro, but the infection efficiencies were improved only marginally (data not shown).

Nevertheless, we were able to obtain positive clones overexpressing BRCA1 variant by infecting MFC-7 and MDA-MB-231 cells with pWZL_Hygro and pWZL_Blast, respectively. Population of surviving cells after selection was analyzed for changes in BRCA1 expression. BRCA1 mRNA levels were analyzed by qRT-PCR using primers common for all BRCA1 variants used. All mutated BRCA1 variants as well as wtBRCA1 were successfully overexpressed 2-5 times in MDA-MB-231 cells (Fig. 7.3.2.1) and MCF-7 cells (data not shown).

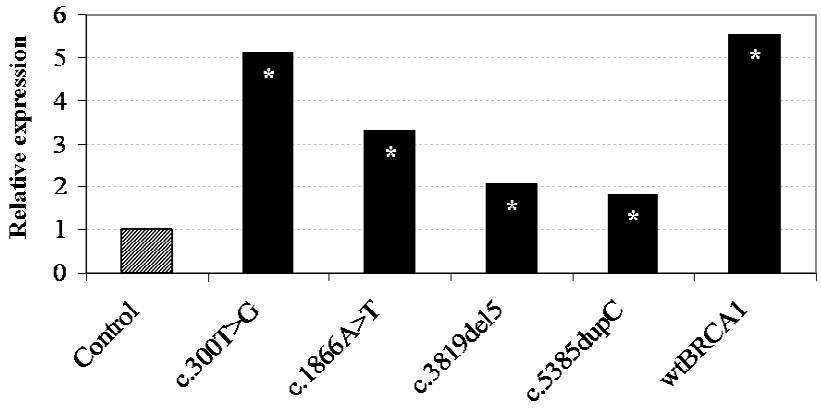

Figure 7.3.2.1. Overexpression of BRCA1 variants in MDA-MB-231 cells. Quantitative real-time PCR (qRT-PCR) analysis of BRCA1 mRNA expression in MDA-MB-231 cells infected pWZL_Blast expressing BRCA1 variants. Housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD) were used as internal controls. qRT-PCR results were evaluated by REST-2005 software and changes in BRCA1 mRNA expression levels relative to housekeeping genes were calculated based on the efficiencies of PCR reactions. BRCA1 relative expression in control MDA-MB-231 cells (treated with empty pWZL plasmid) is equal to 1. Statistical significance of changes in BRCA1 mRNA levels was calculated by pair wise fixed reallocation randomization test using the REST-2005 software and p values (marked by *) are p=0.01. Data from a typical experiment are presented.

Over-expression of BRCA1 variants on the protein level was detected by western blotting. Increase in the expression of wtBRCA1 as well as c.300T>G and c.5385dupC BRCA1 variants was detected in MDA-MB-231 cells (Fig. 7.3.2.2) as well as MCF-7 cells (data not shown). However, we were unable to detect truncated protein expressed from c.1866A>T and c.3819_3823del5 variants. The failure to detect c.1866A>T and c.3819_3823del5 proteins is probably due to technical problems with antibodies directed against N-terminal part of BRCA1 protein as similar problems were reported for several other BRCA1 truncating mutations and different antibodies [336, 380].

Together, we were able to obtain MDA-MB-231 and MCF-7 cells stably over-expressing wtBRCA1 and mutated BRCA1 variants albeit quite low efficiency of retroviral infections.

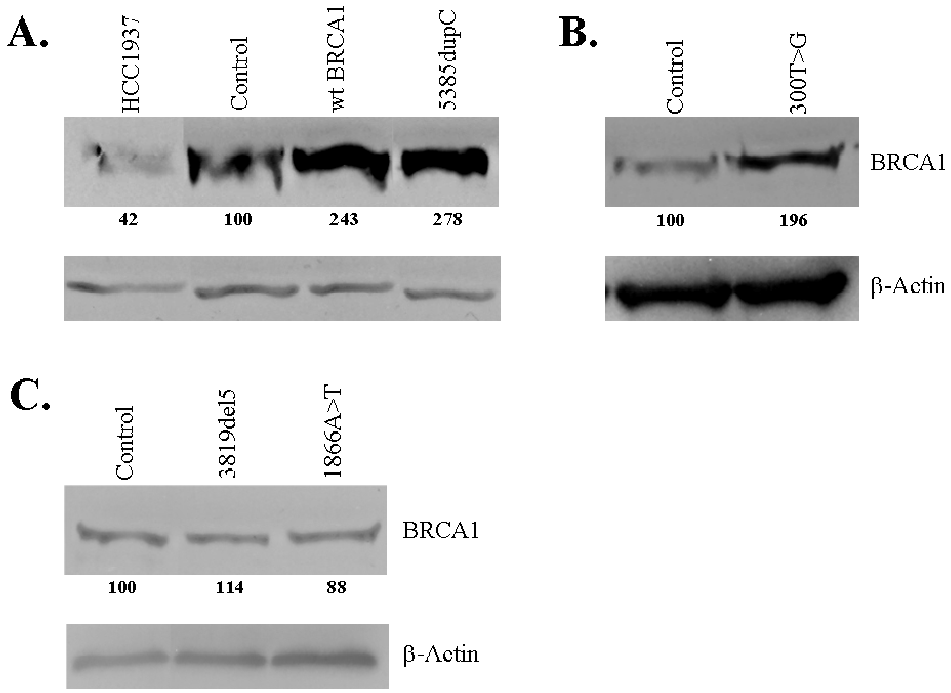

Figure 7.3.2.2. Over-expression of BRCA1 variants in MDA-MB-231 cells. BRCA1 protein expression analysis by Western blotting in MDA-MB-231 cells infected by pWZL_Blast plasmids containing **[A]** wild-type BRCA1 and c.5385dupC variant, **[B]** c.300T>G variant and **[C]** c.3819_3823del5 and c.1866A>T variants. β-actin expression was used as a loading control. Numbers under BRCA1 bands indicated relative band intensity after normalization to corresponding β-actin band

intensity. Intensity of BRCA1 band in control MDA-MB-231 cells (infected with empty pWZL_Blast plasmid) is set to 100. HCC1937 cells expressing c.5385dupC BRCA1 variant and having lower protein levels compared to MDA-MB-231 cells were loaded on the gel as a control for BRCA1 expression levels. Data from a typical experiment are presented. Please note that lanes in panel **[A]** were pasted together using Adobe Photoshop software, but were run on the same gel.

7.3.3. FUNCTIONAL EFFECT OF OVER-EXPRESSION OF MUTATED BRCA1 VARIANTS

We investigated the influence of BRCA1 over-expression on proliferation of MDA-MB-231 and MCF-7 cells. Wild-type BRCA1 slightly reduced the proliferation rate of MCF-7 cells by ~ 20% (p=0.05; Fig. 7.3.3). Similar reduction of proliferation rate was observed for BRCA1 variants c.1866A>T (p=0.05), c.3819_3823del5 (N.S.) and c.300T>G (N.S.). BRCA1 c.5385dupC variant had the most potent inhibitory effect decreasing proliferation of MCF-7 cells by ~ 40% (p=0.01). This inhibition was significantly higher compared to wtBRCA1 (p=0.001) and was thus similar to the effects of shRNA-mediated BRCA1 down regulation (see Fig. 7.2.6.1). Growth inhibitory effect of BRCA1 variants correlated with changes in cell cycle distribution, where growth-inhibited MCF-7 cells had ~ 3-5% less cells in S-phase compared to controls (data not shown). No effect of BRCA1 over-expression, either wild-type or mutated variants, was observed in MDA-MB-231 cells (Fig. 7.3.3) or HeLa cells (data not shown). The exact basis of indifference of MDA-MB-231 cells to the manipulation in BRCA1 levels is not known at present (for discussion see Section 7.2.6). Comparison between MCF-7 and MDA-MB-231 cells may reveal important modifiers of BRCA1 action and is a matter of ongoing research.

Growth inhibitory effect of wtBRCA1 in MCF-7 is in accordance with other published data [336]. However, we did not observed any effect on cell proliferation of MCF-7 cells that may be specifically attributed for mutated BRCA1 variants. The potential higher growth-inhibitory effect of BRCA1 c.5385dupC variant in MCF-7 cells (Fig. 7.3.3) may be due to differences in protein expression levels compared to

other mutants (Fig. 7.3.2.2). However, Fan *et al.* [336] observed that mutant BRCA1 variants, including c.5385dupC, antagonize phenotype of wild-type BRCA1. This antagonism was apparent in several essays but, unfortunately, mutant and wtBRCA1 variants inhibited cell proliferation of MCF-7 cells in similar way. Thus, proliferation assay may not be suitable for detecting functional alterations mediated by mutated BRCA1 variants. Alternatively, BRCA1 variants we used in our study, which differ from variants used in the study of Fan *et al.*, neither antagonize the effect of wtBRCA1 nor have any dominant-negative or gain-of-function effect. The latter possibility is supported by **(a)** Fan *et al.* [336] who observed assay-specific and mutation-specific antagonism between mutated and wtBRCA1; and **(b)** Cousineau and Belmaaza [335] who described that simple BRCA1 haploinsufficiency, not the mutated BRCA1 by itself, is responsible for pathological effect of BRCA1 mutations in HR deregulation.

Our results support the role of BRCA1 haploinsufficiency in altering cellular function(s) with no significant damage introduced by mutated BRCA1 variants, at least in the proliferation assay used. Can “simple” BRCA1 haploinsufficiency play significant role in breast cancer tumorigenesis? Or are potential dominant-negative and/or gain-of-function effects of (some) BRCA1 variants necessary for carcinogenesis? The issue is not fully resolved yet [337]. No doubt that some differences may be due to a position effect of particular mutation within *BRCA1* gene and corresponding alterations in BRCA1 protein structure/function. However, methodological differences may play role as well. For examples, Fan *et al*. [336] and Cousineau and Belmaaza [335] both used MCF-7 cells in their studies. While MCF-7 cells used in the study of Fan *et al.* were obtained from ATTC and expressed high levels of BRCA1 protein (similarly as MCF-7 cells used in our study), Cousineau and Belmaaza used a clone of MCF-7 cells with reduced expression of BRCA1 due to presence of only one wild-type BRCA1 allele [381-383]. The “background” levels of endogenous wild-type BRCA1 may significantly influence overall outcome of functional studies [384, 385].

Together, BRCA1 variants c.300T>G, c.1866A>T and c.3819_3823del5 have no dominant-negative or gain-of-function effect in MCF-7 cells in our proliferation

assay system. BRCA1 variant c.5385dupC may antagonize the function of endogenous wtBRCA1. More functional tests are going to be implemented to verify this conclusion also for other BRCA1 functions besides cell proliferation.

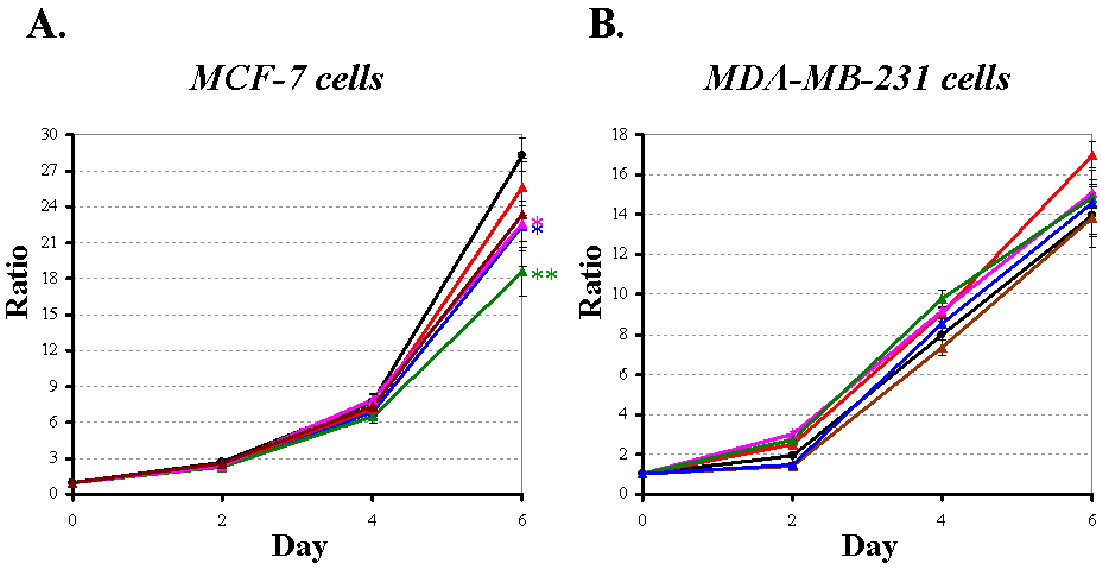

Figure 7.3.3. Effect of BRCA1 up-regulation on proliferation of MDA-MB-231 and MCF-7 cells. [A] MCF-7 cells and **[B]** MDA-MB-231 cells were infected with control (empty retrovirus) or BRCA1-expressing retroviruses, drug selected and used for growth assays. Cell proliferation was monitored over a 6-day period. Each value was normalized to the cell number at day 0. Black line: control; blue line: wtBRCA1; red line: c.300T>G; magenta line: c.1866A>T; brown line: c.3819_3823del5; green line: c.5385dupC. Data are expressed as average ± SEM from at least two independent experiments performed in triplicates. P-values are indicated in color matching particular growth curve, as follows: * p = 0.05; ** p = 0.001 (paired, two-tailed Student's T-test).

7.3.4. Over-Expression of Mutated BRCA1 Variants using Bacterial Artificial Chromosome

Over-expression of proteins using either transfection of conventional plasmids or retroviral infection is associated with apparent problems in interpreting the results. The expression levels are controlled by exogenous promoters (either LTR

in retroviral plasmids or CMV promoter in conventional plasmids) and may be influenced by the site where the plasmid is incorporated into genomic DNA.

BAC (Bacterial Artificial Chromosome) overcomes most of these weaknesses. BACs are large plasmids comprising ~ 300 kb region of genomic DNA. Particular gene expressed from BAC is regulated under physiological conditions, the same way as *in vivo*, since BAC contains all the necessary 5' and 3' regulatory sequences. Thus, BAC permits study of gene function in more physiological setting than is possible with either plasmids or retroviruses. For example, because of the presence of introns in the BAC, alternatively spliced transcripts may be expressed and regulation of alternative splicing and/or nonsense-mediated decay pathway may be studied in this system [356]. BACs were successfully used to dissect the functional effect of c.300T>G mutation which "theoretically" causes cysteine to glycine substitution at the seventh conserved cysteine residue within $Cys_{(3)}$-His-$Cys_{(4)}$ RING finger domain. However, c.300T>G mutation disrupts exon splicing enhancer and leads to exon 5 exclusion, open reading frame shift and production of severely truncated, unstable BRCA1 protein. Thus, c.300T>G mutation *in vivo* behaves as a null one rather than missense one [386].

However, the main disadvantage of BAC is the complicated delivery into cells. Common lipid-based transfection methods cannot be used because of large size of BAC. Electroporation is usually a method of choice, but the efficiency is low and transfection of some cell type (e.g. primary cells) is very difficult.

We constructed BRCA1 variants c.300T>G, c.1866A>T, c.3819_3823del5 and c.5385dupC in HB1-812 BAC which contains genomic region comprising human *BRCA1* gene. Pilot transfection experiments were performed in MCF-7 cells, but we do not have any conclusive results yet.

7.4. VERSATILE SYSTEM FOR BRCA1 FUNCTIONAL STUDIES

To fully test our assays system, we performed rescue experiments. These experiments are based on the up-regulation of RNAi-resistant form of particular gene

under study to confirm the specificity of observed RNAi-mediated phenotype [387, 388]. We used MCF-7 cells, since MDA-MB-231 cells do not respond in our proliferation-based assays neither to BRCA1 up-regulation nor to BRCA1 knock-down by RNAi.

In MCF-7 cells BRCA1 up-regulation caused growth inhibition similarly as BRCA1 down-regulation did. This might exclude exact evaluation of rescue experiments. However, the magnitude of response caused by BRCA1 up-regulation was lower than that caused by BRCA1 RNAi. Thus, we expected to see some response in MCF-7 cell proliferation assay that may be specific to the RNAi-rescue.

We used MCF-7 cells infected with pWZL_Hygro plasmid expressing wtBRCA1* and BRCA1 variants c.300T>G, c.1866A>T, c.3819_3823del5 and c.5385dupC (all these BRCA1 forms are missing 3'-UTR and so are resistant to 3'UTR-directed RNAi) and selected by hygromycine. These cells were infected in the second round by pSUPER.retro.puro plasmids expressing sh_6069 and sh_6073 (control, "non-functional" shRNA) and LMP plasmid expressing sh_6965. In all cases corresponding empty plasmids were used as controls. After selection in puromycine, cells were plated for growth curves experiments. We observed rescue of sh_6069-mediated proliferation block in MCF-7 cells over-expressing wtBRCA1* (Fig. 7.4), but not in cells over-expressing mutated BRCA1 variants or empty plasmid (data not shown). Differences in cell cycle distribution corresponded to changes in the growth of MCF-7 cells (data not shown). This rescue was not observed for LMP-derived sh_6965 (data not shown) probably because sh_6965-mediated block in proliferation of MCF-7 cells is of much higher magnitude than that mediated by pSUPER-derived sh_6069 and wtBRCA1* over-expression is not sufficient to overcome this blockage. Interestingly, BRCA1 variants tested were non-functional and none of them was able to rescue sh_6069 phenotype (Fig. 7.4). This is in an agreement with the up-regulation studies showing that mutated BRCA1 variants effects cell function by haploinsufficiency rather that gain-of-function or dominant-negative effect [335].

Although the rescue experiments based on our proliferation assay have severe limitations, we can conclude that wtBRCA1*, but not mutated BRCA1 variants, is

able to rescue proliferation defect caused by wtBRCA1 knock-down by sh_6069-mediated RNAi. This conclusion has to be confirmed also in other assays and such experiments are in preparation.

Taken together, our results of wtBRCA1 RNAi experiments, BRCA1 up-regulation and combination of both approaches using proliferation assay revealed that mutated BRCA1 variants are defective in BRCA1 function and are not able to rescue proliferation defect mediated by wtBRCA1 knock-down. Concurrently, BRCA1 variants have no dominant-negative and/or gain-of-function effect in this assay. These observations favor the role of BRCA1 haploinsufficiency in tumorigenesis, similarly as described by others.

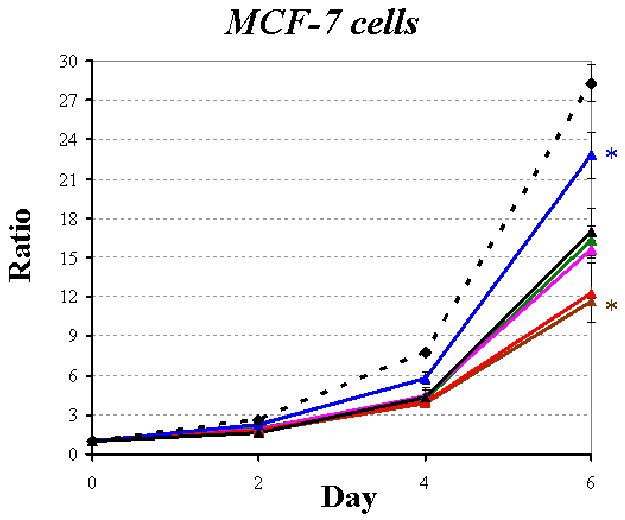

Figure 7.4. BRCA1-mediated rescue of RNAi proliferation defect in MCF-7 cells. MCF-7 cells expressing shRNA-resistant forms of wtBRCA1 and mutated BRCA1 variants were infected with pSUPER.retro.puro retrovirus expressing sh_6069, drug selected and used for growth assays. Cell proliferation was monitored over a 6-day period. Each value was normalized to the cell number at day 0. Black solid line: control; blue line: wtBRCA1; red line: c.300T>G; magenta line: c.1866A>T; brown line: c.3819_3823del5; green line: c.5385dupC. Proliferation of MCF-7 cells infected with empry pWZL_Hygro and empty pSUPER.retro.puro plasmids (dashed black line) is shown for comparison. Data are expressed as average ± SEM from a typical experiment performed in triplicates. Statistically significant differences (P-value p=0.05) are marked by * in colour matching particular growth curve (paired, two-tailed Student's T-test).

7.5. DISCUSSION AND FURTHER PERSPECTIVES

The goal of this study was to design a universal assay system suitable for functional analysis of mutations in *BRCA1* gene and possibly in other genes as well. We advantaged of RNAi and retroviral infections to combine down-regulation of endogenous wild-type BRCA1 with up-regulation of mutated BRCA1 variants, respectively. We used this system to successfully analyse the influence of c.300T>G, c.1866A>T, c.3819_3823del5 and c.5385dupC BRCA1 mutations on the proliferation of breast cancer cells. However, our assay system still suffers some limitations:

(a) Possible non-specific effect of shRNAs. These cannot be absolutely avoided. To minimize the chance of non-specific effects, we followed formulated standards for RNAi experiments [387,388] and designed several shRNA targeting different regions of BRCA1 3'-UTR. New additional shRNAs targeting BRCA1 3'-UTR region will be designed according to up-to-date standards and their performance will be tested in our assay.

(b) Low infection efficiency in BRCA1 over-expression. This is due to large insert size in retroviral vectors and corresponding decrease in packaging efficiency and infecting capacity of viral particles. Advantaneous approaches combining RNAi with "rescue" up-regulation in one plasmid were reported recently [389], but are bases on transfection which is inferior compared to infections in targeting primary cells isolation of permanent clones. Constructing similar "combined" plasmid in retroviral backbone will safe one infection but concurrently will further increase the length of plasmids and attenuated infection efficiency. Independent infections of RNAi and BRCA1 plasmids seem to be necessary for long genes such as *BRCA1*.

(c) Negative selection against cells with variations in BRCA1 expression. Because of the importance of BRCA1 for cell survival and proliferation, changing BRCA1 expression levels (either up- or down-) are likely to induce negative selection against cells with the strongest alterations in BRCA1 expression. Such selection becomes severe problem when it is necessary to passage cells for a longer period of time. Introducing conditional expression of shRNAs and/or BRCA1 can

reduce this effect. Conditional expression is widely used and is recently available also for the expression of shRNAs [347, 363, 390-393]. Construction of such plasmids is underway.

Besides improving our functional assay, we will focus on the following subjects:

(a) Detailed characterization of MCF-7 and MDA-MB-231 cells and their differential reaction to changes in BRCA1 expression levels. We will focus on the role of p53 and estrogen receptor α which expression is impaired in MDA-MB-231 cells compared to MCF-7 cells.

(b) We will implement other assays to our RNAi-based system to characterize the function of wtBRCA1 and BRCA1 variants. These will include dsDNA repair essay after IR and BRCA1 localization studies.

(c) Finally, we will modify our functional assay using BACs to implement BRCA1 expression within more physiological regulations. Dr. S.K. Sharan (MCGP, NCI-Frederick; personal communications) established the proof of the principle of such system for *BRCA2* gene. Such system consists of cells (mouse embryonic stem cells in the case of BRCA2 system) containing only one allele of BRCA2 (the other one is deleted by HR) that can be inactivated by Cre recombination. These cells are transfected by BAC expressing BRCA2 variant and after selection of positive clones, endogenous wild-type allele is inactivated by Cre recombination. Finally, only BAC-derived BRCA2 form is expressed in these cells and can be easily analyzed functionally.

Functional analysis of BRCA1 mutations is currently based mostly on computational modeling of BRCA1 protein structure changes induced by particular mutation. However, such predictions are of limited accuracy. Similarly, simple over-expression of mutated BRCA1 may be inaccurate because of cell-type and background BRCA1 expression-specific effects are strongly influencing observed BRCA1 function. Here, we successfully used our versatile system combining wtBRCA1 down-regulation by RNAi and retroviral-mediated up-regulation of

mutated BRCA1 variants to characterize role of BRCA1 in proliferation of breast cancer cell lines. We continue to improve our assay system to establish a platform for broad functional analysis of BRCA1 variants. This assay system will be an important element in implementing our long-term goal to functionally characterize BRCA1 variants emerging in the population of women with hereditary breast and/or ovarian cancer in the Czech Republic.

8. CONCLUSIONS

The multifunctional BRCA1 tumour suppressor takes part not only in physiological regulations, but also in pathogenesis of several diseases including breast cancer. Exact characterization of the mechanisms regulating BRCA1 activity is a prerequisite for possible therapeutical interference.

1. We set up several methods for characterization of BRCA1 gene and protein previously not available in our laboratory. These include retrovirally-mediated RNA interference, BRCA1 up-regulation using regular plasmids as well as bacterial artificial chromosomes (BACs), western blotting, luciferase-based reporter system and flow cytometry.

2. We designed eight shRNAs sequences targeting 3'-UTR of BRCA1 mRNA and confirmed their potential to down-regulate endogenous wild-type BRCA1 on mRNA and protein levels. This was achieved both by transient transfection (short-term, temporary down-regulation) and infection (long-term, stable down-regulation).

3. No significant functional difference between the expression of shRNAs from H1-driven (RNA polymerase III) and CMV-driven (RNA polymerase II) promoters was observed. However, H1-driven shRNA expression may be more suitable for short-term down-regulation, while the CMV-driven shRNA expression is better for stable, long-term down-regulation.

4. Down-regulation of endogenous BRCA1 as well as over-expression wild-type BRCA1 decreased the growth potential of MCF-7 breast cancer cell line. This effect was cell line-specific, since similar alterations in growth properties were not observed in MDA-MB-231 and HeLa cells.

5. Over-expression of BRCA1 variants c.300T>G, c.1866A>T and c.3819_3823del5 revealed no dominant-negative or gain-of-function effect in MCF-7 and MDA-MB-231 cells. Over-expresssion of BRCA1 variant c.5385dupC decreased the proliferation rate of MCF-7 cells similarly as shRNA-mediated BRCA1 knock-down, indicating possible dominant-negative, antagonistic effect of c.5385dupC variant on wild-type BRCA1. This effect was cell line specific and was not observed in MDA-MB-231 or HeLa cells.

6. RNAi mediated wild-type BRCA1 knock-down was rescued by RNAi-resistant wild-type BRCA1 form but not mutated BRCA1 variants. This observation, together with the lack of dominant-negative or gain-of-function effect in overexpression experiments, favors BRCA1 haploinsufficiency as an important pathological mechanism in breast cancer tumorigenesis.

7. We observed cell-line specific functional consequences of changes in BRCA1 expression level between MCF-7 and MDA-MB-231 breast cancer cell lines. Differences between these cell lines will be analyzed in more details to characterize potential modifiers of BRCA1 function.

8. Pilot experiments of BRCA1 up-regulation in more physiological settings using bacterial artificial chromosomes (BACs) and their incorporation into our assay system were performed.

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