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Transcriptional regulation of Tamoxifen in human and non-human primate endometrium and cultured breast and endometrial cells

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ABSTRACT

Tamoxifen (TAM) is a selective estrogen receptor modulator (SERM) widely used for adjuvant therapy of breast cancer. An important side-effect of TAM is increased risk of endometrial cancer. To investigate mechanisms underlying this effect we examined transcriptional and epigenetic changes in human and monkey uterus, normal human mammary epithelial cells (NHMECs), and human endometrial stromal cells (HESCs). Uterine DNA from women (n = 9), *Erythrocebus patas* monkeys (n = 3), and *Macaca fascicularis* monkeys (n = 6), all exposed to TAM, showed no difference in 5-methyl-cytosine (5-meC) levels compared to unexposed controls (n = 6, 2, and 6, respectively). Microarray comparison of TAM-exposed and unexposed NHMECs and HESCs revealed cell-specific differences, with confirmation by RT-PCR. TAM-exposed NHMECs showed up-regulation of interferon signaling and immune response pathways, while TAM-exposed HESCs showed up-regulation of steroid and fatty acid biosynthesis pathways. Promoter region CpG islands for genes highly up-regulated by TAM in NHMECs (*MX1* and *STAT1*) and in HESCs (*PPARG*, *SREBF2*, *HMCGS* and *Prune2*), did not show significant differences for 5-meC. We did observe a significant depletion of total histone H3, and dimethyl histone H3 lysines K4, K27 and K36, by Western blot, in TAM-exposed HESCs, compared to unexposed controls. Whereas TAM exposure had no discernible effect on 5-meC levels in primate uterus, TAM induced up-regulation of different transcriptional pathways in NHMECs and HESCs, and concomitantly depleted some H3 dimethyl histone lysine levels in HESCs. These findings highlight several features of transcriptional dysregulation by TAM that should be further investigated in the context of TAM-induced endometrial carcinogenesis.

KEYWORDS: microarray, RTq-PCR, 5-methyl-cytosine, histone H3-lysine dimethyl modifications, Western blot, pyrosequencing, normal human mammary epithelial cells, human endometrial stromal cells.

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ABBREVIATIONS

ATCC, American Type Culture Collection; 5-meC, 5-methyl-cytosine; bw, body weight; CHTN, Cooperative Human Tissue Network; CIA, chemiluminescence immunoassay; ER, estrogen receptor; E2, estradiol; E_2, micronized 17β-estradiol; HESCs, human endometrial stromal cells; H3K4me2, histone H3 lysine 4 dimethyl; H3K9me2, histone H3 lysine 9 dimethyl; H3K27me2, histone H3 lysine 27 dimethyl; H3K36me2, histone H3 lysine 36 dimethyl; H3K79me2, histone H3 lysine 79 dimethyl; hTERT, human telomerase reverse transcriptase; IPA, Ingenuity Pathway Analysis; LLD, lower limit of detection; NHMECs, normal human mammary epithelial cell strains; PBS, phosphate buffered saline; PBST, PBS containing 0.05% Tween 20 and 0.02% sodium azide; RTq-PCR, quantitative real-time polymerase chain reaction; RMA, Robust Multi-array Average; SERM, selective estrogen receptor modulator; TAM, tamoxifen, Nolvadex®.

INTRODUCTION

Tamoxifen (TAM) is a synthetic nonsteroidal estrogen analog and selective estrogen receptor modulator (SERM) that is widely used as first line adjuvant therapy for ER-positive breast cancer in both breast cancer survivors and other high-risk women [1]. However, women receiving TAM therapy have a 2- to 7-fold increased risk of endometrial adenocarcinoma [2, 3] and a slight increased risk of uterine sarcoma [4]. TAM exhibits distinctive mixed agonist/antagonist ER effects in the uterus, but the exact mechanisms for its carcinogenic effects remain poorly understood.

TAM-induced endometrial cancer is considered to be associated with an aberrant proliferative stimulus observed in the uteri of patients receiving TAM therapy [5], in combination with the genotoxicity caused by formation of uterine TAM-DNA adducts [6-8]. In the rat, TAM-induced liver carcinogenesis involves TAM-DNA damage and mutagenesis [5, 7, 9-11], and there is strong concordance between the extent of TAM exposure, the levels of hepatic TAM-DNA adducts, and the TAM-induced liver tumor incidence [11]. Furthermore, careful analysis of human clinical studies comparing TAM [1] to other SERMs, such as raloxifene [12] and toremifene [13], which do not bind to DNA or cause increased uterine proliferation, indirectly suggests that both cell proliferation and TAM-DNA adduct formation contribute to TAM-induced uterine cancer risk.

The current study was designed to look beyond these two known causative events (proliferation and DNA damage), by evaluating TAM-induced gene expression changes, and exploring possible TAM-induced epigenetic biomarkers. We were searching for a relationship between TAM-induced gene up-regulation and changes in 5-methyl cytosine (5-meC) methylation. For some of these experiments we used TAM-exposed uterine DNA samples from humans and monkeys, which we previously analysed for TAM-DNA damage [8]. However, because of the scarcity of the non-human primate samples, and the versatility of cell cultures, we also used normal human mammary epithelial cells (NHMECs) and human endometrial stromal cells (HESCs) in order to compare TAM-induced transcriptional events in these two different cell types. In a previous study we analyzed TAM-induced gene expression in the NHMECs [14] and found up-regulation of primarily immune response genes. The current study has allowed us to reproduce and expand those findings, while adding comparison with HESC's and the epigenetic end points.

MATERIALS AND METHODS

Non-human primates, TAM exposures and DNA isolation

Five, 22-year old, female *Erythrocebus patas* (patas) monkeys were bred and reared at BioQual Inc. (Gaithersburg MD) and then housed and treated at the NIH Shared Animal Facility, Dickerson, MD. Maintenance of the patas has been previously detailed [15-17] and is only briefly outlined here. Monkeys were housed in groups of 2 or 3 females under conditions approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International, using protocols reviewed by the Institutional Animal Care and Use Committee of BioQual, Inc., and the Animal Care and Use Committee of the National Cancer Institute, NIH Bethesda, MD. TAM capsules (10 mg, Novaldex,

AstraZeneca Pharmaceuticals) were administered orally to 3 patas monkeys once a day for 3 months. This corresponded to 1.64-1.82 mg TAM/kg bw/day. Using a scaling factor of 4.8, based on the human daily dose of 0.27 mg TAM/kg bw/day for a 75 kg woman, this amount results in a human-equivalent dose of 0.34-0.38 mg TAM/kg bw/day for the patas, 26-40% higher than the standard human daily dose, which would be 1.3 mg TAM/kg/bw in the monkey. Two unexposed patas were used as controls. All patas were euthanized 24 hours after the last TAM dose, and euthanasia was performed in accordance with the 2013 American Veterinary Medical Association Guidelines. The uteri were harvested and snap-frozen for DNA isolation.

Endometrial samples from 12 ovariectomized female *Macaca fasicularis* (macaque) monkeys, with a mean estimated age of 15 years, were also analysed. The full experimental design for this study has been described previously [18]. Macaques were originally imported from the Institut Pertanian in Bigor, Indonesia, and housed in stable social groups of 3 to 4 animals at the Wake Forest University Primate Center. Housing conditions were in compliance with State and Federal laws, and standards of the U.S. Department of Health and Human Services, and all procedures involving these animals were approved by the Wake Forest University Animal Care and Use Committee. The TAM daily dose of 1.3 mg/kg bw was considered equivalent to the human daily dose, and the 16.7 μg of micronized 17β-estradiol (E_2)/kg bw/day (Estrace, Mylan Pharmaceuticals) was considered equivalent to 0.25 mg/woman/day. For four months, 3 animals received TAM, 3 animals received TAM plus E_2, and 6 animals received daily placebo. Monkeys were euthanized 24 hours after the last TAM dose and endometrium was collected and snap-frozen for DNA isolation.

For all samples, DNA was isolated using the QiAamp DNA Blood MaxiKit, (Qiagen, Valencia, CA) and quantified at A_{260} using the *NanoDrop*® ND-1000 spectrophotometer (Thermo Scientific, Asheville, NC).

Human tissues

Under conditions of patient anonymity, the Cooperative Human Tissue Network (CHTN, Birmingham, AL, sponsored by the National Cancer Institute and the National Disease Research Interchange) provided 15 frozen endometrial/uterine samples from women who had (n = 9) and women who had not (n = 6) received TAM therapy. Of the 15 samples from different individuals, 9 samples (6 TAM exposed and 3 unexposed) were designated “malignant” tissue, and 6 samples (3 TAM exposed and 3 unexposed) were designated “normal” tissue based on previous histopathologic analysis of corresponding fixed samples from the same patients. The terms “malignant” and “normal” are used here as general classifiers. Because all the samples were dissected during surgery and flash frozen it was impossible for us to ensure that the “malignant” samples contained no normal tissue, and vice versa. In addition, whereas the medical records were examined by representatives of the CHTN, and we were informed of the TAM status of each individual, there was no information available on the duration of TAM therapy or the interval between stopping TAM therapy and tissue procurement. TAM therapy is recommended for ER-positive breast cancer patients for 5 years at a dose of 20 mg per day. Because we received no unique identifiers for the samples, the NCI Human Institutional Review Board provided exemption status for this study.

Culture of NHMECs and HESCs and TAM exposure

NHMECs were isolated from normal human breast tissue obtained at reduction mammoplasty through the Cooperative Human Tissue Network (CHTN). Tissues were collected by a process involving mechanical and enzymatic disruption [19]. Human Studies Review Board approval was sought at the National Institute for Occupational Safety and Health, where the tissue was received and the cells were derived, and a waiver was granted because no unique identifiers accompanied the tissues. Uniform cultures of epithelial cells were obtained by growing enzyme-disrupted cells for 6 passages in serum-free Mammary Epithelial Growth Medium (MEGM) (Clonetics™, Walkersville, MD). The NHMEC strain used for these studies, M98016 (strain 16), was positive for ERα [20]. Cells were grown to passage 7-13, plated at a density of 1×10^6 cells/15 cm plate or

T-175 flask for DNA preparation, and at a density of 1×10^6 cells/6-well plate for RNA preparation. HESCs, which had been human telomerase reverse transcriptase (hTERT) immortalized, were obtained from the American Type Culture Collection (ATCC, Gaithersburg, MD, Catalogue number CRL-4003) and grown in DMEM/F12 medium with 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (Charcoal/dextran treated), 1% insulin human transferrin and selenous acid, and 500 ng/ml puromycin. Seeding density was the same as for the NHMECs. NHMECs and HESCs were seeded and grown for 48 hours prior to treatment with 10 μM TAM, or vehicle (dimethylsulfoxide), for an additional 48 hours. Previously, exposure to 10 μM TAM for 48 hr was shown to induce no cytotoxicity [14].

Affymetrix microarray

Four replicate TAM exposures were performed on different occasions, and each exposure included 4 separate dishes of vehicle or 10 μM TAM-exposed (48 hr) NHMECs or HESCs. For isolation of RNA, the cells were lysed with 1.0 mL of TRIzol Reagent (Invitrogen Life Technologies), and RNA was extracted using the RNase Easy Mini Kit (Qiagen) according to the manufacturer's protocol. Residual DNA was removed by digestion with DNase I, and the total RNA quantity and purity were assessed by spectrophotometry (Agilent 2100 Bioanalyzer) and gel electrophoresis. cDNA was obtained using the SuperScript III First Strand-Synthesis System (Invitrogen). Whole-Transcript Human Gene 1.0 ST expression arrays (HuGene-1_0-st-v1 array, Affymetrix) were used for the microarrays, which were run by the Genomics Lab and CLIA Molecular Diagnostics Lab Affymetrix Group (Frederick, MD).

Arrays were uploaded to the NIH Microarray database (mAdb) for analysis and data visualization (https://mAdb.nci.nih.gov). Arrays were normalized using Bioconductor Robust Multi-array Average (RMA) (https://www.bioconductor.org) [21, 22]. Exposed and unexposed samples were compared for differential expression using a *t*-test (pooled with equal variance) and presented as a volcano plot (not shown) by Bioconductor. Heat maps were generated using X-Cluster (https://web.stanford.edu/group/sherlocklab/software.shtml) [23]. Data were subsequently analyzed using Ingenuity Pathways Analysis (IPA) software (Qiagen Inc., https://qiagenbioinformatics.com/products/ingenuity-pathway-analysis) to characterize the functions and pathways of genes of interest. The microarray data were deposited at NCBI's Gene Expression Omnibus [24] and are accessible through GEO Series Accession number GSE106892 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106892).

Confirmation of microarrays by RTq-PCR

cDNA was obtained using the SuperScript III First Strand-Synthesis System (Invitrogen). The iQ SYBR Green Supermix (Bio-Rad) was used for quantitative PCR with the Bio-Rad CFX-96 Real Time System. Using the Bio-Rad system, we performed RTq-PCR on the major genes up-regulated by TAM in HESCs, including *SREBF2*, *DHCR7*, *EHP*, *FABP3*, *HMGCS*, *CCL2* and *PPARG*, confirming the changes previously observed. The *CCL2* gene, which was down-regulated by TAM, also showed the same effect by RTq-PCR.

For this study, the genes up-regulated by TAM in the NHMECs were not subjected to RTq-PCR because these data are available from a previous study using the same cells and exposures [14]. As described, the RTq-PCR values for up-regulation of genes *IFIT1*, *IFITM1*, *MX1*, *GIP3*, *KCNJ1 and IFNA1* in NHMEC strain 16 cells were in good agreement with the microarray data.

Methylation analyses

For global 5-meC methylation analysis of DNA isolated from human and non-human primate uterus we used the MethylFlash Global DNA Methylation ELISA Easy Kit (EpiGentek, Farmingdale, NY) according to the manufacturer's instructions.

For methylation analysis by pyrosequencing [25], purified DNA was measured with the Qubit® fluorometric quantitation assay (Fischer Life Technologies). Sample DNA (750 ng) was then used in the bisulfite conversion reaction (Qiagen Epitect Fast Bisulfite Kit, Qiagen Inc., Germantown, MD) following the manufacturer's instructions. The converted DNA was purified, and 20 ng of this material was used in each PyroMark PCR reaction (Qiagen PyroMark PCR Kit). The PCR

Table 1. PyroMark assays used for pyrosequencing.

PyroMark CpG assay	Catalog #	Amplicon length (base pairs)	Number of CpG sites included
Hs_IFITM1_02_PM	PM 00155946	181 b	5
Hs_MX1_02_PM	PM 00202524	187 b	6
Hs_STAT1_02_PM	PM 00097741	176 b	5
Hs_SREBF2_01_PM	PM 00082509	256 b	4
Hs_SREBF2_02_PM	PM 00082516	253 b	5
Hs_SREBF2_03_PM	PM 00082523	84 b	5
Hs_PRUNE2_01_PM	PM 00140217	183 b	5
Hs_PPARGC1B_01_PM	PM 00022526	126 b	4
Hs_PPARGC1B_02_PM	PM 00022533	183 b	4
Hs_PPARGC1B_03_PM	PM 00022540	114 b	7
Hs_PPARGC1B_04_PM	PM 00022547	111 b	4
Hs_HMCGS1_01_PM	PM 00021238	229 b	6

product from this reaction was then used for pyrosequencing analysis on the PyroMark Q96 MD instrument as detailed in the manufacturer's instructions. The individual primer and probe sets were selected from the Qiagen GeneGlobe web-based interface which supplies predesigned assays for quantification of CpG methylation by pyrosequencing. The list of assays tested is shown in Table 1. The analysis of the pyrosequencing reaction was performed with the Pyro Q-CpG 1.0.9 software from Biotage (Charlotte, NC) that was supplied with the pyrosequencing instrument.

Western blots

For the Western blots we used the Di-Methyl-Histone H3 Antibody Sampler Kit (#9847) from Cell Signaling Technologies (Danvers, MA). The kit contained antisera specific for the di-methyl-histone 3 lysines: H3K4, H3K9, H3K27, H3K36, and H3K79, as well as total Histone H3 (D1H2).

Exposed cells were washed twice with PBS and lysed in Cell Lysis Buffer (Cell Signaling Technologies) supplemented with Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, Rockford, IL). After incubation for 30 min on ice and vortexing every 10 min, cell lysates were centrifuged at 10,000 x g for 10 min at 4 ºC (Eppendorf 5430R centrifuge, Eppendorf, Hauppauge, NY) and supernatants were transferred to a clean tube and stored at -80 ºC until use. Samples (5-25 µg of total protein, depending on the cells used), were brought to volume with 5X Laemmili buffer for a final concentration of 2X, electrophoresed at 120 volts in 4-12% SDS-polyacrylamide gradient gels (Bio-Rad, Hercules, CA), and transferred to membranes with 0.22-micron pore size. Membranes were blocked with Starting Block (ThermoFisher) for 1 hour at room temperature, incubated overnight at 4 ºC with primary H3 methylase antibodies (see above) diluted in Superblock (ThermoFisher), washed 3 times with 0.1% Triton X-100 in Tris-buffered saline, and incubated for 2 hours at room temperature with horse radish peroxidase-conjugated secondary antibody (Cell Signaling Technologies). After washing, specific bands were visualized using chemiluminescence (SuperSignal West Pico, Pierce/ Thermo Scientific). After chemiluminescence detection, transparencies were scanned by Image Quant LAS 4000 (General Electric, Boston MA), and band densitometric analysis.

Statistics

Comparisons between TAM-exposed and unexposed groups for global 5-meC, qRT-PCR, and

Western blot were performed by Student's *t*-test using Graph Pad Prism Version 5.04. The Affymetrix statistics are described above. Because the pyrosequencing values were all at background levels [26], no statistics were applied.

RESULTS

Evaluation of global 5-meC in uterine DNA from monkeys and human patients

In a previous publication [8] we reported TAM-DNA adduct formation in uterine tissue from two species of monkey, patas and macaques, and from breast cancer survivors exposed to TAM, but not in samples from unexposed individuals. When the same human DNA samples, (9 TAM-exposed and 6 unexposed), were evaluated for global 5-meC there, was no significant difference between the normal, TAM-exposed *vs*. TAM-unexposed, or between the malignant TAM-exposed *vs*. TAM-unexposed, human tissues. There was higher 5-meC ($p < 0.05$) in the malignant uterine tissue from both TAM-exposed and unexposed women, compared to uterus from women with no uterine cancer, a finding independent of TAM exposure (Figure 1A). In the macaques (6 TAM-exposed and 6 unexposed) there was no difference in global 5-meC values between groups (Figure 1B). In the patas monkeys (Figure 1B) the 3 TAM-exposed females had significantly higher levels of global 5-meC than the two unexposed patas ($p = 0.04$). The patas also appeared to have higher levels of global 5-meC (up to 3%), compared to those seen in the macaques (0.6-1.4%) and humans (0.3-0.8%), though all of the values were low. We conclude that, even though global 5-meC levels may vary slightly with primate species, the determinants of global 5-meC levels in humans and macaques are controlled independently of TAM exposure.

Differential gene expression of TAM-exposed NHMECs and HESCs

To further interrogate potential TAM-induced epigenetic changes, we used normal human mammary epithelial cells (NHMECs) and human endometrial stromal cells (HESCs) to compare TAM-induced gene expression changes by Affymetrix microarray. For these studies, semi-confluent NHMECs and HESCs were exposed to either 10 μM TAM or vehicle for 48 hr. Observation of the heat map for HESCs (Figure 2A) shows that most of the significantly altered genes were up-regulated, with fold change ≥1.5 and $p < 0.001$. Similar data have been published previously for NHMECs and are therefore not shown here [14].

The fold-increase for selected genes significantly altered by TAM exposure, as determined by microarray, is shown in Table 2A for the NHMECs and Table 2B for the HESCs. For the NHMECs, the Affymetrix array showed up-regulation of primarily immune-response related genes, many of which were also up-regulated in a previous study using a different microarray platform with these same cells and TAM exposure [14]. In that study, the microarray data correlated well with qRT-PCR data, and so the confirmatory qRT-PCR was not repeated here. In the TAM-exposed HESCs, the predominate pattern in up-regulated genes comprised cholesterol metabolism and steroid-synthesis genes. Table 3 shows a comparison of microarray and qRT-PCR data for the TAM-exposed HESCs, indicating that the microarray values were confirmed by qRT-PCR.

mAdb and ingenuity analyses

The microarray data were uploaded into the NCI microArray database (mAdb), where differential expression of significantly altered genes was calculated and heat maps were generated for NHMECs (not shown) and HESCs (Figure 2A). Using the microarray data, the major pathways for TAM up-regulation were analyzed by IPA software, and shown to be very different in the two cell types. In the NHMECs (Figure 2B), the Affymetrix array confirmed TAM-induced up-regulation of immune response genes (interferon signaling and complement genes) previously found in the same cells using a different microarray platform [14]. Genes significantly overexpressed here include some reported before (*IFI27, IFIT1, IFIT3, IFI44L, IFITM1, OAS3, MX1 and Stat1*) [14], as well as new genes involved in the same pathway (*IFI44, IFI35,* and *IFIH1*), and new genes in the complement system (*C1S, C1R* and *SERPING1*).

In the HESCs (Figure 2C) the primary genes up-regulated included some involved with biosynthesis of fatty acids and steroids (*FABP3, HMCGS,*

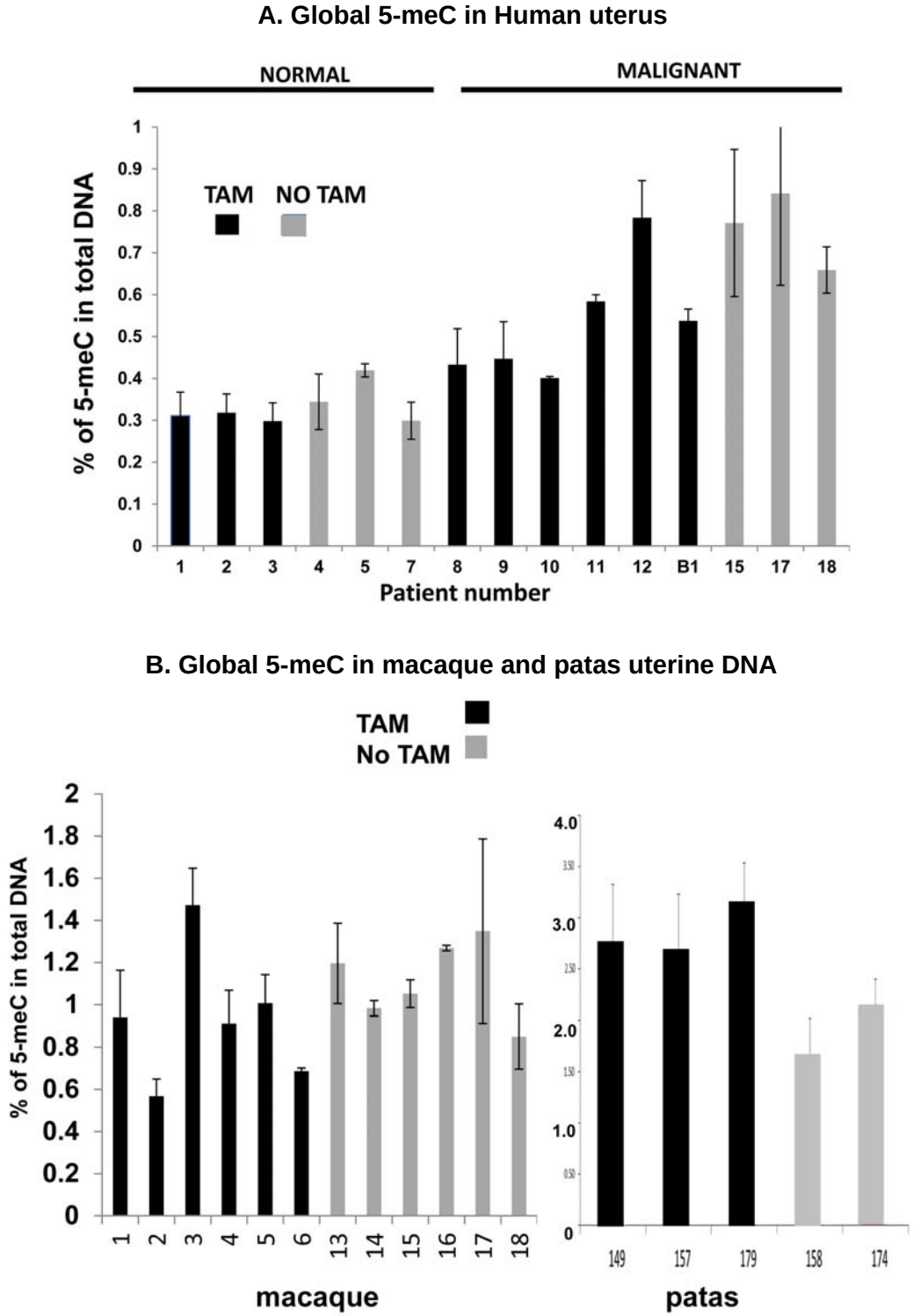


Figure 1. Global 5-meC levels in humans and non-human primates. (A) Normal (n = 6) and malignant (n = 9) human uterine DNA from women receiving TAM therapy (■) and no TAM (□). There were no significant differences between any of the 4 groups. (B) 5-meC in uterine DNA from macaques (n = 6) fed TAM for 4 months and patas (n = 3) fed TAM for 3 months (■). Controls (□) received no TAM. The patas showed a significant difference ($p \leq 0.05$) between TAM and controls.

EBP, *DHCR7*, *FDPS* and *MVD*), and *SREBF2*, a sterol transcription factor. In addition, there was up-regulation of *PPARG*, a gene involved in cell proliferation and cancer, and down-regulation of *CCL2*, a tumor suppressor gene.

Pyrosequencing of CpG islands in promoter regions of selected genes up-regulated by TAM

Because the majority of TAM-altered genes in the breast and endometrial cells were up-regulated we hypothesized that promoter regions of these

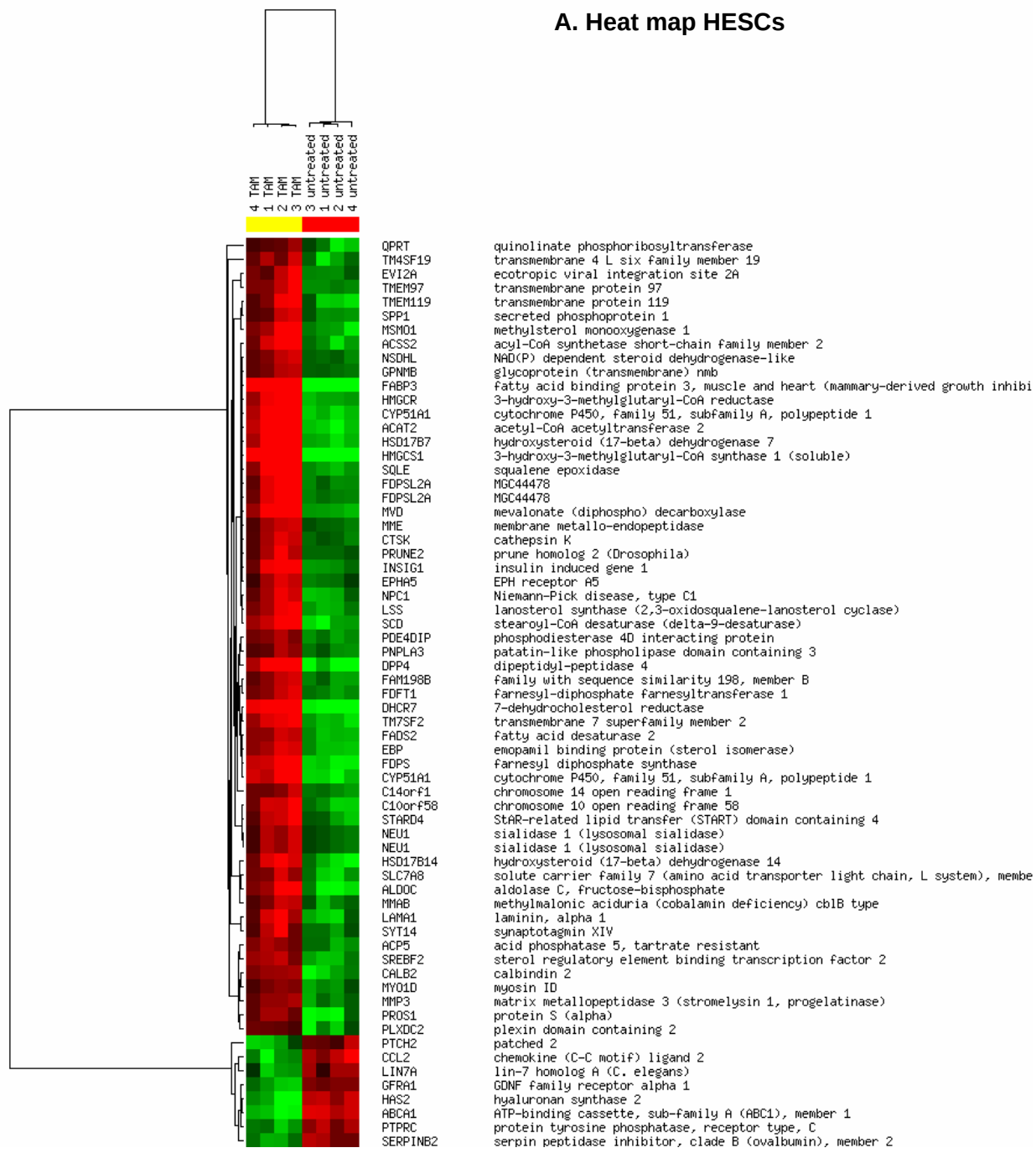
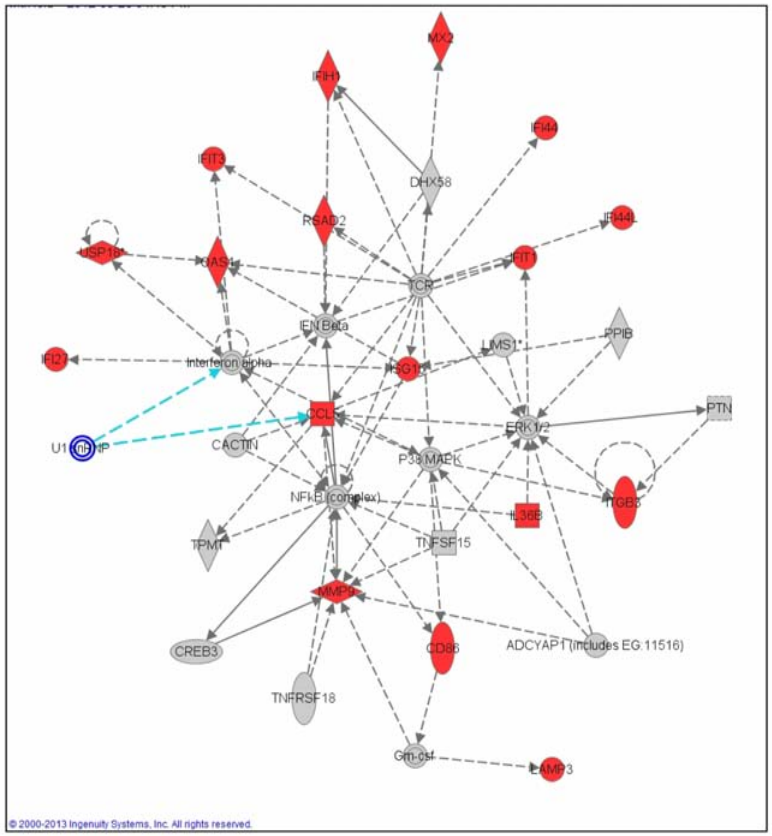


Figure 2A

Figure 2 continued..

B. Ingenuity of TAM-exposed NHMECs



C. Ingenuity of TAM-exposed HESCs

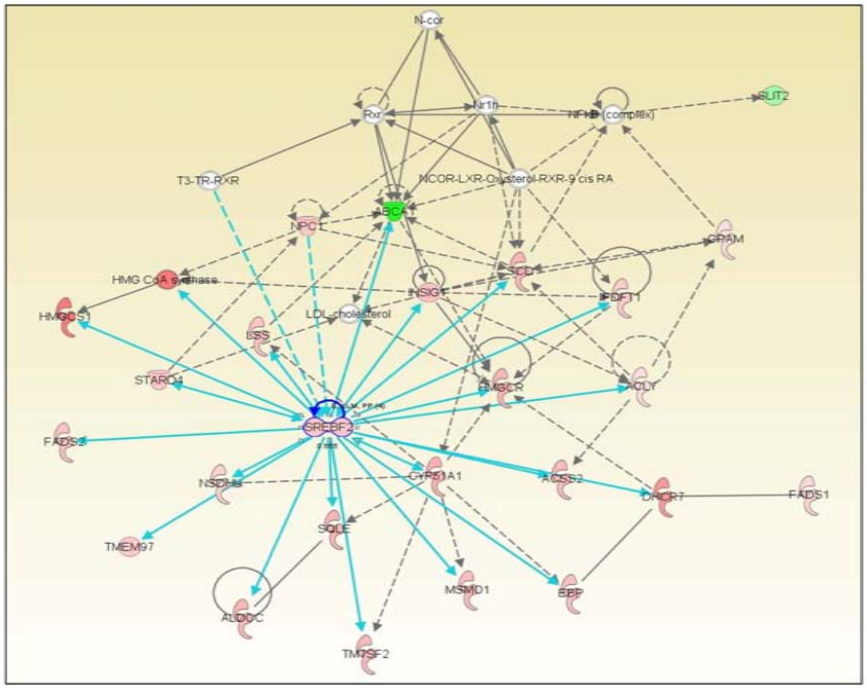


Figure 2. (A) Representative heat map from Affymetrix microarrays of HESCs, where 8 dishes of cells were exposed to 0 (n = 4) or 10 μM (n = 4) TAM for 48 hr. Red shows genes up-regulated and green shows genes down-regulated. (B) Ingenuity Pathway Analysis of Affymetrix microarray of TAM-exposed and unexposed NHMECs (protocol in Figure 2 A) showing up-regula*tion* (in red) of primarily immune-response genes, and genes with no expression changes in grey. (C) Ingenuity Pathway Analysis of Affymetrix microarray of TAM-exposed and unexposed HESCs (protocol in Figure 2A) showing TAM-induced up-regulation (in pink) of primarily cholesterol and fatty acid synthesis genes, and down-regulation (in green) of CCL2, a tumor suppressor gene, while genes in white were not interrogated but are added to show relevant connecting pathways.

Table 2A. Affymetrix microarray of up-regulated genes in TAM-exposed NHMECs.

Gene	Fold change microarray	Gene name/Function
IFIT27	4.26	Interferon-induced protein 27
C1S	3.56	Complement component 1
C1R	3.33	Complement component 1
IFIT1	3.14	Interferon-induced protein 1
IFI44	2.83	Interferon-induced protein 44
IFIT3	2.76	Interferon-induced protein
IFI35	2.71	Interferon-induced protein 35
IFI44L	2.62	Interferon-induced protein 44-like
IFIH1	2.56	Interferon-induced with helicase C
IFITM1	2.19	Interferon-induced transmembrane protein 1
OAS3	2.13	2',5'-oligoadenylate synthetase 3

Table 2B. Affymetrix microarray and qRT-PCR of altered genes in TAM-exposed HESCs.

Gene	Fold change microarray	Normalized expression qRT-PCR (mean ± SEM)	Gene
CCL2	0.61	0.44 ± 0.02	chemokine (C-C motif) ligand 2
DHCR7	2.35	4.81 ± 0.16	7-dehydrocholesterol reductase
EBP	1.68	3.19 ± 0.12	emopamil binding protein
$FABP_3$	5.69	8.06 ± 0.07	fatty acid binding protein 3
HMCGS	3.08	4.85 ± 0.03	3-OH-3-methylglutaryl synthase
PPARG	1.30	2.50 ± 0.20	peroxisome proliferator-activated receptor γ
SREBF2	1.59	1.96 ± 0.09	sterol regulatory element binding transcription factor 2

Table 3A. NHMEC cells, either unexposed (-) or exposed (+) to 10 μM TAM for 48 hr, were subjected to pyrosequencing to reveal % 5-meC in promoter region CpG islands of the TAM-up-regulated genes *MX1* and *Stat1*.

Gene	TAM	% 5-meC in promoter region CpG Site					
		1	2	3	4	5	6
MX1	(-)	3.4	2.2	7.4	3.7	2.6	5.1
	(+)	3.6	2.4	6.7	4.4	2.9	4.7
Stat1	(-)	3.2	2.3	4.1	3.8	3.0	
	(+)	3.2	2.1	4.0	3.0	2.8	

Table 3B. HESC cells, unexposed (-) or 10 μM TAM-exposed (+) for 48 hr, were subjected to pyrosequencing to reveal % 5-meC in promoter region CpG islands of the TAM-up-regulated genes *PPARG, SREBF2, HMCGS and Prune2*.

Gene	TAM	% 5-meC in promoter region CpG Site					
		1	2	3	4	5	6
PPARG	(-)	0	0.9	0.6	0	0	0
	(+)	0	1.7	0.4	0	0	0
SREBF2	(-)	0.6	1.3	0.7	0.6	0.8	
	(+)	0	1.1	1.4	0.5	0.3	
HMCGS	(-)	0	0	0.7	0	0	0
	(+)	0.4	0	0.5	0	0	0
Prune2	(-)	3.1	3.7	4.4			
	(+)	2.9	3.7	4.8			

altered genes might be under-methylated. Clearly global 5-meC appeared not to be altered by TAM, but specific gene regions might show a difference. For the NHMECs we chose 2 genes, *MX1* and *STAT1*, and for the HESCs we chose 4 genes, *PARPG*, *SREBF2*, *HMCGS* and *PRUNE3*. The results are shown in Tables 3A and B. Overall, the 5-meC levels were very low (<7%), and so the methodology employed could not discriminate differences in 5-meC levels between the TAM-exposed and the unexposed samples. Clearly, TAM exposure did not alter 5-meC levels in the promoter CpG islands of the genes interrogated.

Levels of di-methyl histone H3 in NHMECs and HESCs exposed to TAM

Total H3 histone protein (5 μg), evaluated by Western blot with an HSP90 control, was examined in protein extracts from both NHMECs and HESCs exposed in triplicate to 10 μM TAM for 48 hr (Figure 3 A, B). Replicate Western blots showed total histone levels to be generally higher in the HESCs than in the NHMECs. For both cell types the TAM-exposed cultures appeared to have lower levels of total histone H3 than the unexposed cultures; however this difference was statistically significant only in the HESCs (Figure 3B).

Similarly, for Western blots performed with the H3K4me2, H3K27me2, and H3K36me2 antisera, it appeared visually that the levels were reduced in the TAM-exposed HESCs and NHMECs compared to unexposed controls (Figures 3: C, D, for H3K4me2; E, F for H3K27me2; G, H for H3K36me2), but only the TAM-exposed HESCs showed a statistically significant reduction ($p \leq 0.05$). For H3K9me2 and H3K79me2 (not shown) there was no consistent reduction in the TAM-exposed, compared to the unexposed, NHMECs and HESCs.

DISCUSSION

Here we evaluated uterine DNA from women and two different species of monkeys, either unexposed or exposed long-term to oral TAM, for global 5-meC levels. All of the TAM-exposed women and monkeys had previously documented TAM-DNA adduct formation [8]. In the women, the length of TAM exposure was unknown, but it was presumed to be a matter of months, as breast cancer patients are typically given TAM therapy for 5 years. In the monkeys, the length of exposure was 3-4 months. In all three species of primates TAM exposure did not impact/change 5-meC levels. Because we had only DNA from these individuals, our additional transcriptional studies were carried out using cultured human breast and endometrial cells.

In cultured NHMECs and HESCs exposed for 48 hr to 10 μM TAM, gene expression changes were examined in relation to unexposed controls,

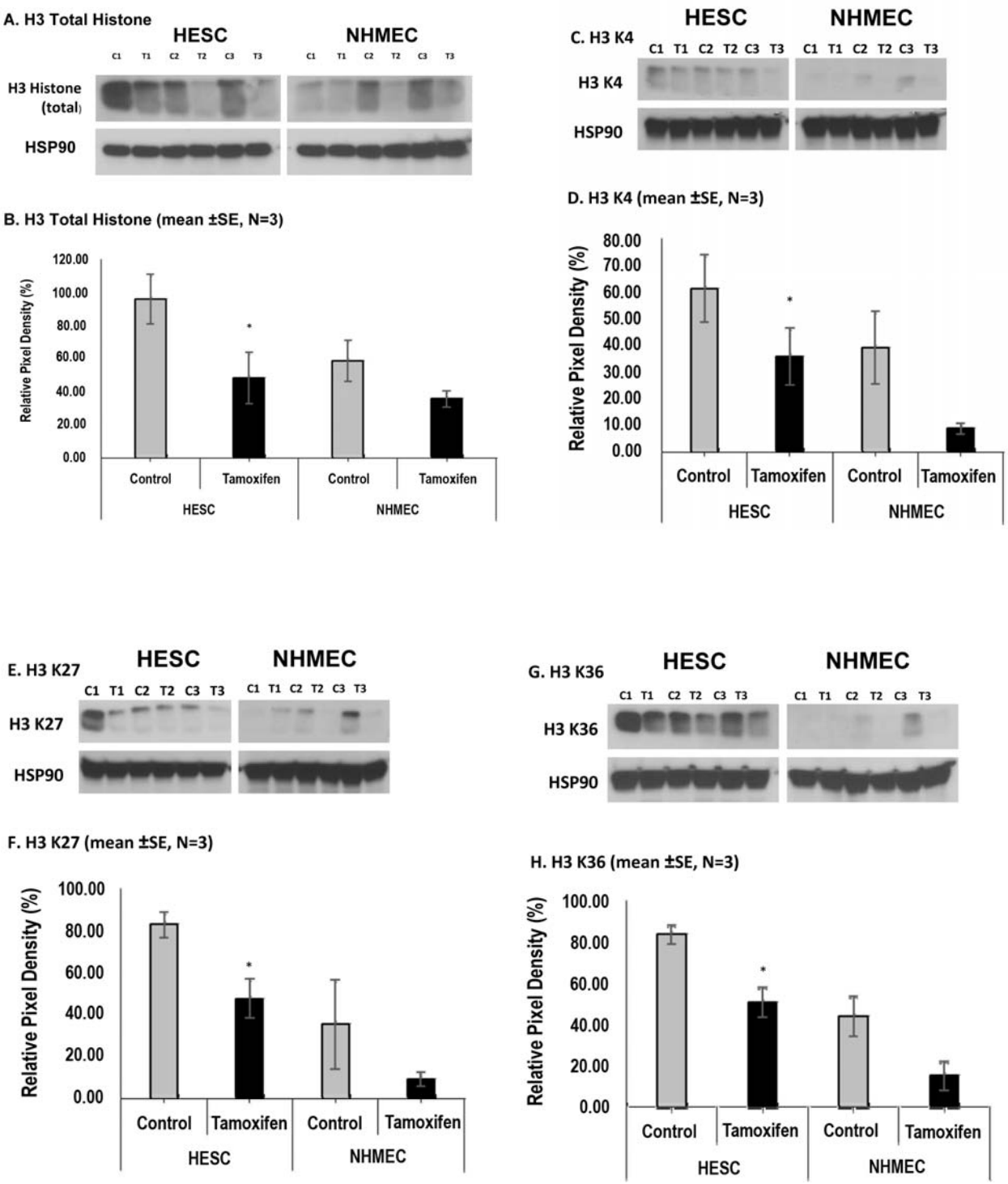


Figure 3. Representative Western blots showing (A) total H3 Histone, (C) H3K4me2, (E) H3K27me2 and (G) H3K36me2 in HESCs and NHMECs, either unexposed (C1, C2, C3) or exposed for 48 hr to 10 μM TAM (T1, T2, T3) in triplicate. Densitometry values calculated from means of each of the triplicate plates are shown for total H3 histone (B), H3K4me2 (D), H3K27me2 (F), and H3K36me2 (H). Statistically significant (* = $p \leq 0.05$) reductions in these H3 histones in TAM-exposed cells were observed in HESCs but not NHMECs.

using Affymetrix microarray with confirmation by qRT-PCR. TAM-exposed NHMECs showed significant up-regulation of interferon signaling and immune response pathways, while the TAM-exposed HESCs showed significant up-regulation of steroid and fatty acid biosynthesis pathways. To examine further the role of cytosine methylation, 5-meC levels at CpG islands in the promoter regions of some of the most highly TAM-up-regulated genes in each cell type were evaluated using pyrosequencing. However, no difference was found between TAM-exposed and unexposed cells, further indicating that 5-meC was not likely to be a relevant regulator of transcriptional effects in TAM-exposed NHMECs and HESCs.

Finally, we examined levels of total H3 histone and multiple di-methyl histone H3 proteins, H3K4me2, H3K9me2, H3K27me2, H3K36me2 and H3K79me2, in TAM-exposed and unexposed NHMECs and HESCs by Western blot. Methylation sites on H3 lysines comprise highly specific enzyme-targeted sites. The corresponding methylases typically modify only a single lysine, and a great deal is known about their effect on transcription. For example, H3 methylases specific for positions K4, K36 and K79 (lysines at positions 4, 36 and 79) are implicated in transcriptional activation and elongation [26]. In contrast, H3K9 and H3K27 are implicated in transcriptional repression and silencing of heterochromatin. Here we investigated the abundance of some H3 di-methylated in specific lysines, using Western blot to evaluate the effect of TAM on levels of these histones. We found a statistically significant depletion of total histone as well as H3K4me2, H3K27me2 and H3K36me2 in TAM-exposed HESCs, compared to the unexposed controls. TAM exposure also resulted in up-regulation of different transcriptional pathways in NHMECs and HESCs. In the NHMECs, major up-regulation was observed in immune response and interferon-related genes, while in the HESCs the predominant up-regulated genes included those involved in steroid synthesis and fatty acid synthesis. Furthermore, TAM exposure in HESCs resulted in depleted levels of total histone H3, H3K4me2, H3K27me2, and HeK36me2. Therefore, significant depletion in some di-methylated lysine H3 histones were observed in TAM-exposed HESCs, but these changes do not appear to be directly related to 5-meC. Clearly, more sophisticated approaches will be required before the epigenetic changes induced by TAM can be understood completely.

Chromatin exists in a dynamic equilibrium between open (transcriptionally active) and closed (transcriptionally silent) states. Regulation of these transitions is accomplished by 5-meC modification of DNA, post-translational modifications (including methylation, acetylation, and phosphorylation) of histone proteins at specific sites, and miRNAs [27]. Only a few studies have described epigenetic effects induced by TAM. In two of these reports, both mice fed TAM in the diet for 12 weeks [28], and rats [29] fed TAM for 6, 12, 18 or 24 weeks, were examined for genotoxic and epigenetic changes. In the rat liver, a target tissue for TAM carcinogenesis, not only did TAM form measurable DNA adducts, but at all time points there were substantial increases in global 5-meC, decreases in selected DNA methyl-transferases, and decreases in H4K20m3. Because similar changes did not occur in non-target tissues, it was suggested that these events may be related to TAM-associated carcinogenesis. In the livers of TAM-exposed mice, where TAM is not a carcinogen despite formation of TAM-DNA adducts, there were no changes in global or repetitive element 5-meC, and histone methylation and acetylation were not altered, compared to the unexposed controls. The methylated H3 examined included H3K4me3, H3K9me3, H3K27me3, and H3K79me3. Microarray of mouse liver indicated that TAM predominantly caused down-regulation of genes associated with hepatic lipid metabolism, while lipocalin 13 and PPARγ were overexpressed. A comparison of these two papers suggests that the lack of epigenome alterations in livers of mice fed TAM may be associated with the resistance of that organ to TAM-induced liver tumors. Conversely, it could be argued that the epigenetic changes in TAM-exposed rat liver may become permanent and contribute to the process of tumor induction.

Epigenetic regulation is an exceedingly complex process and we still know very little about the primary events induced by therapies like TAM, including how they might be related to carcinogenesis.

Previous studies of TAM have shown: an overall reduction in 5-meC in TAM resistant MCF7 cells compared to their non-resistant counterparts [30]; hypomethylation of the *PAX2* promoter induced by TAM or E2 in endometroid carcinoma cells, but not normal endometrial cells, resulting in enhanced uterine proliferation [31]; and increased promoter 5-meC frequency in O^6-methylguanine DNA methyltransferase of endometrial tumors from TAM-exposed patients (78%) compared to unexposed patients (50%) [32]. Finally, TAM-resistant breast cancer can be driven by a histone H3K36 methyltransferase (WHSC1), which facilitates ERα signaling, and it has been proposed that this methyltransferase might be a novel target for therapeutic intervention of TAM-resistant tumors [33]. Here we showed that TAM reduces the levels of some di-methyl-lysine H3 histones in human endometrial cells, but did not alter percentages of 5-meC in human or monkey tissues. This study highlights the need for larger and more targeted epigenetic studies. Fortunately, the tools required for epigenetic analyses are improving rapidly, and the resulting knowledge should enhance biomarker validation and cancer prevention strategies. Meanwhile, knowledge of the epigenetic effects resulting from TAM exposure may elucidate our understanding of TAM carcinogenicity.

CONCLUSION

In this study of the transcriptional and epigenetic effects of TAM exposure, we found that different gene pathways are up-regulated in NHMECs and HESCs exposed to TAM, and that mechanisms involving 5-meC variation are likely not involved. Our finding that TAM-exposed HESCs had a significant loss in total H3 histone, as well as H3K4me2, H327me2 and H3K36me2, and that the NHMECs showed (non-significant) changes in the same direction, indicate that TAM has the potential to dysregulate the epigenome. The exceedingly complex interactions that constitute the whole epigenome, and the current lack of knowledge with respect to the effects of TAM exposure, suggest that we have much to learn. With time and new methods it should be possible to determine whether there are specific epigenetic changes that may be associated with susceptibility to TAM-induced endometrial cancer risk, and whether these targets may also be used in preventive strategies. This study is a first step in that direction.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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