STUDIES OF BRCA1 PROTEIN USING CRISPR/CAS9 GENE EDITING

IN HCC1937 BREAST CANCER CELLS

by

Ali Hassan Baity

B.S. (Jazan University) 2011

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

December 2018

Accepted:

_____	_____
Oney Smith, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
_____	
Georgette Jones, Ph.D. Committee Member	
_____	_____
Rachel Beyer, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

STATEMENT OF USE AND COPYRIGHT WAIVER

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DEDICATION

I dedicate this mock grant proposal to my parents, my wife and my siblings. I hope this achievement meets your expectation and fulfills the dream that you had. Thank you for pushing me hard and believing in me. Thank you for your support with my studies all theses years.

ACKNOWLEDGEMENTS

It would not possible to write this mock grant proposal without the help and support of the kind people around me.

Above all, I would like to thank my lovely wife Anoud for her personal support and great patience at all the time. My parents, sisters, brothers and friends have given me all support.

I would like to thank the professors at Hood College, my advisor Dr. Beyer, and my committee of Dr. Smith and Dr. Jones and the program director Dr. Boyd.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
STUDIES OF BRCA1 PROTEIN USING CRISPR/CAS9 GENE EDITING

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)

3b. DEGREE(S)

3h. eRA Commons User Name: N/A

3c. POSITION TITLE: Graduate Student

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Department of Biology

3f. MAJOR SUBDIVISION: Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: N/A FAX: N/A

3d. MAILING ADDRESS *(Street, city, state, zip code)*
401 Rosemont Ave.
Frederick, MD 21201

E-MAIL ADDRESS:

4. HUMAN SUBJECTS RESEARCH ☐ No ☐ Yes

4a. Research Exempt: N/A

4b. Federal-Wide Assurance No.: N/A

4c. Clinical Trial ☐ No ☐ Yes

4d. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☐ Yes

5a. Animal Welfare Assurance No.: N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name
Title: Graduate Student
Address: Hood College
401 Rosemont Ave
Frederick, MD 21701
Tel: N/A FAX: N/A
E-Mail:

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name
Title: Graduate Student
Address: Hood College
401 Rosemont Ave
Frederick, MD 21701
Tel: N/A FAX: N/A
E-Mail:

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE

PROJECT SUMMARY (See instructions):

The mutation in the BRCA1 gene in HCC1937 breast cancer cell lines produces a truncated protein that prevents interaction between BRCA1 protein and its partner called CtIP protein. The DNA repair processing needs this interaction to initiate double-strand repair. CRISPR/Cas9 system gene editing will be used to correct this mutation by deleting the insertion of cytosine at position 5382 in the wild type BRCA1 protein. Co-immunoprecipitation (Co-IP) and western-blot assays will be used to confirm the protein interaction after correction by CRISPR/Cas9 system gene editing. Moreover, DNA repair and IR sensitivity assays will be conducted study the BRCA1 response in these cell lines. The rate of proliferation, and invasion between mutated, corrected and normal cell lines will be measured by using MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Transwell assays.

RELEVANCE (See instructions):

Breast cancer is the second leading cause of cancer deaths among women, and about 2% of breast cancers are linked to BRCA1 gene mutations. The goal of this study is to use CRISPR/Cas9 system gene editing *in vivo* correction of a mutated gene linked to breast cancer.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Ali Hassan Baity	Full time student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Jazan University, Saudi Arabia	B.S.	2011	Medical Laboratory Science
Hood College, Frederick, MD	M.S.	2018 (anticipated)	Biomedical Science

A. Positions and Honors

NA

B. Selected peer-reviewed publications (in chronological order)

NA

C. Research Support

NA

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Hood College labs

Clinical:

None

Animal:

None

Computer:

Hood College

Office:

None

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges
- Microscope
- Refrigerator
- Miropipettee
- Tissue Culture Hoods

The following consumables will be purchased with grant funding:

- Protein and DNA Ladders
- Petri Dishes
- HCC1937 cell lines
- MCF-10A cell lines
- Antibodies - For western blot and Co-IP assays
- CRISPR and HDR plasmid
- MTT and Transwell assay
- Lipofectamine Reagent
- MTT reagent

SPECIFIC AIMS

Mutation in BRCA1 gene has been associated with breast cancer and prevents the BRCA1 gene to involve in DNA repair. CRISPR/Cas9 system will be used to correct the mutation in BRCA1 gene to rescue BRCA1 protein.

The aims of this study are to correct the BRAC1 mutation in HCC1937 cancer cells employing CRISPR/Cas9 and homology-directed repair followed by:

1. Analysis of BRCA1-CtIP proteins interactions using co-immunoprecipitation and western-blot assays.
2. Assays for DNA double-strand break repair (DSBR) and gamma radiation sensitivity.
3. Studies of the rate of proliferation and invasion using MTT and Transwell assays respectively.

BACKGROUND AND SIGNIFICANCE

Application of CRISPR/Cas9 for Gene Editing in Cultured Cells

The clustered regularly interspaced short palindromic repeats /CRISPR-associated 9 (CRISPR/Cas9) system initially discovered in Escherichia coli in 1987 and then observed in various genome of Bacteria and Achaea (Horvath *et al.* 2010). CRISPR is a system of prokaryotic adaptive immunity that the CRISPR/Cas9 technology comes from (Horvath *et al.* 2010). CRISPR/Cas9 system has found in about 90% of archaea and about 50% of bacteria and it is involving in adaptive system as a defense of viral infection. In this system, in the first viral infection, the viral DNA or invaded plasmid will be cleaved into novel spacer and stored in an array in DNA. When the same virus or plasmid invades as a second infection, the matching invading DNA will be recognized and interfered (Cui *et al.* 2018). Many studies suggest that CRISPR/Cas9 system can be used in eukaryotic cells by co-expressing Cas9 enzyme from *Streptococcus pyogenes* and short guide RNA (gRNA). Simplicity, flexibility, adaptability and ease of use, are the powerful of CRISPR/Cas9 system. Also, CRISPR/Cas9 system can achieve multiplex targeting by the use of multiple gRNAs (White *et al.* 2016). As shown in Figure 1, CRISPR/Cas9 system consists of many parts such as Cas9, gRNA and PAM. Cas9 is an endonuclease enzyme that target DNA sequences and has no basic sequence specificity. Short guide RNA (gRNA) is designed to consist of 20 nucleotides which guide the Cas9/ gRNA complex to the target sequence which also contains a sequence known as the proto-spacer adjacent motif (PAM). Short guide RNA (gRNA) is derived from a fusion of the tracrRNA (trans-activating crRNA) and crRNA (CRISPR RNA) (Cui *et al.* 2018). The proto-spacer adjacent motif (PAM) is trinucleotide sequence immediately following the target sequence (White *et al.* 2016).

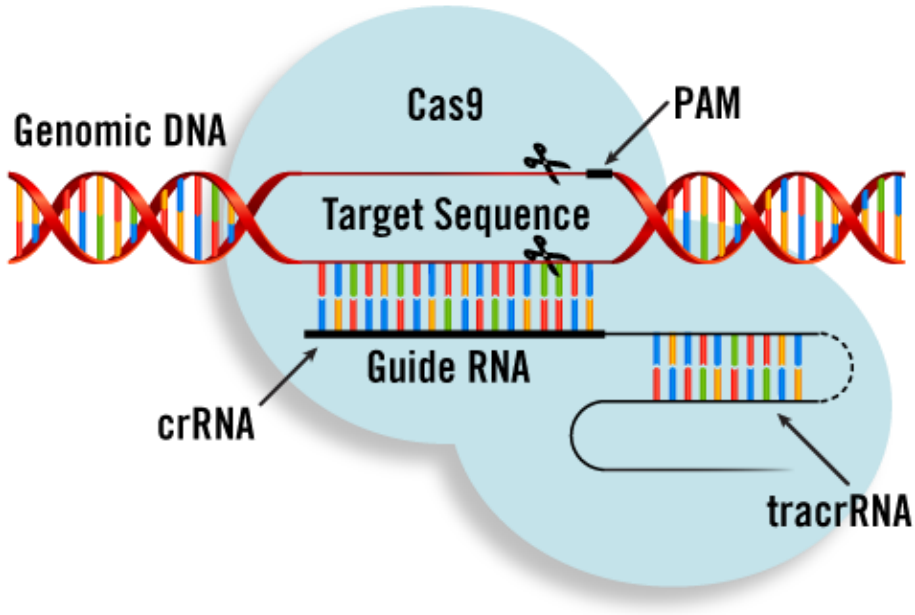

Figure 1. The structure and components of CRISPR/Cas9 systems (Levien 2017).

At the point that lies 3-4 nucleotide upstream of the PAM, Cas9 cuts both DNA strands making a double-strand break (DSB). As shown in Figure 2, repairing the DBSs is made by DNA repair pathways including non-homologous end joining (NHEJ) and homology-directed DNA repair (HDR). NHEJ likely generates insertions /deletions (InDels) at the DSB site and this can lead to frameshift or premature termination codons. Moreover, if the purpose for the cut is to produce loss off function, the site of the cut can be an active promoter or the coding sequence to inactivate the specific gene. HDR needs a homologous DNA template to repair the DBS site by introducing new sequences into the gene of interest. Correcting the protein is usually made by HDR because the DNA donor template has the wild type sequence for the specific protein that has been rescued (Cui *et al.* 2018).

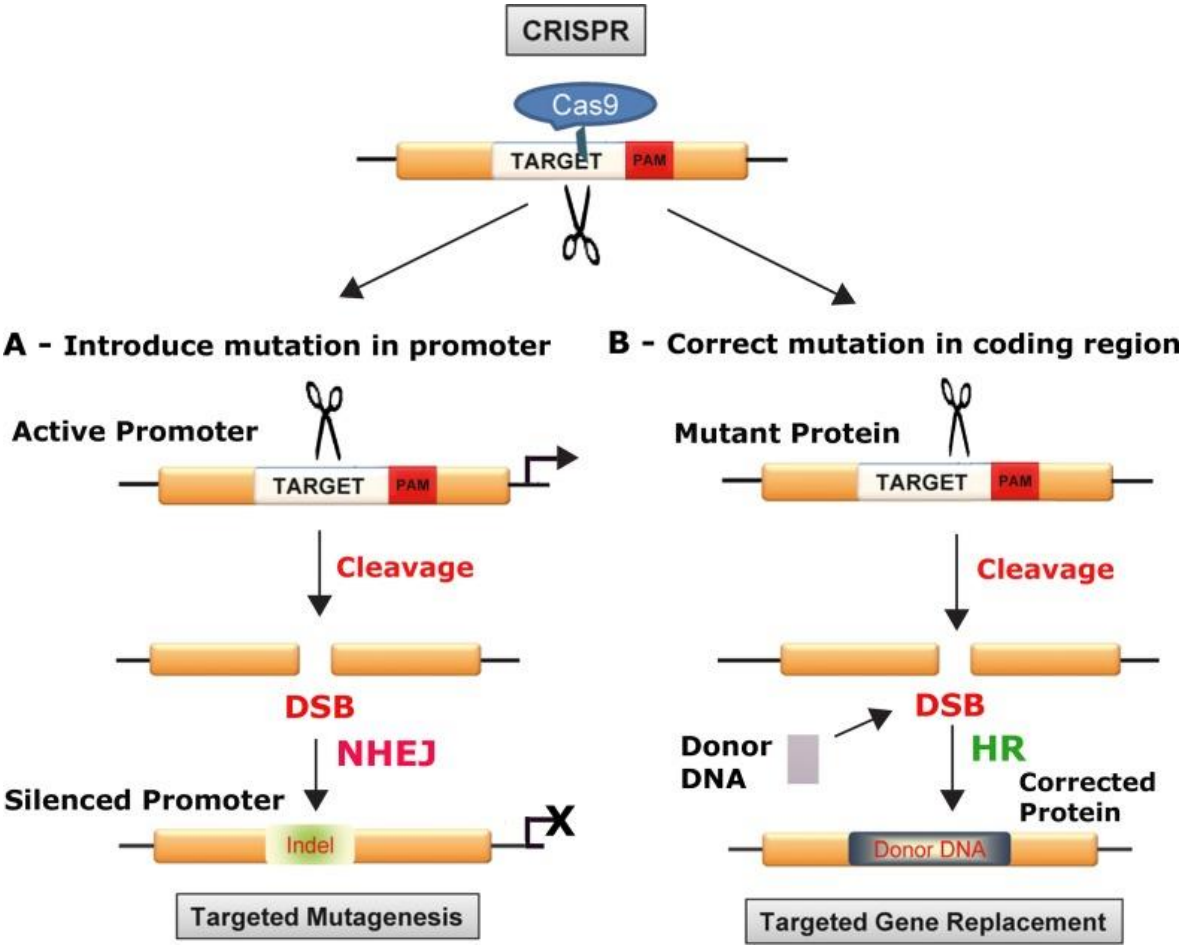

Figure 2. DNA repair pathways after CRISPR/Cas9 system cuts at target site (White *et al* 2016).

The CRISPR/Cas9 system can be used in various ways for genome engineering. As shown Figure 3, there are four examples that apply the CRISPR/Cas9 system in gene editing. For instance, gene knockouts are made by the NHEJ DNA pathway, which introduce InDels and frameshift mutations in the coding the region of the gene. When the gene has mutation, the CRISPR/Cas9 system can correct the mutation, however, this correction requires an exogenous DNA donor template (Lino *et al.* 2018).

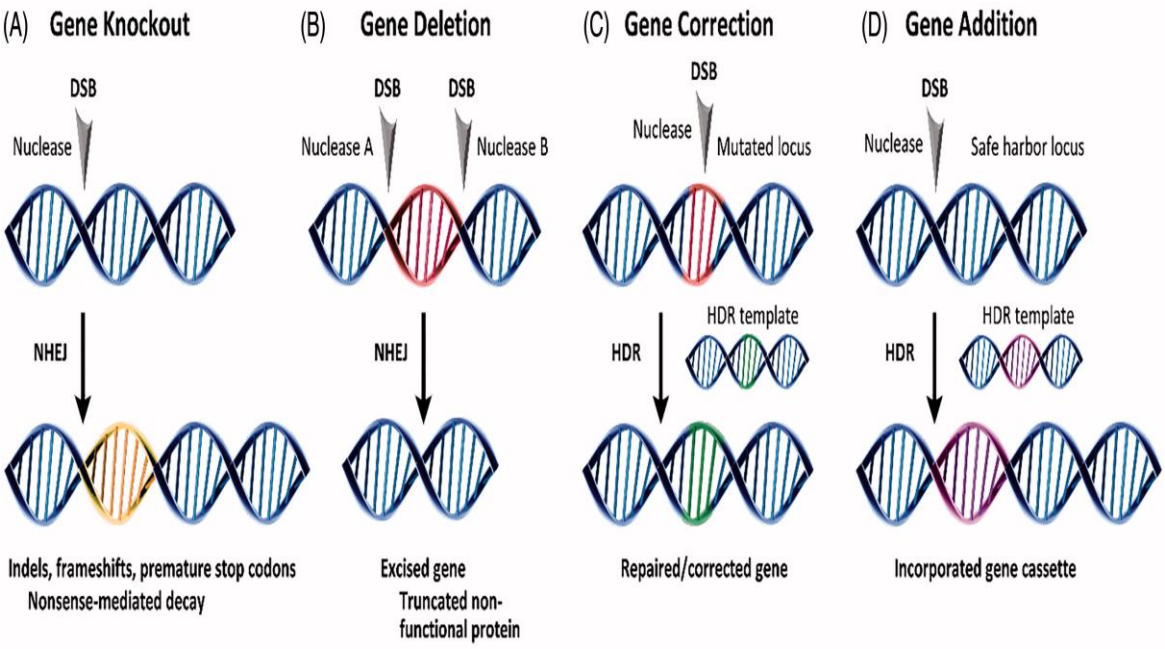

Figure 3. The application of the CRISPR/Cas9 system (Lino *et al.* 2018).

The efficient delivery of CRISPR/Cas9 system to the target sequence is the greatest challenge for genome engineering. There are number of approaches that are possible to use such as viral transduction or non-viral methods (White *et al.* 2016). For the viral transduction approach, adenovirus, adeno-associated virus (AAV) and lentivirus can be used as viral delivery methods. Non-viral delivery methods use liposomes, microinjection, and electroporation (Lino *et al.* 2018).

BRCA1 and Breast Cancer

Breast cancer is a complex disease caused by genetic and/or non-genetic factors (Zhang *et al.* 2012). This cancer accounts for the second leading cause of death among women (Drost *et al.* 2016). Germline mutations in the breast cancer tumor suppressor

gene 1 (BRCA1) increase the risk to develop breast cancer and about 2% of breast cancers are genetically linked to BRCA1. The breast cancer tumor suppressor gene 1 (BRCA1) is located on chromosome 17, specifically at 17q21; it consists of 24 exons and 22 of which are coding exons and 2 of which are non-coding (Miki *et al.*1994; Mullan *et al.* 2006; Silver *et al.* 2012). Figure 4 shows that the BRCA1 full-length protein encodes 1863 amino acids and contains multiple conserved domains. The protein contains a RING domain which encodes about 24–64 amino acids at N-terminus, a coiled-coiled domain that encodes about 73 amino acids and two copies of the BRCT domain at C-terminus (Miki *et al.* 1994; Moynahan *et al.* 2010; Silver *et al.* 2012). The protein also has functional nuclear import and nuclear export signals suggesting it can shuttle between the nucleus and cytoplasm (Rosen *et al.* 2013).

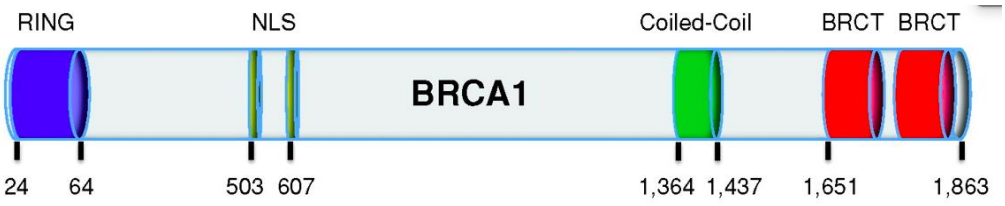

Figure 4. Structure of BRCA1 protein (Silver *et al.* 2012).

The integrity of the genome is maintained partially by BRCA1 protein which involves in DNA repair, and cell cycle control (Mullan *et al.* 2006; Roy *et al.* 2012). The cell cycle checkpoints slow the cell cycle progression when there is a stress to allow the DNA repair to happen. When the damage is too great, the cell undergoes celly cycle arrest

and apoptosis (Roy *et al.* 2012). Interestingly, the BRCA1 protein plays an important role in maintaining genome stability via the homologous recombination repair (HRR) pathway to repair breaks in double-strand DNA (Mylavarapu *et al.* 2018). Also, BRCA1 can be a part of genome surveillance complex named BASC which contains a number of proteins that are important for efficient DNA repair. These proteins include Mre11, Rad50, and Nbs1 (MRN) repair complex, MSH2/MSH6 proteins that has involved in mismatch repair, and the DNA-damage signaling protein kinase ATM (Liu *et al.* 2001). The BRCA1 carboxy-terminal (BRCT) domain consists about 100 amino acids and this domain is involved in the cellular response to DNA damage (Silver *et al.* 2012). Mutations in BRCA1 associated with tumors are reported to have a truncation or complete loss of BRCT motifs. The HCC1937 human breast cancer cell line expresses a truncated BRCA1 protein that has a loss of the C-terminal BRCT domain. Insertion of cytosine at position 5382 (5382insC) causes this mutations resulting from a premature frameshift mutation. This change in the BRC1 protein abolishes the interaction pf the protein with CtIP (Zhang *et al.* 2017). This mutation results in failure of the cell line to undergo apoptosis (Thangaraju *et al.* 2000) therefore, the mutated protein lacks tumor suppressor activity.

Interaction Between BRCA1 and CtIP Proteins

The BRCA1 protein can associate with different proteins namely CtIP, BACH1, BARD1, BAP1, and BACH1. In the Figure 5, CtBP interacting protein (CtIP) consists of 897 amino acids and has BRCA1 binding site (Wang 2012). In addition to binding BRCA1, CtIP also can interact with co-repressor CtBP (C-terminal binding protein) and tumor suppressor Rb (retinoblastoma protein). CtIP protein has a phosphorylation site to bind

BRCA1 protein. The BRCA1 gene can bind to CtIP through its BRCT domains at C-terminus (Chen *et al.* 2008).

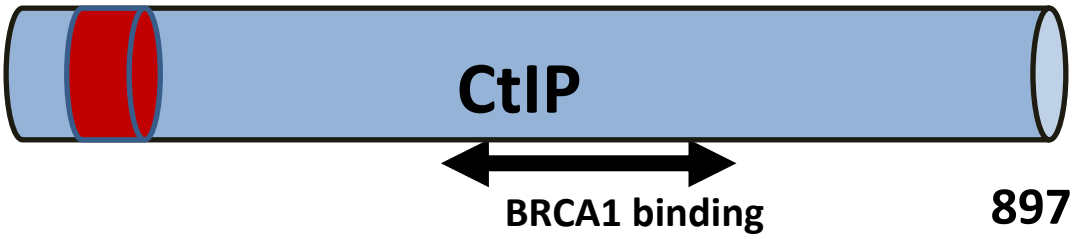

Figure 5. The structure of CtIP protein (Wang 2012).

There are three complexes that BRCA1 proteins forms to make roles in cell cycle and DNA repair including complex A, complex B and complex C as shown in Figure 6. The BRCA1-A complex facilitates DNA damage by inducing signaling for recruitment of BRCA1 to the site of DSBs. The BRCA1-B complex is required to make replication stress, which induces DNA inter-strand crosslink repair. The BRCA1-C complex, which contains MRN complex, and CtIP proteins, is important for DNA end resection to generate single-strand DNA required for homology repair to mediate DSB repair. The CtIP and MRN complex, which interacts with BRCA1, accelerates end processing, and this binding is drives the repair of DSB to the HR pathway (Saha *et al.* 2016). The CtIP protein promotes DNA end resection by interacting and stimulating the nuclease activity of the MRN complex (Wang 2012).

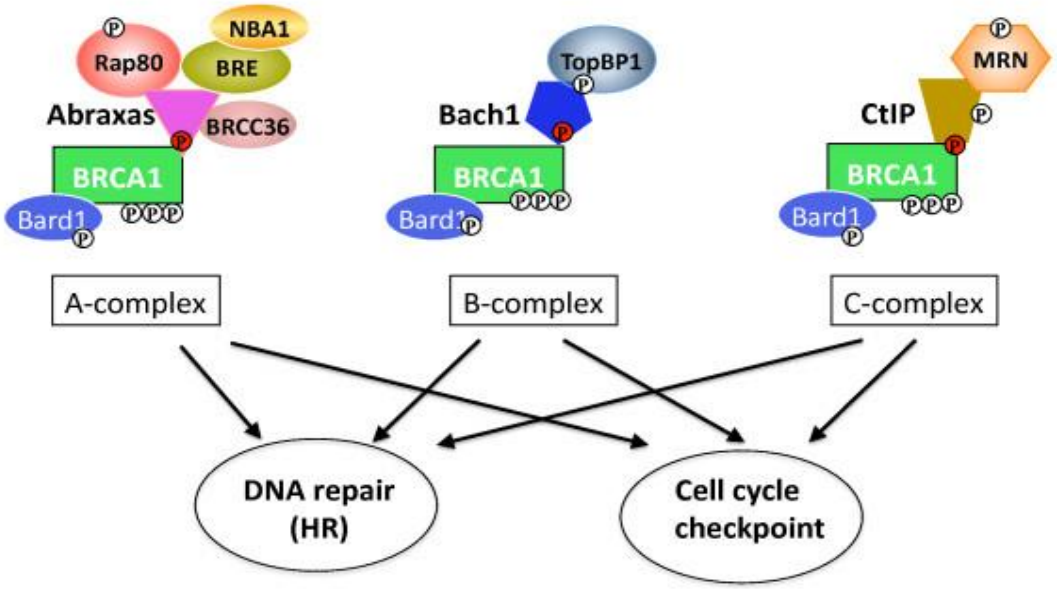

Figure 6. Complexes formed by BRCA1 protein and roles in cell cycle and DNA repair (Wang 2012).

The binding between BRCA1 and CtIP proteins is important for double-strand break repair especially during the cell cycle (Saha *et al.* 2016). The level of the BRCA1 protein varies during the cell cycle; the expression level of BRCA1 protein increases during the S to G2/M phases but it remains in low level in other phases. The CtIP protein expression varies in parallel with BRCA1. The steady-state level of BRCA1 and CtIP proteins expression is low in G1 and resting cycle, but it reaches the peak in late S and G2 phases (Chen *et al.* 1996). During the S phase of cell cycle, BRCA1 plays a critical role by promoting homologous recombination (HR). In addition, it plays a key role attenuating classical non-homologous end-joining (C-NHEJ) to prevent inappropriate repair of double-strand breaks (DSB) (Saha *et al.* 2016).

PRELIMINARY REPORT / PROGRESS REPORT

The BRCA1 protein can keep the genome stable by repairing double-strand DNA breaks using homologous recombination repair (HRR) pathway. The C-terminus BRCA1 (BRCT) domain interacts with CtIP protein and the 5382insC mutation in BRCT abolishes this interaction (Mylavarapu *et al.* 2018). The normal BRCA1 gene can reduce the sensitivity of cells to radiation (IR) which induces breakage of DNA. The mutated BRCA1-HCC1937 cells are more IR sensitive and when wild type BRCA1 is expressed in HCC1937 cells, the cells become less sensitive to IR radiation. As presented in Figure 7, the BRCA1 wild type gene, expressed using a viral vector in HCC1937 breast cancer cells, produces cells that are less sensitive to IR radiation, whereas control HCC1937 cells were more sensitive. At the starting gamma dose, the colony viabilities were the same in both cell types and almost 100%, but when the dose went higher, the cells were starting to die in both cell lines. At the highest dose of 4 Gy, the experimental HCC1937 cells (BRCA1 wild type) were more resistant to gamma radiation. In addition, as shown Fig. 8, the DNA double-strand break repair assay results show the experimental HCC1937 cells (BRCA1 wild type) was more successful restoring double-strand break repair (Scully *et al.*1999). Overall, these results show that experimental HCC1937 cells (BRCA1 wild type) are less sensitive to gamma radiation and have improved times for repairng DNA damage. Therefore, these results suggest that if the BRAC1 mutation in HCC1937 cancer cells is corrected by CRISPR/Cas9 gene editing, the function of protein can restored.

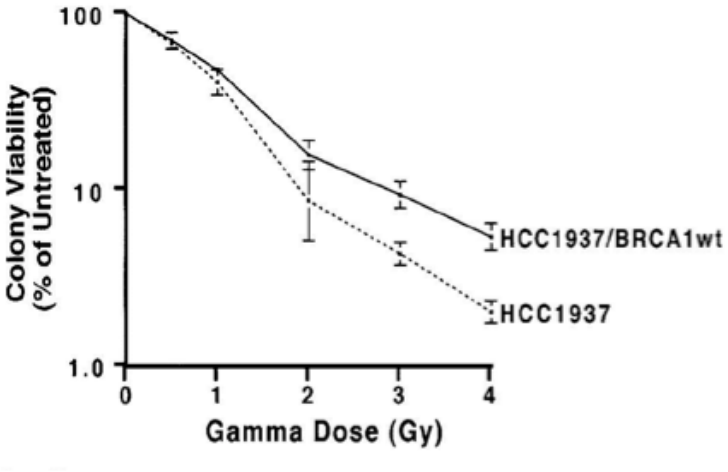

Figure 7. The difference between HCC1937 and HCC1937/BRCA1 wild type in IR sensitivity (Scully *et al*. 1999).

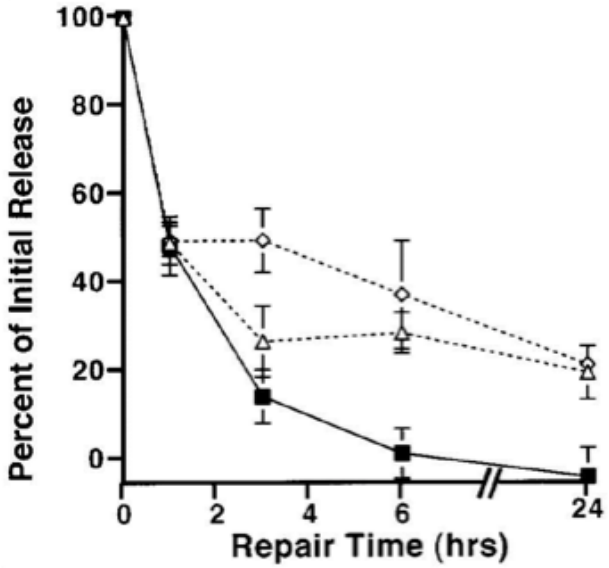

Figure 8. HCC1937/BRCA1 wild types consume less time in DNA repair (Scully *et al.* 1999).

RESEACH DESIGN / METHODS

The CRISPR/Cas9 system will be used to correct the insertion of cytosine at position 5382 (5382insC) in the mutated BRCA1 HCC1937 breast cancer cell line. This system needs elements to achieve the correction of mutation such as two plasmids, delivery methods, indicators, targeting sequence, gRNA and sequencing to confirm that the mutation has been corrected.

The strategy is to deliver the Cas9 and the guide RNA targeting the BRCA1 area of interest on one plasmid and use a second plasmid that expresses a corrected BRCA1 sequence (donor plasmid): the inserted C (position 5382) sandwiched between homology arms to the BRCA1 gene. The design of these plasmids will include the following considerations:

a. The homology arms (donor plasmid) should be as close to the cut site as possible (definitely less than 100 bp).

b. The PAM sequence (NGG) used to direct the Cas9 to the targeted site in the genomic DNA should be mutated in the donor plasmid to avoid re-cutting by Cas9.

c. Off-target effects will be determined by whole genome sequencing and analysis.

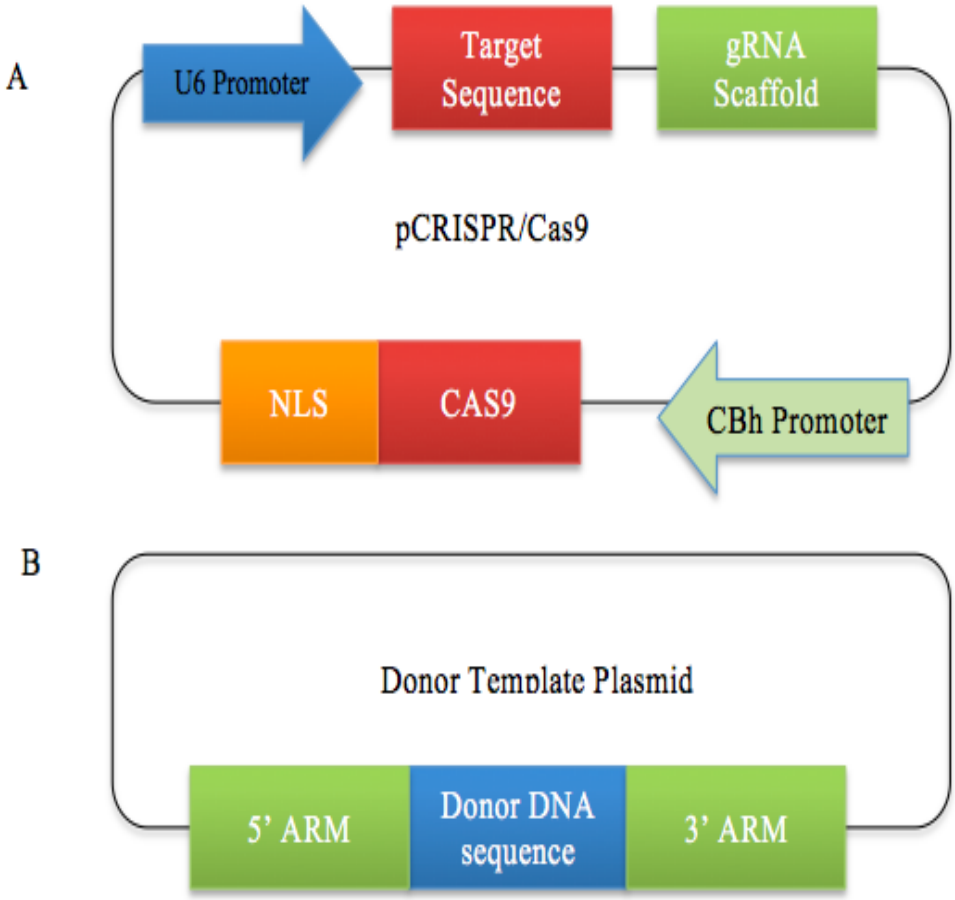

Figure 9. The two components of the CRISPR/Cas9 system used for the DNA repair. A. Schematic of target plasmid. The target sequence will be designed using the bioinformatics tool CHOPCHOP (Labun *et al.* 2016). B. The plasmid containing the donor DNA sequence to correct the 5382insC mutation in the BRCA1 gene by homologous recombination.

Table 1. Critical sequences proposed in this project using the CRISPR Cas9 system. Target sequences will be determined by used bioinformatics tool (CHOPCHOP) (Labun *et al.* 2016). Final sequence determination for donor template (homology arms) and GuideRNA will be optimized as a part of this project. N indicates that the PAM sequence in the donor DNA template will be changed to avoid Cas9 to cut the donor sequence.

Name	**Sequence**
Target Sequence	CAAGGTCCAAAGCGAGCAAGAGAATCCCCAGGACAGAAAGGTAAAGCTCCC
DNA Donor Template	CAAGGTCCAAAGCGAGCAAGAGAATCCCNGGACAGAAAGGTAAAGCTCCC
Guide RNA	AAGCGAGCAAGAGAATCCCCAGG (PAM)

To deliver the two plasmids into HCC1937 breast cancer cell line, non-viral transduction will be used. HCC1937 Transfection Kit will be purchased from Altogen Biosystem (Catalog No. 6807). Figure 10 shows the principle of this transfection method. The charge part of the liposome binds with negative charge of DNA. The complex inserts by the endocytosis and the DNA will escape the endosome and goes toward the nucleus to express and complete the CRISPR/Cas9 system processing. Following the transfection protocol, the HCC1937 cells plate from 1 x 10^4 to 1.5 x 10^4 in each 24-well plate and incubate up to 24 hours with complete medium. The transfection complex, which contains transfection reagent and plasmids, will be added to each well and incubate up to 72 hours.

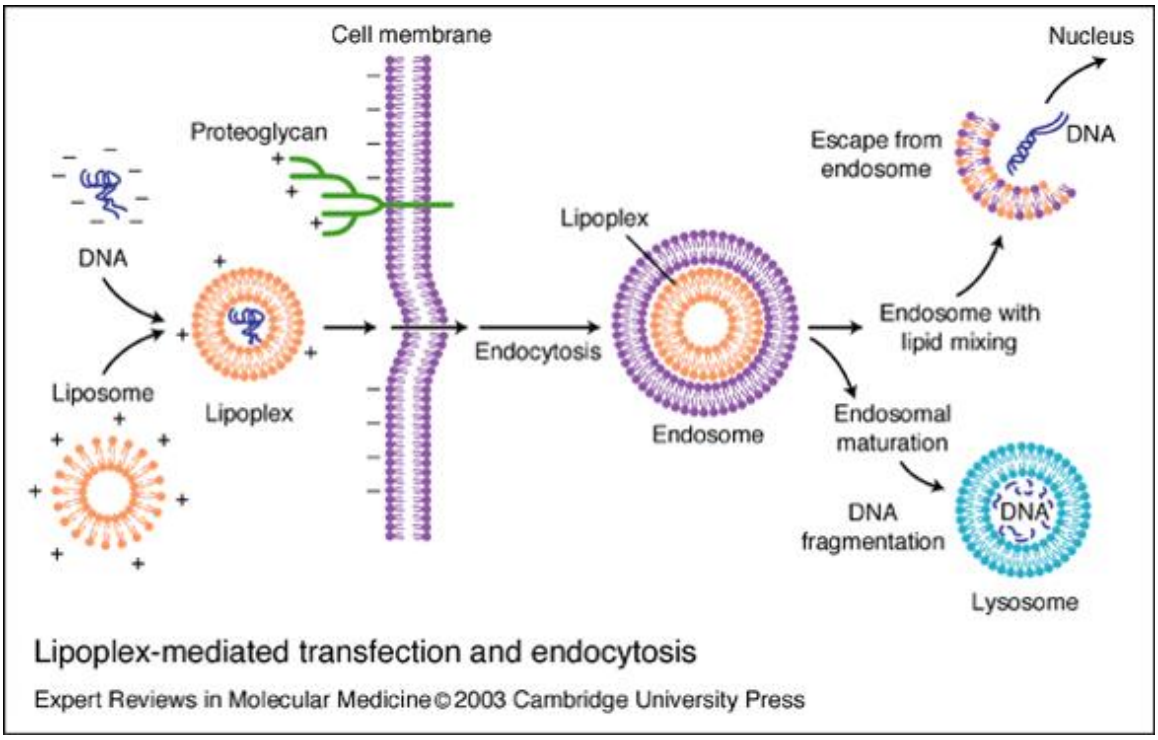

Figure 10. The process of transfecting cultured cells using liposomes (Altogen Biosystems, 2018).

Single cell cloning and culturing will be conducted to screen for cells that have a corrected BRCA1. The hemi-nested polymerase chain reaction (PCR) and computer-generated sequencing electropherogram will be used to confirm the sequence for the corrected BRCA1 in HCC1937 breast cancer cell lines. The hemi-nested PCR products will be used as templates for direct DNA sequencing of corrected BRCA1 gene. Table 2 shows the sequences for the primers for this sequencing strategy based on primary and hemi-nested PCR (Lee *et al*. 2016).

Table 2. Primers that will be used in primary and hemi-nested PCR to produce amplicons for Sanger DNA sequence analysis (Lee *et al*. 2016).

Name	Sequence of Primers
Primary PCR	Forward primer 5'-GTCTGCTCCACTTCCATTGAAG-3'
	Reverse primer 5'-GATGGAAGAGTGAAAAAAGAAC-3'
Hemi-nested PCR	Forward primer 5'-GAAGCTTCTCTTTCTCTTATCC-3'
	Reverse primer 5'GATGGAAGAGTGAAAAAAGAAC-3'

The predicted sequence of the corrected BRCA1 gene from HCC1937 breast cancer cells shown as an electropherogram in Fig. 11. The wild type sequence of corrected BRCA1 gene is CGAGCAAGAGAATCCCAGGACAGAAAGGTAA, and it shows there are only CCC nucleotides rather than CCCC nucleotides in mutated BRCA1 gene (Lee *et al*. 2016).

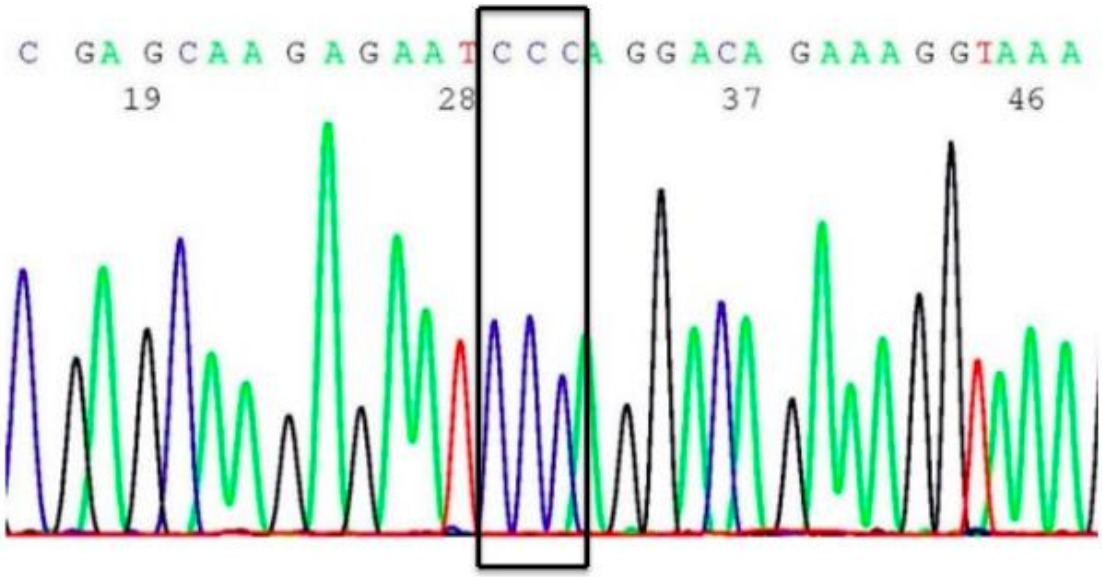

Figure 11. An example of a computer-generated sequencing electropherogram of the hemi-nested PCR product that is expected should the mutation in BRCA1 HCC1937 breast cancer cells be successfully corrected by the CRISPR/Cas9 system (Lee *et al.* 2016).

The whole genome sequencing of corrected cell samples will be conducted to determine the off-target effects of CRISPR/Cas9 system. The extracted DNA will be amplified by PCR and followed by Sanger sequencing. Each fragment will be amplified by primer and the applied the protocol of Sanger sequencing to sequence the whole BRCA1 gene (Lee *et al.* 2016).

Aim 1: Analysis of BRCA1-CtIP Proteins Interaction

HCC1937 and MCF-10A will be obtained from American Type Culture Collection (ATCC). HCC1937 breast cancer cell lines have mutated BRCA1 whereas; MCF-10A is a normal breast cell line and used as positive control. Following the protocol from ATCC, formulated RPMI-1640 Medium of HCC1937 cell line will be removed and will be discarded. The cell layer will rinse with phosphate buffer saline. The complete medium (ATCC-formulated RPMI-1640 Medium and fetal bovine serum 10%) will be added incubate culture at 37°C (ATCC, 2018). The Mammary Epithelial Basal Medium (MEBM) of MCF-10A cells will be discarded and will be removed, and the cells will be rinsed with phosphate buffered saline (PBS). The cells incubate with 0.05% trypsin, 0.53 mM EDTA at 37°C will be added as trypsin inhibitor. The complete medium will be added to the culture.

There are three cell lines including wild type BRCA1 in MCF-10A normal breast cell line as a positive control, corrected BRCA1 in HCC1937 breast cancer cell line and non-corrected BRCA1 in HCC1937 breast cancer cell line. Co-immunoprecipitation (Co-IP) and western-blot assays will be used to confirm the interaction between BRCA1 and CtIP proteins in corrected BRCA1 in HCC1937 breast cancer cell line and non-corrected BRCA1 in HCC1937 breast cancer cell line and compare the result to the positive control MCF-10A cell line. The principle of co-immunoprecipitation (Co-IP) is based on using antibody-antigen complex to detect specific protein-protein interactions. The cell lysates are incubated with the antibody that specific to the protein of interest, and then the unbound antibody is washed. The protein A/G plus agarose bead will be added and the antibody will be bounded to the bead. The binding protein will be eluted from the antibody-bead complex

and the sample transferred to a new tube (Phizicky *et al.* 1995). Co-immunoprecipitation will be carried out using the three cell lines. The cells will be washed with ice-cold phosphate buffered saline (PBS) and will be lysed by RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% deoxycholate) containing protease inhibitors (Zhu *et al.* 2018). The extracted proteins will be incubated with D9-BRCA1 protein antibody (sc-6954, Santa Cruz Biotechnology, MD, USA) and then the Protein A/G PLUS-Agarose Beads (sc-2003, Santa Cruz Biotechnology, MD, USA) will be added. After incubation, the binding proteins will be eluted from the beads with SDS Sample Buffer (62.5 mmol/L Tris pH 6.8, 2% SDS, 10% glycerol, 0.1 mol/L DTT, 0.005% bromophenol blue, 2% 2-mercaptoethanol) (Chiharu *et al*. 2018). Co-immunoprecipitation will be also conducted using corrected BRCA1-HCC1937 and mutated BRCA1-HCC1937 cells for CtIP protein by using D4-CtIP antibody (sc-271339, Santa Cruz Biotechnology, Maryland, USA).

Western-blot analysis is used to identify proteins using antibodies. The proteins mixtures are separated based on molecular weight by the gel electrophoresis. The gel will be transferred into a membrane and this membrane is incubated with antibodies specific to the protein of interest, and the unbound antibody is washed. Bound antibody and protein are then incubated with a secondary conjugate antibody. The bound antibodies are then detected by developing the film and only one band should be visible because the antibodies only bind to protein of interest. The intensity of the band represents the amount of protein present (Mahmood *et al*. 2012). The proteins mixtures of each cell lines will be separated on a 10% SDS-polyacrylamide gel and then transferred onto UltraCruz Polyvinylidene Fluoride (PVDF) transfer membranes (sc-3723, Santa Cruz Biotechnology, Maryland,

USA). The membranes will be blocked with UltraCruz Blocking Reagent, then incubated with primary antibodies D4-CtIP antibody or D9-BRCA1 protein antibody. Tmembrane is washed and then incubated with mouse IgG kappa-binding protein (m-IgGκ BP) conjugated to Horseradish Peroxidase (HRP) (sc-516102, Santa Cruz Biotechnology, Maryland, USA). The protein band will be visualized by using a chemical chemiluminescence imaging system (Zhu *et al*. 2018). The result of both assays will be compared to the positive control MCF-10A cell line.

Expected Results

In MCF-10A cell lines lysate, I expect that the Co-IP and western-blot assays result will show a band for the BRCA1 and CtIP proteins when using antibodies for BRCA1 and CtIP proteins. This result would indicate BRCA1 and CtIP proteins could be detected in MCF-10A cell lines lysate and also in the input lane, which has the whole cell lysate. Moreover, the result would indicate there is an interaction between BRCA1 and CtIP proteins.

In the corrected BRCA1-HCC1937 cell lines lysate, I expect that the Co-IP and western blot result will show a band for the BRCA1 and CtIP proteins and this result would indicate these proteins can be detected in the corrected BRCA1-HCC1937cell line after corrected by CRISPR/Cas9. The input lane, which has the whole cell lysate of the corrected BRCA1-HCC1937cell line, will show there is a band for BRCA1 and CtIP proteins.

In the non-corrected BRCA1-HCC1937 cell lines lysate, I predict that Co-IP and western blot result will show there is no band for BRCA1 proteins and this would indicate this proteins cannot be detected in the non-corrected BRCA1-HCC1937cell line lysates.

The input lane which has the whole cell lysate of the non-corrected BRCA1-HCC1937 cell lines will show there is a band for the BRCA1 and CtIP proteins but the band of the BRCA1 protein is not the same as control.

Aim 2: Analysis of DNA Double-Strand Break Repair and Gamma radiation (IR) sensitivity

The DNA double-strand break repair time and the effect of the BRCA1 on that repair in all three cell lines will be analyzed by using DNA double-strand break repair assay. The assay will be started by 1×10^5 cells (wild type BRCA1-MCF-10A, corrected BRCA1-HCC1937, and non-corrected BRCA1-HCC1937*)* will be embedded into agarose plugs. The cell plugs will be irradiated at 4 °C with 10 Gy ionizing radiation and placed into complete medium at 37 °C to perform double-stranded break repair for the 24h. After the given amount of repair time, the cell plugs will be digested overnight at 55 °C in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% SDS, 50 mM EDTA, and 2 μg/ml proteinase K. The digested cell plugs will be embedded into a 0.7% agarose gel and subjected to pulsed field gel electrophoresis (PFGE) (3 V/cm, 45-s pulse time) for 72 h. The separated DNA will be transferred to a Gene-Screen filter, and Southern blotting will be performed. The probe will be generated by isolation of total genomic DNA from the given cell lines followed by Random Prime labeling using [α-^{32}P] ATP (Prime-It II, Stratagene) (Abbott *et al.* 1998).

The sensitivity of the wild type BRCA1-MCF-10A, the corrected BRCA1-HCC1937, and the non-corrected BRCA1-HCC1937 cell lines to the IR and the response of the BRCA1 to the IR will be analyzed by clonogenic assay. The cells will be plated on six-well at density of 300 cells per well and will be allowed for adhere for 18 hours. IR

will be administered using an orthovoltage X-ray source at different doses of 0-4 Gamma dose. Then the cells will be fixed and stained with crystal violet and counted (Swanner *et al.* 2015).

Expected Results

I expect that the wild type BRCA1-MCF-10A, and the corrected BRCA1-HCC1937 cells will need less time to repair DNA double-strand break, and the non-corrected BRCA1-HCC1937 cells will need more time than other cell lines. Also, the non-corrected BRCA1-HCC1937 cells would have more sensitivity to IR radiation and would produce more DSB than the wild type BRCA1-MCF-10A, and the corrected BRCA1-HCC1937 cells. The result would indicate that the BRCA1 gene plays important roles in DNA repair.

Aim3: MTT and Transwell assays to compare rate of proliferation and invasion in BRCA1 wild type in MCF-10A, mutated and rescued BRCA1 in HCC1937 cell lines

MTT Cell Proliferation Assay is based on the conversion of water-soluble MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product. Living cells with energetic metabolism convert MTT into formazan. Dead cells or slower metabolism in living cells will lose this ability and therefore exhibit no or light signal. The color formazan product serves as a marker of live cells. The measured absorbance at 570 nm is proportional to the number of viable cells (ATCC, USA). All the cell lines namely wild type BRCA1-MCF-10 normal breast cell line, non-corrected BRCA1-HCC1937 breast cancer cell line and corrected BRCA1-HCC1937 breast cancer cell line plate at 50,000 to 100,000 cells per 6-wells with incubation at 37^{o} C up to 72-96 hours. MTT reagent will be added and incubated for 2 to 4 hours.. After that,

detergent reagent will be added and leaved in room temperature in the dark to 2 hours, and record the absorbance at 570 nm (ATCC, USA).

Invasion of the cells will be evaluated by Transwell invasion assays. In this assay, 24-well plate and chemotaxis chambers will be used (Corning cell culture inserts, 8 μm pore size). The cells are washed with phosphate-buffered saline (PBS). Serum-free medium with Matrigel will be added into the upper chambers and the cells will be incubated for 48 hours. The lower chambers fill with medium containing 10% fetal bovine serum and incubate for 24 hours. The cells on the upper side of the chamber will be removed using a cotton swab, and the cells to the lower side of the membrane will be fixed in 4% paraformaldehyde at room temperature, stained with crystal violet, washed with phosphate-buffered saline. The 570 nm value is recorded. (Jia *et al.* 2016).

Expected Results

The expect result of MTT assay will show that the non-corrected BRCA1-HCC1937 cell lines would convert the color at a high rate than corrected BRCA1-HCC1937 and wild type BRCA- MCF-10Acell lines and the result would indicate that the cellular proliferation in mutated cells is higher than the cells in the other cell lines.

The expect results of the Transwell invasion assay for all cell lines will be that the non-corrected BRCA1-HCC1937 cell line would have the ability to invade and the result would indicate that the non-corrected BRCA1-HCC1937 cells invade higher than the corrected BRC1-HCC1937 and the wild type BRCA1-MCF-10A cell lines.

Timeline

Activity	Time
CRISPR/Cas9 system: Cas9 plasmid HDR plasmid GFP plasmid	1-3 weeks
Sanger sequencing	1-3 weeks
DNA repair assay	2 weeks
IR assay	2 weeks
HCC1937 and MCF-10A cell lines	1 week
Co-IP and western-blot supplies	1 week
Co-IP and western-blot assays	1-2 weeks
MTT cell proliferation assay	1 month
Invasion Transwell assay	1 month

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