Targeting Fanconi Anemia/BRCA2 Pathway Defects in Cancer: The Significance of Preclinical Pharmacogenomic Models

Eike Gallmeier and Scott E. Kern

Abstract

Defects in the Fanconi anemia (FA) pathway occur in subsets of diverse human cancers. The hypersensitivity of FA pathway-deficient cells to DNA interstrand cross-linking and possibly other agents renders these genes attractive targets for a genotype-based, individualized anticancer therapy. A prerequisite before clinical trials is the validation and quantification of this hypersensitivity in suitable preclinical pharmacogenomic models. In addition, the effects of combination therapy need to be evaluated and novel agents sought. We discuss here the pitfalls and limitations in the interpretation of common FA models when applied to the validation of FA gene defects as therapeutic targets. In general, all preclinical models are prone to certain artifacts and, thus, promising results in a single or few models rarely translate into clinical success. Nevertheless, the extraordinary robustness of FA pathway-deficient cells to interstrand cross-linking agents, which are observable in virtually any model independent of species, cell type, or technique used to engineer the gene defect, in various in vitro and in vivo settings, renders these gene defects particularly attractive for targeted therapy. Clinical trials are now under way.

Background

The Fanconi anemia (FA) gene family consists of at least 12 genes (FANCA, FANCB, FANCC, BRCA2/FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCIJ, FANCL, FANCM; refs. 1–12). Biallelic mutations of these genes cause FA, a rare recessive disorder comprising congenital skeletal abnormalities, progressive bone marrow failure, and increased cancer risk (13). FA gene defects also occur in solid tumors among the general population; BRCA2 is mutated in pancreatic cancers (14–16); and epigenetic gene silencing of FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM occurs in a variety of tumors (17–19).

The FA proteins act in a common pathway, distal parts of which interact with regulators of cell cycle control and DNA repair, especially the repair of DNA interstrand cross-links and double-strand breaks (Fig. 1). The formation of the nuclear FA core complex, comprising Fanca, Fancb, Fancd, Fance, Facl, Fancm, and one yet unidentified 100-kDa protein (Faa100), depends on the integrity of all proteins involved (11, 20, 21). The FA complex becomes activated on DNA damage, as coordinated by DNA damage sensor proteins ATM, ATR, and p53, which interact with regulators of cell cycle control and DNA repair, especially the repair of DNA interstrand cross-links and double-strand breaks (29–34). A direct function of the FA pathway in DNA repair is further supported by Fancj being identical to the DNA helicase Brip1 (35) and Fancm representing the human orthologue of the bacterial DNA repair protein Hef (11, 37).

Clinical Translational Advances

In contrast to tumors of FA patients, FA pathway-deficient tumors arising in the general population have a biallelic FA gene defect whereas the patients’ other cells do not, thus representing a tumor-specific absolute biochemical difference (38). FA pathway-deficient cells are hypersensitive to certain therapeutics, particularly interstrand cross-linking agents (13) and some poly(ADP-ribose) polymerase inhibitors (39–44). Their sensitivity to irradiation remains controversial (45, 46). An important challenge will be the identification and development of novel agents to which FA pathway-deficient cells are hypersensitive. If those agents elicited hypersensitivity via a different mechanism of action than interstrand cross-linking agents, they might permit synergistic effects when used in combination. This would allow a reduction of dosage and thus of the harmful side effects of interstrand cross-linking agents. Likewise, combinational uses of the known agents exerting hypersensitivity deserve investigation.

Potential options for genotype-specific anticancer therapies arising from FA pathway inactivation in tumors will require suitable preclinical models, for which stringent and universal validation criteria have not yet been established (47). Various models of FA gene defects have been developed, each of which has certain limitations when used for pharmacogenomic studies.

References


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Pharmacogenomic FA models

**Human versus other species.** The progress in understanding the molecular functions of the FA genes is largely owed to the successful development and analysis of models of FA gene defects in a variety of species (including human, mouse, hamster, chicken, frog, and zebrafish). For pharmacogenomic studies, the use of human cells may be desirable to eliminate any artifacts attributable to interspecies variability, as most proximal FA genes (constituting the FA core complex) are structurally and functionally not highly conserved. As an example, the chicken *FANCG* gene sequence shares only 39% similarity to human *FANCG*, and its function is not complemented by the human gene (48). In contrast, the more distal *FANCD2* is better conserved (9) and *BRCA2* is highly conserved within the BRC-repeat regions (49).

**Malignant versus nonmalignant cells.** The FA genes are involved in the regulation of cell cycle controls, DNA repair, and genome maintenance, features that are expected to differ between malignant and nonmalignant cells. FA models that use cancer cells may therefore be preferable for pharmacogenomic studies because it is uncertain whether the FA phenotype of nonmalignant cells can be fully extrapolated to malignant cells (46). Table 1 lists many of the presently available human cancer FA lines.

**Natural selection versus artificial engineering.** Gene defects conferring a selective cellular advantage can cause coincidental detrimental effects (“reduction of fitness”), which, if evolving naturally, must be outweighed by the gained advantage. The mutational profile of a cancer thus represents the balance of selective pressures for and against any given mutation; both directions of selection can operate on a given mutated gene (50, 51). Accordingly, cancer cells that have been naturally selected for a FA gene defect are thought to differ significantly from cells on which this defect was superimposed. The extent of a gene knockout–induced reduction of fitness varies strongly among the FA genes, with only a few being essential under many experimental circumstances. Mice having a gene knockout of the proximal genes *FANCA*, *FANCC*, and *FANCG* have normal neonatal viability and no gross growth abnormalities (52–57). In contrast, mice with null mutations of the distal gene *BRCA2* have embryonic lethality, whereas truncating mutations that retain the conserved BRC repeats can confer a viable phenotype (58–64). Some mice having defects in the distal *FANCD2* gene are perinatally lethal, depending on the background strain, which suggests the presence of a modifier locus (65). Fancd2-deficient zebrafish embryos develop a smaller body due to extensive apoptosis (66). In human cancer cells, we found the engineered disruption of the proximal FA

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The development of isogenic cell lines, in which a gene cells.

nonphysiologic influences. Despite these concerns, robust FA models. Nevertheless, the confirmation of a relationship using outbred nonisogenic cells provides a useful assurance of robustness and argues against the results being dominated by idiosyncratic phenomena.

Some FA models compare conditions of constitutive gene overexpression to null expression. In neither condition is physiologic gene function necessarily assessed, for it may be exaggerated, mitigated, or not present at all. In addition, when using human lymphocytes or fibroblasts from FA patients, even the immortalization step, which involves viral tumorigenic proteins, could introduce nonphysiologic influences. Despite these concerns, robust FA phenotypes, such as deficient Fancd2 monoubiquitination, loss of Fancd2 focus formation, and hypersensitivity to interstrand cross-linking agents, are routinely reversed by complementation through gene overexpression.

Specific (targeted) gene knockout and knock-in. The preferable comparison of physiologic to null expression of FA genes can be achieved by either endogenously creating a gene defect in cells proficient (knockout) or reverting a gene defect in cells deficient for the respective gene (knock-in). As these defects are artificially created, there is little opportunity for compensatory evolution in these models and, thus, effects due to a reduction of fitness sometimes predominate, depending on the gene of interest. In addition, somatic cell gene targeting is mainly done (diploid) cancer cells having microsatellite instability. Microsatellite unstable cells, however, do not display chromosomal instability and therefore do not reflect the majority of tumors. Furthermore, the mismatch repair defect of

Table 1. Human FA cancer cell models

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue</th>
<th>Gene defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKO FANCC&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Colorectal adenocarcinoma</td>
<td>FANCC (homozygous Δexon 10)</td>
</tr>
<tr>
<td>RKO FANCG&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Colorectal adenocarcinoma</td>
<td>FANCG (homozygous Δexon 8)</td>
</tr>
<tr>
<td>Hs766T</td>
<td>Pancreatic adenocarcinoma</td>
<td>FANCG (313G&gt;T + LOH&lt;sup&gt;*&lt;/sup&gt;)</td>
</tr>
<tr>
<td>PL11</td>
<td>Pancreatic adenocarcinoma</td>
<td>FANCA2 (617ΔT + LOH&lt;sup&gt;*&lt;/sup&gt;)</td>
</tr>
<tr>
<td>CAPAN1</td>
<td>Pancreatic adenocarcinoma</td>
<td>BRCA2 (8415G&gt;T, 8732C&gt;A, reverting mutation 8731T&gt;G)</td>
</tr>
<tr>
<td>TOV-21G</td>
<td>Ovarian adenocarcinoma</td>
<td>FANCF (epigenetic silencing)</td>
</tr>
<tr>
<td>OHSU-974</td>
<td>Head and neck squamous cell carcinoma</td>
<td>FANCA&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>VU1365</td>
<td>Head and neck squamous cell carcinoma</td>
<td>FANCA&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>VU1131</td>
<td>Head and neck squamous cell carcinoma</td>
<td>FANCC (67ΔG)</td>
</tr>
<tr>
<td>FA-AML1</td>
<td>Acute myelocytic leukemia cells</td>
<td>BRCA2 (3827ΔGT, IVS7 + 2T&gt;GT)</td>
</tr>
<tr>
<td>SB1690CB</td>
<td>Acute myelocytic leukemia cells</td>
<td>BRCA2 (3827ΔGT, IVS7 + 2T&gt;GT)</td>
</tr>
</tbody>
</table>

<sup>*</sup>LOH, loss of heterozygosity.

<sup>1</sup>Complementation analysis was not conclusive (100).

<sup>1</sup>Complementation group was determined by retroviral complementation (100).

genes FANCC and FANCG, but not of the distal FANCD2 and BRCA2, to yield viable clones (46). Consistently, mutations of the proximal FANCC and FANCG, but not of the distal FANCD2, are reported. Despite BRCA2 being mutated in some tumors, its complete inactivation might predominantly be an adverse event (51, 67) because BRCA2 mutations occur late during tumorigenesis (68), almost exclusively yield truncated Brca2 proteins that retain partial function, and are mainly of germ line origin, even in apparently sporadic tumors (69–72). Therefore, tissue culture models in which FA gene defects are artificially created might not accurately reflect the phenotype of this defect in a natural setting, depending on the extent of reduction of fitness and compensatory evolution associated (i.e., high for the distal FANCD2 and BRCA2 genes, low for the proximal FA genes). Consequently, depending on the gene of interest, models using cells that had been naturally selected for a FA defect are preferable for pharmacogenomic studies.

On-target versus off-target effects. On-target effects need to be distinguished from off-target effects, although a specific FA gene defect defines the model. Whereas on-target effects are immediate and directly caused by the FA gene defect, off-target effects comprise artifacts resulting from the particular technique applied to manipulate FA gene function, including unintended drug interferences, oligonucleotide interactions, squelching, threshold effects, etc. Whereas on-target effects are reproducible, off-target effects can seem to be random and are not reliably reproducible. FA models using random chemical mutagenesis, small interfering RNA technology, or nonphysiologic exogenous gene overexpression seem to be particularly prone to off-target effects.

Options for manipulating FA gene function in isogenic cells. The development of isogenic cell lines, in which a gene defect is artificially created or, conversely, the function of a defective gene is artificially restored, overcomes the problem of managing uncontrolled variation inherent to nonisogenic models. Nevertheless, the confirmation of a relationship using outbred nonisogenic cells provides a useful assurance of robustness and argues against the results being dominated by idiosyncratic phenomena.

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microsatellite unstable cells might complicate the interpretation of DNA repair-related FA phenotypes. Another largely neglected problem of knockout models, which is particularly obvious in cancer cells, is clonal variability. “Isogenic” derivatives can comprise variable genotypes among different clones. Thus, more than one gene knockout clone needs to be created and compared with parental and heterozygous control cells. Technical improvements have recently simplified this formerly highly laborious approach (73–75).

**Unspecific (random) gene knockout.** Models using chemical random mutagenesis have lesser gene specificity than single gene knockout models. For example, N-ethyl-N-nitrosurea–mutagenized Chinese hamster cells having defects in the hamster homologues of FANCA, FANCG, and BRCA2 (76–82) are expected to involve many, perhaps hundreds of thousands, simultaneous genetic mutations. Thus, gene specificity of a phenotypic alteration can only be inferred when multiple independent clones can be analyzed. Even then, the magnitude of these phenotypic alterations can potentially be misleadingly high (82).

**RNA interference.** RNA interference models, comparing physiologic gene expression to acutely reduced, but not absent, gene expression (75, 83), have become increasingly popular, having “true” FA gene defects are readily available from FA patients.

### The robust hypersensitivity of FA pathway-deficient cells to interstrand cross-linking agents

Pharmacogenomic FA models differ with regard not only to the above features but also to where the FA defect evolved (in vitro or in vivo) and whether the cellular drug sensitivity was tested in vitro or in vivo through the xenografting of cell lines in mice (86, 87). Each combination offers a different constellation of effects and artifacts. Notably, despite the huge variety of unique FA models, which are all prone to different artifacts, the hypersensitivity of FA pathway-deficient cells to interstrand cross-linking agents holds true in virtually all of these models. The extent of hypersensitivity is best described by the IC$_{50}$ ratios between FA pathway-proficient and FA pathway-deficient cells, here termed the pharmacogenomic window. The width of this pharmacogenomic window depends on the specific interstrand cross-linking agent used and might reflect the proportional contribution of interstrand cross-links to the overall toxicity of the drug (46). IC$_{50}$ ratios of ≥10-fold, generally regarded as promising for further clinical evaluation, can be achieved in preclinical FA cancer models on treatment with several interstrand cross-linking agents (e.g., melphalan, mitomycin C, and carboplatin; ref. 46).

**The particular problem of modeling BRCA2 defects in tumors**

CAPAN1, the only BRCA2-deficient human cancer cell line. Only one cancer cell line identified to date harbors a naturally selected, inactivating BRCA2 mutation (6174ΔT and loss of the second allele). The pancreatic cancer line CAPAN1 is

### Table 1. Human FA cancer cell models (Cont’d)

<table>
<thead>
<tr>
<th>Origin of null state</th>
<th>Available controls</th>
<th>Limitations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Engineered in vitro</td>
<td>Parental/heterozygote clones (physiologic gene expression)</td>
<td>Defect not naturally selected, microsatellite unstable cancer cells</td>
<td>(46)</td>
</tr>
<tr>
<td>Engineered in vitro</td>
<td>Parental/heterozygote clones (physiologic gene expression)</td>
<td>Defect not naturally selected, microsatellite unstable cancer cells</td>
<td>(46)</td>
</tr>
<tr>
<td>Somatically acquired in vivo</td>
<td>Derivatives overexpressing FANCG or other lines</td>
<td>Either gene overexpression or lack of isogenic controls</td>
<td>(15, 86)</td>
</tr>
<tr>
<td>Somatically acquired in vivo</td>
<td>Derivatives overexpressing FANCC or other lines</td>
<td>Either gene overexpression or lack of isogenic controls</td>
<td>(15, 86)</td>
</tr>
<tr>
<td>Somatically acquired in vivo</td>
<td>Derivatives overexpressing BRCA2 or other lines</td>
<td>Either gene overexpression or lack of isogenic controls</td>
<td>(15, 86, 88, 89)</td>
</tr>
<tr>
<td>Unknown</td>
<td>Derivatives overexpressing FANCF or other lines</td>
<td>Either gene overexpression or lack of isogenic controls</td>
<td>(19)</td>
</tr>
<tr>
<td>Germ line (i.e., before tumorigenesis)</td>
<td>Other lines</td>
<td>Lack of isogenic controls</td>
<td>(100)</td>
</tr>
<tr>
<td>Germ line (i.e., before tumorigenesis)</td>
<td>Derivatives overexpressing FANCA or other lines</td>
<td>Either gene overexpression or lack of isogenic controls</td>
<td>(100)</td>
</tr>
<tr>
<td>Germ line (i.e., before tumorigenesis)</td>
<td>Derivatives overexpressing FANCC or other lines</td>
<td>Either gene overexpression or lack of isogenic controls</td>
<td>(100)</td>
</tr>
<tr>
<td>Somatically reverted germ line null state during tumorigenesis</td>
<td>Other lines</td>
<td>No FA phenotype due to reversion of the null state</td>
<td>(101)</td>
</tr>
<tr>
<td>Germ line (i.e., before tumorigenesis)</td>
<td>Other lines</td>
<td>Lack of isogenic controls</td>
<td>(102)</td>
</tr>
</tbody>
</table>
cumbersome for pharmacogenomic studies because it has low transfectability, poor clonogenicity, and slow growth in culture, the latter presumably representing an on-target, reduction-of-fitness phenotype. Perhaps for similar reasons, CAPAN1 cells are more sensitive to a variety of agents than other pancreatic cancer lines,2 constituting in most instances a false positive result. Therefore, nonisogenic cancer lines do not represent suitable controls for CAPAN1. Isogenic controls were provided through the exogenous overexpression of wild-type BRCA2 (88). Paradoxically, the growth rate in these derivatives is even lower, which may be an off-target effect due to unphysiologically high levels of BRCA2 expression. Very recently, additional BRCA2-overexpressing CAPAN1 derivatives were reported and follow-up studies are anticipated (89). Due to the above, the CAPAN1 cell line is thus best suited as a positive control in pharmacogenomic studies, whereas tests of specificity are not afforded.

**BRCA2 knockout models in nonhuman species.** No engineered human gene knockout model exists for BRCA2, probably because the BRCA2-null state usually is lethal (46, 51).1 BRCA2 knockout models in mouse, hamster, and chicken cells do exist, but are of unclear relevance for pharmacogenomic studies of human cancers. In mice, null mutations of BRCA2 are embryonic lethal, a phenotype seeming to be less severe in a p53-null background (61). In contrast, truncating mutations that retain certain BRCA repeats can confer a viable phenotype (58–64). In hamster cells, the compound heterozygote BRCA2 line V-C8, derived from (random) chemical mutagenesis of V79 cells, harbors two different nonsense BRCA2 mutations (82). In chicken DT40 cells, the complete disruption of BRCA2 did not yield viable clones (90), whereas truncating mutations, which spared the first two BRCA repeats, only decreased the cell growth rate in these cells (91). Engineered BRCA2 loss thus variably affects different species, presumably due to the different balance of cellular selection, reduction of fitness, and compensatory evolution, which operates after gene targeting.

**A model mimicking a non-FA patient having a BRCA2-deficient cancer.** A model that might closely reflect non-FA patients having BRCA2-deficient cancer was established by Ludwig et al. (92). BRCA20/0 mammary tumors in BRCA2-proficient mice were obtained through conditional tissue-specific gene targeting. This model has not yet been applied to pharmacogenomic studies.

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2 Unpublished observations.

**References**

15. van der Heijden MS, Brody JR, Gallmeier E, et al. Requirement of rapid diagnostic assays for FA defects in cancer

Defects in the distal FA pathway are diagnosed by direct sequencing for germ line mutations in blood samples, which are commercially available for BRCA2 (such as Myriad Genetics, Salt Lake City, UT). Screening for proximal FA pathway inactivation in tumors remains problematic. As a clinical test, direct sequencing would be laborious and expensive due to the number and size of the many proximal FA genes. Furthermore, one would miss large deletions (especially relevant for FANCA; ref. 93) or epigenetic inactivation (especially relevant for FANCF; refs. 17–19, 94). Chromosome breakage on treatment with diepoxybutane, which is the clinical test applied for the diagnosis of FA, is only feasible for pure and actively growing cancer cells (i.e., cell lines). Similarly, Fancd2 immunoblots using primary cancer tissue are hampered by contaminating normal tissue and therefore also require cell lines. Cell lines, however, cannot yet be quickly and reliably grown from primary tumors. Thus, other techniques to enable screening for proximal FA gene defects in tumors need to be developed.

**Conclusions**

Despite increasing interest in targeted therapies in recent years, there are few examples of successful genotype-based anticancer therapy, including imatinib (Gleevec) for the treatment of chronic myeloid leukemia (95, 96) and gefitinib (Iressa) for the treatment of non–small-cell lung cancers harboring EGFR mutations (97, 98). As discussed in this review, any preclinical pharmacogenomic model has limitations and is prone to certain artifacts. Most pharmacogenomic findings are therefore strongly model-dependent and are rarely generalizable over several models. Thus, they often translate poorly into clinical success. In stark contrast, the hypersensitivity of FA pathway-deficient cells to interstrand cross-linking agents holds true in virtually any FA model, using different species, cell types, and techniques, in various in vivo and in vitro settings. This extremely robust pharmacogenomic phenomenon might therefore represent one of the most promising avenues for individualized anticancer therapy to date. Clinical trials are now under way.

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