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Transcriptional Responses to Estrogen and Progesterone in Mammary Gland Identify Networks Regulating p53 Activity

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Estrogen and progestins are essential for mammary growth and differentiation but also enhance the activity of the p53 tumor suppressor protein in the mammary epithelium. However, the pathways by which these hormones regulate p53 activity are unknown. Microarrays were used to profile the transcriptional changes within the mammary gland after administration of either vehicle, 17β-estradiol (E), or progesterone (P) individually and combined (EP). Treatment with EP yielded 1182 unique genes that were differentially expressed compared to the vehicle-treated group. Although 30% of genes were responsive to either E or P individually, combined treatment with both EP had a synergistic effect accounting for 60% of the differentially regulated genes. Analysis of protein-protein interactions identified p53, RelA, Snw1, and Igfals as common targets of genes regulated by EP. RelA and p53 form hubs within a network connected by genes that are regulated by EP and that may coordinate the competing functions of RelA and p53 in proliferation and survival of cells. Induction of early growth response 1 (Egr1) and Stratifin (Sfn) (also known as 14–3-3σ) by EP was confirmed by reverse transcription-quantitative PCR and shown to be p53 independent. In luciferase reporter assays, Egr1 was shown to enhance transcriptional activation by p53 and inhibit nuclear factor κB activity. These results identify a gene expression network that provides redundant activation of RelA to support proliferation as well as sensitize p53 to ensure proper surveillance and integration of their competing functions through factors such as Egr1, which both enhance p53 and inhibit RelA. (*Endocrinology* 149: 4809–4820, 2008)

ESTROGENS and progestins induce a broad spectrum of changes within the mammary epithelium that are essential for both normal development and function. The sustained increases in levels of 17β-estradiol (E) and progesterone (P) during pregnancy induce differentiation of terminal end buds and terminal ducts (1–5). In addition, pregnancy levels of exogenous E and P are sufficient to render the mammary gland resistant to mammary tumorigenesis (6–8).

The p53 tumor suppressor pathway appears to be a critical target of hormone-mediated prevention of breast cancer. Basal levels of p53 protein are normally below the limit of detection due to its rapid degradation, but it is stabilized and accumulates rapidly after DNA damage (9, 10). However, ionizing radiation induces only modest levels of p53-dependent apoptosis in the mammary epithelium of nulliparous mice (11). Radiation-induced apoptosis increases dramatically within the first 4 d of pregnancy (12) in concert with the increasing levels of proliferation stimulated by estrogens and progestins (13). Treatment with E and P for 4 d is sufficient to increase p53-dependent responses to ionizing radiation (14). The increase in p53 activity during pregnancy appears to persist in mammary epithelium of parous mice (15). The hormone-induced increase in p53 activity appears critical for parity induced protection from mammary tumors because the protective effect of parity was diminished markedly in mammary tissues from p53-deficient mice (16, 17).

As the responsiveness of p53 to ionizing radiation increases rapidly after exposure to E and P (14), the transcriptional responses in the mammary gland after acute stimulation with these hormones provide a method to elucidate hormone-responsive pathways that regulate p53 function. In these experiments mice were treated with E and P, individually and combined (EP), for 4 d to define the transcriptional changes that are associated with the enhanced sensitivity of p53. Although transcriptional responses to estrogen or P alone were significant, 60% of the differentially expressed genes required combined treatment with E and P, indicating synergistic interactions between these signaling pathways. The expression profiles showed an up-regulation of genes associated with proliferation and differentiation, whereas

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Abbreviations: CFP, Cleared fat pad; E, 17β-estradiol; Egr1, early growth response 1; EP, 17β-estradiol and progesterone; ERE, estrogen-responsive element; GO, Gene Ontology; Gpx, glutathione peroxidase; ID, identification; IGFBP, IGF binding protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; NF-κB, nuclear factor κB; P, progesterone; PPI, protein-protein interaction; qPCR, quantitative PCR; Rbp1, retinol binding protein 1; RT, reverse transcription.

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expression of genes involved in lipid metabolism and mitochondrial respiration were diminished. Protein interaction networks identified RelA as a common target of genes induced by EP, which is consistent with the essential role of nuclear factor κB (NF-κB) in proliferation of the mammary epithelium. Despite the pronounced effects on proliferation, p53 itself was also overrepresented as a common target of genes that were up-regulated by EP. The protein interaction networks identified targets that may mediate cross talk between these pathways to balance the proliferative responses with the need to ensure genomic integrity in the mammary epithelium. Reporter assays demonstrated that early growth response 1 (Egr1) simultaneously inhibits the transcriptional activity of NF-κB while enhancing the activity of p53 in MCF-7 cells. Thus, the transcriptional profiles induced by E and P reveal redundant networks that initiate proliferation while sensitizing p53 to ensure proper genomic surveillance.

Materials and Methods

Animal husbandry and surgery

There were 17 (8 wk old) virgin BALB/c mice ovariectomized followed by a period of 1 wk to clear endogenous hormones. Hormones were administered by ip injections in a total volume of 100 μl repeated daily for 4 d, and included four animals receiving 2 μg E, four animals receiving 1 mg P, five animals receiving both E and P (EP), and four animals receiving sesame oil (vehicle). To distinguish responses in the stroma, three mice with epithelial-free fat pads [referred to as "cleared fat pads" (CFP)] were prepared by surgically removing the primary duct at 21 d of age (16, 18, 19) and treated with EP when mice reached 8 wk of age. Additional BALB/c animals were treated with either E, P, EP, or vehicle for confirmation of gene expression. These treatments were also administered to ovariectomized BALB/c-*Trp53*−/− mice to determine whether responses to hormones were p53 dependent. Lymph nodes were removed from mammary glands, and the tissue was flash frozen in liquid nitrogen. The hormones were purchased from Sigma-Aldrich (St. Louis, MO). All procedures involving animals were in accordance with institutional and national guidelines for the use of animals, and were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts-Amherst.

Microarray hybridization and data normalization

The total RNA from each lymph node-free mammary gland was extracted and purified using UltraSpec (Biotecx Laboratories, Inc., Houston, TX). Of total RNA, 10 μg were biotinylated, then fragmented into approximately 35–200 bases. Fragmented, biotinylated cRNA was hybridized to the Affymetrix U74v2 mouse genome chipset using standard protocols (Affymetrix, Inc., Santa Clara, CA). Background correction and normalization of gene expression data were computed directly from the Affymetrix .CEL files using the Bioconductor package for R [R 2.4.0; (20)] implementation of GC-Robust Multichip Average (21). The resulting expression values for each sample were compared with the average expression value of the appropriate treatment group by calculating their correlation coefficients. All samples had correlations of $R > 0.9$ and were included in the analysis. The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series accession no. GSE5483.

Data filtering

ANOVA for each of the 36,899 probe sets among all treatments was used to select those that were differentially expressed ($P < 0.05$). Initial analysis identified one animal in the E-treatment group (no. 3021) that formed a unique cluster and had an overrepresentation of immune-related genes suggesting a subclinical infection. Therefore, data from this animal were excluded from further analysis. ANOVA was repeated using the 16 remaining animals, and 2899 probe sets were identified as differentially expressed ($P < 0.05$). Hierarchical clustering was conducted using the 2899 probe sets. Pairwise comparisons of each treatment relative to the vehicle-treated group were performed on these 2899 probe sets by applying a *t* test. Distribution of expression values for each treatment data set was computed using 1000 permutations. Treatment with E or P individually identified differential expression of 1605 probe sets, but no difference in expression was detected in mammary glands from EP-treated mice. Because EP is necessary for the induction of p53 responsiveness, the 1294 probe sets differentially expressed in response to EP ($P < 0.05$) were selected for further analysis. ANOVA, *t* tests, and hierarchical clustering were implemented using the Institute for Genomic Research's MeV package, version 4.0.1 (22).

Classification of differentially expressed genes

Average expression signals for each treatment group were calculated for the 1294 differentially expressed probe sets. Ratios of the treatment averages relative to the vehicle-treated group were calculated to describe the direction and fold change in expression for each probe set. The fold change identified genes that were regulated by either E alone, P alone, or required both EP. Mean log2 expression ratios for E-responsive, P-responsive, EP-responsive, and E- or P-responsive probe sets are provided in supplemental data, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Analysis of biological relationships

The 1294 probe sets that were differentially expressed in the EP treatment represent 1182 unique genes. Biological relationships among the genes that were up-regulated or down-regulated by hormone treatments were assessed. Statistical overrepresentation of Gene Ontology (GO) terms (23), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (24), and first-neighbor interactions among proteins (ftp://ftp.ncbi.nih.gov/gene/GeneRIF; accessed May 30, 2006) within a study set of genes was obtained using the hypergeometric distribution with a modified Bonferroni correction (e score) as implemented in GeneMerge (25). Only unique genes with a National Center for Biotechnology Information gene identification (ID) number were used in the analysis. Identifiers for the GO and KEGG analyses were unique gene IDs for *Mus musculus*, whereas identifiers for the first-neighbor protein interactions were unique gene IDs for *Homo sapiens*. The Homologene database (build 5.1) was used to convert mouse gene IDs to human gene IDs. The population to which the study sets were compared was all unique genes with a gene ID on the U74v2 chip set.

Northern analysis

Total RNA from mammary glands was isolated using UltraSpec and RNA Tack Resin (Biotecx Laboratories). Northern blotting was performed following standard procedures using ^{32}P-labeled probes generated by random priming. Template cDNAs were amplified by RT-PCR using primers described in Table 1 and were cloned into pCR2.1-TOPO plasmids (Invitrogen Corp., Carlsbad, CA). Each template sequence was confirmed by at least two different restriction digests. The blots were scanned by the Cyclone phosphorimager system (PerkinElmer Life And

TABLE 1. Primers used for RT-PCR

Transcript	Primers
Gapdh	gapdh5F: 5′-TTC ACC ACC ATG GAG AAG GC-3′
	gapdh3R: 5′-GGC ATG GAC TGT GGT CAT GA-3′
Areg	Areg-3−5 F: 5′-CAA CTG GGC ATC TGG AAC C-3′
	Areg-3–3R: 5′-GCA GAG ACC GAG ACG CT-3′
Gpx3	Gtpx-1−5 F: 5′-GGA CCA CAG TCA GCA ACG T-3′
	Gtpx-1–3R: 5′-AGT GAG AGG ATA GCA TGT CCT-3′
Rbp1	Rbp-1−5 F: 5′-CAG CAA CCG TCC GGG C-3′
	Rbp-1–3R: 5′-GTG GGT ATG CGT TTC GGT CC-3′
Gsn	Gels-1−5 F: 5′-TTC TGT ACA ACT ACC GCC ACG-3′
	Gels-1–3R: 5′-TTT TCC AAC CCA GAC AAA GAC C-3′

F, Forward; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; R, reverse.

TABLE 2. Primers and probes used for RT-qPCR

Transcript	Primers	Probe
Sfn	Sfn-new-left: 5′-CCA CTG GCG ATG ACA AGAA-3′	79
	Sfn-new-right: 5′-GTT GGT AGG CGG CAT CTC-3′	
Egr1	Egr1-left: 5′-CCT ATG AGC ACC TGA CCA CA-3′	22
	Egr-right: 5′-TCG TTT GGC TGG GAT AAC TC-3′	
Pgk1	Pgk1-left: 5′-TAC CTG CTG GCT GGA TGG-3′	108
	Pgk1-right: 5′-CAC AGC CTC GGC ATA TTT CT-3′	

Analytical Sciences, Inc., Waltham, MA) and quantified by OptiQuant software (Parkard Bioscience, Downers Grove, IL). Statistical differences between means were calculated using paired *t* tests.

Quantitative RT-PCR

Total RNA was extracted with QIAzol (QIAGEN, Inc., Valencia, CA) following the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically, and RNA quality was confirmed visually on an agarose gel. The RNA was heated to 65 C for 10 min, then the reverse transcription (RT) (20 μl reactions) was performed on 2 μg RNA using Transcriptor First Strand cDNA synthesis kit (Roche, Mannheim, Germany) following the manufacturer's instructions for transcription using both random hexamers and oligo(deoxythymidine) primers. Real-time PCR [quantitative PCR (qPCR)] was performed in duplicate using Fast Start Universal Probe Master (Roche). Each 20-μl reaction contained a final concentration of 1 μM each of forward and reverse primers, 0.2 μM probe (Universal Probe Library; Roche), 1× master mix, and 2 μl cDNA. cDNA was diluted 1:10 for Pgk1 reactions. Standard curves were run for each transcript to ensure exponential amplification, and "no RT" controls were run to exclude nonspecific amplification. Expression of genes was normalized to phosphoglycerate kinase (Pgk1) expression. Primers and probes used are listed in Table 2. The reactions were run on an MX300 real-time PCR machine (Stratagene, La Jolla, CA) with

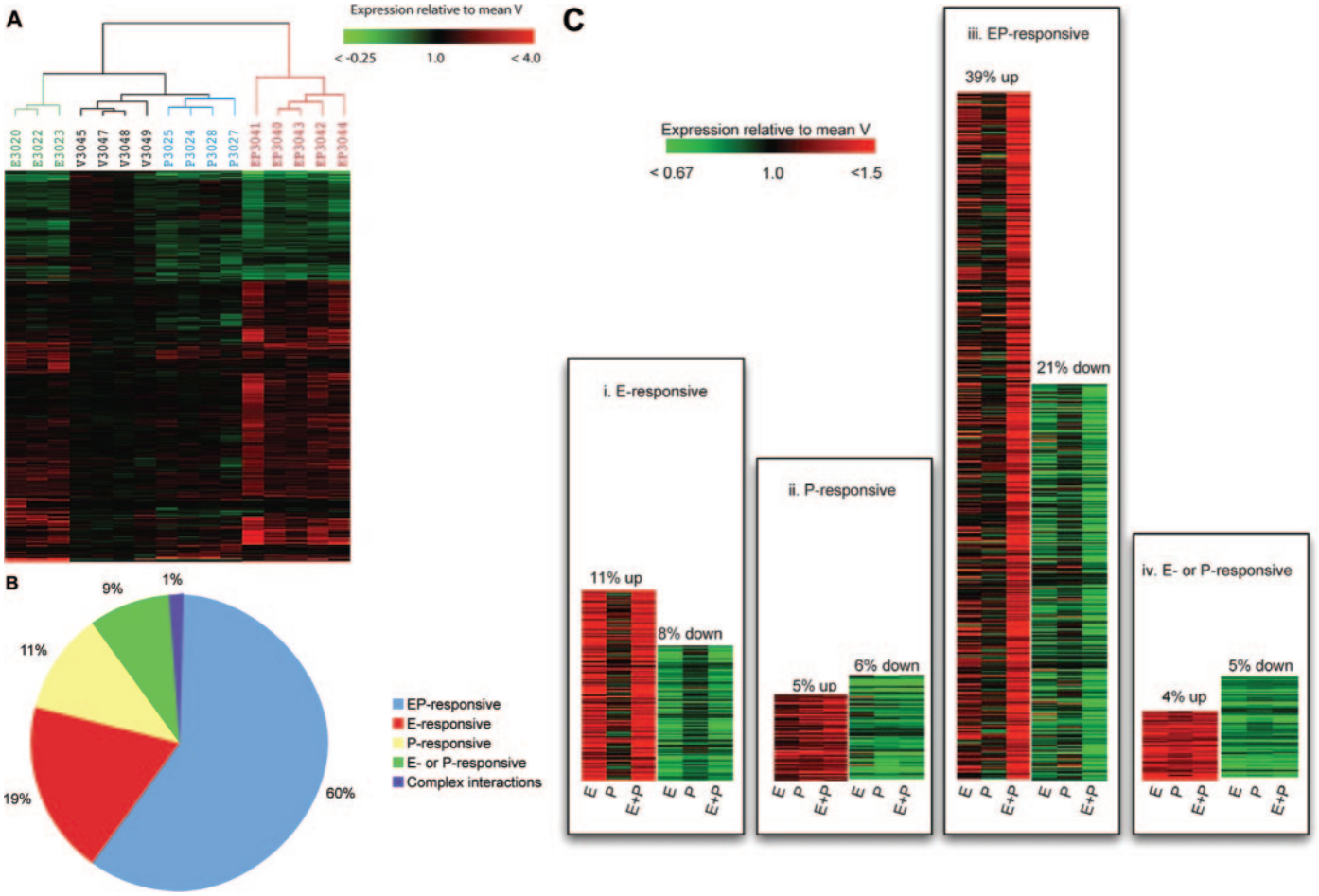

FIG. 1. Gene expression patterns induced by E and P. A, Hierarchical clustering was performed using the probe sets differentially expressed among treatments. The results for probe sets with more than 4-fold difference in expression are shown. *Red and green* indicate more than 4-fold increase and decrease in expression levels, respectively. Mouse IDs and treatment are indicated for each sample: E, 17ß-estradiol; EP, estrogen plus P; V, vehicle. B, Classification of differentially regulated genes by hormone responses. "E-responsive" indicates genes that were increased or decreased to similar extents in both E and EP treatments. "P-responsive" indicates genes that were increased or decreased to similar extents by both P and EP treatments. "EP-responsive" indicates genes that were not altered by either hormone alone but were significantly increased or decreased when both hormones were administered. "E or P-responsive" indicates genes showing significant increase or decrease in levels when either E or P was administered. "Complex interactions" indicates a small group of genes for which expression was increased by one treatment but reversed by another treatment. C, Heat maps of average expression levels for each treatment (E, P, EP) relative to the vehicle for genes that were differentially expressed.

a temperature profile of 95 C for 10 min, then 45 cycles of 95 C for 15 sec and 57 C for 1 min.

Luciferase reporter assays of transcriptional activity

Telomerase-immortalized breast epithelial cells, hME-CC (26), were used as the source of mRNA to obtain the full-length human Egr1 cDNA by RT-PCR. The PCR primers were 5′-GGTACCAAGCTTTCGCCGCCT-GCACGCTTCTCAGTGT-3′ and 5′-CTCGAGGCGGCCGCACCCCAA-GAAAAACGAAATCCAT-3′. The cDNA was cloned into the pcDNA3.1/V5-His-TOPO (Invitrogen) to generate the pcDNA-Egr1 expression plasmid. The cDNA sequence was confirmed by sequencing. The p53-Luc and pNFκB-Luc reporter plasmids were purchased from Stratagene. pRL-CMV was purchased from Promega Corp. (Madison, WI). MCF-7 cell were cultured in DMEM:F12 supplemented with 25 mM HEPES, 1.2 g/liter $NaHCO_3$, 10 μg/ml insulin, 10% adult bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were plated in 12-well plates at 300,000 cells per well 24 h before transfection. Mixtures of the pcDNA-Egr1 expression plasmid together with reporter plasmids (0.5 μg p53-Luc or pNFκB-Luc) and 0.05 μg pRL-null were transfected into MCF-7 cells using Lipofectamine 2000 (Invitrogen). At 6 h after transfection, the media were replaced with DMEM:F12 medium containing 0.1% serum. At 48 h the cells were harvested in 1× luciferase lysis buffer. Luciferase activity was determined and normalized to the pRL-null luciferase activity using the Dual Luciferase Assay Kit (Promega) to account for differences in transfection efficiency.

Results

Classification of transcriptional responses

E and P initiate a host of changes in the mammary gland with the onset of pregnancy. The transcriptional changes may simply reflect additive effects of each hormone in-

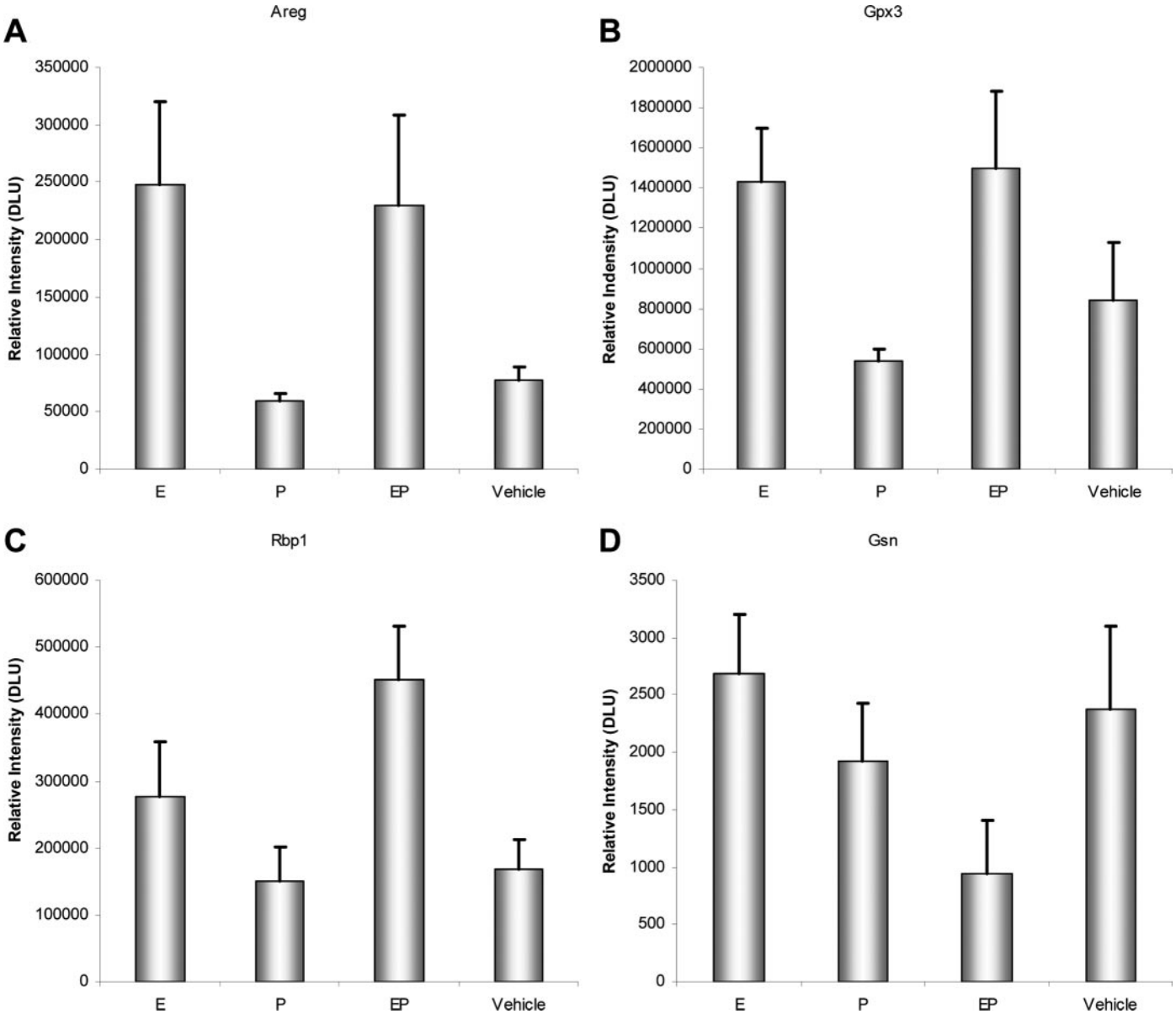

FIG. 2. Validation of gene expression patterns. Genes were selected representing the estrogen responsive (E-responsive) or those requiring both E and P (EP-responsive) patterns as described in Fig. 1C. Both Areg and Gpx3 are E responsive being induced to similar levels by estrogen and unaffected by the presences of P (A and B). EP-responsive genes such as Rbp1 and Gsn required both EP for induction or repression (C and D, respectively).

dividually but may also require more complex interactions. Hierarchical clustering provides a Euclidean distance measure and, thus, can be used to evaluate relationships among treatments. Clustering was performed on the 2899 probe sets that were differentially expressed among treatments (Fig. 1A). Although each hormonal treatment formed distinct branches, P was closely related to vehicle. Tissues from E-treated mice were distinct but remained on the same branch of the dendrogram. In contrast, tissues from mice treated with both EP formed a distinct branch.

The genes differentially expressed by treatment with EP were of primary interest because both hormones are necessary to enhance the responsiveness of p53 (14). A total of 1294 probe sets were differentially expressed in response to treatment with both hormones (EP). The proportions of these genes that were altered by treatment with estrogen or P individually were 19 and 11%, respectively (Fig. 1B). Although similar in the proportion of genes affected, comparing the heat maps for these treatments (Fig. 1A) indicates that the fold changes in expression was much less for P compared with the responses to E. Promiscuous responses were detected for 9% of the genes with either E or P being sufficient to induce changes in expression. Complex interactions were detected for a small number of the genes (1%, Fig. 1B). In contrast, 60% of the genes required both hormones for the transcriptional responses (EP-responsive, Fig. 1B). Therefore, E and P act cooperatively to regulate a large proportion of the genes.

Steroid hormone action is mediated by transcriptional activation as well as repression mediated by their receptors. Indeed, the EP-induced changes in mRNA levels were balanced with gene expression being both enhanced and inhibited to similar extents. Among the genes classified as E responsive, treatment with E induced expression of 11% and decreased expression of 8% of the genes (Fig. 1C, panel i). Among the genes classified as P responsive, treatment with P induced expression of 5% and decreased expression of 6% of the genes (Fig. 1C, panel ii). A small group of genes was sensitive to stimulation with either hormone (Fig. 1C, panel iv) with 4% of genes up-regulated and 5% down-regulated. Therefore, treatment with E or P transcriptionally activated and repressed near equal numbers of targets. In contrast, among the EP-responsive genes, transcriptional activation was far more prevalent, with 39% showing increased expression compared with 21% that were diminished (Fig. 1C, panel iii).

The expression of genes representing E-responsive and EP-responsive classes was validated using a separate set of animals (Fig. 2). The amphiregulin (Areg) gene contains an estrogen-responsive element (ERE) and is known to be estrogen responsive in the mammary gland (27). Indeed, expression was increased by 3.2-fold by E, and similar levels were detected when both EP were administered (Fig. 2A). This represents the clas-

TABLE 3. Estrogen receptor targets conserved targets in mouse mammary gland and human MCF-7 cells

Human gene name	Human gene ID	Increased at 0-3 h[a]	Promoter ERE[b]	Chromosome	Strand	Transcript start	Transcript end
ACTN4	81	−	+	19	1	43,830,166	43,913,010
BMF	90427	+	−	15	−1	38,167,386	38,188,367
C15ORF38	348110	+	−	15	−1	88,244,836	88,257,163
CCND1	595	+	−	11	1	69,165,054	69,178,422
CD164	8763	+	−	6	−1	109,794,416	109,810,340
CKS1B	1163	+	−	1	1	153,213,781	153,218,346
CXCL12	6387	+	−	10	−1	44,186,808	44,200,548
DMPK	1760	−	+	19	−1	50,964,818	50,977,655
DNAPTP6	26010	+	−	2	1	200,985,153	201,051,475
DNTTIP1	116092	+	−	20	1	43,853,983	43,873,471
DPP7	29952	+	−	9	−1	139,124,814	139,129,016
FAM100B	283991	+	−	17	1	71,773,003	71,778,944
FOS	2353	+	−	14	1	74,815,284	74,818,685
HBP1	26959	+	−	7	1	106,596,696	106,630,209
HMGCL	3155	+	−	1	−1	24,000,955	24,024,536
IKBKG	8517	+	−	X	1	153,423,673	153,446,437
IRX2	153572	+	−	5	−1	2,799,880	2,804,776
JDP2	122953	+	−	14	1	74,968,590	75,006,908
LSR	51599	+	−	19	1	40,431,399	40,450,703
MYC	4609	+	+	8	1	128,817,498	128,822,853
PFDN1	5201	−	+	5	−1	139,604,819	139,662,873
PHLDA1	22822	+	−	12	−1	74,705,494	74,711,823
RAB43	339122	+	−	3	−1	130,289,108	130,323,309
SLC25A36	55186	−	+	3	1	142,143,378	142,178,843
SLC38A2	54407	+	−	12	−1	45,038,239	45,052,824
SLC7A2	6542	−	+	8	1	17,440,683	17,472,296
TAF9	6880	+	−	5	−1	68,696,327	68,701,596
TFRC	7037	+	−	3	−1	197,260,553	197,293,343
TMEM64	169200	+	−	8	−1	91,704,778	91,727,309
VDR	7421	−	+	12	−1	46,521,589	46,585,081
WWC1	23286	+	−	5	1	167,651,670	167,829,334

[a] Transcripts induced in MCF-7 cells at 3 h after treatment with E (30) and in mouse mammary glands after treatment for 4 d with EP are indicated as "+."

[b] Genes for which estrogen receptor was shown to bind by chromatin immunoprecipitation (30) and were increased in mouse mammary glands after treatment for 4 d with EP are indicated as "+."

TABLE 4. Analysis of biological relationships among genes up-regulated by estrogen and/or P

Functional category	Term	Population fraction	Subset fraction	e score
Biological process	Protein biosynthesis	285/17730	31/687	0.0001
	DNA replication	98/17730	16/687	0.0006
	Cell cycle	330/17730	32/687	0.0010
	Cell division	169/17730	19/687	0.0162
	Ribosome biogenesis	90/17730	13/687	0.0217
Cellular component	Ribonucleoprotein complex	245/17730	29/687	1.32e-05
	Nucleus	3209/17730	197/687	4.14e-10
	Ribosome	168/17730	22/687	8.35e-05
	Chromatin	58/17730	11/687	0.0017
	Cytoplasm	1081/17730	68/687	0.0075
	Cytosolic ribosome	28/17730	7/687	0.0106
	Chromosome	95/17730	13/687	0.0106
	Chromosome, pericentric region	24/17730	6/687	0.0350
KEGG	Ribosome	70/17730	16/687	1.03e-06
	Cell cycle	103/17730	16/687	0.0178
PPI	First-neighbor of p53	155/13627	20/604	0.0240
	First-neighbor of Snw1	15/13627	6/604	0.0375
	First-neighbor of RelA	78/13627	13/604	0.0496

GO terms, KEGG pathways, and first-neighbor protein interactions statistically overrepresented within the E- and/or P-up gene set (subset fraction) relative to all unique genes on the U74v2 chips (population fraction) are shown.

sical estrogen-response pathway. Because glutathione peroxidase (Gpx) had been shown previously to be p53 responsive (28), the increase in Gpx3 detected by microarray was of interest. Although not known to be regulated by estrogen, levels were increased by 1.7-fold compared with vehicle by E and EP (Fig. 2B). Among the large group of genes requiring the combined effects of EP, retinol binding protein 1 (Rbp1) was selected because its expression is increased among parous mice (29). Levels of Rbp1 mRNA were increased modestly by E but were increased 2.6-fold in mammary tissue by EP compared with vehicle-treated mice (Fig. 2C). Conversely, expression of gelsolin (Gsn) was decreased in the microarray data by EP and was confirmed by Northern blot (Fig. 2D). Treatment with EP also reduced expression of inhibitor of DNA binding 1 (Id1) by 40 and 70% in the microarray data and RT-qPCR, respectively (data not shown).

Because hormones were administered for 4 d, these genes include both direct targets of E and P receptors, as well as secondary targets. To define which may be direct transcriptional targets of estrogen receptors, the list of EP genes were compared with the transcriptional responses in MCF-7 cells during the first 3 h after treatment with E (30). A total of 24 genes was common to both data sets (Table 3). Of this group of 24 genes, only *MYC* had an ERE within the 1-kilobase promoter region. In addition to *MYC*, seven additional genes (*ACTN4*, *DMPK*, *PFDN1*, *SLC7A2*, *VDR*, *HBP1*, and *SLC25A36*) showed increased levels of mRNA in mouse mammary glands after EP treatment and bound estrogen receptor within the 1-kilobase promoter region of MCF-7 cells (Table 3). Given that only a small fraction of the EP-responsive genes in mouse mammary gland were E responsive in MCF-7 cells, it is likely that EP treatment induces expression of transcriptional activators and/or repressors that, in turn, alter expression of a much broader class of genes that are secondary targets. Actions of EP in the stroma are also likely to contribute to the modest overlap between these data sets. Overall, these results identify a large class of genes that require both E and P for their regulation, and underscore

TABLE 5. Analysis of biological relationships among genes down-regulated by estrogen and/or P

Functional category	Term	Population fraction	Subset fraction	e score
Biological process	Lipid metabolism	152/17730	15/463	0.0039
	Antigen presentation	36/17730	7/463	0.0123
	Glycogen biosynthesis	9/17730	4/463	0.0187
Cellular component	Mitochondrion	703/17730	48/463	1.16e-07
	MHC class I protein complex	26/17730	7/463	0.0003
	Membrane	3520/17730	129/463	0.0016
	Mitochondrial inner membrane	220/17730	18/463	0.0019
	Integral to membrane	3108/17730	116/463	0.0023
	Plasma membrane	429/17730	27/463	0.0024
	Mitochondrial envelope	22/17730	5/463	0.0210
Molecular function	MHC class I receptor activity	35/17730	8/463	0.0008
KEGG	Oxidative phosphorylation	105/17730	14/463	7.39e-05
	Cell adhesion molecules	206/17730	18/463	0.0010
	PPAR signaling pathway	65/17730	9/463	0.0057
	Type 1 diabetes mellitus	52/17730	8/463	0.0070
	Antigen processing and presentation	71/17730	9/463	0.0116
PPI	First-neighbor of Igfals	3/13627	3/391	0.0167

GO terms, KEGG pathways, and first-neighbor protein interactions statistically overrepresented within the E- and/or P-down gene set (subset fraction) relative to all unique genes on the U74v2 chips (population fraction) are shown. MHC, Major histocompatibility complex; PPAR, peroxisome proliferator-activated receptor.

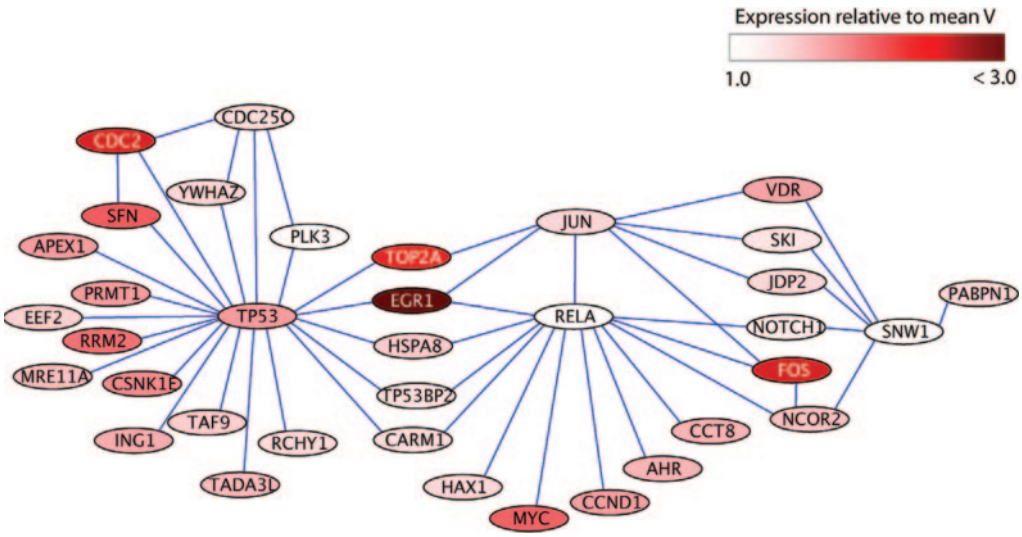

FIG. 3. Network of protein interactions among genes that are up-regulated by E and P. Analysis of PPIs among the genes up-regulated by E and P identified p53, RelA, and Snw1 as common targets that are statistically overrepresented, as described in Tables 4 and 5. The relationships among the genes were graphed to demonstrate interaction networks. TP53, RelA, and Snw1 form nodes but are connected by genes that are coregulated, providing pathways for cross talk. Genes connecting p53 and RelA include topoisomerase II α (TOP2a), Egr1, heat shock 70-kDa protein 8 (HSPA8), tumor protein p53 binding protein 2 (TP53BP2), and coactivator-associated arginine methyltransferase 1 (CARM1). Genes connecting RelA and Snw1 include vitamin D (1,25-dihydroxyvitamin D3) receptor (VDR), v-ski sarcoma viral oncogene homolog (SKI), jun dimerization protein 2 (JDP2), notch gene homolog 1 (NOTCH1), v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), and nuclear receptor co-repressor 2 (NCOR2). The *colors* indicate the relative expression level of the mRNA in EP-treated compared with the vehicle-treated controls, and range from *white* (no difference from control) to *dark red* (>3-fold increase compared with control).

the extensive cross talk and collaboration among these hormones and their signaling pathways.

Analysis of biological relationships

Global changes in transcriptional profiles in response to EP provide insights into the alterations in cellular and metabolic pathways that regulate p53 activity in the mammary epithelium. Therefore, the entire set of genes regulated by EP was analyzed for overrepresentation of specific GO terms or KEGG pathways. The genes up-regulated by EP show strong changes associated with cellular proliferation (Table 4). GO annotations for biological processes showed changes in genes associated with DNA replication, cell cycle, and cell division. The protein synthetic capacity also was prominently up-regulated. Similarly, the cellular components, molecular functions, and KEGG pathways also reflect synthesis of nucleotides and proteins associated with proliferation. These were expected given the potent mitogenic roles of estrogen and P. In contrast, the genes down-regulated by E and P revealed metabolic changes (Table 5). Genes involved in lipid metabolism and electron transport and localized in the mitochondria were all decreased. The decrease among genes associated with the KEGG pathway for oxidative phosphorylation was especially striking. Therefore, E and P appear to promote proliferation while diminishing aerobic metabolism.

These changes represent the global responses to E and P but can obscure the modest changes in regulatory pathways that may determine p53 activity in mammary tissue. Furthermore, gaps in signaling pathways can result from both low levels of expression of any particular gene and events that rely on post-translational modifications of proteins rather than transcriptional responses. Therefore, analysis of protein-protein interactions (PPIs) among genes that are differentially expressed was used to identify common targets and pathways. This approach identified an overrepresentation of genes that were first neighbors interacting with p53, Snw1, and RelA among the up-regulated genes (Table 4) and IGF binding protein (IGFBP), labile subunit (Igfals) among the down-regulated genes (Table 5). These genes form hubs within an interaction network (Fig. 3). Although E and P stimulated expression of numerous proteins that interact with RelA, Snw1, and Igfals, the expression levels of these were unchanged by the hormonal treatments. One probe set in the microarray data showed induction of p53 mRNA by E and EP. However, this does not appear to be reproducible because no difference in expression was detected by three other probe sets for p53 mRNA, and no difference in expression was detected by Northern blot (14). Because overrepresentation of proteins interacting with p53 had the highest significance level (e score = 0.0240), it suggests that EP induces a complex of genes that interact with p53 and augment its activity.

Hormone-induced expression of Egr1 and Stratfinin (Sfn) in Trp53+/+ and Trp53−/− tissues

The fold changes in mRNA for p53-interacting genes and their responses to each of the treatments are summarized in Table 6. These genes represent potential effectors that are downstream of E and P. Egr1 and Sfn (also known as 14-3-3σ) were among the highest fold increases and have been implicated previously as enhancing p53 activity. Therefore, these genes were examined in greater detail. Pgk1 was used as a housekeeping gene to normalize the microarray expression data. Expression of Egr1 was increased 3-fold after treatment with EP compared with vehicle (Fig. 4A). The induction by EP was primarily via the mammary epithelium because no induction by EP was observed in epithelium-free mammary fat pads

TABLE 6. Genes that are up-regulated by estrogen and/or P and interact directly with p53

Probe set ID	Gene symbol	Regulatory pattern[a]			E plus P/sham	
		E	P	EP	Expression ratio	*P* value
98579_at	EGR1	↑	—	↑	3.025	0.001
100128_at	CDC2	—	—	↑	2.787	0.001
99578_at	TOP2A	—	—	↑	2.592	0.032
96704_at	SFN	—	—	↑	2.071	0.023
102001_at	RRM2	—	—	↑	1.896	0.001
97925_at	CSNK1E	↑	—	↑	1.716	0.012
104275_g_at	TP53	↑	—	↑	1.621	0.001
93559_at	APEX1	—	—	↑	1.617	0.001
96696_at	PRMT1	—	—	↑	1.611	0.012
94396_at	ING1	↑	—	↑	1.503	0.036
113636_at	TADA3 liter	↑	↑	↑	1.409	0.012
94376_s_at	MRE11A	↑	↑	↑	1.388	0.012
93918_at	TAF9	↑	—	↑	1.312	0.018
96564_at	HSPA8	↑	↑	↑	1.305	0.016
97559_at	EEF2	—	—	↑	1.285	0.033
97544_at	YWHAZ	↑	—	↑	1.244	0.019
106647_at	RCHY1	—	—	↑	1.217	0.016
99169_at	CARM1	—	—	↑	1.212	0.016
102934_s_at	CDC25C	—	↑	↑	1.199	0.001
106238_at	TP53BP2	↓	—	↑	1.181	0.044
161636_r_at	PLK3	—	↑	↑	1.024	0.038

↓, Down-regulated; —, no significant change; ↑, up-regulated.
[a] Changes in levels of mRNA for the genes are indicated relative to vehicle-treated controls.

(CFP). Sfn expression was increased 2-fold over vehicle when treated with EP (Fig. 4A). Expression of Sfn was decreased significantly in the epithelium-free fat pads, suggesting that the majority of Sfn is expressed in the mammary epithelium.

Although Egr1 and Sfn are potential activators of p53, it is possible that these are targets of p53 and reflect an increase in basal p53 activity. Therefore, it was important to determine whether Egr1 and Sfn may be targets of EP and upstream of p53 or downstream targets that are reporters of p53 activity, but not key regulators of p53. Responses to EP were examined in both wild-type and p53-deficient tissues (*Trp53*+/+ and *Trp53*−/−, respectively) to ascertain whether these genes are upstream of p53 or downstream targets. The mice were ovariectomized then treated with EP for 4 d as described in *Materials and Methods*. Pgk1 was used to normalize levels of Egr1 and Sfn because its expression was unaffected by the treatments. The levels of Egr1 mRNA were increased by EP treatment in both the *Trp53*+/+ and *Trp53*−/− mice (2.0- and 2.3-fold, respectively). Therefore, the induction of Egr1 is not p53 dependent. Similarly, levels of Sfn mRNA were increased 1.5-fold compared with the vehicle control in *Trp53*+/+ mammary tissues. Although the level of Sfn mRNA was reduced dramatically in *Trp53*−/− mammary tissues, levels of Sfn mRNA were increased 3-fold by EP in *Trp53*−/− tissues (0.3 *vs.* 1.0 in vehicle and EP, respectively). These results suggest that p53 activity is important for the maintenance of the basal level of Sfn but that E and P act upstream of p53 to enhance its expression in both the wild-type and p53-deficient mammary tissues. These results demonstrate that Egr1 and Sfn are among a group of genes that are induced within the mammary epithelium after treatment with EP.

Cross-regulation of p53 and NF-κB transcriptional activity by Egr1

The PPIs (Fig. 3) suggest that a subset of the proteins induced by EP treatment interacts with both NF-κB and p53 networks. Egr1 was selected for further study because it has both inhibited NF-κB activity and enhanced p53 activity in other cell systems (31, 32). Luciferase reporter constructs were used to examine the effects of Egr1 expression on the transcriptional activity of NF-κB and p53 in MCF-7 breast cancer cells. Expression of Egr1 resulted in a significant reduction of NF-κB activity ($P < 0.001$) and a simultaneous increase in p53 activity ($P < 0.05$). These results confirm the ability of Egr1 to regulate reciprocally the transcriptional activities of both NF-κB and p53.

Discussion

The p53 pathway responds to a variety of cellular stresses, and its induction by DNA damage has been studied extensively. Although a critical pathway for the maintenance of genomic stability, p53-dependent responses to ionizing radiation vary dramatically among tissues. Ionizing radiation induces apoptosis in the lymph nodes and intestinal crypts, whereas arrest and repair is the prevalent response in liver and kidney, while brain and muscle are nearly devoid of a p53 response (33, 34). In mammary epithelium the activity of p53 is regulated by hormonal exposures and varies across developmental stages (12, 14, 15). In particular, treatment with E and P leads to enhanced responsiveness of p53 to ionizing radiation (14). Neither steady-state levels of p53 mRNA (14) nor basal levels of p53 protein (12) are altered by hormonal stimulation. Therefore, it appears that E and P alter cellular targets that sensitize p53 by either enhancing posttranslational modifications of p53 or promoting interactions with factors that enhance the activity of p53. Direct analyses of p53 in the mammary epithelium are hampered by the fact that the epithelium comprises only a small fraction of the total nulliparous mammary gland (<20%). Attempts to isolate the epithelium by collagenase digestion led to induction of p53 (Jerry, D. J., unpublished data). *In vitro* approaches are hampered by the fact that the

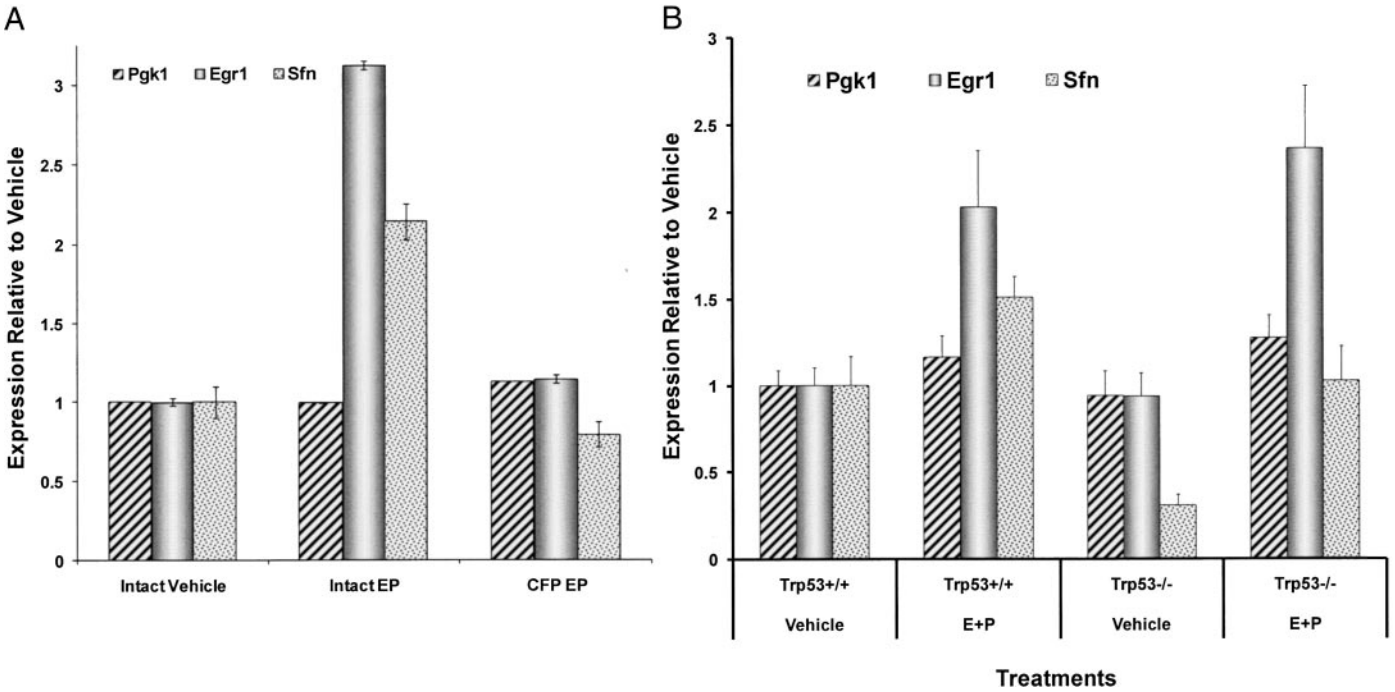

FIG. 4. Egr1 and Sfn expression in mammary tissues. A, Data from the microarray studies were plotted to show the fold changes in Egr1, Sfn, and Pgk1 in mammary tissues from mice with intact mammary glands treated with vehicle or both estrogen and P (designated Intact-vehicle and Intact-EP, respectively). The expression levels in mammary fat pads that have been cleared of epithelium and treated with both E and P (designated CFP-EP) are also shown. Both Egr1 and Sfn are induced by more than 2-fold by EP in intact tissues. In the absence of the epithelium (CFP-EP), levels of Egr1 were not different from the vehicle treated, indicating that the epithelium is required for induction of Egr1 by EP. The decreased level of Sfn mRNA in CFP-EP tissues indicates that this gene is predominantly expressed in the epithelium. Levels of mRNA for the housekeeping gene, Pgk1, were unchanged across treatments. B, Responses to treatment with vehicle or EP were confirmed by RT-qPCR using an independent set of wild-type mice (*Trp53*+/+) as well as with p53-deficient mice (*Trp53*−/−). Mice were ovariectomized and administered hormones for 4 d as described in *Materials and Methods*. Levels of both Egr1 and Sfn were significantly increased by EP treatment. Egr1 was similarly increased by EP in *Trp53*−/− tissues. Basal levels of Sfn were decreased in vehicle-treated *Trp53*−/− tissues by 70% relative to the vehicle-treated *Trp53*+/+ controls but was induced 3.3-fold by EP treatment (0.3 *vs.* 1.0 for *Trp53*−/−, vehicle and *Trp53*−/−, EP, respectively). Expression levels were normalized to expression of Pgk1 within each animal. Means for each treatment are expressed relative to the means for the *Trp53*+/+, vehicle-treated controls. All comparisons are relative to the *Trp53*+/+, vehicle, and significant differences are indicated by *, $P < 0.05$ or **, $P < 0.01$.

receptors for E and P are diminished when normal mammary epithelial cells are placed in culture.

Known breast cancer susceptibility genes provide candidates that may be subject to hormonal stimuli and regulate p53 activity. Among the 10 recognized breast cancer susceptibility genes (35), *ATM* and *CHK2* are significant because they regulate p53 activity by inducing posttranslational modifications. Similarly, polymorphisms that increase *MDM2* levels and promote degradation of p53 protein have been linked to increased risk of breast cancer (36, 37). However, the expression microarray analysis failed to detect changes in these factors in a pattern consistent with the hormone-induced increase in p53 activity. Similarly, none of the recognized breast cancer susceptibility genes has been shown to be changed in parous mammary tissues (29, 38–40). Therefore, it appears that treatment with E and P engages alternate pathways to sensitize p53 in the mammary epithelium.

The transcriptional responses in the mammary gland detected by the microarrays demonstrate the breadth of alterations induced by E and P. Although the magnitude of the effect was expected, comparisons of the transcriptional changes in mammary tissue with those detected in MCF-7 cells resulted in only 24 genes that were shared in both data sets (Table 3). This underscores the many distinctions between *in vitro* and *in vivo* conditions. Analysis of the biological relationships among EP-induced genes provided further insights into the responses within the tissue (Tables 4 and 5). Mapping the EP-responsive genes onto first-neighbor PPIs provided insights into the networks that are altered by the hormones. The overrepresentation of proteins that interact with p53 identified a broad complement of genes, suggesting that redundant mechanisms are present to ensure proper regulation of p53. The induction of Egr1 and Sfn expression after EP treatment was confirmed by RT-qPCR and was shown to be p53 independent (Fig. 4). Thus, both of these factors are responsive to EP and represent potent activators of p53 that can contribute to the sensitization of p53 to enhance responsiveness to DNA damage (41–46).

Among the genes up-regulated by EP, there was also a significant overrepresentation of transcriptional targets interacting with RelA, some of which can also influence the activities of p53. RelA contains a transactivation domain and is a subunit of the NF-κB transcription factor. NF-κB and p53 can cooperate to enhance apoptosis in some settings (47–49) but serve antagonistic roles in most instances. Cross-regulatory mechanisms of p53 and NF-κB subunits have been demonstrated on promoters of target genes, including p21, DR5, and PUMA (50). This cross-regulation appears to be via a competition for coregulatory

factors such as p300 and CRE binding protein (50, 51). NF-κB complexes increase during pregnancy (52). The induction of NF-κB during pregnancy appears to be important for expansion of the mammary epithelium because blocking the pathways by knockout of CHUK (conserved helix-loop-helix ubiquitous kinase, also known as IKKα) resulted in a failure of mammary gland development (53, 54). In addition to promoting proliferation of the normal mammary epithelium, NF-κB appears to play a prominent role in inducing mammary tumors in mice (55, 56) and is constitutively activated in a majority of human breast cancers, as well as promoting epithelial-mesenchymal transformation (57, 58). NF-κB pathways are also induced during involution of the mammary gland, a period characterized by rapid apoptosis (59, 60). However, NF-κB expression appears to be restricted to the cells that are surviving involution (61). Thus, the overrepresentation of RelA-associated protein appears to reflect the proliferative responses induced by EP treatment, rather than regulating p53 activity.

Although the activities of p53 and RelA appear to be antagonistic, the interaction network (Fig. 3) provides insights into how cross talk between p53 and NF-κB is integrated to balance the need for proliferation and genomic surveillance. Both p53 and RelA bind Egr1, heat shock 70-kDa protein 8, tumor protein p53 binding protein 2, and coactivator-associated arginine methyltransferase 1. Of these, Egr1 may be especially important in integrating the responses to EP and DNA damage. EP treatment promotes expression and activity of NF-κB (52) to allow proliferation of the mammary epithelium. However, Egr1 expression is also enhanced, which can bind and temper transcriptional activation mediated by RelA (31). Expression of Egr1 is further enhanced in response to DNA damage (62), leading to increased p53 activity. Egr1 can interact with multiple tumor suppressors and, thus, may have tumor suppressive activities that extend beyond sensitizing p53 (32). The reciprocal actions of Egr1 on transactivation by p53 and NF-κB were demonstrated directly in breast epithelial cells (Fig. 5). The increase in Sfn serves to reinforce and amplify the responsiveness of p53. Together, Egr1 and Sfn can collaborate to increase the activity of p53 that competes for the pool of transcriptional coactivators, and, thus, can limit the effects of NF-κB and sway the balance of responses toward p53-dependent apoptosis when cells encounter DNA damage.

Additional signaling pathways may collaborate with p53 and NF-κB to enhance the responsiveness of p53. Rbp1 was among the genes induced by EP treatments. Because retinoids have reduced the risk of breast cancer and are being examined in breast cancer chemoprevention trials (63), this would suggest a relevant pathway. Furthermore, retinoids enhanced p53 responses to ionizing radiation in mouse mammary tissues to an extent that was similar to that observed for EP (64). Retinoids enhance secretion of TGF-β in MCF-7 cells (65), and TGF-β influences p53 activity in mammary tissue (14). Expression of TGF-β is induced by ovarian hormones (66), and both Rbp1 and TGF-β3 are persistently elevated in mammary tissue from parous mice (29). Conversely, Igfals was identified as being overrepresented in the EP-treated tissues because of decreased expression of IGF-I, IGFBP-3, and IGFBP-5. Reductions in IGF-I are associated with a reduced risk of mammary tumors in women (67) as well as in rodents (29). IGF-I levels are persistently decreased in mammary tissue from parous rodents (68), and elevated IGF-I can overcome the protective effect of parity with respect to mammary tumors (69). Because IGF-I can antagonize p53 (70), the decrease in this pathway would likely also enhance p53 activity. Therefore, increases in Rbp1 and TGF-β

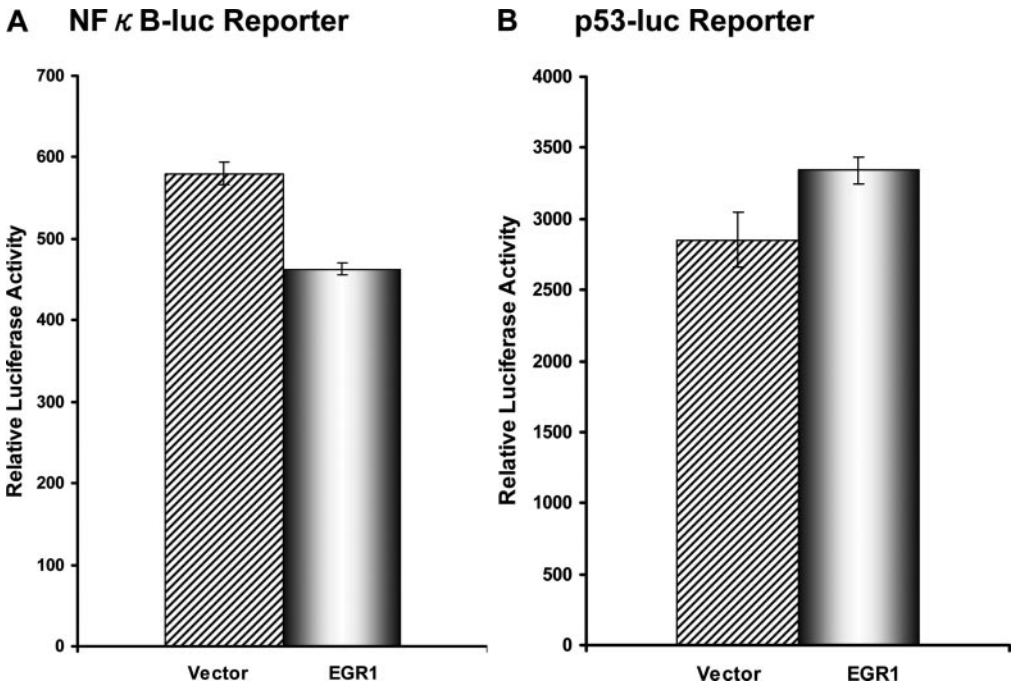

FIG. 5. Effect of Egr1 expression on transcriptional activity of p53 and NF-κB. MCF-7 cells were transfected with the control plasmid (pcDNA vector) or an Egr1 expression plasmid (pcDNA-Egr1) together with luciferase reporter plasmids with responsive elements for either NF-κB (A) or p53 (B). The mean activity is shown ± SEM for samples run in triplicate. The differences in reporter activity were significant for both NF-κB ($P < 0.001$) and p53 ($P < 0.05$) using a one-tailed t test.

signaling together with decreases in IGF-I appear to be targets of EP treatment in the mammary gland that can persistently enhance the activity of p53 in parous individuals.

The transcriptional profiles provide an integrated view of the responses to estrogen and P in the mammary gland, and establish a framework with which to understand the complex signaling underlying the balance between proliferation and the need for genomic surveillance. Combined treatment with E and P engage signaling pathways that enhance expression of a variety of factors that interact with p53 and RelA. The increased expression of Egr1 is significant because it can coordinate responses to cellular stresses and proliferative signals by enhancing p53 and diminishing NF-κB transcriptional activities. In addition, expression of Rbp1 was increased, and expression of IGF-I-related proteins was decreased. Together, these genes and pathways identify redundant mechanisms by which estrogen and P regulate p53 activity in the mammary epithelium and render it resistant to tumors. The results also provide targets for chemoprevention as well as surrogate endpoints with which to evaluate therapeutic agents.

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