Breast Cancer Risk after Diagnostic Gene Sequencing (BRIDGES)

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Contents

Executive Summary

The objective of WP4 is to develop a high-throughput *in vitro* assay, termed RIgORouS, that should allow us to test the effect of large numbers of missense variants on BC-HR protein function. As already explained in the first periodic report, unforeseen technological advances have prompted us to reconsider and adapt the steps leading to RIgORouS. The alternative technology we have implemented allows us to develop RIgORouS as originally planned. In addition, RIgORouS is expected to be a much more robust assay pipeline than previously envisaged and will offer more flexibility with regard to the inclusion of genes that are expected to be identified in WP2/WP3 during the course of our project. The major change in the plan was that we now focus on the generation of a generic cell line which can be used for all BC-HR genes in this project. Here we report on the establishment of this generic cell line and its use in functional assays towards setting up RIgORouS. Deliverable 4.3 has been achieved in time.

This is a descriptive deliverable. Because of confidentiality issues, details and figures have been omitted in this report. As soon as data are published this information will become publically available.

Introduction and Overview

The objective is to develop a high-throughput *in vitro* assay to allow rapid testing of the effect of missense variants on BC-HR protein function. In first instance, the BC-HR gene of interest was to be silenced using siRNA technology, after which an siRNA-resistant mCherry-tagged BC-HR gene would be re-introduced (see section 1.3.3.10). We successfully applied this approach to analyse variants in the XRCC2 (one of the BC-HR genes in the set) in hamster cells and human HEK293t cells. However, when we assessed the robustness of the functional assays by examining the correlation between the RAD51 foci and homologous recombination (HR) assay in hamster cells to that of the HR assay in human cells, we only found moderately high Pearson correlation coefficients for these assays ranging from 0.57 to 0.72 (Hilbers et al., 2016). This likely stems from the incomplete and variable XRCC2 knockdown levels, as well as the large differences in transient expression levels of mCherry-tagged XRCC2, causing partial and variable complementation of the XRCC2 knockdown phenotypes following expression of mCherry-tagged XRCC2. To address these issues, we have introduced several improvements in our original approach as described below.

Science and Technology

We will construct a generic mouse cell line containing all necessary requirements to perform semi-automated high throughput functional analysis of our variants in the proposed BC-HR genes. Such a cell line will allow much more flexibility regarding the genes to be tested.

1. A DR-GFP reporter will be integrated into the *Pim1* locus to enable the measurement of HR efficiency.

2. Instead of transiently expressing siRNA-resistant mCherry-tagged BC-HR genes, we will stably express epitope-tagged BC-HR genes from a specific site in the mouse genome. This can be achieved by introducing the FLP-based Recombination-Mediated Cassette Exchange (RMCE) system into one allele of the ROSA26 locus of mouse embryonic stem cells. In this way, epitope-tagged BC-HR constructs are efficiently targeted into the ROSA26-RMCE locus by a FlpO recombinase-based integration reaction.

3. At the time of writing the BRIDGES proposal, siRNA-based knockdown of genes was the most straightforward and fastest way of knocking down the expression of any given gene. However, the disadvantage was that knockdown efficiency varies strongly from gene to gene and does not always reach 100%. The recently developed CRISPR/Cas9-based genome editing technology on the other hand, allows efficient generation of complete knockouts of the BC-HR genes in eukaryotic cells, and therefore strongly improves the read-out window of functional assays. Knockouts of the BC-HR genes will therefore be generated in the mouse ES (mES) cell line carrying the DR-GFP reporter and the RMCE cassette. In the event that knockout cells for a given BC-HR gene are not viable, the knockdown will be executed in a p53 deficient background. The CRISPR/Cas9 knock out techniques works extremely efficient in these cells, as demonstrated for the p53 gene in our previous report.

4. After knockout cells have been generated, complementation will be performed by targeted integration of cDNA constructs in the RMCE cassette. Constructs integrated in this locus are much more stably expressed than plasmid-based constructs introduced by transfection, improving the reliability of the assay.

5. A vector for transient expression of the SceI nuclease, which is necessary to induce a DNA double-strand break and measure HR in the DR-GFP reporter, will be introduced.

Once the generic cell line has been created we will follow our original proposal and design the analysis pipeline RIgORouS, which exploits multiple readouts to assess the effect of a VUS on the BC-HR gene function (e.g. homologous recombination (HR), sensitivity to PARP inhibitors and RAD51 foci formation).

Description of results

- DR-GFP has been integrated into mES cells (single copy at Pim1 locus, as confirmed by PCR and Southern blot).
- transient expression of SceI nuclease results in high levels of HR as previously reported (Pierce et al., Genes Dev, 1999; DOI: 10.1101/gad.13.20.2633), indicating that the DR-GFP reporter is fully functional.
- RMCE system has been integrated into mES cells containing DR-GFP (see first point; single copy at ROSA26 locus, as confirmed by PCR and Southern blot)
- RMCE vectors containing human CHEK2 and PALB2 cDNAs were generated
- co-transfection of these vectors and a FlpO expression plasmid lead to highly efficient integration of these vectors at the RMCE locus at ROSA26
- gene-knockouts p53, CHEK2 and PALB2 were generated with high efficiency using the CRISPR/CAS9-system in mES cells

CHEK2

- CHEK2 knockout leads to a defect in IR-induced G2/M arrest as demonstrated by flow cytometry and as reported previously (Hirao et al., Science 2000; DOI: 10.1126/science.287.5459.1824)
- the checkpoint defect in CHEK2 knockout cells could be complemented by introducing wildtype,

illustrating the validity of the assay

PALB2

- p53 knock out mES cells do not have a defect in HR
- PALB2 knockout mES cells are only viable in the presence of p53 knock out
- PALB2 loss leads to a defect in HR as measured by flow cytometry and as reported previously (Shy et al., PNAS 2009; DOI: 10.1073/pnas.0811159106)
- the HR defects in the PALB2 knockout cells could be complemented by introducing wildtype PALB2 cDNA or PALB2 cDNA containing benign variants (n=6), but not PALB2 cDNA containing truncating (n=6), illustrating the validity of the assay
- about 60 RMCE vectors with truncating, benign (SNPs) or missense variants in the human PALB2 cDNA were generated
- an ongoing analysis of these variants already identified several missense variants (VUS) that affect PALB2 functionality (n=6)

Expected outcome for the next period

- testing of the effect of variants in PALB2 in HR
- testing the effect on PARP inhibitor treatment and RAD51 foci formation for missense variants in PALB2 that affect HR
- presenting these effects in VUSplot (MS14)
- generating at least 60 RMCE vectors with truncating, benign (SNPs) or missense variants in the human CHEK2 cDNA
- testing of the effect of these variants in CHEK2 on G2/M checkpoint activation

Summary

By implementing state-of-the-art technology, we have introduced several improvements to generate a much more robust pipeline to analyse the functional consequences of VUS in BC-HR genes. Integrated constructs will allow for stable expression of BC-HR cDNAs in BC-HR knockout cells, which will improve the robustness, sensitivity and reliability of the pipeline. Indeed, we were able to generate PALB2 and CHEK2 knockout mouse ES cells that display defects in HR and G2/M checkpoint activation, respectively. Integrated constructs with human cDNA for PALB2 or CHEK2 complemented these phenotypes, while several loss-of-function variants failed to do so. Thus, we have setup RIgORouS, a robust cellular system for the functional analysis of VUS in BC-HR genes. RIgORouS is flexible with regard to testing large numbers of variants using multiple read-outs, and the inclusion of (HR) genes that may be identified as breast cancer susceptibility genes during the course of our project.

Conclusion

The Deliverable 4.3 has been achieved in time.