Received: 18 October 2022 | Revised: 27 January 2023 | Accepted: 15 February 2023

DOI: 10.1002/ijc.34506

INNOVATIVE TOOLS AND METHODS

IJC INTERNATIONAL JOURNAL of CANCER

Fanconi anemia-isogenic head and neck cancer cell line pairs: A basic and translational science resource

Hiep Tai Nguyen[1] | Weiliang Tang[1] | Andrew L. H. Webster[2] | Jeffrey R. Whiteaker[3] | Christopher M. Chandler[1] | Ricardo Errazquin[4,5] | Khashayar Roohollahi[6] | Madeline Fritzke[1] | Elizabeth E. Hoskins[6] | Erica Jonlin[1,7] | Leslie Wakefield[8] | Lucas B. Sullivan[9] | Eleanor Y. Chen[1] | Josephine Dorsman[6] | Ruud Brakenhoff[6] | Amanda G. Paulovich[3] | Markus Grompe[8] | Ramon Garcia-Escudero[4,5,10] | Susanne I. Wells[11] | Agata Smogorzewska[2] | Raymond J. Monnat Jr[1,12]

[1]Department of Laboratory Medicine and Pathology, University of Washington, Seattle, Washington, USA

[2]Laboratory of Genome Maintenance, Rockefeller University, New York, New York, USA

[3]Fred Hutchinson Cancer Center, Clinical Research Division, Seattle, Washington, USA

[4]Instituto de Investigación Sanitaria Hospital 12 de Octubre (imas12), Madrid, Spain

[5]Biomedical Oncology Unit, CIEMAT (Centro de Investigaciones Energéticas, Medio-ambientales y Tecnológicas), Madrid, Spain

[6]Amsterdam University Medical Center, Amsterdam, The Netherlands

[7]University of Washington Institute for Stem Cell and Regenerative Medicine, Seattle, Washington, USA

[8]Departments of Pediatrics and Molecular and Medical Genetics, Oregon Health and Sciences University, Portland, Oregon, USA

[9]Fred Hutchinson Cancer Center, Human Biology Division, Seattle, Washington, USA

[10]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain

[11]Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

[12]Department of Genome Sciences, University of Washington, Seattle, Washington, USA

Correspondence
Raymond J. Monnat, Jr., University of Washington, Departments of Laboratory Medicine/Pathology and Genome Sciences, Box 357705, 1959 N.E. Pacific Street HSB K-wing Room K-072C, Seattle, WA, 98195, USA.
Email: monnat@uw.edu

Present addresses
Hiep Tai Nguyen, National Cancer Institute, Laboratory of Cell Biology, Bethesda, Maryland, USA; Weiliang Tang, Umoja Biopharma, Seattle, Washington, USA; Andrew L. H. Webster, Institute for Systems Biology, Seattle, Washington, USA;
Christopher M. Chandler, Billings Clinic,

Abstract

Fanconi anemia (FA) is a heritable malformation, bone marrow failure and cancer predisposition syndrome that confers an exceptionally high risk of squamous carcinomas. These carcinomas originate in epithelia lining the mouth, proximal esophagus, vulva and anus: their origins are not understood, and no effective ways have been identified to prevent or delay their appearance. Many FA-associated carcinomas are also therapeutically challenging: they may be multi-focal and stage-advanced at diagnosis, and most individuals with FA cannot tolerate standard-of-care systemic therapies such as DNA cross-linking drugs or ionizing radiation due to constitutional DNA damage hypersensitivity. We developed the Fanconi Anemia Cancer Cell Line Resource

Abbreviations: % v/v, percent by volume; CC3, cleaved caspase 3; CCLE, Cancer Cell Line Encyclopedia Project; *cis*-PT, *cis*-diamminedichloroplatinum; CK5, cytokeratin 5; CNV, copy number variant; DAPI, 4′,6-diamidino-2-phenylindole; DDR, DNA damage response; DepMap, the Cancer Dependency Map Project; FA, Fanconi anemia; FA-, Fanconi anemia genetic complementation group; FA-CCLR, Fanconi Anemia Cancer Cell Line Resource; FACS, fluorescence-activated cell sorting; *FANC*, Fanconi anemia complementation group genes; GDSC, Genomics of Drug Sensitivity in Cancer Project; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; indel, insertion-deletion nucleotide variant; MEM/D-MEM, minimal essential cell culture media; MRM-MS, multiple reaction monitoring mass spectrometry; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; SNV, single nucleotide variant; STR, short tandem repeat DNA locus/marker; TCGA, The Cancer Genome Atlas Project; WES, whole exome sequencing; WGS, whole genome sequencing.; γ-H2AX, serine139-phosphorylated histone H2AX.

Billings, Montana, USA; and
Elizabeth E. Hoskins, Halloran Consulting Group, Boston, Massachusetts, USA.

Funding information
Aven Foundation; Brotman-Baty Institute for Precision Medicine; Fanconi Anemia Research Fund; National Cancer Institute (NCI), Grant/Award Number: CPTAC Grant U01CA214114; Pershing Square Sohn Prize for Young Investigators; US National Cancer Institute, Grant/Award Number: R50CA211499; V Foundation for Cancer Research, Grant/Award Number: grant T2019-013

(FA-CCLR) to foster new work on the origins, treatment and prevention of FA-associated carcinomas. The FA-CCLR consists of Fanconi-isogenic head and neck squamous cell carcinoma (HNSCC) cell line pairs generated from five individuals with FA-associated HNSCC, and five individuals with sporadic HNSCC. Sporadic, isogenic HNSCC cell line pairs were generated in parallel with FA patient-derived isogenic cell line pairs to provide comparable experimental material to use to identify cell and molecular phenotypes driven by germline or somatic loss of Fanconi pathway function, and the subset of these FA-dependent phenotypes that can be modified, complemented or suppressed. All 10 *FANC*-isogenic cell line pairs are available to academic, non-profit and industry investigators via the "Fanconi Anemia Research Materials" Resource and Repository at Oregon Health & Sciences University, Portland OR.

KEYWORDS
cancer cell line models, cancer predisposition syndrome, Fanconi anemia, genome engineering, head and neck squamous cancer

What's new?

People with Fanconi anemia are at high risk of squamous cell carcinomas (SCC), but it is not well understood how these cancers originate and there is no effective prevention strategy. Here, the authors describe a new tool for studying the origin, treatment and prevention of these carcinomas. The Fanconi Anemia Cancer Cell Line Resource consists of 10 new *FANC*-isogenic cell line pairs generated from 5 people with FA-associated head and neck SCC and 5 with sporadic HNSCC. These cell lines are available for distribution to investigators studying FA or sporadic HNSCC.

1 | INTRODUCTION

Fanconi anemia (FA) is a heritable bone marrow failure, malformation and cancer predisposition syndrome that results from loss of function of any of 23 functionally linked Fanconi (*FANC*) genes.[1,2] The first recognized FA cancer predisposition was leukemia, often in conjunction with bone marrow failure. The risk of bone marrow failure and leukemia were substantially reduced once highly effective bone marrow transplantation protocols were developed. This changed the natural history of Fanconi anemia, and there are now more adults living with FA than newly diagnosed children and young adults.

An unanticipated consequence of the success in treating FA by bone marrow transplantation has been the emergence of a second, extraordinarily high lifetime risk of squamous cell carcinomas (SCC) arising in epithelia lining the upper aerodigestive tract (oropharynx, neck and proximal esophagus), anus and vulva. These FA-associated SCC may be the first clinical presentation of FA at any age.[1,3] An elevated risk of cutaneous squamous carcinomas has also been documented in individuals with FA, together with a persistent elevated risk of leukemia regardless of prior bone marrow/stem cell transplantation.[4,5] Many additional cancer types have been reported in individuals with FA, but only a small subset—liver and brain tumors, lymphomas and embryonal tumors such as Wilms' tumor and neuroblastoma—are observed at higher-than-expected frequencies vs population controls,[4] and with much lower risk than observed for squamous carcinomas.

The origin of FA-associated carcinomas is not well-understood. The loss of function of any of the 23 genes and proteins that constitute the FA functional pathway promotes constitutional genomic instability and perturbs other cellular pathways that may potentiate cancer risk.[2,5,6] Prior bone marrow transplantation, smoking, excessive alcohol consumption and human papillomavirus (HPV) infection may also contribute, though appear to be less prominent drivers of HNSCC risk in FA,[4,7] There are no effective ways to prevent FA-associated carcinomas, and effective therapy remains elusive for many patients: cancers are often multi-focal or late stage at diagnosis offering few opportunities for surgical cure, and FA patients cannot tolerate effective doses of standard-of-care therapies including DNA cross-linking drugs and ionizing radiation.[3] The combination of elevated risk and a lack of effective preventions or treatments has made SCC the leading cause of premature morbidity and mortality in adults with FA.

In order to foster new work on the origins, treatment and prevention of FA-associated SCC, we generated 10 new *FANC*-isogenic cell line pairs from HNSCC cell lines from FA patients or from sporadic, non-FA-associated HNSCC. FA patient-derived cell lines were from complementation groups FA-A, FA-C and FA-L that collectively encompass ~75% of FA patients.[1,2] Comparable *FANC*-isogenic sporadic HNSCC cell line pairs were generated as experimental controls by *FANCA* gene disruption followed by transgene complementation. The generation and distribution of these *FANC*-isogenic HNSCC cell line pairs to academic, non-profit and industry investigators was made

TABLE 1 Fanconi anemia patient-derived and sporadic head and neck cancer cell lines used to develop the Fanconi anemia cancer cell line resource (FA-CCLR)

ICLAC name[a]	Abbrev	RRID name[b]	Age/sex	Site	Genotype	Transgene(s)[c]
FA HNSCC						
CCH-SCC-FA1[d]	FA1	None	?/M	Tongue	*FANCA*	Lenti
CCH-SCC-FA2[d,e]	FA2	None	?/F	Oropharynx	*FANCA/F*	Lenti
OHSU-SCC-974[f]	974	CVCL_XX23	29/M	Pharyngeal	*FANCA*	Safe harbor/lenti
VU-SCC-1131[g]	1131	CVCL_XX18	34/F	Mouth floor	*FANCC*	Safe harbor/lenti
VU-SCC-1365[g]	1365	CVCL_XX19	22/M	Oral cavity	*FANCA*	Lenti
VU-SCC-1604[g]	1604	CVCL_XX20	?/F	Tongue	*FANCL*	Safe harbor/lenti
Sporadic HNSCC						
JHU-SCC-FaDu[h]	FaDu	CVCL_1218	56/M	Tongue	*FANC+*	Safe harbor/lenti
UM-SCC-01[h]	SCC1	CVCL_7707	73/M	Tongue	*FANC+*	Safe harbor
SFCI-SCC-09	SCC9	CVCL_1685	25/M	Oral cavity	*FANC+*	Safe harbor
CAL-SCC-27[i]	CAL27	CVCL_1107	56/M	Tongue	*FANC+*	Retro
CAL-SCC-33[i]	CAL33	CVCL_1108	69/M	Tongue	*FANC+*	Retro

Abbreviations: F, female; M, male; "site", SCC primary site of origin (Data S1); ?, no information.

[a]Cell line names following systematic naming guidelines established by the International Cell Line Authentication Committee (ICLAC, see: https://iclac.org/resources/cell-line-names/) and cell line use best practices. abbrev = short cell line designation used in manuscript Figures and Tables for clarity and readability.

[b]RRID cell line designations in Release 44 of the Expasy Cellosaurus Cell Line Resource viewed December 2022 (https://www.cellosaurus.org/).

[c]FANC transgene complementations were performed by chromosome 4 "safe harbor" site SHS231-targeted transgene insertion ("safe harbor"), or by lentiviral (lenti) or retroviral (retro) transgene transduction. See Figures 3 and S3 for additional detail.

[d]Feeder cell-independent derivatives were established by Susanne Wells (Children's Hospital Medical Center, Cincinnati OH), then further characterized by Josephine Dorsman (Amsterdam University Medical Center, Amsterdam, The Netherlands) who provided cell line cultures to develop FA-CCLR isogenic pairs. Donor sex was inferred from cell line sequencing data.

[e]FA complementation group assignment and mutation identifications were based on cell line sequencing data.

[f]The isogenic cell line pair was developed from a genome-sequenced isolate of 974 provided by Agata Smogorzewska (Rockefeller University, New York NY).

[g]VU FA patient-derived cell lines 1131, 1365 and 1604 were provided by Josephine Dorman and Ruud Brakenhoff (Amsterdam University Medical Center, Amsterdam, The Netherlands).

[h]FaDu cells contain homozygous nonsense mutations in *FANCM*. SCC1 contains a deleterious *FANCA* variant in exon 21, c.A1891T, leading to protein-truncating stop-gain mutant p.K631X.

[i]Sporadic HNSCC *FANCA* knockout/retroviral transgene-complemented isogenic derivatives were originally generated and characterized by Ricardo Errazquin and Ramon Garcia-Escudero (CIEMAT, Madrid Spain).

possible by generous support from the Fanconi Anemia Research Fund, Eugene, OR.

2 | MATERIALS AND METHODS

2.1 | Cell line sources and culture

Eleven existing FA patient-derived or sporadic HNSCC cell lines were initially identified and used to develop the FA-CCLR. All were given systematic names following International Cell Line Authentication Committee (ICLAC) guidelines[8] for unambiguous identification together with abbreviated names to improve Figure labeling. Cell line designations are cross-correlated with RRID designations where they existed in Table 1. All cell lines were authenticated using short tandem repeat (STR) profiling within the last 3 years. All experiments were performed with *Mycoplasma*-free cells. The human osteosarcoma-derived cell line U-2 OS was purchased from the American Type Culture Collection (ATCC) as an FA-proficient cell line for methods development. Cell line sources and suppliers of all culture reagents and media are detailed in Supporting Information (Data S1).

FA-patient derived OHSU-SCC-974 and sporadic JHU-SCC-FaDu cell lines were grown in Eagle's Minimum Essential Media (MEM) supplemented with 10% v/v fetal bovine serum (FBS; Hyclone Laboratories) and 1% penicillin-streptomycin (Gibco #10378016). Sporadic cell line SFCI-SCC-9 was grown in a 1:1 mixture of Dulbecco-Modified Eagle's Medium (D-MEM) and Nutrient Mixture F12 supplemented with 10% FBS, 0.5 mM sodium pyruvate, 400 ng/ml hydrocortisone and 1% penicillin-streptomycin. All other cell lines were grown in D-MEM supplemented with 10% FBS, 1% non-essential amino acids (Gibco #11140050) and 1% penicillin-streptomycin unless otherwise noted. Cell growth was at 37°C in a 5% CO_2/ambient (20%) oxygen, water vapor-saturated incubator.

2.2 | Cell line genomic characterization

Prior reports and recent, comprehensive genomic profiling data were used to assemble cell line-specific lists of single nucleotide (SNV),

copy number (CNV) and indel (insertion-deletion) variants.[9-13] Somatic SNV/indel and CNV data for FA patient-derived cell lines OHSU-SCC-974, CCH-SCC-FA1 and CCH-SCC-FA2 were from Webster et al,[14] where somatic SNV/indel calls were made using CaVEMAN[15] and Pindel[16] to analyze tumor cell line and patient-matched control whole-genome sequencing (WGS) data. Copy-number amplification/deletions in OHSU-SCC-974, CCH-SCC-FA1 and CCH-SCC-FA2 were called by using CNVkit[17] to compare tumor cell line and patient-matched WGS data. SNV/indel and copy-number amplification/deletions were called for FA patient-derived cell lines VU-SCC-1604, VU-SCC-1365 and VU-SCC1131 using, respectively, Mutect2[18] and CNVkit to compare tumor cell line and patient-matched fibroblast whole-exome sequencing (WES) data.[12] Our primary focus was on gene-centric variant calling in significantly mutated genes considered to be HNSCC drivers.[9,19,20] Somatic mutation data for sporadic HNSCC cell lines JHU-SCC-FaDu, CAL-SCC-27, CAL-SCC-33, SFCI-SCC-09 and UM-SCC-01 were extracted from the Broad Institute Cancer Cell Line Encyclopedia (CCLE)[10] employing Mutect2 and Absolute[21] for SNV/indel and copy-number analyses. For all cell lines, the reporting threshold for copy-number amplification/deletions was $\log_2(CN) > 0.5$ or $\log_2(CN) < -0.5$.

2.3 | Generation and transgene complementation of FANC-deficient cell lines

FANCA-mutant sublines were generated from sporadic HNSCC cell lines by dual guide RNA-targeted Cas9-mediated deletion of a 5′ portion of *FANCA*, or by single guide-targeted *FANCA* exon 4 cleavage to promote mutagenic end joining.[22] Clonally derived *FANCA*-mutant sublines were identified and characterized by deletion-specific colony PCR and sequencing prior to transgene complementation.[22] *FANC* transgene complementations were performed by Cas9 (or Cas12a/CpfI)-gRNA-targeted *FANC* transgene insertion into chromosome 4q Safe Harbor Site 231 (SHS231[23]), or by lentiviral or retroviral transduction.

Chromosome safe harbor site SHS231-targeted transgene cassettes were electroporated into *FANC*-deficient cells with expression plasmids encoding SHS231-specific gRNAs and Cas9 or Cas12a/Cpf1 nuclease. Three days after electroporation hygromycin B (400 μg/ml; Millipore Sigma, Catalog #400050) or puromycin (1 μg/ml, Invivogen) was added followed by 10 to 14 days additional growth, then dilution cloning and PCR screening to identify transgene-positive colonies. Lentiviral transductions were performed at low MOI (multiplicities-of- infection) using third generation HIV-1-based *FANCA*, *FANCC*, *FANCF* or *FANCL* transgene vectors that were CsCl gradient-purified and packaged according to Addgene-archived pLKO.1 vector protocols. Retroviral *FANCA* transgene vector S11FAIN transductions were performed as previously described.[22]

2.4 | Cell proliferation

Cell population doubling times were quantified as previously described[24] in different growth media to identify potential metabolic constraints on proliferation. Cell cycle phase distributions were determined by 4′,6-diamidino-2-phenylindole (DAPI)/ethidium bromide staining of exponentially growing control or mitomycin-C-treated cultures, followed by DNA content profiling on a BD Biosciences LSR II Flow Cytometer using chicken erythrocytes as a DNA content standard. Cell cycle phase fractions were extracted from FACS/flow cytometric data using FlowJo (FlowJo, Ashland, OR) as previously described.[25]

2.5 | Drug and small molecule response profiling

All cell lines and derivatives were profiled for the canonical FA-dependent cellular phenotype of MMC sensitivity. Additional exploratory analyses used two representative FA patient-derived and two sporadic, isogenic cell line pairs, for dose-sensitivity to additional drugs or small molecules including *cis*-Pt, carboplatin and oxaliplatin; formaldehyde; the kinase inhibitors gefitinib and afatinib; inhibitors of ATR kinase (M6620, VX-970/VE822) and WEE1 (AZD-1775); and metformin and rapamycin.

In brief, triplicate wells were seeded in 96-well plates with 2500 cells/well in 150 μl of complete growth medium, followed by the addition of two- or 3-fold serial dilutions of drugs or small molecules in 50 μl of medium. Cell survival was quantified 4 days later by WST-1 (Takara Bio, Catalog #MK400) or AlamarBlue (ThermoFisher, Catalog #DAL1025) assay to compare treated with control wells that were either mock-treated or received vehicle or carrier only. Data were plotted using GraphPad PRISM (GraphPad Software, San Diego, CA) to determine dose-dependent growth suppression, and to estimate drug/small molecule IC_{50} values.

2.6 | Protein expression analyses

Western blot analyses of FANC protein expression were performed using total cell protein extracts (~40 μg from ~1-2.5 × 10^6 cells) prepared in RIPA Lysis Buffer (Boster Biological Technology, Catalog #AR0105) containing protease inhibitors as previously described.[26] Proteins were size-fractionated on precast 10 cm 4% to 12% Bis-Tris or Tris-acetate gels (Thermo Fisher Catalog #NP0336BOX), then electro-blotted onto nitrocellulose membranes (Thermo Fisher Catalog #88025). Membranes were blocked in Tris-buffered saline solution containing 5% non-fat dry milk for 1 h at room temperature or overnight at 4°C, followed by the addition of FANC protein-specific rabbit polyclonal or mouse monoclonal primary antibodies. Primary antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit IgG goat or anti-mouse antisera. All transgenes apart from *FANCA* had in-frame N-terminal HA (hemagglutinin) epitope tags that could be detected by an anti-HA epitope tag-specific mouse monoclonal antibody. All antibodies, suppliers, dilutions and blot protocols are further detailed in Data S1.

Peptide immuno-enrichment coupled with targeted multiple reaction monitoring mass spectrometry (immuno-MRM) was used as a

complementary approach to detect and quantify expression and modification status of 66 DNA damage response (DDR) proteins including 10 individual FANC proteins. In brief, triplicate 10 cm dishes were seeded with 2 to 5 × 10^6 cells prior to the addition of 0.2 μM mitomycin C for 24 h. Whole cell lysates were prepared and processed in a blinded fashion as previously described, using two antibody panels that targeted 126 peptides and 59 post-translational modification sites.[27-29] MRM-MS data were analyzed using Skyline.[30] Peak integrations were reviewed manually to confirm and align peptide transitions of endogenous and stable mass isotope-labeled peptide standards. Means and standard deviations of peptide-specific peak area ratios were calculated from three complete biological replicates of a cell line and genotype ± treatment, then combined and reported as single values.

2.7 | In vitro three-dimensional "tumoroid" cultures

Three-dimensional (3D) in vitro "tumoroids" were grown from isogenic sporadic (JHU-SCC-FaDu) and FA patient-derived (VU-SCC-1131) cell line pairs by modifying a previously reported protocol.[31] In brief, single cells in Matrigel (Corning Life Sciences) were plated onto bare plastic culture dishes to form 40 μl "domes" that each contained ~1000 cells. After Matrigel domes had solidified, plates were flooded with media for seven additional days prior to gentle mechanical dissociation to allow continued growth in suspension. The MMC responsiveness of tumoroids was assessed using Day 40 tumoroids in suspension cultures that were supplemented for 3 days with 10, 50 or 100 nM MMC. Tumoroid samples were then harvested by unit gravity sedimentation, fixed, paraffin-embedded and sectioned for staining and immunostaining to characterize structure (hematoxylin and eosin) and to detect differentiation/disease-specific (cytokeratin 5 (CK5) and the p40 fragment of p63) and functional state (Ki67; Ser139-phosphorylated histone H2AX (γ-H2AX); and cleaved caspase 3 (CC3)) markers.

2.8 | Statistical analyses

Cell proliferation and dose-dependent survivals were plotted with mean ± the SE of the mean (SEM) values. The statistical significance of differences in cell proliferation rate as a function of genotype was determined by one-way ANOVA. Cell cycle phase analyses were extracted from a minimum of 10 000 gated and flow-analyzed single cell events per culture.

2.9 | Results

A systematic work flow was used to generate isogenic cell line pairs from five FA patient-derived and five sporadic HNSCC cell lines: FA patient-derived lines were complemented with transgenes for *FANCA*, *FANCC* or *FANCL*, while sporadic *FANC*-isogenic cell line pairs were generated by disrupting, and then transgene-complementing, *FANCA* (Table 1 and Figure 1). All cell line identities were confirmed and/or newly established by STR DNA fingerprinting, and all lines were verified to be free of cross-species contamination or *Mycoplasma* infection upon receipt and again prior to distribution (Table S1).

Both FA patient-derived and sporadic HNSCC cell lines contained previously reported genomic alterations.[7,9,12,13,32,33] Figure 2 summarizes these data for cell line-specific single nucleotide (SNV) and copy number (CNV) variant data for three FA patient-derived and five sporadic HNSCC cell lines in genes relevant to HNSCC. Full data access for these lines allowed direct comparisons to each other, and to 426 HPV-negative HNSCC samples included in TCGA data.[20] We had more limited data access for FA patient-derived cell lines VU-SCC-1131, 1365 and 1604, though were also able to call potential HNSCC driver gene variants in these lines using a similar workflow (Figure S1).[19,20] *TP53* pathogenic variants were present in all FA patient-derived and three of five sporadic HNSCC cell lines, together with additional deleterious variants in significantly mutated, putative HNSCC driver genes (Figures 2 and S1). Of note, none of the FA patient-derived cell lines contained identifiable HPV-derived DNA sequences. Driver gene alterations in FA patient-derived HNSCC cell lines were accompanied by many additional gene deletion/amplification/rearrangement events that are the dominant genomic signature observed in FA patient-derived primary HNSCC.[14]

2.10 | Generation of FANC-isogenic HNSCC cell line pairs

FANC-isogenic cell line pairs were generated from five sporadic HNSCC cell lines in order to provide directly comparable experimental material for investigators using FA patient-derived isogenic cell lines. Isogenic, sporadic pairs were generated by Cas9 dual guide RNA-targeted deletion of *FANCA* exon 2 and a portion of exon 3, or by Cas9 single guide RNA-targeted cleavage of *FANCA* exon 4 to promote mutagenic end-joining. *FANCA* mutant clones were verified by PCR deletion or mismatch cleavage screening, DNA sequencing, Western blot analysis and eventual phenotypic analysis (Figure 3A, B).[22]

FANC-complemented derivatives of FA patient-derived and sporadic *FANCA*-mutated HNSCC cell lines were generated by transgene insertion into chromosome 4 safe harbor site SHS231, and/or by lentiviral or retroviral transduction (Figures 3B-D and S3). Viral complementation led to consistently higher transgene protein expression levels than did safe harbor site targeting, and was required to fully restore MMC resistance in FA patient-derived, but not in newly generated sporadic, *FANCA*-mutant HNSCC cell lines. We were able to generate a fully complemented isogenic cell line pair for all FA patient-derived cell lines with the exception of CCH-SCC-FA2: neither sequential or simultaneous safe harbor site insertion or viral transduction of *FANCA* and *FANCF* transgenes fully restored MMC resistance. This result remains unexplained, and as a result we did not

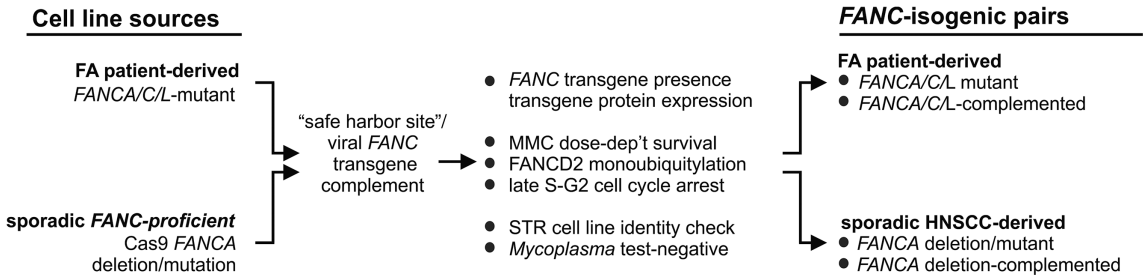

FIGURE 1 Work flow for generation of Fanconi-isogenic cell line pairs from FA patient-derived and sporadic head and neck squamous carcinoma cell lines.

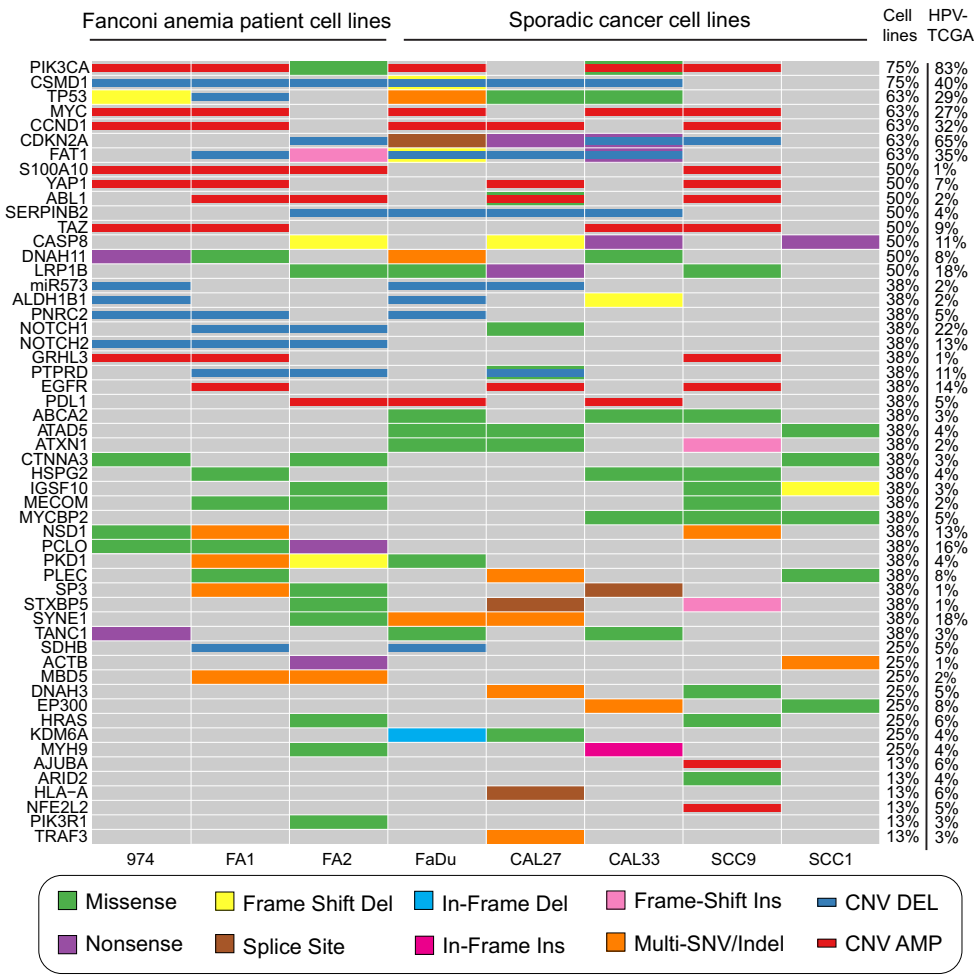

FIGURE 2 Gene-specific alterations in Fanconi anemia patient-derived and sporadic HNSCC cell lines. Columns display Oncoplot data for eight head and neck cancer cell lines, including three Fanconi anemia patient-derived and five sporadic HNSCC cell lines, with cell lines identified under each column. Rows indicate specific genes frequently mutated in, and considered HNSCC driver genes. Gene-specific variants are shown using the type-specific color code shown at bottom. Right margin columns indicate frequencies with which each gene was altered in our eight HNSCC cell lines, and the corresponding frequency for HPV-negative HNSCC samples in TCGA data. The reporting thresholds for copy-number amplifications and deletions are log2(CN) > 0.5 or log2(CN) < −0.5 respectively. See Table 1 for additional cell line information.

complete an isogenic FA2 cell line pair for distribution as part of the FA-CCLR resource (Table 1).

A concern in all cancer cell line work is clonal heterogeneity that may confound many types of cell-based analyses. We addressed this issue by comparing mutant and transgene-complemented cell line clones and pools vs their parental cell line of origin. Figure S2 provides an example, in which we compared the MMC sensitivity of parental FA patient-derived cell line OHSU-SCC-974 with 11 independent, transgene-complemented clonal derivatives, and with a complex pool of independent, transgene-complemented cells. The consistent functional equivalence of clones and pools of transgene-complemented, *FANC*-deficient cells as assessed by MMC dose/sensitivity led us to distribute transgene-complemented pools of cells with clonal complexities of ≥200 as the most versatile starting point for end users.

2.11 | Characterization of FANC-isogenic HNSCC cell line derivatives

FANC gene- and pathway activity-dependent phenotypes were characterized in isogenic cell line pairs to establish causal relationships between *FANC* genotype, FANC protein expression and cellular

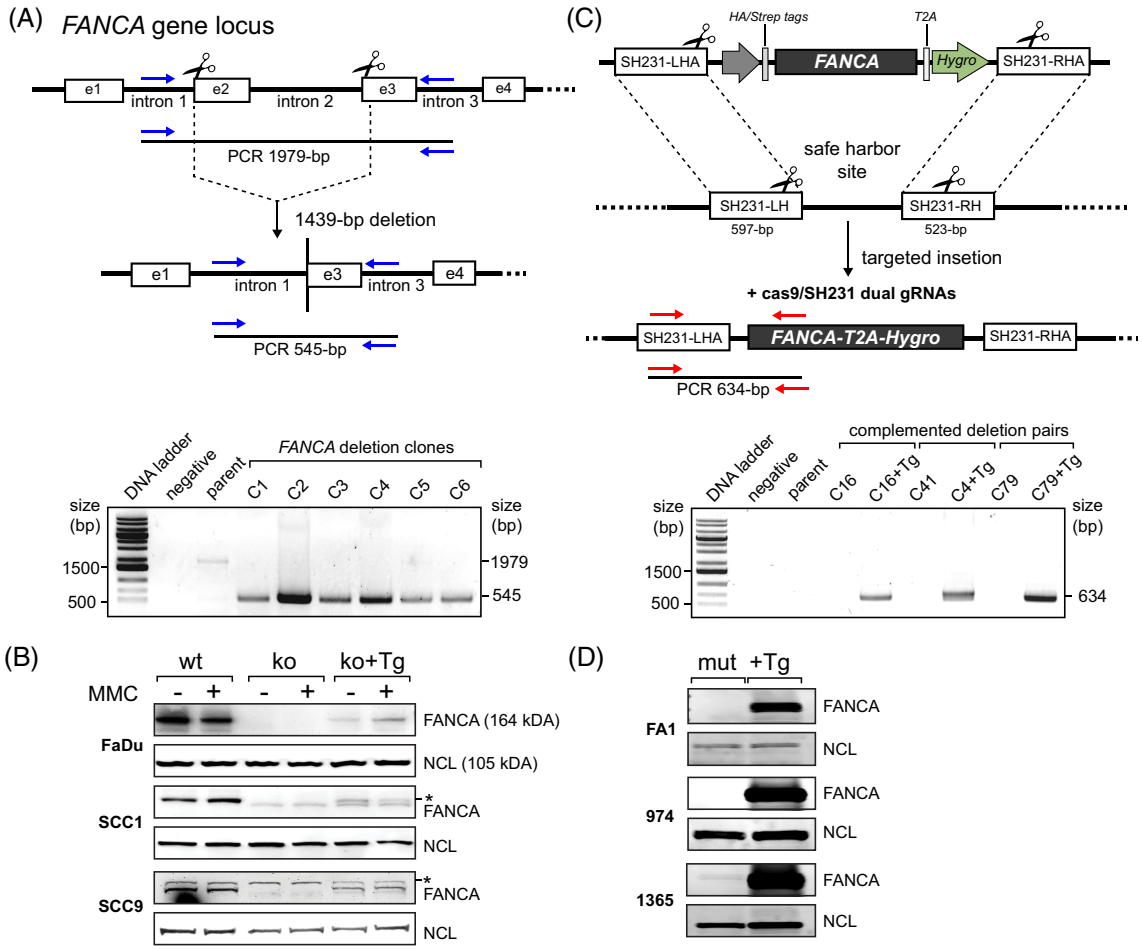

FIGURE 3 Generation and transgene complementation of FANC-deficient HNSCC cell lines. (A) *FANCA* inactivation by dual guide RNA-targeted bi-allelic *FANCA* 5′ gene deletions (upper panel) with PCR detection of intact (upper panel) and deleted (lower panel) alleles. (B) FANCA protein expression in sporadic HNSCC cell lines FaDu, SCC1 and SCC9 prior to (wt, ko = knockout) and after (ko + Tg) *FANCA* transgene insertion and MMC treatment. (C) *FANCA* transgene insertion into chromosome 4q safe harbor site SHS231 by nuclease cleavage-targeted, homology-mediated break repair (upper panel). Transgene insertion and orientation were confirmed by site- and orientation-specific PCR assay (lower panel). (D) FANCA protein expression in three FA patient-derived and *FANCA* transgene-complemented cell lines FA1, 974 and 1365. LH, left homology; LHA, left homology arm; RH, right homology; RHA, right homology arm; ko, gene knockout line; mut, mutant; NCL, nucleolin, a loading control; Tg, transgene; wt, wild-type. *Non-specific band variably detected by FANCA antiserum. [Color figure can be viewed at wileyonlinelibrary.com]

phenotype. We looked initially for a proliferative advantage of *FANC*-proficient or complemented cells, as this phenotype provided a strong rationale for treating FA with bone marrow or hematopoietic stem cell transplantation.[34] Figure 4A provides a representative example of the proliferation of two isogenic, FA patient-derived cell line pairs grown in different media at 37°C in ambient (20%) oxygen. Proliferation rates varied by over 2-fold across cell lines (range 0.47-1.2 doublings/day) with FA patient-derived cell line VU-SCC-1131 having the fastest, and FA patient-derived cell line VU-SCC-1365 the slowest, doubling rates. We did not observe genotype-specific proliferation rate differences between cells grown in ambient (20%) vs low (5%) oxygen in pilot experiments (additional results not shown). These experiments identified cell line- and growth medium-specific proliferative differences between *FANC*-isogenic cell line pairs, but no consistent relationship between cell line proliferation, *FANC* genotype or protein expression. Parallel observations have been reported by Errazquin et al. for the sporadic *FANCA*-isogenic CAL-SCC-27 and CAL-SCC-33 cells included in this Resource.[22]

In contrast to cell proliferation, *FANC* genotype modified MMC sensitivity in a highly reproducible and specific way in all isogenic cell line pairs (Figure 4B and Table S2). FA patient-derived cell lines were MMC-sensitive, with MMC IC_{50} values of 2.8-13.4 nM (mean 6.5 nM) or ~ 4-fold *lower* than FA-proficient sporadic HNSCC cell lines (range 4.4-36.8 nM, mean 22.9 nM). *FANC* transgene complementation of these FA patient-derived cell lines conferred MMC resistance with 3-to-10-fold *higher* MMC IC_{50} values. *FANCA* deletion in sporadic HNSCC cell lines, in contrast, sensitized cells to MMC, with 14-to-44-fold *lower* MMC IC_{50} values that could again be *FANCA* transgene-complemented (Figure 4B and Table S2).

FA patient-derived cell lines display a strong MMC dose-dependent increases in late S/G_2 cell cycle fractions after treatment with 10 or 40 nM MMC. This cell cycle phenotype is so robust that

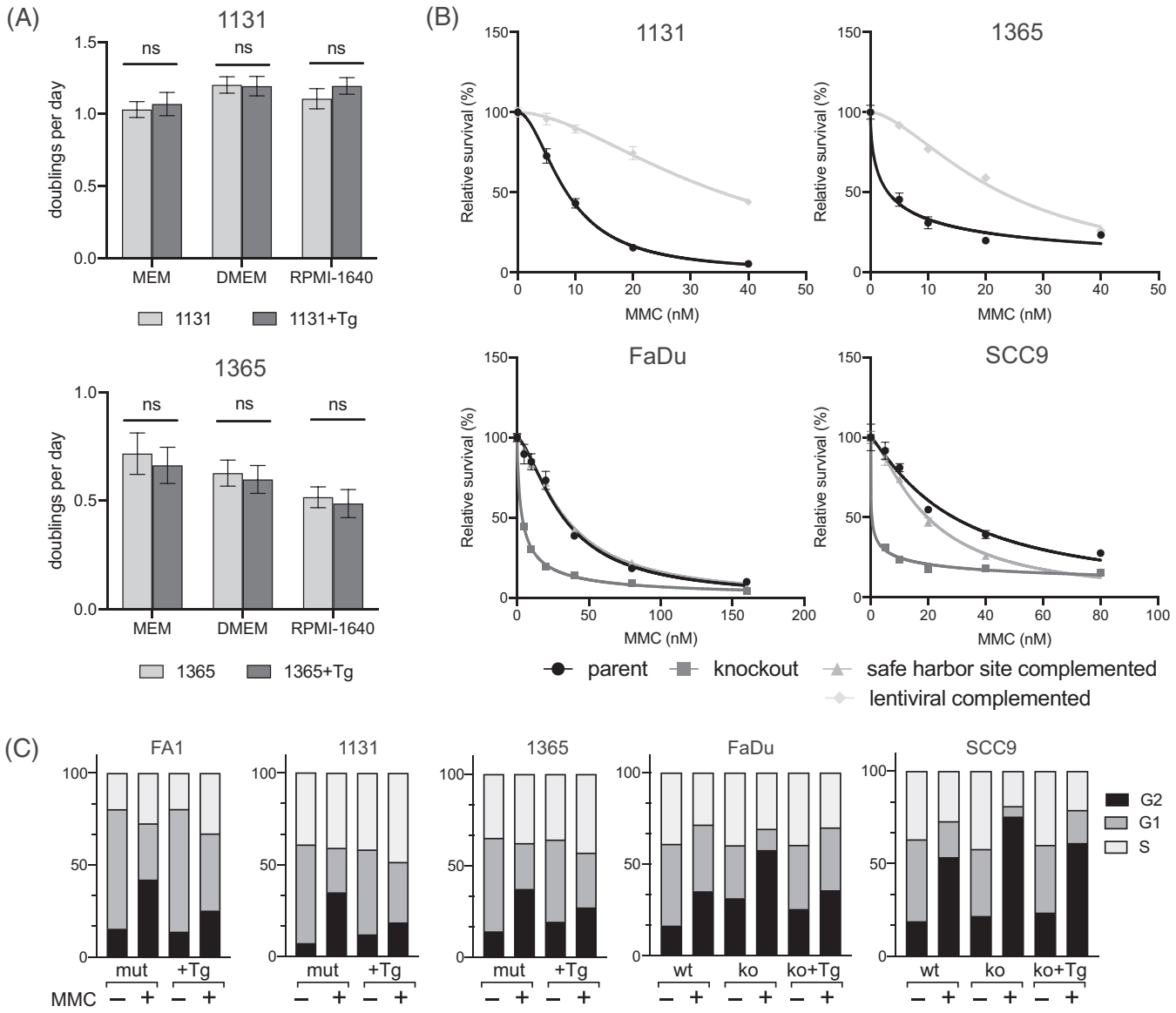

FIGURE 4 FA-dependent phenotypes of *FANC*-isogenic HNSCC cell lines. (A) Proliferation rates of isogenic FA patient-derived cell line pairs 1131 and 1365 in three different growth media. (B) Mitomycin-C dose-dependent survival for the same two isogenic FA patient-derived cell line pairs and two sporadic (FaDu, SCC9) HNSCC cell line pairs. (C) Cell cycle phase distributions quantified for three FA patient-derived (FA1, 1131 and 1365) and two sporadic (FaDu and SCC9) isogenic cell line pairs prior to and after MMC treatment. Left margin is a % scale for cell cycle phase fractions indicated by shading key to the right. Representative flow histograms for the isogenic cell line pair 1131 are shown in Figure S6. All analyses used data from 10 000 events (cells), with data summarized as mean % ± SEM.

it is widely used in FA diagnosis as well as in many mechanistic analyses.[1,2,6,35] This phenotype, readily apparent in FA patient-derived isogenic 1131 cells as an elevated G_2/M fraction in flow profiles (Figures 4C and S6), ranged from 16.4% (range 13.1% to 25.2%) in untreated cells up to 41.3% (range 23.6% to 65.7%) in cells treated with 40 nM MMC. *FANC* transgene complementation substantially reduced G_2/M fractions in MMC-treated, but not in control, cells (Figures 4C and S6). Errazquin et al. reported similar results for *FANCA*-mutant derivatives of the sporadic HNSCC cell lines CAL-SCC-27 and CAL-SCC-33 cells[22] using diepoxybutane (DEB), another DNA damaging agent to which FA-deficient cells are sensitive.[35]

MMC treatment or other types of DNA damage induce monoubiquitylation of FANCD2 in FA-competent cells. This key post-translational modification, mediated by the FA core complex protein and E3 ubiquitin transferase FANCL, is a biochemical marker of FA pathway activity that is lost in ~90% of FA patients.[2,6,36] In order to further explore this biochemical phenotype, we used peptide immuno-enrichment coupled with targeted multiple reaction monitoring mass spectrometry (immuno-MRM) (Figure 5A) and Western blotting to analyze FANCD2 expression and monoubiquitylation in isogenic cell lines, together with the abundance and post-translational modification status of peptides derived from 65 additional DNA damage response proteins that included nine other FANC proteins.[27-29]

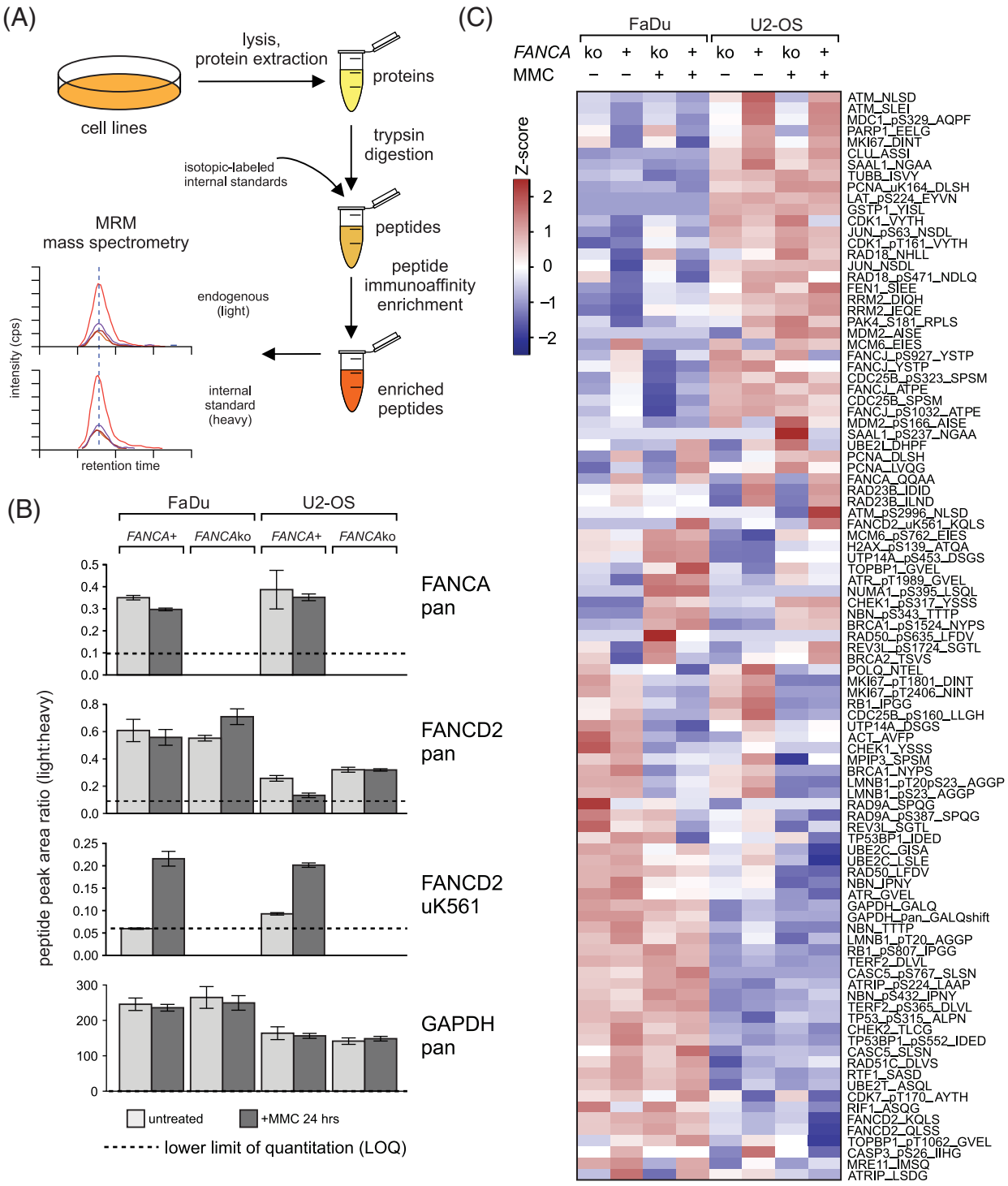

FIGURE 5 Immuno-MRM analysis of FANCA protein expression and FANCD2 ubiquity-lation. (A) Experimental outline for immuno-MRM mass spectrometry using added mass labeled internal standard peptides. (B) Bar plots of peak area ratios of light: heavy peptides for FANCA, FANCD2 and FANCD2 ubiquitylated (uK561) peptides. GAPDH is a protein loading control. Error bars show the SD of triplicate analysis, and the dashed horizontal line indicates the experimentally determined, peptide-specific lower limit of quantification (LOQ). (C) Heatmap of unsupervised clustering of 100 analyte peptides detected above LOQ in FaDu and U2-OS cells after 24 h of MMC treatment, where peak area ratios (light: heavy) were again normalized for each peptide analyte. A subset of peptides near the center of the heatmap show coordinate increases or decreases in abundance in response to MMC treatment.

We were able to quantify 98 of 129 potential target peptides by this approach in FA-proficient and FA-deficient cell line pairs using experimentally defined lower limits for quantification (LOQ; Figure 5 and Table S3). FANCA protein was readily detected in parental JHU-SCC-FaDu and control U-2 OS cells, but not in *FANCA*-mutant derivatives of either cell line. FANCD2 protein expression was unchanged in

FANCA-proficient or mutant cells (Figure 5B). FANCD2 ubiquitylation was detected as a ubK561-modified peptide induced by MMC in parental, but not in *FANCA*-mutant, cells (Figure 5B). Our ability to detect FANCD2 ubiquitylation by immuno-MRM, though not consistently by Western blot, in nominally *FANCA*-proficient JHU-SCC-FaDu cells may reflect differences in sensitivity of detection of these two assays, together with the presence of homozygous *FANCM* mutations in FaDu[37] that may blunt a MMC-induced DNA damage response without conferring a clear FA-deficient cellular phenotype.

Immuno-MRM also detected other functionally important DNA damage-dependent post-translational modifications in these experiments such as ATRpT1989, H2AXpS139 (γH2AX) and CHEK1pS317. Cell line-specific protein expression and/or modification differences together with *FANC* genotype appeared to drive clustering of MMC treatment-responsive peptides in both cell line backgrounds (Figure 5C and Table S3). These results demonstrate the ability of immuno-MRM to simultaneously detect and quantify the expression and the DNA damage-dependent modification of dozens of proteins in parallel, in a common assay format.

2.12 | Isogenic cell line responses to additional drugs and small molecules

We determined the MMC sensitivity of all isogenic cell line pairs as noted above. These experiments were supplemented by additional exploratory analyses in which a subset of FA patient-derived and sporadic HNSCC isogenic cell line pairs were used to determine *FANC* genotype-dependent responses to additional drugs or small molecules. These additional agents were chosen based on their mechanism of action, and prior reports of *FANC* genotype-dependent activity.

The loss of FA pathway activity did not consistently sensitize FA-deficient patient- or sporadic, *FANCA*-mutant HNSCC lines to formaldehyde dose-dependent cell killing over up to 4 days of continuous low dose exposure (Figure S4). This may reflect cell line-specific differences that collectively determine formaldehyde toxicity, a potent driver of FA pathway activity.[38-40] In contrast, loss of FA pathway function sensitized cells regardless of origin to platinum-based chemotherapeutic drugs, with *cis*-Pt displaying the strongest and oxaliplatin the least dose-dependent reductions in FA-deficient cell survivals. Sensitization could be suppressed by *FANC* transgene expression in FA patient-derived cell line VU-SCC-1365 and in sporadic *FANCA*-mutant HNSCC cell lines (Figure S5A,B). Comparable results using *cis*-Pt have been observed in *FANCA*-mutant derivatives of the sporadic HNSCC cell lines SCC-CAL-27 and SCC-CAL-33.[22]

The kinase inhibitors gefitinib and afatinib have been reported to modify the growth of FA-deficient HNSCC cell lines, both in culture and in xenograft experiments.[41] Neither inhibitor displayed appreciable *FANC* genotype-dependent growth suppressive activity in two FA patient-derived and one sporadic, *FANCA*-deficient HNSCC cell line pair (Figure S5C,D). Similar results were observed for the WEE-1 kinase inhibitor AZD-1775 (Figure S5E), for the ATR inhibitor VE-822 and for metformin and rapamycin, two small molecules being explored to prevent, delay or treat bone marrow failure and head and neck cancer in FA (additional results not shown).

2.13 | Generation of cell line-derived 3D "tumoroids"

Many HNSCC cell lines can form mouse xenografts, and HNSCC tumor tissue can be used to generate HNSCC-derived organoid cultures.[31,42,43] We thus determined the ability of isogenic FA patient-derived VU-SCC-1131 and sporadic line JHU-SCC-FaDu cell line pairs to form three-dimensional (3D) in vitro "tumoroids." Both *FANC*-isogenic cell line pairs readily formed tumoroids after single cell suspensions were initially seeded in Matrigel, then freed for subsequent growth in suspension to form mm-sized tumoroids after 40 days growth. Cell numbers and sizes of 40 day tumoroids paralleled cell line proliferation rates, time in culture and the extent of central necrosis, with little apparent dependence on *FANC* genotype (Figure 6A).

FANC-isogenic tumoroids displayed diffuse squamous differentiation with strong cytokeratin 5 cytoplasmic (CK5) and p40 antigen nuclear staining (Figure 6B). Continuous cell proliferation and death, detected by robust Ki67 and cleaved caspase 3 (CC3) immunostaining (Figure 6C), were observed in tumoroids grown for up to 43 days regardless of origin or genotype. Both parental and *FANCA*-deleted JHU-SCC-FaDu Day 40 tumoroids also displayed a DNA damage response after 3 days of continuous low dose MMC treatment, detected by both CC3 and γH2AX immunostaining (Figure 6C). These experiments establish the feasibility of generating and using *FANC* isogenic tumoroid pairs to explore 3D growth, gene expression and therapeutic responses as a function of *FANC* genotype.

3 | DISCUSSION

Fanconi anemia confers a remarkably high risk of developing carcinomas in the squamous epithelia lining the upper aero-digestive tract (oropharynx and proximal esophagus), vulva and anus[4,44,45] and, to a lesser extent, cutaneous squamous carcinomas.[5] Work to better understand these FA-associated cancer risks and to identify preventions and treatments have been hampered by the lack of facile, widely available disease models. In order to address these challenges, we generated 10 new *FANC*-isogenic cell line pairs from independent FA patient-derived and sporadic HNSCC cell lines to foster new research on the origins, treatment and prevention of FA-associated cancers.

Patient-derived isogenic HNSCC cell line pairs were generated for FA patient-derived cell lines representing complementation groups FA-A, FA-C and FA-L. We used the same approach to generate *FANCA*-isogenic pairs from sporadic HNSCC cell lines (Figure 1) in order to provide investigators with directly comparable non-FA experimental material. *FANCA* was chosen to generate these isogenic sporadic cell line pairs as it is the *FANC* gene mutated in ~2/3 of all FA patients[1,2] (Table 1). The encoded FANCA protein plays a central role as part of the heteromeric FA "core complex" that orchestrates FANC

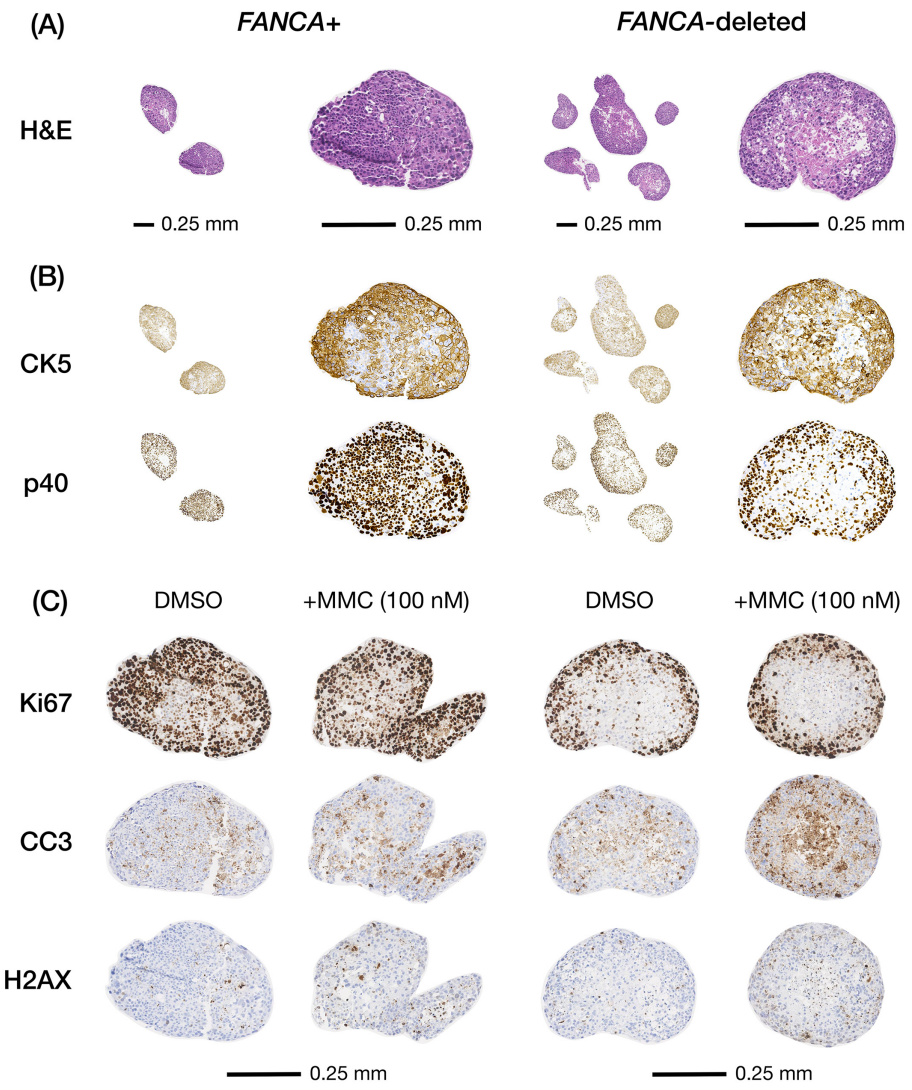

FIGURE 6 In vitro HNSCC "tumoroids" grown from *FANCA*-isogenic sporadic FaDu cells. (A) Hematoxylin and eosin (H&E) staining reveals viable squamoid cells with pleomorphic nuclei, plentiful cytoplasm and conspicuous mitotic figures surrounding areas of variable central necrosis (×4 original magnification, left; ×16 original magnification right). (B) *FANCA*+ and *FANCA*-mutant tumoroids both display squamous/epidermoid differentiation with diffuse nuclear p40 and cytoplasmic cytokeratin 5 (CK5) staining (original magnification ×16). (C) Cell proliferation revealed by Ki67 immunostaining; cell death by cleaved caspase 3 (CC3) staining; and DNA damage response induction by staining for γ-H2AX (H2AX) after 3 days of continuous MMC treatment of 40 day tumoroids in suspension culture (original magnification ×16). All scale bars are 0.25 mm.

DNA damage-dependent responses.[36] We reasoned this combination of patient-derived and *FANCA*-isogenic sporadic HNSCC cell line pairs would be the most useful experimental material to provide investigators, and would provide results broadly representative of all FA complementation groups.

FANC transgene complementation by chromosomal safe harbor site insertion or viral transduction restored FANC protein expression in FA patient-derived and sporadic, HNSCC cell lines. Higher levels of transgene protein expression were observed after viral transduction, and were required to fully restore MMC resistance and other FA-associated cellular phenotypes in FA patient-derived, though not in sporadic, HNSCC cell lines. The requirement for higher levels of transgene protein expression to confer full MMC resistance in FA patient-derived cell lines may reflect the many additional genomic and other differences in FA-patient-derived cell lines when compared with newly generated, *FANCA*-mutant sporadic HNSCC cell lines.[9,14] A decision to distribute transgene-complemented cell pools as opposed to clones was made after early experiments revealed little evidence of clonal heterogeneity in FA-relevant phenotypes in either FA patient-derived or sporadic HNSCC cell lines.

We did observe considerable heterogeneity between cell lines in responses to drug and small molecule treatments. Only MMC and *cis*-platinum treatment conferred displayed consistent, reproducible *FANC* genotype-dependent differences in cell survival and cell cycle perturbation. Among the additional drugs and small molecules profiled, oxaliplatin may be the most interesting: it displayed little *FANC* genotype-dependent cell killing, but has been reported to show little cross-resistance with *cis*-Pt in HNSCC cell lines[46] together with the ability to potentiate anti-PD-1 immunotherapy in preclinical models.[47] These results may reflect a different mechanism of action by oxaliplatin, and the potential utility of oxaliplatin as an alternative to other platinum-based chemotherapeutic agents.[48,49]

Cell line heterogeneity of the types we observed has been well-documented for HNSCC and for other cancer types in CCLE,[10,50] GDSC[51-53] and DepMap (depmap.org/portal/) data. These potentially confounding differences need to be recognized and taken into account in experiments that use multiple different cell lines. Cell line

heterogeneity may, to the extent it is understood, be practically useful. For example, detailed knowledge of genomic and phenotypic differences among our 10 FA-CCLR HNSCC isogenic cell line pairs may allow better experimental design and the selection of cell line pairs for hypothesis testing, gene dependency or high throughput screens.

Cell lines continue to play a central role in cancer research[54-56]: they are widely available and experimentally tractable disease models that capture and propagate many cell-autonomous features of neoplasia. Cell lines are often easy and inexpensive to use when compared with alternative disease models, and have enabled many high-throughput drug, small molecule and genomic screens (see, for example, CCLE[10,50]; GDSC[51-53]; DepMap resources (depmap.org/portal/)). The resulting data from these cell line-based screens have become an increasingly valuable resource and cancer discovery tool. The limitations of cancer cell line models are also well-recognized, including lack of immune/inflammatory cells and a vascularized tumor microenvironment. Many of these limitations are now being imaginatively addressed by combining xenograft, tissue engineering and organoid protocols.

The 10 new *FANC*-isogenic cell line pairs described here can be used for many different types of experimental analyses, and will provide highly reproducible results if care is taken to minimize passage-associated phenotypic drift or genomic evolution.[55-57] Our goal in generating these *FANC*-isogenic cell line pairs and distributing them widely to appropriate investigators is to promote and enable new research to benefit individuals with FA and their families, as well as individuals with sporadic HNSCC.

AUTHOR CONTRIBUTIONS

All work reported in the paper has been performed by the authors unless otherwise clearly specified in the text. The contributions of individual authors are listed below in alphabetical order using CRediT taxonomy terms. Ruud Brakenhoff: Project administration, Resources, Writing - original draft. Christopher M. Chandler: Methodology, Visualization, Writing - original draft. Eleanor Y. Chen: Methodology, Supervision, Visualization, Writing - original draft/review & editing. Josephine Dorsman: Resources, Supervision. Ricardo Errazquin: Investigation, Methodology, Resources, Validation, Visualization. Madeline Fritzke: Investigation, Visualization. Ramon Garcia-Escudero: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft/review & editing. Markus Grompe: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision. Elizabeth E. Hoskins: Investigation, Methods. Erica Jonlin: Methodology, Project administration, Supervision. Raymond J. Monnat, Jr.: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft/review & editing. H. Tai Nguyen: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing - original draft/review & editing. Amanda G. Paulovich: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft/review & editing. Khashayar Roohollahi: Formal Analysis, Investigation, Methodology, Visualization, Writing - original draft. Agata Smogorzewska: Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft/review & editing. Lucas B. Sullivan: Investigation, Methodology, Resources, Supervision, Visualization, Writing - original draft. Weiliang Tang: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization. Leslie Wakefield: Conceptualization, Data curation, Methodology, Project administration, Resources, Supervision, Andrew L. H. Webster: Data curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing - original draft/review & editing. Susanne I. Wells: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft/review & editing. Jeffrey R. Whiteaker: Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft.

ACKNOWLEDGEMENTS

We thank members of the FA research community for help locating and providing FA cancer cell lines, and for resolving questions of cell line origins or identity. Hui Li assembled our initial *FANC* gene open reading frame vectors from reference gene sequences; Helmut Hanenberg provided detailed information on retroviral complementation vector structure; and Bryan Johnson of the UW-ISCRM Histology and Imaging Core provided expert guidance for tumoroid histologic analyses. We dedicate this manuscript to the Fanconi Anemia community of patients, families and scientists, and to our late colleague Eddie Mendez (Fred Hutchinson Cancer Research Center, Seattle, WA USA). Eddie was a physician-scientist and inspiring colleague, dedicated to helping patients and families facing HNSCC and FA. Many of the ideas for a FA-CCLR were first discussed with Eddie.

FUNDING INFORMATION

This work was supported by a research grant and supplement from the Fanconi Anemia Research Fund to Raymond J. Monnat Jr.; by National Cancer Institute (NCI) CPTAC Grant U01CA214114 to Amanda G. Paulovich; by NCI Research Specialist Grant R50CA211499 to Jeffrey R. Whiteaker; by the Brotman-Baty Institute for Precision Medicine, Seattle WA and a generous donation from the Aven Foundation to Amanda G. Paulovich. Agata Smogorzewska received support from a Pershing Square Sohn Prize for Young Investigators in Cancer Research and a V Foundation grant T2019-013 (Agata Smogorzewska).

CONFLICT OF INTEREST

Amanda G. Paulovich is founder of Precision Assays, LLC; Markus Grompe is founder of Yecuris Corporation and founder and C.S.O. of Ambys Medicines. Rocket Pharmaceuticals provided research funding and partial salary support to Agata Smogorzewska for an unrelated project. None of these associations creates a conflict of interest with

work reported in this manuscript. None of the authors have disclosed other potential conflicts of interest involving work reported in this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Cell line use was considered "Not Human Subjects Research" in light of NIH and University of Washington Institutional guidelines defining human subjects research. No patient-identifying information has been associated with the resulting FA-CCLR cell lines.

ORCID

Ramon Garcia-Escudero https://orcid.org/0000-0001-5640-6542
Susanne I. Wells https://orcid.org/0000-0002-7234-676X
Raymond J. Monnat Jr https://orcid.org/0000-0001-7638-7393

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nguyen HT, Tang W, Webster ALH, et al. Fanconi anemia-isogenic head and neck cancer cell line pairs: A basic and translational science resource. *Int J Cancer*. 2023;153(1):183-196. doi:10.1002/ijc.34506