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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

c-Abl and BRCA1 have certain overlaps that have led us to speculate of a functional relationship. In murine knockout studies each gene deficiencies affects spermatogenesis at the same stage of differentiation. In addition numerous nuclear proteins have been shown to interact both with c-Abl and BRCA1. In fact a recent publication points to a direct interaction resulting an association that can be disrupted by ionizing radiation.

In this proposal we sought to identify a functional link between these two gene products by first characterizing an interaction during spermatogenesis and secondly to determine if modulating c-Abl activity had any effect on BRCA1 function. Our initial study on localization of c-Abl and BRCA1 in murine testis failed to provide us with evidence of a co-localization during spermatogenesis. The second experimental approach was to investigate the biological consequences of c-Abl on BRCA1 function. Towards this goal we have generated HCC19437 cells reconstituted with BRCA1, standardized an transfection protocol to test this hypothesis.

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INTRODUCTION

Evidence in the literature suggests a functional relationship exists between the c-Abl tyrosine kinase and the BRCA1 breast cancer susceptibility gene product. First, a defect in spermatogenesis results in arrest at the pachyetene stage of differentiation in both c-*abl* and *brca1* null mice. This indicates not only a potential temporal overlap during spermatogenesis but also suggests a functional necessity for each for each protein for the successful completion of this stage. In addition there is considerable overlap between Brca1- and c-Abl-interacting proteins including ATM (Baskaran et al., 1997 and Shafman et al., 1997), Rad51 (Yuan et al., 1998), and p53 (Goga et al., 1995) . The goal of this project was to first determine if c-Abl and BRCA1 were co-localized in sectioned murine testis by immunofluorescence and to determine the role of c-Abl tyrosine kinase activity in this association. Secondly, we proposed to investigate the regulation of BRCA1 signaling by c-Abl in a somatic cell line derived from a breast neoplasia. The cell line HCC1937 is a unique established cell line that it does not encode a functional BRCA1 gene product (Tomlinson et al., 1998). A single mutated BRCA1 allele is expressed and the affected gene suffers from a 3' mutation that renders the expressed gene product non-functional. That is, the cells exhibit a hypersensitivity to killing by ionizing irradiation which can be reversed by reconstitution of the cells with full-length BRCA1 (Abbott et al., 1999 and Scully et al., 1999).

BODY

Research accomplishments

Localization of c-Abl in sectioned murine testis.

Murine testis sections were used to investigate the localization of c-Abl by immunostaining with a c-Abl antibody. We first standardized the immunofluorescence protocol using c-Abl transfected MCF-7 cells. As shown in Fig 1 c-Abl is readily detected in the nucleus of these somatic cells by confocal laser microsopy.

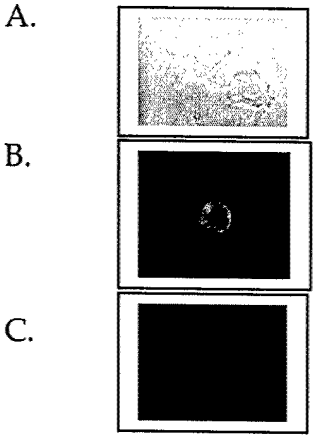


Fig. 1. Nuclear localization of c-Abl in MCF-7 breast cancer cells. Transfected c-Abl was visualized by indirect fluorescence using confocal laser micrscopy. The cells were plated on coverslips, tranfected with pcDNA3/Abl1b and first incubated with anti-Abl and then with a fluorescein-conjugated secondary antibody. Nuclear staining was with DAPI. Panel A; brightfield microscopy, B; c-Abl indirect immunofluorescence and C; nuclei staining with DAPI.

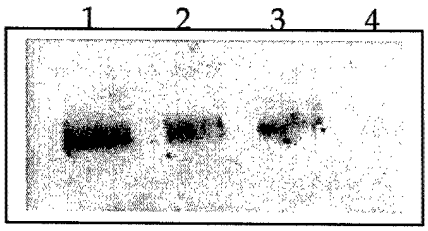
Using primary antibodies against either c-Abl and BRCA1with fluorescent conjugated secondary antibodies and confocal laser scanning microscopy we examined sections of murine testis for c-Abl localization. There are different reports in the literature concerning the localization of c-Abl in the testis. One study uses rat samples and they reported localization in nuclei during the pachytene stage of differention (Kharbanda et al., 1998) whereas a report published during the award period reported an exclusive cytoplasmic distribution in murine testis (Hamer et al., 2001). We examined c-Abl localization in murine testis because of our concern of the ability of our antibodies to recognize the rat protein. As shown in the Appendix, c-Abl expression is limited to the cytoplasm in the developing sperm in the seminiferous tubules. This is consistent with the report by Hamer et al., 2001, suggesting a species-dependent expression profile. When we counterstained with anti-BRCA1 and performed overlay analysis we failed to observe co-localization. As expected BRCA1 foci are present within the nucleus (Scully et al., 1997). Two typical sections are shown in the Appendix. While it is clear that the testis expresses high levels of c-Abl and as we noted years ago predominantly as a novel transcript (Oppi et al., 1987) its function is still unclear and the species-dependent localization adds to the uncertainty of its function.

HCC1937 cells

Isolation of BRCA1 reconstituted HCC1937 cells

The second goal in the proposal was to utilize the HCC1937 cell line to investigate if c-Abl modulated BRCA1 function. Our experimental approach is to examine the consequences of modulating c-Abl on BRCA1-induced radiation sensitivity in these cells. It has been shown that reconstitution of HCC1937 cells with BRCA1 restores radiation resistance indicating the loss of BRCA1 is fully responsible for the phenotype (Abbott et al., 1999 and Scully et al., 1999). We first obtained the HCC1937 cell line from ATCC and confirmed the loss of the c-terminus by immunoprecipitating the protein using a mAb recognizing the C-terminal 17 amino acids (Oncogene Science Ab-3 SG118). Fig. 2 Panel A shows that in contrast to Cos7 cells, BRCA1 in HCC1937 is not immunoprecipitated by Ab-3. We next sought to reconstitute full-length BRCA1 in these cells by transfecting wt BRCA1 cDNA into the cells and selecting transfected cells by culturing them in the presence of G418. To limit clonal variation we isolated 3 different mass cultures of drug resistant cells after a 3 week course of hygromycin exposure (300μg/ml) and assayed for expression of the BRCA1 transgene. As shown in Fig. 2 Panel B, all 3 cultures express the wt gene as the protein can be immunoprecipitated with the Ab3 mAb. The expression of BRCA1 is increased relative to the parental HCC1937 cells compare to panel A, yet has not exceeded the level observed in the Cos cells.

A.



BRCA1

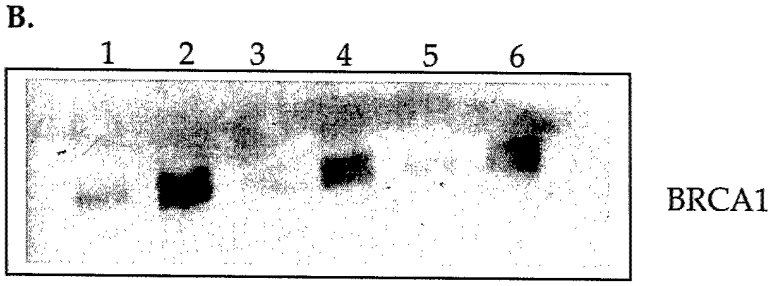


Figure 2. Isolation of BRCA1 reconstituted HCC1937 cells. **A.** Cos7 and HCC1937 cell extracts were immunoprecipitated with BRCA1 mAb (AB3) and then the resulting blots were probed with BRCA1 AB1 mAb. Lane 1; Cos 7 total cell extract, lane 2 Cos immunoprecipitated sample, lane 3; HCC1937 total cell extract and lane 4; HCC1937 immunoprecipitated sample. **B.** Expression of full-length BRCA1 in HCC1937 cells transfected with pcDNA3/BRCA1. The gel represents BRCA1 expression in 3 separately derived mass cultures run as paired samples (1 and 2, 3 and 4, 5 and 6). Lanes 1, 3 and 5 are total cell extracts and lanes 2, 4, and 6 are BRCA1 Ab3 immunoprecipitated samples.

c-Abl-BRCA1 co-immunoprecipitation

One possible mechanism for c-Abl modulation of BRCA1 function is through a direct interaction between these two proteins. A paper published at the end of the initial funding period reported that such an interaction does indeed occur in cells (Foray et al., 2002). Foray et al., detected a constitutive association between c-Abl and BRCA1 both by immunoprecipitation and GST pull down assays. We were also pursuing such an association and were unsuccessful. We then began studies to repeat their findings. Using nuclear extracts prepared from the breast cancer cell line MCF-7 (expresses nuclear c-Abl as well as wt BRCA1) we tested for the presence of c-Abl in BRCA1 immunoprecipitates. BRCA1 antibodies raised against unique epitopes in the protein (Ab1 N-terminus and Ab3 C-terminus) for the immunoprecipitations and immunoblotted for c-Abl. A typical experiment is shown in Fig.3. Even with the extended exposure shown, we are unable to detect c-Abl in the BRCA1 immunoprecipitate. The successful immunoprecipitation of BRCA1 is shown in the top panel. While the results from Foray et al. clearly indicate that only a small percentage of BRCA1 and c-Abl are associated, our inability to detect this association suggest that if c-Abl modulates BRCA1 function it may be through an indirect mechanism.

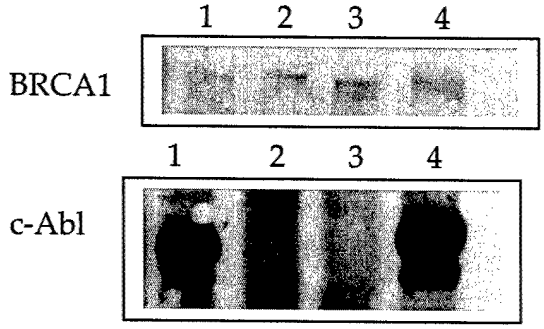


Figure 3. Co-immunoprecipitation between BRCA1 and c-Abl. Nuclear extracts were prepared and immunoprecipitated with BRCA1 mAb Ab-1 and 3 (Oncogene Science). The samples were electrophoresed and immunoblotted with BRCA1 Ab-1 in the top panel and c-Abl (k-12) in the bottom. Lane 1; MCF-7 whole cell extract, lane 2 Ab-1 and 3 Ab-3 750 μg nuclear extract immunoprecipitated with BRCA1 mabs, and lane 4 nuclear extract.

Standardization HCC1937 transfection

In order to examine a the biological consequence of modulating c-Abl activity on BRCA1 function we proposed to use as a readout HCC1937 response to irradiation. To establish the recipient cells we generated wt BRCA1 reconstituted HCC1937 cell lines as described above. To modulate c-Abl function in these cells we will express various c-abl cDNAs and the score the cells survival following exposure to IR. We standarized a transient transfection protocol using Lipofectamine 2000 (Invitrogen). Under the optimal conditions we can achieve a transfection efficiency of approximatelt 25%. As shown in Fig. 4, B-gal staining of transfected (B) versus non-transfected(A) dishes of 10^6 HCC1937 cells illustrating the highly introduction and expression of transgenes in these cells.

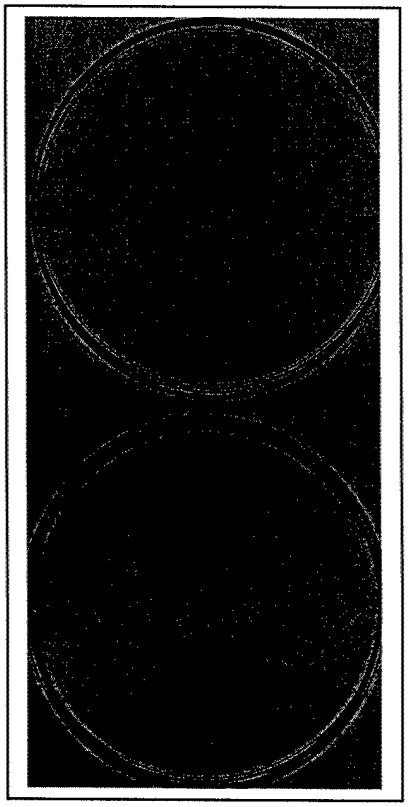


Figure 4. 10^6 HCC1937 cells were seeded the in 100mmplates and transfected with 1 μg B-gal plasmid DNA and 20 μl Lipofectamine 2000. Expression of B-gal activity was detected by incubating fixed cells with X-galfor 2 hours 48 hours after exposure to the DNA.

KEY RESEARCH ACCOMPLISHMENTS

- Observation of exclusive cytoplasmic localization of c-ABL in murine testis
- Lack of co-localization of c-Abl and BRCA11 in murine testis
- Establishment of HCC1937 cells and standardization of transfection procedure

REPORTABLE OUTCOMES

Development of HCC1937 pcDNA3/hygro/BRCA1 cells

CONCLUSIONS

The observation of a lack of co-localization of BRCA1 and c-Abl in the testis was somewhat unexpected. Due to the circumstantial evidence in the literature and the report of nuclear c-Abl in rat testis we were surprised to observe the exclisively cytoplasmic nature of c-Abl in murine testis. The report published during the award period corroborating our observation suggests c-Abl exhibits species-dependent differences in localization and significantly perhaps function. Because of our failure to observe co-localization, we did not pursue additional studies on c-Abl kinase activity and BRCA1 in the testis as we had proposed in our Concept Award plan. We initiated our second group of studies which utilize the BRCA1-deficient tumor cell line HCC1937. The key observation for this plan is the restoration of radiation resistance following reconstitution of HCC1937 cells with wt BRCA1 (Abbott et al., 1999 and Scully et al., 1999). We have developed the reconstituted cells by transfecting and selecting mass cultures of wt BRCA1 expressing cells that are resistant to hygromycin. These cells and the parental line will be transfected with c-Abl transgenes and the effect on BRCA1 function will be examined. In addition, we have attempted to detect a direct association between BRCA1 and c-Abl, as was recently reported (Fornay et al., 2002), but have been unsuccessful. Despite using nuclear extracts from cells expressing both c-Abl and wtBRCA1 we did not observe c-Abl in BRCA1 immunoprecipitates. This is not inconsistent with our original hypothesis though, c-Abl may regulate BRCA1 function by affecting BRCA1 protein complexes indirectly. This strategy represents our current research efforts.

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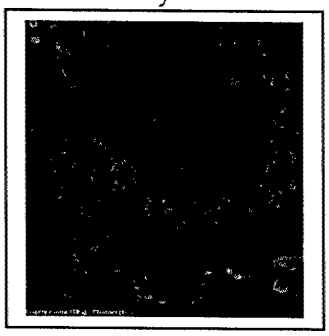
Appendix

BRCA1 c-Abl Overlay



BRCA1 c-Abl Overlay

Deparaffined murine testis samples were boiled in 10 mM sodium citrate for 10 minutes by heating in a microwave oven. After epitope recovery, the slides were blocked in PBS containing 5% FBS. The slides were then incubated in a humidified chamber with c-Abl antibody (K-12 Santa Cruz) a rabbit polyclonal and BRCA1 mAb (AB-1) (Oncogene Sciences). Incubation with the secondary goat anti-rabbit or horse anti-mouse (Vector labs). The slides were washed in PBS and coverslips were mounted with Vectashield. Negative controls were treated identically but the primary antibody was omitted (not shown). Sections showing seminiferous tubules are shown.