Malignant Transformation of Fanconi Anemia Complementation Group D2-deficient (*Fancd2*^{-/-}) Hematopoietic Progenitor Cells by a Single HPV16 Oncogene

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Abstract. Aim: To demonstrate that Fanconi anemia complementation group D2-deficient (*Fancd2*^{-/-}) hematopoietic progenitor cell lines can be transformed by transfection with a plasmid containing either the E6 or E7 oncogene of human papillomavirus (HPV) to generate malignant plasmacytoma-inducing cell lines. Materials and Methods: In order to determine whether a single HPV type 16 (HPV16) oncogene induced malignant transformation, *Fancd2*^{-/-} and *Fancd2*^{+/+} interleukin 3 (IL3)-dependent hematopoietic progenitor cell lines were transfected with plasmids containing E6 or E7 oncogene, or control empty plasmid. Results: *Fancd2*^{-/-} but not *Fancd2*^{+/+} cells were transformed into malignant IL3-independent cells by both E6, and E7 oncogenes, but not by empty plasmid. Hematopoietic cell lines and tumors induced by *Fancd2*^{-/-} E6 and *Fancd2*^{-/-} E7 cell lines were positive for each respective HPV RNA and protein. Conclusion: A single HPV16 oncogene is adequate to produce malignant transformation of *Fancd2*^{-/-} hematopoietic cells.

Patients with Fanconi anemia (FA) with intrinsic DNA repair defects (1-11) demonstrate an increased risk for development of squamous cell carcinomas, with an increasing frequency with age (12-16). Patients with FA, both with and without bone marrow transplant, are at high risk for development of

cancer of the oral cavity, oropharynx, esophagus, and vulvar vaginal region (2), suggesting a role of human papillomavirus (HPV) (17-33). Cytokeratin 14, HPV E6/E7 (K14E6/E7) Fanconi anemia complementation group D2-deficient (*Fancd2*^{-/-}) mice have been demonstrated to develop oral laryngeal squamous cell carcinomas when placed on drinking water containing the chemical carcinogen 4-nitroquinoline oxide (4-NQO) and female mice with estrogen pellets implanted at the cervix subsequently demonstrate squamous cell carcinoma of the cervix (2, 3).

Continuous culture of bone marrow from K14E6/E7 *Fancd2*^{-/-} mice, a condition producing continual oxidative stress over 20-22 weeks in culture, has been demonstrated to generate malignant plasmacytoma-inducing cell lines *in vitro*, while similar cultures from K14E6/E7 *Fancd2*^{+/+} marrow produces no such changes (1). Furthermore, interleukin 3 (IL3)-dependent hematopoietic progenitor cell lines derived from long-term bone marrow cultures from K14E6/7 *Fancd2*^{-/-} mice, when transfected with a plasmid containing E6/E7 oncogenes of HPV, demonstrate similar malignant transformation, indicating that the stromal cells of the hematopoietic microenvironment are not required for oncogenic transformation (1).

In the present studies, we determined whether a single oncogene of HPV, E6 or E7 was adequate for induction of malignant transformation in FA hematopoietic cells.

Materials and Methods

Mice. *Fancd2*^{-/-}, *Fancd2*^{+/-}, and *Fancd2*^{+/+} (129/Sv background) mice were bred according to published methods (1).

Cell lines. Bone marrow stromal cell lines and IL3-dependent cell lines from *Fancd2*^{-/-} (129/Sv) (1), *Fancd2*^{-/-} (C57BL/6) (1), and control mouse strains have been reported. The human HPV-induced cytokeratin 14-positive squamous cell tumor cell (CaSki) line was obtained from the American Type Culture Collection (Manassas, VA, USA).

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Establishment of IL3-dependent hematopoietic progenitor cell lines and clonal cell sub-lines. Non-adherent cells were harvested from long-term cultures of bone marrow from each mouse genotype at week 4 and cultured in six-well tissue culture plates in Iscove's modified Eagle's medium (IMDM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS) and 10 ng/ml recombinant mouse IL3 (Peprotech, Rocky Hill, NJ, USA). The cell lines were passaged weekly for 12-14 weeks to establish primary IL3-dependent cell lines using published methods (1).

Clonal cell sub-lines were established from each of the genotype parent cell lines by expansion of single cell-derived colonies. Cells were then re-plated in methylcellulose, colonies selected at 14 days, and cultured as above, to establish subclonal lines (1). Confirmation of genotype after repeated subcloning was carried out for each cell line.

Transfection of 129/Sv *Fancd2*^{+/+} and *Fancd2*^{-/-} IL3-dependent cell lines (1) was carried out by growing cells in 10 ng/ml recombinant IL3 as published elsewhere (1). Cell lines were transfected with HPV16 *E6* or *E7* containing plasmids or empty plasmids (Figure 1). 129/Sv *Fancd2*^{+/+} and 129/Sv *Fancd2*^{-/-} IL3-dependent nonadherent cell lines derived from long-term bone marrow cultures were transfected with MSCV-C16E6 or MSCV-C16E7 (Addgene, Watertown, MA, USA) plasmids containing *E6* or *E7* gene, respectively, according to Clontech's Retroviral Gene Transfer and Expression User Manual. In brief, cell-free supernatants containing *E6* or *E7* viral particles were produced by transient transfection of 293T packaging cells using the packaging plasmids pRSVRev, as well as, pVSV-G. After infecting for 48 hours, selection using puromycin (4 µg/ml) was carried out for 1 week. IMDM with 20% FBS and 10 ng/ml IL3 was used for all cell lines.

Characteristics of cell lines, tumors, and explanted tumor-derived cell lines from E6-carrying or E7-carrying cell lines. Athymic nude mice with deletion of forkhead box protein N1 (*Foxn1*^{nu}) (Envigo, Indianapolis, IN, USA) in groups of 10 were injected in the flank with 10⁶ *E6*- or *E7*-transfected IL3-independent *Fancd2*^{-/-} cells. Tumors formed four weeks after injection of either *E6* or *E7* transfected *Fancd2*^{-/-} IL3-dependent cell lines in all mice, but not in mice injected with non-transfected cell lines. Once the tumors reached 1 cm in diameter, the tumors were removed, half was prepared for histology, while the other half was grown *in vitro*.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for expression of E6 and E7. RNA was extracted from *Fancd2*^{-/-} cells, *E6*-transfected *Fancd2*^{-/-} cells grown *in vitro* or tumors grown in athymic male mice, *E7*-transfected *Fancd2*^{-/-} cells or tumors grown in nude mice, and *E6*- and *E7*-positive CaSki cells (American Type Tissue Collection) using triazol (Thermo Fisher Scientific, Waltham, MA, USA). Primers specific for *E6* and *E7* were used to demonstrate mRNA expression for *E6* and *E7* using RT-PCR, as previously described (1).

Western blot for proteins. *E6*- and *E7*-transfected and control non-transfected *Fancd2*^{-/-} and *Fancd2*^{+/+} IL3-dependent cell lines, as well as, the *E6*- and *E7*-positive CaSki cell line were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA), 1% L-glutamine (Thermo Fisher Scientific), and 1% antibiotics antimycotic solution (Thermo Fisher Scientific). Total cellular protein was extracted using protein extraction buffer (IP Lysis

Buffer; Thermo Fisher Scientific), containing protease inhibitor and phosphatase inhibitor cocktails (Thermo Fisher Scientific). Protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA, USA). The proteins (15 µg per lane) were separated on denaturing polyacrylamide gels (Mini-Protean TGX Gels; Bio-Rad Laboratories) and then transferred to Immobilon-P polyvinylidene fluoride membranes (Bio-Rad Laboratories) by electrophoresis. Blots were blocked with 5% fat-free dry milk in tris-buffered saline Tween 20 buffer (TBST) for 1 h and then incubated overnight with primary antibodies for *E6* or *E7* (Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary anti-mouse IgG horseradish peroxidase (HRP) conjugate (Promega, Fitchburg, WI, USA). The proteins were exposed to x-ray film (5 to 30 sec) using enhanced chemiluminescence detection reagent SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA, USA). To ensure equal protein loading for quantitation, the same blot was subsequently developed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (mouse anti-GAPDH; Millipore Sigma, Burlington, MA, USA).

E6 and E7 immunofluorescence. *E6*-transfected *Fancd2*^{-/-} and *E7*-transfected *Fancd2*^{-/-} cell line-derived tumors were removed and digested into single-cell suspensions using collagenase and pepsin. Immunofluorescence was performed on tissue culture cell lines including *Fancd2*^{-/-} cells grown *in vitro*, *E6*- or *E7*-transfected *Fancd2*^{-/-} cells grown *in vitro*, single-cell suspensions of explanted tumor cells, T-cell-positive Jurkat cells and RPC5.4 (American Type Culture Collection). Cells were incubated with antibodies to T-cells or B-cells (Life Span Biosciences, Seattle, WA, USA) at 4°C overnight, and washed three times in phosphate-buffered saline (PBS) for 5 min each. Secondary antibodies to mouse IgG HRP (Promega, Madison, WI, USA) or anti-rabbit IgG-HRP (Promega, Madison, WI, USA) were added for 1 h at room temperature then cells were washed in three times in PBS for 5 min each. The cells were incubated in Elite ABC Kit (#PK-6100; Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min, washed three times in PBS for 5 min each, incubated in DAB kit (#SK-4100; Vector Laboratories) at room temperature for 1 min, and washed in running water for 5 min. Hematoxylin was added followed by Methylene Blue Reagent Solution for 1 min. RPC5.4 cells were used as positive controls for B-cell staining, while Jurkat cells were used for positive controls for T-cell staining. The slides were mounted and analyzed as described elsewhere (1).

Statistical methods. Comparisons were performed using a *t*-test if data were normally distributed, or with Wilcoxon rank-sum test otherwise. In all these tests, a *p*-value of less than 0.05 was regarded as significant. As an exploratory analysis, *p*-values were not adjusted for multiple comparisons.

Results

E6 and E7 plasmid transfection of Fancd2^{-/-} cell lines and derivation of IL3-independent cell lines. Cell lines were derived from both *E6*- and *E7*-transfected IL3-dependent cell lines from *Fancd2*^{-/-}, as well as *Fancd2*^{+/+} long-term

Figure 1. Murine stem cell virus (MSCV) plasmids used for transfection of Fanconi anemia complementation group D2 (*Fancd2*^{-/-} and *Fancd2*^{+/+} interleukin 3 (IL3)-dependent hematopoietic progenitor cell lines. A: pMSCV puro plasmid map; B: pMSCV puro plasmid showing E6 and E7 transgene insertion sites in plasmid.

Figure 2. Interleukin 3 (IL3)-dependent Fanconi anemia complementation group D2 (*Fancd2*^{-/-} cells, but not *Fancd2*^{+/+} cells are transformed by human papillomavirus type 16 (HPV16) E6 and E7 transgene insertion. IL3-dependent *Fancd2*^{+/+} (A) and *Fancd2*^{-/-} (B) cells were transfected with pMSCV puro plasmid containing either HPV16 E6 or E7 transgene. IL3-dependent *Fancd2*^{-/-} cells were transformed, but the IL3-dependent *Fancd2*^{+/+} cells were not.

Figure 3. Human papillomavirus type 16 E6- and E7-transfected interleukin 3 (IL3)-dependent Fanconi anemia complementation group D2 (*Fancd2*)^{-/-} cells grown in the absence of IL3. IL3-dependent *Fancd2*^{-/-} but not *Fancd2*^{+/+} cells transfected with either E6 or E7 become IL3-independent and malignant in appearance. Non-transfected *Fancd2*^{-/-} cells (A) were dead by day 7 (magnification ×40). *Fancd2*^{-/-} cells transfected with either E6 (B) or E7 (C) (but not control plasmid) transformed to adherent cells grown in the absence of IL3 and grew to high density. *Fancd2*^{-/-} cells transfected with empty plasmid grown in the absence of IL3 were also dead by day 7 (magnification ×40).

bone marrow cultures (1) using plasmid vectors shown in Figure 1. *Fancd2*^{-/-} cells but not *Fancd2*^{+/+} cells became IL3-independent and grew without IL3 (Figure 2). The *Fancd2*^{-/-} lines became IL3 independent by 4 weeks (Figures 2 and 3). Results are from triplicate experiments. The IL3-independent *Fancd2*^{-/-} cell lines formed adherent

cell clusters (Figure 2). No *Fancd2*^{+/+} cells that were E6- or E7-transfected became IL3-independent (Figure 3). The E6- and E7-transfected *Fancd2*^{-/-} cell lines all became IL3-independent (Figure 3). No IL3-independent cell lines were derived from empty plasmid-transfected *Fancd2*^{+/+} cell lines or any *Fancd2*^{+/+} cell lines.

Figure 4. Gross pathological appearance of tumors in athymic nude mice at day 30 after subcutaneous injection of 1×10^6 human papillomavirus type 16 E6-(A) or E7-(B) transfected interleukin 3 (IL3)-independent Fanconi anemia complementation group D2 (*Fancd2*)^{-/-} cells ($\times 10$). Appearance of IL3-independent cells cultured from tumor in panel A (C) and tumor in panel B (D) ($\times 40$).

Figure 5. Detection of human papillomavirus type 16 E6 and E7 oncogenes by reverse transcriptase-polymerase chain reaction (RT-PCR) in 129/Sv mouse Fanconi anemia complementation group D2 (*Fancd2*)^{-/-} cell lines transfected with human papillomavirus type 16 (HPV16) E6 or E7 oncogenes and in explanted tumors. Tumors that developed from the injection of *Fancd2*^{-/-} E6- or *Fancd2*^{-/-} E7-bearing cells into athymic nude mice were analyzed for the expression of E6 or E7 by RT-PCR. RNA was extracted from *Fancd2*^{-/-} cells, E6- or E7-transfected *Fancd2*^{-/-} cells grown in culture, and from tumors derived from these cell lines. CaSki cells, which are positive for E6 and E7, grown in culture and plasmid DNA containing the E6 or E7 transgene were used as positive controls. RT-PCR was performed using primers specific for HPV16 E6 and E7. N.C.: Empty plasmid DNA, negative control.

Figure 6. Detection of human papillomavirus type 16 (HPV16) E6 and E7 oncogene proteins in HPV16 E6- and E7-transfected Fanconi anemia complementation group D2 (*Fancd2*)^{-/-} cell lines and derived tumors. Western blot analysis for FANC2, E6 and E7 was performed on *Fancd2*^{-/-} cells, E6- and E7-transfected *Fancd2*^{-/-} cells grown in culture or from tumors. CaSki cells, which are positive for E6 and E7, were used as the positive control.

Biological properties of factor independent cell lines derived from E6- and E7-transfected Fancd2^{-/-} cells. The IL3-independent *Fancd2*^{-/-} cell lines formed tumors *in vivo* in nude mice (Figure 4). Explanted tumor cell-derived cell lines remained IL3-independent (Figure 4). Both injected cell lines and explanted tumors showed

expression of E6 or E7 oncogene sequenced by RT-PCR (Figure 5) and proteins by western blot (Figure 6). Explanted tumor-derived cell lines were IL3-independent (Figure 4), while the parent *Fancd2*^{-/-} cell line transfected with empty plasmid died in the absence of IL3 (Figure 3).

Cell lines and cells from cell line derived explanted tumors demonstrated markers of T-cell and B-cell lymphoma. E6- and E7-transfected IL3-independent *Fancc2*^{-/-} cell lines displayed T-cell and B-cell markers (CD3 and CD19, respectively) *in vitro* (Figure 7). Tumor cell explants remained positive for the B-cell marker (CD3) by immunohistochemistry (Figure 7). The appearance of hematoxylin and eosin-stained tumor was consistent with lymphoma or plasmacytoma (Figure 8).

Discussion

Patients with FA have experienced an improved survival following bone marrow transplantation (9). Patients commonly live well into their second or third decade (9). Patients with FA, both with and without bone marrow transplants have demonstrated an increased frequency of malignant squamous cell carcinomas of the head and neck region, and female patients with FA demonstrate an increased frequency of cervical and vulvar vaginal cancer, as well as esophageal cancer (12-15). These data demonstrate an alarming age-dependent increase in frequency of appearance of these cancer types, the etiology of which remain unknown. Despite the search for HPV as a potential cofactor in oncogenic transformation (16-33), and for other bacterial and viral pathogens, no etiological agent has yet been identified.

A recent publication demonstrated that transgenic expression of HPV E6 and E7 oncogenes in *Fancc2*^{-/-} mice, but not control mouse strains, was associated with an induction of squamous cell carcinomas of the oral cavity by continuous administration of the chemical carcinogen 4-NQO (3). Furthermore, the female mice of this strain demonstrated evolution of squamous cell cervix cancer following implantation of estrogen pellets at the cervix (3). No carcinogenesis was demonstrated in control mouse strains, nor were squamous cell carcinomas detected in K14E6/7 *Fancc2*^{-/-} mice that were not exposed to 4-NQO or estrogen pellets (3). The question of whether HPV oncogenes were sufficient for malignant transformation was evaluated in a recent publication (1) in which continuous bone marrow culture from these K14E6/7 *Fancc2*^{-/-} mice, as well as *Fancc2*^{-/-} IL3-dependent hematopoietic progenitor cells maintained in the absence of a hematopoietic microenvironment, generated malignant plasmacytoma-inducing cell lines. While this model system tested the effect of HPV16 oncogenes in a different target organ (bone marrow), the system also provided the unique ability to study multilineage stem cell populations of the marrow in organ culture with an intact microenvironment. No such long-term organ culture system exists for squamous cell/stem cell progenitors of the oral cavity or cervix; however, the experiments with bone marrow demonstrated the capacity for oncogenic transformation of two human HPV16 oncogenes

Figure 7. Human papillomavirus type 16 E6- and E7-transfected Fanconi anemia complementation group D2 (*Fancc2*^{-/-}) cell lines expressed both T-cell (CD3) and B-cell (CD19) markers, and cell line-derived tumors also expressed B-cell-specific marker. Human papillomavirus type 16 E6- and E7-transfected interleukin 3-independent *Fancc2*^{-/-} cells, grown in tissue culture or isolated from explanted tumors grown in athymic nude mice, were stained with an antibody to a T-cell-specific protein (CD3) or B-cell-specific protein (CD19) followed by a fluorescent secondary anti-mouse IgG or anti-rabbit IgG antibody. Cells were examined by fluorescent microscopy (×40).

(E6 and E7) in the *Fancc2*^{-/-} genetic background *in vitro* (1). The question of whether a single HPV16 oncogene was adequate to induce malignant transformation was tested in the present study.

The present study demonstrates that the presence of either HPV16 E6 or E7 oncogene alone is sufficient to induce malignant transformation of *Fancc2*^{-/-} IL3-dependent cell lines

Figure 8. Histopathological appearance of tumors formed by human papillomavirus type 16 E6- (A) and E7- (B) transfected interleukin 3-independent Fanconi anemia complementation group D2 (*Fancd2*)^{-/-} cell lines stained with hematoxylin and eosin (×40).

in vitro. No such transformation was detected with control cell lines including those from long-term cultures of bone marrow from *Fancd2*^{+/+} mice. The present cell lines were consistent with formation of malignant plasmacytoma or lymphoma *in vivo*.

The pathway for DNA repair in FA consists of 23 separate proteins, many of which have multiple functions (8). The FA core complex assembles at the site of DNA double-strand breaks, and results in communication of a signal along the scaffold for DNA-repair enzymes leading to mono-ubiquitination of FANCD2 and FANCI, both of which are required to initiate the process of repair of DNA double-strand breaks. Inactivating mutations or deletion of one or more FA proteins are found in all patients with FA (8). The FANCD2 component of the final activation of duplex step in DNA double-strand break repair is critical for appropriate management of DNA double-strand breaks. The mechanism of malignant transformation in patients with FA is not known, but accumulations of mutations resulting from continuous repair of DNA by non-homologous end joining rather than homologous recombination may be one contributing factor (8). Insertion of a specific viral oncogene into progenitor cells of squamous epithelium leading to squamous cell carcinomas has been hypothesized to be a contributory mechanism of malignant transformation (34-37). No such mechanism has been demonstrated with squamous tissue organoids *in vitro* in mouse models of or patients with FA.

Patients with FA demonstrate evolution of acute myeloid leukemia resulting from the malignant transformation of bone marrow of hematopoietic stem cells (9). The use of the long-term bone marrow culture system mouse model of FA has a great potential value for elucidation of the sequential mutation mechanisms involved in evolution of hematopoietic malignancies (5-7). Further studies should elucidate the sequential mutational changes in *Fancd2*^{-/-} long-term bone marrow cultures and derived hematopoietic cell lines which lead to final malignant transformation by a single HPV or other viral or microbial factors including other oncogenes.

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Conflicts of Interest

Drs. Greenberger and Epperly have a conflict of interest due to the issuance of patents for JP4-039 and related compounds, as radiation protectors and mitigators.

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