SUPPLEMENTAL MATERIAL: Upregulation of Fanconi Anemia DNA Repair Genes in Melanoma Compared to Non-Melanoma Skin Cancer, Kao *et al.*

MATERIALS AND METHODS

Tissue specimens

Harvesting of tumors and purification and hybridization of RNA has been previously described(Riker *et al.*, 2008). Briefly, 40 metastatic melanomas (MM), 16 primary cutaneous melanomas (PCM), 11 squamous cell carcinomas (SCC), and 15 basal cell carcinomas (BCC) were obtained under a protocol approved by the Institutional Review Boards of the Moffitt Cancer Center and the Ponce School of Medicine (MCC#13448, IRB#101751; PSM# 990914-JM, 020318-JM) and frozen in liquid nitrogen. The study was conducted according to Declaration of Helsinki Principles, and written informed consent was obtained from participants. Melanomas were staged according to the *American Joint Committee on Cancer Staging System for Cutaneous Melanoma*(Balch *et al.*, 2001), based on Breslow depth(Breslow, 1979) and the presence of lymph node involvement and/or metastasis. Four samples of normal human skin were used as negative controls.

RNA isolation, purification, and hybridization

Cryopreserved tissue was dissolved in TRIzol® (Invitrogen, Carlsbad, CA) and purified using RNeasy columns (Qiagen Inc., Valencia, CA). 5 μg of RNA was processed using established Affymetrix protocols for the generation of biotin-labeled

cRNA and the hybidization, staining, and scanning of arrays as outlined in Affymetrix technical manuals(Van Gelder *et al.*, 1990; Warrington *et al.*, 2000). Briefly, RNA was converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Invitrogen). An oligo$(dT)_{24}$ primer (Affymetrix, T7-oligo(dT) Promoter Primer kit) was then used to generate cRNA, which was then purified using an Affymetrix GeneChip Sample Cleanup module. RNA integrity was verified by gel electrophoresis and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Further procedural details have been published previously (Dobbin *et al.*, 2005). The processed RNA was then hybridized to Human Genome U133 Plus 2.0 arrays from Affymetrix, Inc. (Santa Clara, CA), and scanned on an Affymetrix GeneChip® scanner 3000 at 2.5 μm resolution.

DNA microarray analysis

Initial characterization of global gene expression profiles in metastatic and primary skin cancers has been previously published(Riker *et al.*, 2008). The microarrays used in this study are available on-line at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7553). Scanned output files were visually inspected for hybridization artifacts. MAS 5.0 analysis software was then utilized to generate signal values for all probe sets based upon a mean intensity of 500, Signal values below 25 were truncated to 25(Li and Wong, 2001). The tissue samples were processed in three independent groups. Expression levels of each gene in each tumor sample were then normalized with respect to mean intensity in the 4 normal skin samples. A 235-probe subset corresponding to known human DNA repair genes was selected as the starting point for this study(Wood *et al.*, 2005; Wood *et al.*, 2001). Visual

inspection of raw microarray data was used initially to identify candidate genes for possible differential expression in melanoma vs. non-melanoma samples. Differences from normal skin were then formally evaluated using a two-tailed Student's *t*-test, assuming unequal variance between samples (Microsoft Excel, Redmond, WA). A p-value of less than .05 was used as the cutoff for statistical significance.

Quantitative RT-PCR

For qRT-PCR experiments, cDNA synthesis was done using the Oligo dT primers cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNAs were purified using PCR purification kit (Qiagen). The cDNAs were subsequently analyzed by qPCR using SYBR Green (Bio-Rad) and the Bio-Rad Chromo 4 DNA Engine Thermal Cycler. FANCD2 expression levels were normalized to beta actin mRNA levels. Gene specific primer sequences were as follows: FANCD2 FP- ACGGTGCTAGAGAGCTGCTT, FANCD2 RP- TGTTCTCAGCACACTGGCAT, ACTB FP-TGAAGTGTGACGTGGACATC, and ACTB RP-GGAGGAAGCAATGATCTTGAT.

Imnunohistochemistry

Paraffin sections were washed 3 times in xylene (Sigma, St. Louis MO) and then rehydrated in 100% ethanol, 95% ethanol, followed by double distilled water (ddH_2O). Antigen retrieval was performed by incubation in 10 mM sodium citrate (pH 6.0) at 85°C for 10 minutes. Slides were cooled at room temperature for 30 minutes, washed with ddH_2O, treated with 3% hydrogen peroxide, and again rinsed with ddH_2O. The region of interest was washed with 1x PBS/0.1% Tween-20 (PBST), blocked for one hour at room temperature with PBST + 5% goat serum (Vector Laboratories, Burlingame, CA), and

incubated with 1:50 anti-FANCD2 (H-300) (sc28194, Santa Cruz Inc, Santa Cruz, CA) at 4°C overnight. The specificity of this antibody has been characterized and validated previously(Alexander *et al.*, 2010). The slides were washed with PBST, incubated with biotinylated anti-rabbit antibody (Vector Laboratories, Burlingame CA) for 30 minutes at room temperature, and following 3 additional PBST washes, developed using ABC method (Vector Laboratories, Burlingame CA). Sections were counterstained with Mayer's hematoxylin and mounted with Permount™ Mounting Medium (Electron Microscopy Sciences, Hatfield, PA). Representative 40X images were taken and analyzed using Image J (http://rsbweb.nih.gov/ij/). Approximately 2,500 tumor cells were analyzed.

Supplemental Table S1. Characteristics of tumors included in this study.

Tumor	Subtype (if applicable)	Number of cases
SCC	All	11
BCC	All	15
PCM	*in situ*	2
	Thin (<1 mm)	2
	Intermediate (1-4 mm)	3
	Thick (>4 mm)	9
MM	Lymph node	22
	Subcutaneous	16
	Solid organ metastases	2

SCC, squamous cell carcinoma
BCC, basal cell carcinoma
PCM, primary cutaneous melanoma
MM, metastatic melanoma

Supplemental Table S2. Statistical analysis of FA genes from DNA microarray shown in Figure 1a.

	Metastatic Melanoma			Squamous Cell Carcinoma		
Gene	**Avg.**	**95% CI**	**P-value**	**Avg.**	**95% CI**	P-value
FANCA	**2.16**	**1.20; 3.12**	**0.013†**	**1.22**	**0.35; 2.08**	**0.473**
FANCB	**4.58**	**3.38; 5.77**	**<.001†**	**2.16**	**1.08; 3.23**	**0.023†**
FANCC	**1.02**	**0.86; 1.18**	**0.843**	**0.73**	**0.43; 1.03**	**0.126**
FANCE	**0.71**	**0.21; 1.20**	**0.284**	**1.35**	**0.74; 1.96**	**0.274**
FANCF	**1.40**	**1.13; 1.67**	**.003†**	**0.94**	**0.70; 1.17**	**0.579**
FANCG	**1.69**	**1.46; 1.92**	**<.001†**	**1.11**	**0.82; 1.39**	**0.627**
FANCL	**2.13**	**1.80; 2.47**	**<.001†**	**0.96**	**0.75; 1.17**	**0.764**
FANCM	**1.09**	**0.76; 1.42**	**0.499**	**1.03**	**0.69; 1.36**	**0.885**
FANCI	**3.33**	**2.83; 3.83**	**<.001†**	**2.32**	**1.84; 2.81**	**<.001†**
FANCD2	**2.01**	**1.54; 2.48**	**0.002†**	**1.01**	**0.54; 1.48**	**0.974**
FANCD1/BRCA2	**3.04**	**2.17; 3.90**	**<.001†**	**2.43**	**1.25; 3.62**	**0.004†**
FANCN/PALB2	**1.47**	**1.16; 1.77**	**<.016†**	**1.20**	**0.90; 1.50**	**0.195**
FANCJ/BRIP1	**3.31**	**2.09; 4.54**	**<.001†**	**2.20**	**1.23; 3.17**	**0.020†**

Avg., average gene expression relative to normal skin; 95%CI, 95% confidence interval
† P-value < .05.

Supplemental Table S3. Aggregate FANCD2 and FANCL mRNA expression in BCC relative to normal skin based on DNA microarray data

BCC Sample number	FANCD2+ FANCL levels relative to normal skin
1	1.24
2	0.88
3	1.37
4	1.42
5	1.00
6	1.09
7	1.01
8	1.41
9	1.21
10	1.38
11	1.42
12	1.38
13	0.92
14	0.93
15	1.10

Supplemental Table S4. Statistical analysis of genes from the NER pathway from DNA microarray shown in Figure 1a.

	Metastatic Melanoma			Squamous Cell Carcinoma		
Gene	**Avg.**	**95% CI**	**P-value**	**Avg.**	**95% CI**	**P-Value**
XPA	**0.611**	**0.42; 0.80**	**.012†**	**0.83**	**0.61; 1.06**	**.148**
XPB	**1.378**	**1.20; 1.55**	**< .001†**	**0.90**	**0.70; 1.09**	**0.255**
XPC	**0.973**	**0.69; 1.26**	**0.843**	**0.59**	**0.32; 0.87**	**0.031†**
XPD	**1.451**	**0.60; 2.30**	**0.301**	**1.66**	**0.77; 2.55**	**0.160**
RAD23A	**1.185**	**0.91; 1.46**	**0.052**	**1.10**	**0.84; 1.35**	**0.584**
RAD23B	**0.583**	**0.48; 0.68**	**< .001†**	**1.20**	**1.08; 1.32**	**.003†**
XPE	**0.556**	**0.27; 0.84**	**0.024†**	**1.07**	**0.77; 1.36**	**0.517**
XPF	**1.018**	**0.36; 1.68**	**0.947**	**0.80**	**0.11; 1.48**	**0.490**
XPG	**0.669**	**0.56; 0.78**	**< .001†**	**0.98**	**0.75; 1.20**	**0.816**

Avg., average gene expression relative to normal skin; 95%CI, 95% confidence interval
† P-value < .05.

Supplemental Table S5. Statistical analysis of FA genes in PCM from DNA microarray in Figure 1b.

	Melanoma *in situ*			Thin			Intermediate			Thick		
Breslow Thickness	n/a			< 1mm			1-4 mm			>4 mm		
Gene	**Avg**	**95% CI**	**P**	**Avg**	**95% CI**	**P**	**Avg**	**95% CI**	**P**	**Avg.**	**95% CI**	**P**
FANCA	1.20	0.04; 2.34	0.319	2.01	0.46; 3.55	0.202	1.82	0.35; 3.29	0.108	3.35	1.26; 4.71	0.01 †
FANCB	2.53	-0.42; 5.48	0.346	0.78	0.12; 1.44	0.849	1.72	1.06; 2.38	0.043†	5.15	3.09; 8.39	< .001†
FANCC	0.55	0.08; 1.03	0.058	0.60	0.16; 1.05	0.084	0.91	0.52; 1.30	0.637	1.44	1.06; 2.49	0.004 †
FANCE	0.70	0.25; 1.16	0.277	0.82	0.34; 1.31	0.983	0.74	0.20; 1.27	0.373	0.99	0.37; 1.36	0.959
FANCF	0.78	0.42; 1.14	0.053	0.75	0.37; 1.13	0.275	0.94	0.57; 1.30	0.575	1.23	0.73; 1.79	0.316
FANCG	0.98	0.90; 1.06	0.654	1.12	0.66; 1.59	0.675	1.20	1.06; 1.34	0.044†	2.15	1.31; 2.99	0.016†
FANCL	1.02	0.89; 1.15	0.741	1.10	0.77; 1.44	0.456	1.54	1.23; 1.84	0.031†	2.17	1.23; 3.43	0.028†
FANCM	1.49	0.72; 2.26	0.176	0.79	0.13; 1.45	0.402	0.89	0.27; 1.51	0.634	1.25	0.81; 2.06	0.227
FANCI	1.45	0.86; 2.03	0.210	1.17	0.74; 1.60	0.284	1.21	0.81; 1.62	0.194	3.40	2.44; 4.36	< .001†
FANCD2	0.50	0.08; 0.92	0.048	1.23	0.40; 2.06	0.838	1.29	0.77; 1.81	0.275	2.44	1.27; 3.79	0.027†
FANCD1/ BRCA2	1.63	0.08; 3.17	0.502	1.13	-1.05;3.31	0.924	1.85	0.52; 3.17	0.254	3.12	1.51; 4.74	0.027†
FANCN	1.26	1.03; 1.49	0.024†	1.01	-0.08;2.10	0.826	1.01	0.05; 1.97	0.644	1.44	1.01; 1.86	0.032†
FANCJ	1.57	0.89; 2.25	0.144	0.87	-0.54;2.29	0.891	0.82	-0.64;2.28	0.832	2.12	0.80; 3.44	0.127

Avg., average gene expression relative to normal skin; 95%CI, 95% confidence interval

† P-value < .05.

Supplemental Table S6. Statistical analysis of NER pathway genes from DNA microarray in Figure 1b.

	Melanoma *in situ*			Thin			Intermediate			Thick		
Breslow Thick.	**n/a**			**<1 mm**			**1-4 mm**			**>4 mm**		
Gene	**Avg.**	**95% CI**	**P**	**Avg.**	**95% CI**	**P**	**Avg.**	**95% CI**	**P**	**Avg**	**95% CI**	**P**
XPA	**0.97**	**0.77; 1.17**	**0.763**	**1.06**	**0.79; 1.34**	**0.642**	**1.12**	**0.863; 1.37**	**0.360**	**0.62**	**0.36; 0.88**	**0.015†**
XPB	**0.79**	**0.68; 0.91**	**0.027†**	**1.01**	**0.29; 1.73**	**0.976**	**1.29**	**1.07; 1.51**	**0.027†**	**1.33**	**0.92; 1.74**	**0.105**
XPC	**1.01**	**0.46; 1.56**	**0.972**	**0.76**	**0.34; 1.17**	**0.308**	**1.11**	**0.742; 1.49**	**0.532**	**0.86**	**0.51; 1.20**	**0.380**
XPD	**1.87**	**-0.47; 4.21**	**0.575**	**1.67**	**0.88; 2.47**	**0.166**	**1.64**	**0.829; 2.45**	**0.182**	**2.62**	**-0.11; 5.35**	**0.224**
RAD23A	**1.11**	**0.70; 1.52**	**0.564**	**0.95**	**0.57; 1.33**	**0.782**	**0.77**	**0.475; 1.06**	**0.084**	**1.44**	**0.56; 2.32**	**0.320**
RAD23B	**1.04**	**0.87; 1.22**	**0.602**	**1.07**	**0.65; 1.49**	**0.718**	**0.83**	**0.567; 1.08**	**0.167**	**0.52**	**0.41; 0.64**	**< .001†**
XPE	**1.18**	**0.61; 1.62**	**0.664**	**0.79**	**0.42; 1.17**	**0.313**	**0.82**	**0.61; 1.18**	**0.450**	**0.61**	**0.24; 0.98**	**0.046†**
XPF	**0.54**	**0.05; 1.04**	**0.156**	**0.74**	**0.04; 1.45**	**0.976**	**0.81**	**0.264; 1.35**	**0.489**	**1.29**	**0.49; 1.95**	**0.532**
XPG	**1.57**	**0.87; 2.28**	**0.316**	**1.26**	**0.93; 1.60**	**0.074**	**1.15**	**0.856; 1.45**	**0.355**	**0.78**	**0.49; 1.09**	**0.158**

Avg., average gene expression relative to normal skin; 95%CI, 95% confidence interval
† P-value < .05.

Supplemental Table S7. Characteristics of tumors included in immunohistochemistry analysis.

Diagnosis	Age	Sex	Location	Breslow
Melanoma	74	F	Back	0.4
Melanoma	56	F	R Arm	0.75
Melanoma	60	F	R Knee	1.75
Melanoma	54	M	R Arm	2.0
BCC	49	F	R NLF	N/A
SCC	60	M	Chest	N/A
SCC	69	M	Back	N/A
SCC	58	M	L Arm	N/A

Breslow, depth in mm; NLF, nasolabial fold; N/A, not applicable

Supplemental Table S8. Statistical analysis of Histone H3 and Cyclin A/E gene expression from DNA microarray analysis in PCM and NMSC.

	Melanoma *in situ*			Thin			Intermediate			Thick		
Breslow Thick.	n/a			<1 mm			1-4 mm			>4 mm		
Gene	**Avg.**	**Std. Dev.**	**P**	**Avg.**	**Std. Dev.**	**P**	**Avg.**	**Std. Dev.**	**P**	**Avg**	**Std. Dev.**	**P**
Cyclin E2	1.14	0.64	0.479	1.51	0.85	0.980	1.25	0.55	0.320	0.87	0.46	< .001†
Cyclin A1	2.10	1.20	0.370	0.67	0.56	0.06	1.00	0.48	0.01†	1.56	0.84	0.070
Hist H3	1.04	0.82	0.012†	1.56	1.91	0.308	2.48	2.51	0.229	1.19	1.15	0.028†

	NMSC	
	Avg.	**Std. Dev**
Cyclin E2	1.54	0.96
Cyclin A1	2.34	1.44
Hist H3	1.76	2.76

Avg., average gene expression relative to normal skin; Std. Dev, Standard Deviation.
Note: P-values listed for melanomas are in comparison to NMSC; † P-value < .05.; N/A, non-applicable

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