What is wrong with Fanconi anemia cells?

Sharon B Cantor[1,*] and Robert M Brosh, Jr[2]

[1]Department of Cancer Biology; University of Massachusetts Medical School; UMASS Memorial Cancer Center; Worcester, MA USA; [2]Laboratory of Molecular Gerontology; National Institute on Aging; NIH, NIH Biomedical Research Center, Baltimore; MD USA

Figuring out what is wrong in Fanconi anemia (FA) patient cells is critical to understanding the contributions of the FA pathway to DNA repair and tumor suppression. Although FA patients exhibit a wide range of disease manifestation as well as severity (asymptomatic to congenital abnormalities, bone marrow failure, and cancer), cells from FA patients share underlying defects in their ability to process DNA lesions that interfere with DNA replication. In particular, FA cells are very sensitive to agents that induce DNA interstrand crosslinks (ICLs). The cause of this pronounced ICL sensitivity is not fully understood, but has been linked to the aberrant activation of DNA damage repair proteins, checkpoints and pathways. Thus, regulation of these responses through coordination of repair processing at stalled replication forks is an essential function of the FA pathway. Here, we briefly summarize some of the aberrant DNA damage responses contributing to defects in FA cells, and detail the newly-identified relationship between FA and the mismatch repair protein, MSH2. Understanding the contribution of MSH2 and/or other proteins to the replication problem in FA cells will be key to assessing therapeutic options to improve the health of FA patients. Moreover, loss of these factors, if linked to improved replication, could be a key event in the progression of FA cells to cancer cells. Likewise, loss of these factors could synergize to enhance tumorigenesis or confer chemoresistance in tumors defective in FA-BRCA pathway proteins and provide a basis for biomarkers for disease progression and response.

It remains a mystery why Fanconi anemia (FA) patient cells are exquisitely sensitive to agents that induce DNA interstrand crosslinks (ICLs). This sensitivity is common to cells from the 16 known FA complementation groups (FANCA through FANCQ) of which patients suffer a range of maladies including congenital abnormalities, developmental defects, anemia, and cancer (recently reviewed in[1,2]). Even in the absence of exogenous genotoxic stress, FA hematopoetic stem cells exhibit elevated DNA damage and abnormal checkpoint responses, explaining the increased prevalence for bone marrow failure in FA patients.[3] Thus, FA cells have an underlying deficiency in repairing not only ICLs but also endogenous DNA damage.[4] A basic understanding of the underlying replication problem and the resulting increased sensitivity to ICLs could therefore provide an opportunity for therapeutic intervention, not only for bone marrow failure but also for treatment of malignancy that develops in FA

*Correspondence to: Sharon B Cantor; Email: Sharon.Cantor@umassmed.edu

patients. In a larger context, this understanding could provide insight into how the FA pathway modulates the response of tumors to a broad range of crosslinking chemotherapy agents, such as platinum salts and alkylating agents.

To protect the genome from replication stress and potentially throughout the cell cycle, the FA-BRCA pathway functions with a diverse set of proteins including: structure-specific endonucleases which bind and incise DNA structures; error tolerating translesion DNA synthesis (TLS) polymerases, which bypass certain DNA lesions; and DNA recombinases. In the most basic model, the pathway is triggered when a DNA replication fork collides with a DNA lesion, such as an ICL.[5] An upstream core complex functions to mono-ubiquitinate FANCD2 and FANCI ("ID" complex), thereby triggering changes in the FA complex that facilitates the coordination of the FAN endonuclease with other nucleases (XPF-ERCC1, MUS81-EME1, SLX1-SLX4, SNM1A, and SNM1B) implicated in ICL repair that incise and unhook DNA crosslinks to generate a double-stranded DNA break (DSB). The unhooked ICL is bypassed by TLS polymerases and the replication fork is restored via recombination events mediated in part by downstream FA proteins such as FANCJ/BACH1, FANCD1/BRCA2, and FANCN/PalB2. Elegant studies continue to reveal many new details of FA pathway function in ICL repair,[5] including the recent finding that FANCM can traverse an ICL and therefore separate repair from replication.[6] Moreover, additional functions of the FA pathway continue to emerge[2,7] as well as a greater understanding of modifiers of FA phenotypes. In particular, an endogenous source of DNA crosslinks that disrupts replication in FA cells comes from aldehydes.[8] Correspondingly, loss of aldehyde dehydrogenases, such as ALDH2 in an FA mouse, exacerbates many features of FA, including bone marrow failure.[9-11] Here, we review other factors that contribute to defects in FA cells that could provide insight toward therapeutic intervention, balancing out defects to suppress bone marrow failure or targeting pathways in FA cancer progression to limit tumorigenesis.

Overactive Nonhomologous End-Joining Pathway

Clues to understanding the defects in FA cells have come from the identification of proteins or pathways whose inactivation improves the fitness of FA cells. One of the first pathways identified to contribute to both genomic instability and ICL hypersensitivity in FA cells is the nonhomologous end-joining (NHEJ) pathway. Aside from its role in rearranging immunoglobulin,

NHEJ fixes broken DNA ends and is important for genome stability. In FA cells, several groups report that NHEJ is overactive in S phase and interferes with the preservation of replication forks. Thus, inhibition of NHEJ can improve FA cells in many respects. For example, loss of NHEJ factor, Ku in cells mutant for FA pathway protein, FANCC enhanced ICL resistance and reduced genomic instability.[12] Similarly, inhibition of the NHEJ factor, DNA-PKcs, enhanced ICL resistance in cells deficient for FA pathway proteins FANCD2, FANCA or FANCC.[13] One of the best examples of rescue by elimination of NHEJ comes from studies in BRCA1-deficient cells, which share common defects with FA cells. Most notably, loss of 53BP1, a DNA repair protein functioning in part with the NHEJ pathway, restores homologous recombination (HR) and rescues the lethality in *Brca1* deficient mice.[14-17] These promising findings suggest that elimination of NHEJ could restore fitness in FA/BRCA pathway-deficient cells by reinstating DNA repair, albeit with the cost of impaired NHEJ. However, in some FA/BRCA pathway-deficient cells, elimination of NHEJ failed to completely suppress crosslink sensitivity. In particular, *Brca1* deficient mouse cells that have 53BP1 loss remain sensitive to ICL-inducing agents. Combined loss of 53BP1 and Ku was able to enhance resistance, but not to the level observed in cells with wild-type BRCA1.[18] Likewise loss of NHEJ did not enhance ICL resistance in worms or human FA-J patient cells that are deficient for the BRCA1-interacting helicase FANCJ (BACH1/BRIP1).[13,19] The relationship between FA and NHEJ pathways appears to be species-specific given that in contrast to the rescue in human cells,[13] inactivation of NHEJ exacerbated ICL sensitivity in *Fancd2*$^{-/-}$ mouse cells and double knockout mice had more severe developmental defects.[18,20] Thus, BRCA-FA proteins likely have several functions in the repair of ICLs that extend beyond balancing HR and NHEJ pathways.

Aberrant DNA Damage Responses and Opportunistic DNA Nucleases

In addition to NHEJ, several other factors have been suggested to contribute to the proliferative impairment of FA cells. First, the p53-p21 axis has been linked to the elevated DNA damage response (DDR) in FA cells. Consistent with this interpretation, depletion of p53 suppresses haematopoietic stem and progenitor cell (HSPC) defects observed in *Fancd2*$^{-/-}$ mice.[3] Again, rescue appears to fall short of a panacea for FA cells as elimination of the p53-p21 axis did not suppress the underlying replication stress and genomic instability that accumulates in FA cells.[3] Second, nucleases have been linked with an overactive DDR and genomic instability in FA cells. The nuclease-helicase DNA2 over-resects ICLs in FANCD2-deficient cells and depletion of DNA2 suppresses cisplatin sensitivity.[21] Moreover, the MRE11 nuclease aberrantly degrades replication forks and contributes to genomic instability in BRCA2/FANCD1-, BRCA1-, and FANCA-deficient cells.[22,23] In fact, in FA cells, inhibition of the MRE11 nuclease can preserve fork integrity similar to overexpression of a mutant Rad51 that binds and coats stalled forks.[22]

However, given that HR is not also restored, preserving stalled forks alone may not improve ICL repair. Thus, it may be critical to both protect replication forks from nucleases and also enhance recombination to comprehensively improve the fitness of FA cells. Accordingly, overexpression of wild-type Rad51 improved recombination and resistance to DNA damaging agents in BRCA2 mutant cells.[24,25] Moreover, depletion of the anti-recombinase PARI reduced chromosomal aberrations and sensitivity of FA cells to PARP inhibitors.[26]

MSH2 and Defects in Cells Lacking FANCJ-MLH1 Interaction

More recently, enhanced replication stress and an overactive DDR in FA cells have been linked to proteins of the mismatch repair (MMR) pathway. The link to MMR came from studies designed to understand why ICL resistance requires the direct interaction between FANCJ and the MMR protein, MLH1.[27-29] We found that the MMR protein, MSH2 underlies the ICL processing defects resulting from loss of the FANCJ-MLH1 interaction.[19] Consistent with this finding, depletion of MSH2 suppresses defects found in cells deficient for the FANCJ-MLH1 interaction, including ICL-induced sensitivity, chromosomal aberrations, abnormal G2/M accumulation, and as well as an over-active NHEJ pathway. Depletion of MSH2 did not appear to alter recombination. Instead, the restored ICL resistance was dependent on Rad18-dependent pathway and the Rev1 bypass polymerase suggesting MSH2 depletion enhanced TLS.[19] These outcomes are not unprecedented. In fact, MSH2 deficiency reverses proliferation defects due to short telomeres by a mechanism independent of recombination.[30] Moreover, deficiency in MMR is associated with fewer DSBs at stalled forks and enhanced bypass.[31-35] Furthermore, loss of MMR is linked to enhanced ICL resistance.[36] While the function of MMR proteins in ICL processing is not entirely clear, they bind ICL lesions and other DNA structures that form at stalled replication forks through either the heterodimer, MutSβ (composed of MSH2 and MSH3), or MutSα (composed of MSH2 and MSH6), which subsequently recruit the MutLα complex (composed of MLH1 and PMS2).[34,37-40] Our data indicate that at stalled replication forks, MSH2 interferes with replication restart in cells lacking the FANCJ-MLH1 interaction.[19] Therefore, the interaction between FANCJ and MLH1 could serve to coordinate the DDR and to prevent unproductive MMR processing that impedes the recovery of cells following replication stress.

A conceptual model predicts that FANCJ might serve to catalytically displace the MSH2 protein complex bound to damaged or alternatively structured DNA to facilitate ICL repair (**Fig. 1**). This scenario is supported by recent biochemical data showing that FANCJ partners with the single-stranded DNA binding protein Replication Protein A (RPA) to efficiently disrupt high affinity interactions of proteins bound to duplex DNA driven by FANCJ's motor ATPase.[41] The co-localization of FANCJ with RPA after replication stress also suggests that FANCJ is well situated at forks to help them resume DNA synthesis potentially in

an RPA-stimulated manner.[42] Conceivably, FANCJ contributes to replication restart similar to other RPA-interacting factors that have DNA metabolizing functions such as the SMARCAL1 translocase or RecQ helicases BLM, WRN, and the Fanconi Anemia FANCM/FAAP24 complex.[43-50] Understanding the molecular mechanism whereby FANCJ promotes ICL repair and the restart of stalled replication forks, mediated by its interaction with MLH1 in a pathway suppressed by MSH2, should provide valuable insight to how FANCJ operates with other downstream factors to enable appropriate processing of ICLs or stalled forks so that HR can proceed to preserve genomic stability.
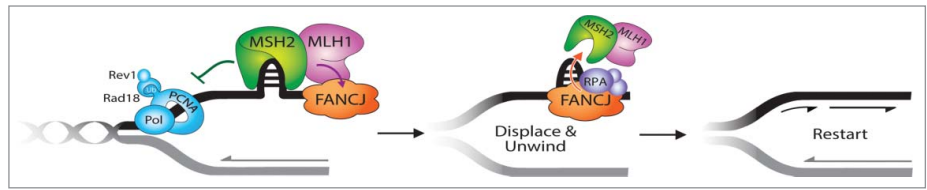

Figure 1. FANCJ-MLH1 interaction suppresses MSH2 to restart stalled replication forks. The timely localization of FANCJ to a fork blocking lesion is mediated by its MLH1 interaction, which places FANCJ in position to subsequently dismantle MSH2 or the DNA structures bound by MSH2 at stalled replication forks. This MSH2 displacement activity is dependent on FANCJ ATPase activity and is facilitated by FANCJ binding to RPA. Failure to displace MSH2 disrupts replication restart and underlies the "problem" in FA cells that lack FA and MMR pathway connections.

Linking MMR and FA-BRCA Pathways for Mutual Regulation

In addition to the FANCJ-MLH1 interaction, FA and MMR pathways are linked at several nodes, suggesting this crosstalk has functional importance. These interactions include BRCA1-MSH2, FANCD2-MLH1/MSH2, SLX4/FANCP-MSH2, and FANCD2-associated nuclease, FAN1-MLH1,[27,51-61] but the functional importance of these interactions has not been fully addressed. A simplified FA pathway in yeast reveals an interaction between the FANCM-like helicase, Mph1, and MutSα functions to repair ICL-induced breaks through the recruitment of Rad51. Notably, this Mph1-MutSα ICL repair pathway involves Chl1, a putative FANCJ helicase.[61] In human cells, it was demonstrated that MSH2 promotes FA pathway function including the chromatin localization of FA core components, the monoubiquitination of FANCD2,[1,51,52] and the localization of FANCJ to sites of DNA crosslinks.[62,63] Given the relationship between the FANCJ-MLH1 interaction and MSH2, a central question is whether other components of the FA pathway also function to balance the activity of MSH2 or other MMR proteins. Supporting this idea, MSH2 loss also suppresses defects in cells deficient for BRCA1 or FANCD2, and this rescue was confirmed in $Msh2^{-/-}$ $Fancd2^{-/-}$ mouse cells. Rescue included not only enhanced ICL resistance, but also suppression of an aberrant DDR including the NHEJ factor, DNA-PKcs.[19] The mechanism linked to suppression of these FA defects was not fully explored, but it is worth considering that similar to the rescue in cells lacking the FANCJ-MLH1 interaction, MSH2 depletion enhances restart pathways.[19] Alternatively, MSH2 loss could reduce fork collapse by enabling FANCM to traverse ICLs and ensure replication forks are uncoupled from ICL repair.[6] This model could explain why MSH2 depletion did not suppress ICL sensitivity in FANCM-null chicken cells.[52] Likewise, MSH2 depletion did not suppress ICL sensitivity in FANCA deficient cells.[19] Since neither FANCM nor FANCA have known MMR interactions,

MSH2 depletion may only rescue FA cells that have defects in FA-MMR interactions but otherwise have intact ICL processing enzymes. Collectively, these findings suggest that cross talk between the MMR and FA pathways enables not only the FA pathway to modulate the MMR pathway, but also for the MMR pathway to modulate the FA pathway and generates a full-regulatory circuit.

Modulators of FA Cell Fitness and Clinical Potential

Ideally the health of FA patients will also be improved by the inactivation or suppression of proteins or pathways that reduce the fitness of FA cells. In particular, it will be important to consider strategies that reduce sources of endogenous DNA damage. Incubation of FA cells under low oxygen conditions can enhance proliferation,[64] and treatment with antioxidants delays carcinogenesis in FA mice.[65] Moreover, enhancing aldehyde catabolism through agonists or balancing their loss with free radical quenchers are promising future directions.[66] In addition, the expression and activity levels of factors that promote replication stress in FA cells could provide biomarkers for disease onset and progression. For example, in FA patients, MSH2 loss could signify that bone marrow failure is improbable but that leukemia is developing. Indeed, MMR-deficient mice are predisposed to hematologic malignancy[67,68] and MMR loss is implicated in acute myeloid leukemia in humans.[69-71] Not only does loss of MMR protein expression and function contribute to cancer susceptibility, it also contributes to chemoresistance.[36,72] Given that the FA-BRCA pathway is essential for tumor suppression in a range of tumors, including breast and ovarian cancer,[73] MMR loss could contribute to a diverse set of cancers and affect patient response. Significantly, if cancers evolve or develop chemoresistance through loss of MSH2 function, several therapeutic options can be considered.[74] In particular, methotrexate induces oxidative DNA damage and is selectively lethal to cancer cells with defects in MSH2[75] and may prove efficacious on FA-associated cancers in which free radicals already reduce fitness. Developing novel

and directed cancer therapies is of high priority, especially since FA patients are exquisitely sensitive to the DNA damage induced by traditional therapies. Ideally, if we are able to right what is wrong in FA patient cells, there will be hope to mitigate bone marrow failure and the development of cancer in patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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