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14. ABSTRACT During the first year of the funding period 04/01/2011 until present, we have devoted the majority of our efforts towards obtaining pairs of mutant and functionally corrected telomerase-immortalized (hTERT) Fanconi anemia patient-derived fibroblast lines. Specifically, we have focused on the following FA complementation groups: FA-A, FA-G, and FA-D2. For the FA-D2 complementation group, we have obtained two pairs of mutant and corrected lines from collaborators. We have subsequently hTERT-immortalized these pairs during the funding period. For the FA-A complementation group, we had previously generated hTERT-immortalized mutant and corrected lines. During the funding period we have performed numerous functional assays, including mitomycin C cytotoxicity, clastogenicity, and G2-M accumulation assays, to confirm functional correction. For the FA-G complementation group, we purchased a primary FA-G line from Coriell Cell Repositories, hTERT-immortalized this line, generated a stable line expressing FANCG and are currently in the process of performing functional assays to confirm functional correction. In addition, for all of these mutant and corrected lines we are in the process of generating monoclonal cell populations to control for heterogeneity in pre-existing copy number variation within polyclonal cell populations.					
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Appendix 2. Expansion of monoclonal populations of FA-G hTERT and FA-G + FANCG hTERT cells

Introduction

Fanconi anemia (FA) is a rare disease characterized by developmental defects, progressive bone marrow failure (BMF) and pronounced cancer susceptibility. The FA proteins and the major breast cancer susceptibility gene products BRCA1 and BRCA2 function cooperatively in the FA-BRCA pathway to repair damaged DNA. Recent studies have demonstrated that the FA-BRCA pathway plays an important role in the response of hematopoietic stem and progenitor cells to cellular stresses, and in particular oxidative stress caused by elevated levels of reactive oxygen species (ROS). In our research proposal, we have hypothesized that the FA-BRCA pathway may play an important role in the prevention of genome-wide *de novo* copy number variation. Chromosome copy number variation refers to gains or losses of large (>10 kb) genomic DNA segments. While copy number variation is a feature of normal genetic variation it is also strongly associated with genetic disease, including autism and psychiatric disorders. In addition, several recent studies have demonstrated that hematological malignancies show large numbers of *de novo* somatically acquired copy number variants (CNVs). As with all classes of mutation, an important role for *de novo* CNVs in cancer initiation and progression, as well as BMF, is highly likely. **Importantly, the biological pathways that prevent *de novo* CNV formation, as well as the endogenous and exogenous agents that promote *de novo* CNV formation, remain largely unknown.** We hypothesize that the FA-BRCA pathway, through its role in the suppression and repair of oxidative DNA damage, plays a central role in the prevention of genome-wide *de novo* CNVs. These mutational events are likely to be highly relevant to FA-associated BMF, myelodysplasia (MDS) and progression to acute myeloid leukemia (AML). The major goals of this research proposal are to systematically test the hypothesis that the FA-BRCA pathway plays a major role in the prevention of *de novo* pathogenic CNVs.

Body

With reference to our approved Statement of Work:

Specific Aim1: Determination of the role of the FA-BRCA pathway in the suppression of spontaneous *de novo* CNVs

Task 1. Correction of FA-A, FA-C, and FA-D2 hTERT cells with pLenti6.2/V5-FANCA, -FANCC, and FANCD2, respectively.

Sub-task 1. Selection and expansion of clonal populations

Sub-task 2. Mitomycin C cytotoxicity and clastogenicity assays, assessment of FANCD2 mono-ubiquitination status *via* immunoblotting and nuclear foci formation – *to determine if the FA-BRCA pathway is functionally restored*

During the first year of this research project we have focused the majority of our efforts on Specific Aim 1 to obtain pairs of telomerase (hTERT)-immortalized mutant and corrected FA patient-derived fibroblast lines. Specifically, we have focused on the following FA complementation groups: FA-A, FA-G, and FA-D2. In our original proposal we indicated that we would use FA-C patient-derived cells, however we have chosen to focus on FA-G cells instead because of the availability of FA-G lines and the *FANCG* cDNA in our laboratory.

FA-A complementation group: We previously generated hTERT-immortalized mutant FA-A and FA-A + FANCA skin fibroblasts. During the funding period we have determined that the FA-A + FANCA hTERT cells express robust levels of FANCA protein and that this leads to a restoration of DNA damage-inducible FANCD2 monoubiquitination in these cells, compared with mutant FA-A hTERT cells (Figure 1). In addition, we have used several complementary approaches to determine that the FA-A + FANCA hTERT cells are indeed functionally corrected. FA patient-derived cells are known to accumulate in the G2/M phase of the cell cycle following treatment with the DNA interstrand crosslinking agent mitomycin C (MMC) (Naf et al., 1998). FA-A hTERT and FA-A + FANCA hTERT cells were incubated in the absence or presence of MMC for 48 h and the cell cycle stage distribution was determined by FACS analysis/flow cytometry (Figure 2). Following treatment with MMC, a clear shift in DNA content is observed for the mutant FA-A hTERT cells compared with the FA-A + FANCA hTERT cells, suggesting a severe defect in the repair of MMC-induced DNA damage in the mutant FA-A hTERT cells and, more importantly, functional correction of this defect in the FA-A + FANCA hTERT cells. Taken together, these results indicate that the FA-A + FANCA hTERT cells are indeed functionally corrected and that these FA-A hTERT and FA-A + FANCA hTERT cells represent an excellent isogenic system for the study of the role of the FA-BRCA pathway in the prevention of *de novo* pathogenic CNVs.

In consultation with our collaborators in the Department of Human Genetics at the University of Michigan, we have decided that it is important to isolate monoclonal populations of FA-A hTERT and FA-A + FANCA hTERT cells prior to beginning the CNV analyses experimentation. All cells will have a measurable background level of CNVs and polyclonal cell populations will be mosaic for background CNV levels. For the purposes of our experiments, it is important to isolate genetically homogenous populations, with respect to CNV, prior to initiating our experiments. Therefore, *de novo* CNVs will be clearly distinguishable from pre-existing CNVs in the parental cell population. Thus, over the past few months we have sought to isolate monoclonal populations of FA-A hTERT

and FA-A + FANCA hTERT cells. This has proven to be quite a challenge as in initial experiments the vast majority of clones, of both cell types, have failed to progress past the 10-20 cell clone size. However, with modifications to the serum composition and content of tissue culture growth medium we have made considerable progress over the past few weeks and are currently in the process of expanding multiple viable

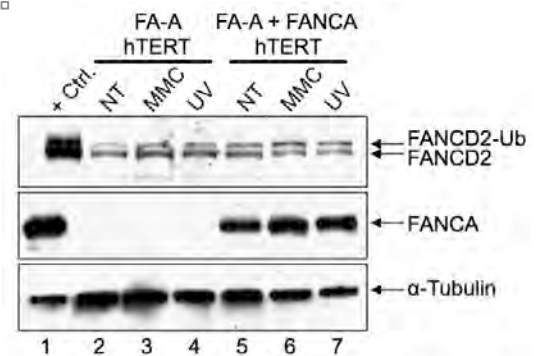


Figure 1. Restored FANCA protein expression and DNA damage-inducible FANCD2 monoubiquitination in FA-A + FANCA hTERT-immortalized cells. NT, no treatment; MMC, 200 nM mitomycin C for 18 h; UV, 6 h post 20 J/m² UV-C irradiation.

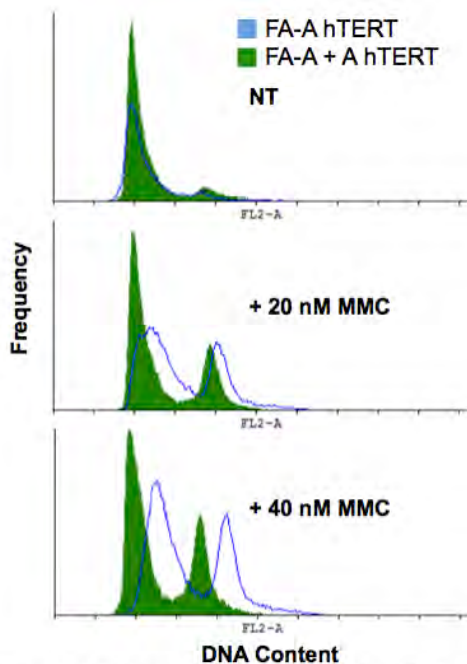


Figure 2. Functional correction of mutant FA-A cells by re-expression of FANCA. Cells were incubated in the absence (NT) or presence of mitomycin C (MMC) and DNA content was assessed by flow cytometry.

clones. A table summarizing our progress in isolating monoclonal cell populations of the FA-A hTERT and FA-A + FANCA hTERT cells is included in appendix 1.

FA-G complementation group: At the start of the funding period we purchased the GM02361 FA-G patient derived line from Coriell Cell Repositories. We immortalized this line with hTERT and subsequently infected the FA-G hTERT cells with the murine moloney leukemia retroviral vectors pMMP-Empty or pMMP-FANCG. Recent immunoblotting experiments have confirmed re-expression of FANCG in the FA-G + FANCG hTERT cells (Figure 3). We could not however detect FANCD2 protein expression by immunoblotting but could detect its paralog FANCI (Figure 3 and results not shown). As the monoubiquitination of FANCD2 and FANCI are interdependent (Sims et al., 2007; Smogorzewska et al., 2007) we are currently in the process of performing DNA damage experiments with the FA-G hTERT and FA-G + FANCG hTERT cells to confirm restoration of DNA damage-inducible FANCI monoubiquitination in the FA-G + FANCG hTERT cells. In addition, similar to that described for the FA-A complementation group above, we are currently performing several functional assays with these cells including MMC cytotoxicity, clastogenicity and G2/M cell cycle stage accumulation analyses. Furthermore, similar to that described above for the FA-A hTERT and FA-A + FANCA hTERT cells, we are currently in the process of isolating and expanding monoclonal populations of the FA-G hTERT and FA-G + FANCG hTERT cells, summarized in Table 2 of the appendix.

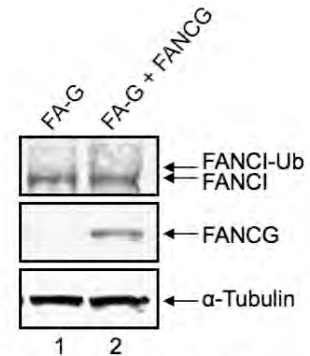


Figure 3. Restored FANCG protein expression in FA-G + FANCG hTERT-immortalized cells.

FA-D2 complementation group: We had previously obtained the PD733 primary FA-D2 line from the Fanconi Anemia Research Fund cell repository. We immortalized this line with hTERT to generate FA-D2 hTERT cells and subsequently infected these cells with pMMP-Empty or pMMP-FANCD2, to generate the FA-D2 hTERT and FA-D2 + FANCD2 hTERT lines. We had planned to use these lines for the CNV experiments. The PD733 FA-D2 line is reported to harbor compound heterozygous *FANCD2* mutations: an intron 3 point mutation (IVS3-2 A→T) and an exon 17 459 bp deletion mutation (g.22875_23333del459) (Kalb et al., 2007). Early in the funding period we sought to confirm the presence of these mutations. Unfortunately, we could not detect the presence of the *FANCD2* exon 17 459 bp deletion mutation in these cells and thus could not confirm the complementation group of this line. As an alternative approach we recently obtained two mutant and functionally corrected FA-D2 primary lines from Detlev Schlinder of the University of Wuerzburg in Germany. We have hTERT immortalized these lines and have recently confirmed FANCD2 protein re-expression in both of the FA-D2 + FANCD2 hTERT lines (Figure 4). We are also currently in the process of performing DNA damage experiments with the FA-D2 hTERT and FA-D2 + FANCD2 hTERT cells to confirm restoration of DNA damage-inducible FANCD2 and FANCI monoubiquitination in the FA-D2 + FANCD2 hTERT cells. In addition, similar to that described for the FA-A complementation group above, we are currently performing MMC cytotoxicity, clastogenicity and G2/M cell cycle stage accumulation assays to confirm functional correction of these cells. Furthermore, we are also currently in the process of isolating and expanding monoclonal populations of both pairs of FA-D2 hTERT and FA-D2 + FANCD2 hTERT cells. Unfortunately this is proving particularly challenging. In general, compared with other FA complementation groups, FA-D2 cells tend to have a more severe chromosomal instability phenotype (Garcia-Higuera et al., 2001; Reliene et al., 2010), and this may be contributing to the difficulty in obtaining monoclonal cell populations.

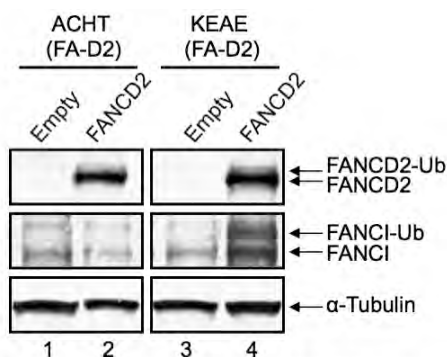


Figure 4. Restoration of FANCD2 protein expression in two independent FA-D2 + FANCD2 hTERT-immortalized lines.

Isolation of monoclonal cell populations: As described above, in consultation with our collaborators at the University of Michigan who routinely perform SNP-based CNV analysis, we have agreed that our CNV experiments need to be performed with genetically homogenous monoclonal cell populations. Towards this goal, we have been attempting to isolate monoclonal populations of mutant and functionally corrected FA hTERT lines with varying degrees of success. Numerous reports have indicated that primary/hTERT-immortalized cells display significantly improved growth/proliferation when cultured under physiological oxygen concentrations ($[O_2]_{\text{Phys}} = 5\%$), as opposed to atmospheric oxygen concentrations ($[O_2]_{\text{Atmos}} = 20\%$) (Atkuri et al., 2007). We believe that an increased oxidative burden may be contributing to our difficulties in isolating monoclonal cell populations. This may also be exacerbated in the case of FA, as it is well known that FA cells are particularly sensitive to reactive oxygen species (Du et al., 2008; Zhang et al., 2007). To overcome this obstacle we have recently purchased a New Brunswick Galaxy 170R tri-gas incubator enabling us to grow cells at physiological oxygen concentrations. We anticipate that this will greatly improve our ability to isolate monoclonal populations of mutant and corrected FA hTERT cells.

Key Research Accomplishments

- We have determined that FA-A + FANCA hTERT cells express robust levels of FANCA protein
- We have determined that DNA damage-inducible FANCD2 monoubiquitination is restored in the FA-A + FANCA hTERT cells
- We have established that the FA-A + FANCA hTERT cells are functionally corrected, i.e. are no longer hypersensitive to the DNA interstrand crosslinking agents MMC
- We have generated FA-G hTERT and FA-G + FANCG hTERT-immortalized cells
- We have determined that the FA-G + FANCG hTERT cells express robust levels of FANCG protein
- We have generated two pairs of FA-D2 hTERT and FA-D2 + FANCD2 hTERT-immortalized cells
- We have determined that the FA-D2 + FANCD2 hTERT cells express robust levels of FANCD2 protein that can undergo monoubiquitination
- We have successfully begun to isolate monoclonal populations of FA-A hTERT, FA-A + FANCA hTERT, FA-G hTERT and FA-G + FANCG hTERT cells

Reportable Outcomes

The major reportable outcomes achieved during the first year of funding for this award are the development of multiple cell lines critical for our experimental approach, as described above.

Conclusion

Task 1 of our original Statement of Work was to develop several sets of mutant and functionally corrected hTERT-immortalized FA lines. We have made considerable progress in this endeavor. In addition, following consultation with our collaborators at the University of Michigan we have initiated experiments to isolate monoclonal populations of all of these lines. This is an arduous and time-consuming process, yet is essential for our planned CNV experiments (*for rationale see above*). While this has proven challenging thus far, we believe that we are on the verge of overcoming these challenges and will very soon have several monoclonal populations of mutant and functionally corrected FA-A, FA-G, and FA-D2 lines to directly test the hypothesis that the FA-BRCA pathway plays a major role in the prevention of *de novo* pathogenic CNVs.

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Appendix 1. Expansion of monoclonal populations of FA-A hTERT and FA-A + FANCA hTERT cells

Clone Name	Cell Type	Dish Size	Days In Culture*	Growth
CORA001	1309 hTERT + RVA	6cm ²	19	Fast
CORA002	1309 hTERT + RVA	6 Well	19	Fast
CORA003	1309 hTERT + RVA	6 Well	19	Fast
CORA004	1309 hTERT + RVA	6 Well	19	Fast
CORA005	1309 hTERT + RVA	12 well	19	Few healthy cells
CORA006	1309 hTERT + RVA	12 well	19	Slowed
CORA007	1309 hTERT + RVA	12 well	19	Few healthy cells
CORA008	1309 hTERT + RVA	12 well	19	Not Progressing
CORA009	1309 hTERT + RVA	12 well	19	Slowed
CORA010	1309 hTERT + RVA	12 well	19	Steady
CORA011	1309 hTERT + RVA	12 well	19	Few healthy cells
CORA012	1309 hTERT + RVA	24 well	35	No Growth
CORA013	1309 hTERT + RVA	24 well	35	Not Progressing
CORA014	1309 hTERT + RVA	24 well	35	Steady
CORA015	1309 hTERT + RVA	24 well	35	Few healthy cells
CORA016	1309 hTERT + RVA	24 well	28	Steady
CORA017	1309 hTERT + RVA	24 well	28	Steady
CORA018	1309 hTERT + RVA	24 well	28	No Growth
CORA019	1309 hTERT + RVA	24 well	28	Steady
CORA020	1309 hTERT + RVA	24 well	28	Not Progressing
CORA021	1309 hTERT + RVA	24 well	28	Steady
FAA001	1309 hTERT	24 well	35	Few healthy cells
FAA002	1309 hTERT	24 well	28	Not Progressing
FAA003	1309 hTERT	24 well	28	Not Progressing
FAA004	1309 hTERT	24 well	28	Not Progressing
FAA005	1309 hTERT	24 well	28	Not Progressing

1309 hTERT = FA-A hTERT

1309 hTERT + RVA = FA-A + FANCA hTERT

Appendix 2. Expansion of monoclonal populations of FA-G hTERT and FA-G + FANCG hTERT cells

Clone Name	Cell Type	Dish Size	Days In Culture*	Growth
CORG001	GM02361A hTERT + RVG	12 well	28	Slow growth
CORG002	GM02361A hTERT + RVG	12 well	28	Not Progressing
CORG003	GM02361A hTERT + RVG	12 well	28	Slow growth but look healthy
CORG004	GM02361A hTERT + RVG	12 well	28	Slow growth but look healthy
CORG005	GM02361A hTERT + RVG	T-25	28	Fast growth
CORG006	GM02361A hTERT + RVG	12 well	28	Not Progressing
CORG007	GM02361A hTERT + RVG	T-25	28	Fast growth
CORG008	GM02361A hTERT + RVG	T-25	28	Fast growth
CORG009	GM02361A hTERT + RVG	12 well	28	Not Progressing
CORG010	GM02361A hTERT + RVG	12 well	28	Not Progressing
CORG011	GM02361A hTERT + RVG	12 well	28	Not Progressing
CORG012	GM02361A hTERT + RVG	12 well	28	Not Progressing
CORG013	GM02361A hTERT + RVG	12 well	22	Slow growth
CORG014	GM02361A hTERT + RVG	12 well	22	Slow growth
CORG015	GM02361A hTERT + RVG	12 well	22	Slow growth
CORG016	GM02361A hTERT + RVG	12 well	22	Slow growth
CORG017	GM02361A hTERT + RVG	12 well	22	Not Progressing
CORG018	GM02361A hTERT + RVG	T-25	18	Fast growth
CORG019	GM02361A hTERT + RVG	12 well	18	Not Progressing
CORG020	GM02361A hTERT + RVG	12 well	18	Slow growth
FA-G001	GM02361A hTERT + Empty retrovirus	12 well	22	Not Progressing
FA-G002	GM02361A hTERT + Empty retrovirus	12 well	22	Not Progressing
FA-G003	GM02361A hTERT + Empty retrovirus	12 well	22	Not Progressing
FA-G004	GM02361A hTERT + Empty retrovirus	12 well	22	Not Progressing
FA-G005	GM02361A hTERT + Empty retrovirus	12 well	22	Not Progressing
FA-G006	GM02361A hTERT + Empty retrovirus	12 well	22	Not Progressing
FA-G007	GM02361A hTERT + Empty retrovirus	12 well	18	Slow growth but look healthy
FA-G008	GM02361A hTERT + Empty retrovirus	12 well	18	Not Progressing
FA-G009	GM02361A hTERT + Empty retrovirus	12 well	18	Not Progressing
FA-G010	GM02361A hTERT + Empty retrovirus	12 well	18	Slow growth

GM02361A hTERT + Empty retrovirus = FA-G hTERT

GM02361A hTERT + RVG = FA-G + FANCG hTERT