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14. ABSTRACT
The major goal of our research proposal was to determine the role of the FA-BRCA pathway in the suppression of spontaneous and reactive oxygen species (ROS)-induced de novo copy number variation (CNV). A major challenge of our experimental approach has been the isolation of monoclonal populations of hTERT-immortalized FA-A, FA-D2, and FA-G patient-derived cells. This considerable challenge significantly delayed our experimental progress. Nevertheless, through persistent efforts we successfully isolated small numbers of monoclonal populations of FA-D2 and FA-D2 + FANCD2 cells. We performed preliminary studies on these cells and determined that the frequency of spontaneous de novo CNVs is statistically significantly increased in the absence of FANCD2. We also developed an alternative approach to study the role of the FA proteins in the suppression of spontaneous and ROS-inducible CNV using short-interfering RNA (siRNA) in the HCT116 colorectal carcinoma cell line. Using this approach, we have also established that the FANCD2 protein, and not FANCA, is required for the suppression of spontaneous de novo CNV. These findings support a model whereby the FANCD2 protein, possibly independent of the FA core complex proteins, plays a critical role in the prevention of de novo pathogenic CNVs. Future studies will seek to elucidate the molecular mechanism(s) by which FANCD2 performs this key function.

15. SUBJECT TERMS
Fanconi anemia, Copy number variation, Monoclonal cell populations, Functional assays

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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 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, may play 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.

Keywords
Aphidicolin (APH), Copy number variation (CNV), DNA repair, Fanconi anemia (FA), Mitomycin C (MMC), Reactive oxygen species (ROS), Ubiquitination

Overall Project Summary/Body
With reference to our approved Statement of Work:
Specific Aim 1: 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 monoubiquitination status via immunoblotting and nuclear foci formation – to determine if the FA-BRCA pathway is functionally restored

Outcome: As described in our year 1 progress report, in year 1 of the funding period we focused the majority of our efforts on the generation of telomerase (hTERT)-immortalized mutant and corrected FA patient-derived fibroblast lines. Specifically, we focused on the following FA complementation groups: FA-A, FA-D2, and FA-G. In our original proposal we indicated that we would use FA-C patient-derived cells, however we chose to focus on FA-G cells instead because of the availability of FA-G lines and the FANCG cDNA in our laboratory. We successfully generated hTERT-immortalized FA-A and FA-A + FANCA cells and, 1) confirmed re-expression of FANCA protein in the FA-A + FANCA cells, 2) demonstrated correction of mitomycin C (MMC)-inducible FANCD2 monoubiquitination in the FA-A + FANCA cells, and 3) demonstrated correction of MMC-sensitivity in the FA-A + FANCA cells. Thus, the hTERT-immortalized FA-A and FA-A + FANCA cells represent an excellent isogenic system for the study of the role of the FANCA protein in the prevention of de novo pathogenic CNVs.

With regard to the FA-D2 complementation group, in year 1 of the funding period, we obtained two mutant and functionally corrected FA-D2 primary lines from Detlev Schlinger of the University of Wuerzburg in Germany. We hTERT-immortalized these lines and confirmed re-expression of the FANCD2 protein in both the ACHT and KEAE FA-D2 + FANCD2 hTERT lines. Similar to that described for the FA-A complementation group above, during year 2 we performed MMC-inducible G2/M cell cycle stage accumulation assays with these cells and confirmed functional correction of the FA-D2 + FANCD2 cells, consistent with that reported by the Schlinger group (1). Therefore, similar to the hTERT-immortalized FA-A and FA-A + FANCA cells, the hTERT-immortalized FA-D2 and FA-D2 + FANCD2 cells represent an excellent isogenic system for the study of the role of the FANCD2 protein in the prevention of de novo pathogenic CNVs.

With regard to the FA-G complementation group, we hTERT-immortalized FA-G patient-derived fibroblasts and subsequently infected these cells with the murine moloney leukemia retroviral vectors pMMP-Empty or pMMP-FANCG. While we confirmed re-expression of FANCG in the FA-G + FANCG hTERT cells, we could not detect FANCD2 protein expression or FANCD2 and FANCI monoubiquitination in these cells by immunoblotting, raising some concerns as to whether the FA-G + FANCG cells are fully functionally corrected.

Task 2 Determination of the frequency of spontaneous de novo CNVs in mutant and corrected FA-A, FA-C, and FA-D2 patient cells

Sub-task 1. Selection and expansion of clonal populations of FA-A, FA-A + FANCA, FA-C, FA-C + FANCC, FA-D2 and FA-D2 + FANCD2 cells
Sub-task 2. SNP array analysis of genomic DNA from clonal cell populations – to be performed at the University of Michigan

Outcome: Task 2 of specific aim 1 was to determine the frequency of spontaneous de novo CNVs in mutant and corrected FA-A, FA-C (revised to FA-G), and FA-D2 patient cells. In consultation with our collaborators at the University of Michigan, we decided that it was critical to isolate monoclonal populations of hTERT-immortalized FA-A and FA-A + FANCA and FA-D2 and FA-D2 + FANCD2 cells prior to beginning the CNV analyses experimentation. The reasons for this are that 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
background levels of CNVs, prior to initiating our experiments. Therefore, de novo CNVs will be clearly distinguishable from pre-existing CNVs in the parental cell population. Thus, in year 2 of the funding period we sought to isolate monoclonal populations of these cells. This proved to be an extremely challenging endeavor. Nevertheless, in collaboration with the Glover Laboratory, we successfully managed to isolate a small number of monoclonal populations of the hTERT-immortalized FA-A and FA-A + FANCA, FA-D2 and FA-D2 + FANCD2, and FA-G and FA-G + FANCG cells. However, it must be emphasized that for the mutant FA-A, FA-D2, and FA-G cells, yet not the corrected cells, this was a remarkably inefficient process, discussed in more detail below.

**Isolation of monoclonal cell populations for analysis of de novo CNV:** Upon generating these monoclonal cell populations we commenced experiments to select and expand clonal populations of FA-A, FA-A + FANCA, FA-D2, FA-D2 + FANCD2, FA-G and FA-G + FANCG cells for the analysis of spontaneous de novo CNV. Multiple experiments were conducted in our laboratory towards this goal with limited success. The following are some of the variables that we modified over the course of our experiments in an effort to isolate appreciable numbers of monoclonal cell populations. We used several different commercially available tissue culture dishes and flasks, e.g. Corning, Fisher BioLite, and CellTreat. We tried several different brands, grades and percentages of fetal bovine serum in our tissue culture medium. We varied the frequency at which we changed our media during the clonal isolation process. We plated cells at various cell densities. We also purchased a New Brunswick Galaxy 170R tri-gas incubator enabling us to grow cells at physiological oxygen concentrations ([O₂]Phys = 5%), as opposed to atmospheric oxygen concentrations ([O₂]Atmos = 20%). Numerous reports have indicated that primary/hTERT-immortalized cells display significantly improved growth/proliferation when cultured under physiological oxygen concentration, as opposed to atmospheric oxygen concentration (2). We hypothesized that an increased oxidative burden might be contributing to our difficulties in isolating monoclonal cell populations, and that this might be particularly relevant to FA patient-derived cells, as they are well known to be sensitive to reactive oxygen species (3,4). Unfortunately, culturing these cells at [O₂]Phys did not lead to an improved yield of monoclonal cell populations. Thus, despite multiple attempts using multiple variations in experimental conditions, we were unsuccessful in generating appreciable numbers of additional monoclonal populations of the mutant FA cells. We believe that this phenotype may be innately linked to the underlying DNA repair and/or cell proliferation defects of FA cells. Interestingly, the Glover Laboratory has previously attempted to isolate monoclonal populations of hTERT-immortalized Werner syndrome (WS) patient-derived cells without success. WS is characterized by cancer predisposition and accelerated aging and is caused by biallelic mutations in the WRN/RECQL2 gene (5). Similar to FA, WS patient-derived cells have innate DNA repair defects suggesting that defective DNA repair may negatively impact the clonal expansion potential of hTERT-immortalized cells in general.

**Figure 1.** Monoclonal populations of ACHT FA-D2 + FANCG (mutant) (ACHT + G) and ACHT FA-D2 + FANCD2 (corrected) (ACHT + D2) cells were isolated and analyzed for spontaneously arising de novo CNVs by SNP array analysis using Illumina HumanOmni2.5 BeadChip microarrays. Fifteen clones of each line were analyzed.

Nevertheless, following persistent experimental efforts, in collaboration with the Glover Laboratory, we finally generated 15 monoclonal populations each of hTERT-immortalized ACHT FA-D2 + FANCG (mock corrected/mutant) and ACHT FA-D2 + FANCD2 (corrected) cells. Genomic DNA was isolated from these clones and SNP array analysis was performed using Illumina HumanOmni2.5 BeadChip microarrays. Consistent with our hypothesis that the FA proteins suppress the formation of spontaneous de novo CNVs,
only 2 de novo CNVs were detected in 15 clones of ACHT FA-D2 + FANCD2 cells, while 12 de novo CNVs were detected in 15 clones of ACHT FA-D2 + FANCG cells ($P = 0.003$) (Figure 1). These results strongly suggest that the FANCD2 protein plays an important role in the prevention of spontaneous de novo CNVs.

**Alternative approach:** As a result of the considerable difficulties in isolating monoclonal cell populations from our FA-A, FA-D2, and FA-G patient-derived cells, in collaboration with the Glover Laboratory, we devised an alternative approach for assessing the role of the FA proteins in the suppression of spontaneous and ROS-inducible de novo CNV. The Glover Laboratory had recently successfully used an RNA interference approach (RNAi or short-interfering RNA (siRNA)) to interrogate the role of several DNA repair proteins, e.g. RAD51, in the molecular origins of CNV (Thomas W. Glover and Martin F. Arlt, personal communication). The Glover Laboratory used two cell types for these experimental approaches: 090 hTERT and HCT116. 090 hTERT are a normal hTERT-immortalized skin fibroblast while HCT116 are a colorectal carcinoma cell line. The Glover Laboratory shared monoclonal populations of both cells. We subsequently optimized experimental conditions for the knockdown of the FANCA and FANCD2 proteins in the HCT116 colorectal carcinoma cell line (Figure 2).

**Figure 2.** A monoclonal population of the HCT116 colorectal carcinoma line was transiently transfected with control non-targeting siRNA (siCtrl.) or siRNA targeting FANCA (siFANCA) or FANCD2 (siFANCD2) for 72 h. Cells were incubated in the absence (-) or presence (+) of mitomycin C (MMC) for the final 24 h. Whole-cell lysates were prepared, electrophoresed on a 3-8% Tris-Acetate gel, and immunoblotted with anti-FANCD2, anti-FANCA, and anti-Tubulin antibodies.

Next, using these monoclonal HCT116 cells, we initiated experiments to determine the role of FANCA and FANCD2 in the suppression of spontaneous de novo CNVs using this siRNA-based system. We isolated and expanded 10 monoclonal populations each of HCT116 transiently transfected with control non-targeting siRNA and siRNA targeting FANCA, and 11 monoclonal populations of HCT116 transiently transfected with siRNA targeting FANCD2. Genomic DNA was purified from these monoclonal populations and SNP array analysis was once again performed using Illumina HumanOmni2.5 BeadChip microarrays. Similar to that observed for the ACHT FA-D2 + FANCG and ACHT FA-D2 + FANCD2 cells, HCT116 cells transiently depleted of the FANCD2 protein displayed an approximate 2-fold increase in spontaneously arising de novo CNVs ($P = 0.07$), compared to cells transfected with control non-targeting siRNA. In addition, interestingly, no increase in spontaneously arising de novo CNVs was observed for HCT116 cells transiently depleted of the FANCA protein. Taken together, the results of our experiments with hTERT-immortalized FA-D2 patient-derived cells and HCT116 cells transiently depleted of FANCA or FANCD2 using siRNA, strongly suggest that the FANCD2 protein, and not FANCA, plays an important role in the prevention of spontaneous de novo CNVs.

**Table 1.** Monoclonal populations of the HCT116 colorectal carcinoma line transiently transfected with control non-targeting siRNA (siC), siRNA targeting FANCA (siA) or FANCD2 (siD2) were isolated and analyzed for spontaneously arising de novo CNVs by SNP array analysis using Illumina HumanOmni2.5 BeadChip microarrays.

<table>
<thead>
<tr>
<th>Sample</th>
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<td>10</td>
<td>0.5</td>
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<tr>
<td>siA</td>
<td>6</td>
<td>10</td>
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<tr>
<td>siD2</td>
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<td>11</td>
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Specific Aim 2: Determination of the role of the FA-BRCA pathway in the suppression of ROS-induced de novo CNVs

Task 1. Determination of the frequency of H₂O₂- and menadione-induced CNVs in mutant and corrected FA patient cells.

Outcome: As described in our original submission, our collaborators the Glover laboratory had established an in vitro system for the study of de novo somatic CNV formation and demonstrated that aphidicolin (APH), an inhibitor of the replicative DNA polymerases, induces a high frequency of genome-wide CNVs in human cells that resemble both polymorphic variants and pathogenic CNVs (6). These findings strongly suggest that DNA replication stress is a fundamental mechanism in de novo CNV formation (6). In our application, we proposed the determination of the frequency of H₂O₂- and menadione-induced CNVs in mutant and corrected FA patient cells. However, prior to performing experiments with H₂O₂ and menadione, we initiated experiments with APH, a validated and potent inducer of de novo CNVs (6). For this experiment, we once again used a siRNA approach with HCT116 cells. Cells were transfected with control non-targeting siRNA or siRNA targeting FANCA or FANCD2. On day 4 of the experiment, cells were untreated or treated with 0.4 µM APH for 72 h. Multiple monoclonal populations of untreated and APH-treated cells were isolated and expanded. Genomic DNA was purified from these monoclonal populations and SNP array analysis was once again performed using Illumina HumanOmni2.5 BeadChip microarrays.

Unfortunately, for this experiment, in contrast to both previous experiments, we did not observe any appreciable differences in spontaneous or APH-inducible de novo CNVs between cells transiently transfected with control non-targeting siRNA or cells transfected with siRNAs specific for FANCA or FANCD2 (Figure 3). We can only speculate that these particular results are attributable to incomplete siRNA-mediated knockdown of FANCA and FANCD2 in this experiment.

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 confirmed DNA damage-inducible FANCI monoubiquitination in the KEAE FA-D2 + FANCD2 cells
• We have successfully isolated a small number of monoclonal populations of hTERT-immortalized FA-A, FA-A + FANCA, FA-D2, FA-D2 + FANCD2, FA-G and FA-G + FANCG cells
• We have optimized conditions for depleting FANCD2 and FANCA in monoclonal populations of 090 hTERT and HCT116 cells
• We have established that ACHT FA-D2 hTERT cells exhibit an increased frequency of spontaneously arising de novo CNVs, compared with corrected ACHT FA-D2 + FANCD2 hTERT-immortalized cells
• We have established that HCT116 cells transiently depleted of FANCD2 via siRNA, and not FANCI, exhibit an increased frequency of spontaneously arising de novo CNVs, compared with HCT116 cells transfected with a control non-targeting siRNA

Conclusion
A major challenge of our experimental approach has been the isolation of monoclonal populations of hTERT-immortalized FA-A, FA-D2, and FA-G patient-derived cells. This considerable challenge has significantly delayed our experimental progress. Nevertheless, through persistent efforts in collaboration with the Glover laboratory at the University of Michigan, we have successfully isolated small numbers of monoclonal populations of FA-D2 and FA-D2 + FANCD2 cells. We have performed preliminary studies on these cells and determined that the frequency of spontaneous de novo CNVs is statistically significantly increased in the absence of FANCD2. We have also developed and optimized an alternative approach to study the role of the FA proteins in the suppression of spontaneous and ROS-inducible CNV using siRNA in the HCT116 colorectal carcinoma cell line. Using this approach, we have also determined that the FANCD2 protein, and not FANCA, is required for the suppression of spontaneous de novo CNV. These findings support a model whereby the FANCD2 protein, possibly independent of the FA core complex proteins, plays a critical role in the prevention of de novo pathogenic CNVs. Future experiments will include determining the role, if any, of FANCD2 monoubiquitination and phosphorylation in this function, as well as determining the underlying molecular mechanisms by which FANCD2 performs this key function. We will also determine if FANCI, a FANCD2 paralog and heterodimeric partner (7-9), also plays a role in the prevention of de novo CNVs. In addition, as proposed in our original application, to gain insight into the origins and molecular mechanisms of de novo CNV formation, we will systematically analyze the distribution and genomic architecture of experimentally detected CNVs. CNVs will be annotated with respect to gene content, overlap with known polymorphic variants, CNVs associated with congenital disorders, as well as de novo CNVs recently catalogued in cancer genomes (10-12). In addition, future studies will use long-range genomic PCR to sequence the breakpoints of several (~10 per treatment) detected CNVs (6), as breakpoint sequences are highly informative in predicting the DNA repair mechanisms involved in their formation (13).

Publications, Abstracts, and Presentations/Bibliography of Meeting Abstracts
Nothing to report, currently

Inventions, Patents, and Licenses
Nothing to report, currently

Reportable Outcomes
Nothing to report, currently

Other Achievements
As a direct result of this research, numerous cell lines have been generated that will be of considerable benefit for the FA and greater DNA repair communities. For example, monoclonal and polyclonal populations of hTERT-immortalized ACHT and KEAE FA-D2 and FA-D2 + FANCD2 cells.
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References