PROPOSAL: DISSECTING BRCA2

CIG Final report: PUBLISHABLE SUMARY

5-10% of breast and ovarian cancer patients are genetically predisposed to the disease, the majority of which are attributed to mutations in BRCA1 and BRCA2 genes (Wooster, 1994) (Welcsh and King, 2001) BRCA2 tumor suppressor gene encodes a protein of 3,418 amino acids. BRCA2 protein is required for homologous recombination (HR), the predominant mechanism employed by cells to accurately repair double-stranded DNA breaks (DSBs). In addition, BRCA2 can associate with other proteins and participate in related pathways such as cell cycle control or meiotic recombination. As a consequence, a defect in this protein leads to uncontrolled cell replication, and genomic instability, both hallmarks of tumorigenesis. However, many questions are still unanswered regarding the specific functions that trigger tumor formation in a BRCA2 mutated background.

Almost half of the BRCA2 missense mutations identified in families at high risk of breast cancer don't have a defined clinical significance and therefore, those unclassified variants (UVs) remain a challenge for the genetic counseling of patients. There is an international effort to compile information based on genetic and functional data to reveal the pathogenicity of those mutations of which we are part of (Spurdle et al., 2011).

Thus, a better understanding of BRCA2 function(s) can serve to many purposes relevant to the clinic: 1. It can help define the specific molecular signature of inherited breast cancer linked to BRCA2 mutations. 2. It can reveal new protein partners that may be used for targeted cancer therapy. 3. It will help to functionally evaluate BRCA2 variants of unknown clinical significance (VUS) to improve genetic counselling.

In this project, we set out to ‘dissect the function(s)’ of BRCA2 protein to understand its link to tumorigenesis. The specific aims we proposed are: 1) exploring the least conserved parts of BRCA2 protein 2) by studying the functional impact of BRCA2 variants of unknown clinical relevance identified in families at high risk of breast cancer. 3) In addition, we are developing new tools based on BRCA2-partner interference peptides as a mean to inhibit the HR pathway and new approaches to target those inhibitors into tumor cells to be used in anticancer therapy.

Current achievements on the three main aspects of the project:

1. **Revealing new functions of BRCA2**

The high sequence variability of BRCA2 protein on one hand, and the scarce information on its structure on the other, represents a challenge to understand its function.

Among the least conserved regions of the protein is the N-terminus, which comprises 1/3 of BRCA2. The first 40 amino acids of this region (of 1000) are conserved and contain several protein-binding sites, like PALB2, (Partner and Localizer of BRCA2). The rest of this part of the protein remains unexplored. Therefore, we have chosen the N-terminal region of BRCA2 to generate an interactome and focus on the interacting partners that are enriched under DNA damage conditions.

Gene ontology analysis and functional clustering revealed that the BRCA2 interactors fall mainly into these categories: DNA repair, chromatin remodeling, cell cycle regulation and RNA binding proteins, helicases and ubiquitin/SUMO proteins. We have also identified new putative post-translational modifications that we would like to characterize.

We have verified two of the interactions in the DNA repair and RNA binding protein categories in vitro using the endogenous proteins and we are extensively characterizing them using cell biology tools: PLA (Proximity Ligation Assay), recruitment to DNA damage sites using laser induced microirradiation and Immunofluorescence as well as biochemistry tools: Co-immunoprecipitation, in vitro pull-down with purified proteins. We also plan to use ChIP-seq (Chromatin Immunoprecipitation-sequencing) of genome-wide induced double strand breaks to analyze the recruitment of BRCA2 and its partners to these sites. One of the interactions is well advanced and the manuscript is in preparation.

- We have also characterized an interaction outside the N-terminus for which its functional relevance is unclear. The interaction between BRCA2 and DMC1, the homolog of RAD51 recombination protein in meiosis. We have revealed a direct interaction between the BRC motifs of human BRCA2 and DMC1 that results in the stimulation of the recombination function of DMC1. Importantly, we have confirmed this stimulation with the full length BRCA2 protein. These results suggest that BRCA2 regulate both meiotic and mitotic recombination through the BRC motifs. This work is under revision in PNAS.

1. **Functional Impact of BRCA2 variants of unknown clinical relevance identified in families at high risk of breast or ovarian cancer**

Many missense variants, in-frame insertions and deletions, considered as unclassified variants (VUS) affecting *BRCA2* have been identified in high-risk breast and ovarian cancer patients. However, it is not known whether any of these changes actually alter BRCA2 function sufficiently to predispose carriers of these mutations to cancer. Thus, the carriers of these variants cannot receive any specific cancer risk measures. A substantial number of those variants may have a direct impact on cancer risk.

Many BRCA2 VUS lack sufficient family information for their accurate classification thus, an alternative method like functional assays, can assess the impact of variants on the activity of the protein and thus, help interpret VUS pathogenicity.

Over the course of the CIG period, we have generated several stable clones in a BRCA2 deficient hamster cell line, VC8, extensively used and described in the literature, to evaluate the function of VUS located in the N-terminal region of BRCA2. However, we have come to realize that an isogenic inducible system in human cells would be much more appropriate to assess accurately the phenotype of these variants as the expression levels would be comparable and the background of the cells would be intact. Thus, we are now validating this more physiological method based on the FLP-IN system and generated the tools to facilitate an accurate measure of the ability of the cells carrying these variants to survive after DNA damage, to repair correctly the damaged DNA, located at DNA damage sites, etc.

A new project funded by INCa (Institut National de Cancer) in collaboration with members of the Department of Constitutional Genetics in Curie Hospital and the group of Sophie Zinn-Justin at CEA, Saclay, will follow up this project and enlarge it to a bigger set of BRCA2 VUS with the main objective of providing a comprehensive functional approach to evaluate missense VUS using an interdisciplinary and systematic approach.

- We also have an ongoing collaboration with Fergus Couch (Mayo Clinic, Rochester, MN, US), Maaike Vreeswijk (Leiden Univ. Medical Center) and the ENIGMA Consortium for the study of variants at the C-terminal region of BRCA2 that display an intermediate phenotype in functional assays. The goal of this project is to determine if the intermediate phenotype in functional assays corresponds to intermediate cancer risk. This work is under submission.

1. **Exploit regions of the protein as a therapeutic tool for tumor treatment**

The idea behind this part of the project is to use the newly characterized interacting partners of BRCA2 revealed in aim 1 to then generate inhibitor molecules that will allow this interaction for its possible use as adjuvants for anti-cancer therapy. This part of the project is therefore the last, and it has not been accomplished yet as we want to make sure we have selected the right target.

Once the last experiments confirm the potential of disrupting the interactions validated in aim 1 we will use the Innopharma early drug discovery platform (innopharmaplatform.com) to screen compounds that will specifically disrupt this interaction (using the purified proteins) and test their effect in tumor cells alone or in combination with radio or chemotherapy.

**CONCLUSIONS & SOCIO-ECONOMIC IMPACT**

**1.** Our work will unravel new layers of complexity and connections between different DNA repair pathways from the careful study of the structural domains of BRCA2 protein, and its partners, which will likely reveal new functionalities of this complex protein. The identified interacting partners, in turn, are potential targets for anti-cancer therapy.

2. The successful classification of missense VUS will represent a major benefit for patient care and that of their relatives. When a VUS gets classified as pathogenic, genetic testing becomes available for the other members of the family. The presence of a deleterious mutation leads to the recommendation of a specific breast follow-up and to a prophylactic oophorectomy at age 40, and maybe, to personalized therapy. In contrast, the presence of a neutral variant will allow considering that person as the general population in terms of patient care. **Therefore, the evaluation of VUS has a direct impact in the clinic.**