The Octamer Binding Transcription Factor Oct-1 Is a Stress Sensor

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Abstract

The POU-domain transcription factor Oct-1 is widely expressed in adult tissues and has been proposed to regulate a large group of target genes. Microarray expression profiling was used to evaluate gene expression changes in Oct-1-deficient mouse fibroblasts. A number of genes associated with cellular stress exhibited altered expression. Consistent with this finding, Oct-1-deficient fibroblasts were hypersensitive to γ radiation, doxorubicin, and hydrogen peroxide and harbored elevated reactive oxygen species. Expression profiling identified a second group of genes dysregulated in Oct-1-deficient fibroblasts following irradiation, including many associated with oxidative and metabolic stress. A number of these genes contain octamer sequences in their immediate 5′ regulatory regions, some of which are conserved in human. These results indicate that Oct-1 modulates the activity of genes important for the cellular response to stress. (Cancer Res 2005; 65(23): 10750-8)

Introduction

The archetypal POU (*P*it-1, *O*ct-1/2, *U*nc-86) domain transcription factor Oct-1/Pou2f1 was identified through its ability to interact with conserved regulatory sequences in viral, cell cycle–dependent, and immunoglobulin genes (1–5). Oct-1 interacts with an 8-bp sequence termed the octamer motif (5′-ATGCAAAT-3′) and related sequences (6). Evidence gathered over the last two decades indicates that Oct-1 associates with a number of proteins including a B-cell cofactor known as OCA-B (7, 8), a multisubunit cofactor termed OCA-S (9), host cell factor-1 (10), DNA-dependent protein kinase (11), BRCA1 (12, 13), and the MAT1 component of the TFIIH cyclin-dependent kinase activating kinase subcomplex (14). Oct-1 is widely expressed in adult tissues and is the only known POU family member not expressed in a specific temporal or spatial pattern. For example, Oct-2, a related protein with an identical DNA binding specificity *in vitro*, is expressed in the B-cell lineage, in the central nervous system, and in some monocytes/macrophages (15).

A number of laboratories have defined a role for Oct proteins in the B-cell–specific expression of immunoglobulin. Because of the wide expression pattern of Oct-1, initial thinking favored Oct-2 as the primary mediator of B-cell–specific immunoglobulin promoter activity. However, immunoglobulin expression and B-cell development were essentially unperturbed in Oct-2-deficient mice (16). Recent studies have also shown that although Oct-2 levels generally increase as B cells mature, in plasma cells (when immunoglobulin expression is highest) Oct-2 is down-regulated by the transcriptional repressor protein Blimp-1 (17). Together, the data indicate little direct role for Oct-2 in mediating immunoglobulin expression.

Mice harboring two disrupted *Oct-1* alleles die *in utero* relatively late in gestation (E13.5-18.5), possibly due to an anemic condition; however, the expression of reputed Oct-1 target genes such as *H2B* and *U6 snRNA* was unaffected, indicating that Oct-1 is nonessential for the expression of these genes. Oct-1-deficient cells proliferate at normal rates (18). Oct-1 is also dispensable for B-cell development and immunoglobulin gene expression (19). The number of immunoglobulin-positive B cells, the amount of immunoglobulin expression per B cell, and the usage of κ variable region gene segments were largely normal in Oct-1 mutants. Lentiviral knockdown of Oct-2 in Oct-1-deficient Abelson-transformed pre–B-cell lines indicates that in the absence of Oct-1, and with 1/8th the normal levels of Oct-2, immunoglobulin heavy chain gene expression remains unperturbed.[4] Therefore, the role of Oct-1 and Oct-2 in directing baseline immunoglobulin gene expression is unclear but may be minimal.

Other *cis*-acting elements within the regulatory regions of octamer-containing genes help explain their expression patterns and robust activity in the absence of Oct-1. For example, a factor known as NPAT interacts with histone core promoters and is a critical regulator histone gene expression (20). The proximal sequence element–binding protein interacts with regulatory sequences in the *U6 snRNA* gene and its abundance correlates with gene activity (21). Immunoglobulin promoters and enhancers contain *cis*-acting elements, including Ets sites, E boxes, and CCAAT/enhancer binding protein (C/EBP) sites, which can interact with PU.1 and Spi-B, HEB, and C/EBPβ (22–24). Functional immunoglobulin promoter regulatory elements located downstream of the transcription initiation site have also been recently described that interact with TFII-I family transcription factors (25).

Although Oct-1 is dispensable for the expression of histones and snRNAs, there are nevertheless canonical octamers in the promoters of many of these genes. Oct-1 has also been shown to interact with the H2B promoter in cycling cells *in vivo* using chromatin immunoprecipitation assays (9). The octamer site is present many times throughout the immunoglobulin heavy chain and κ loci, including the variable region promoters (1, 2). Thus, a number of questions arise: What is the function of the octamer sites in genes such as *H2B, U6 snRNA*, and *IgH*? If Oct-1 is nonessential for the normal expression of many reported targets, then what is its function *in vivo*?

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[4] D. Tantin and P.A. Sharp, unpublished observations.

Here we show that Oct-1-deficient cells respond abnormally to cellular stress. Fibroblasts lacking Oct-1 are hypersensitive to ionizing radiation, doxorubicin, and hydrogen peroxide. Microarray analysis indicates that Oct-1-deficient fibroblasts treated with γ radiation fail to properly regulate a number of genes, many of which are associated with oxidative and metabolic stress. These data suggest a new model of Oct-1 function whereby this factor recognizes cellular stress and modulates the expression of target genes, producing the appropriate cellular response.

Materials and Methods

Cell culture. Primary, 3T3-immortalized, and retrovirally transduced fibroblasts have been previously described (18, 19, 26). Phase-contrast microscopic images were generated using a Nikon inverted microscope at ×40 magnification and an Olympus digital camera. Fibroblasts treated with doxorubicin (Sigma, St. Louis, MO) or carrier (PBS) for 1 hour were plated onto 24-well clusters and cultured for 4 days. On the 4th day, media was aspirated and the cells were washed with PBS, fixed with 3.7% formaldehyde in PBS, stained with 0.5% crystal violet in water, and rinsed with water thrice. Staining was quantified by elution of crystal violet with 1 mL of 33% acetic acid, followed by measurement of absorbance at 570 nm. The amount of dye in the untreated controls was taken as 100%.

Gene expression analysis. Affymetrix MOE430A/B GeneChips were used. RNA was prepared from matched-passage control (WT and MU) or γ-irradiated wild-type and Oct-1-deficient primary fibroblasts (WTR and MUR) according to the protocols of the vendor. RNA quality was assessed using an Agilent 2100 Bioanalyzer. Raw data were imported into the S-plus Array analyzer module for normalization by quantile (WT, WTR, MU, MUR) Robust Multichip Analysis using GC-content empirical Bayes background and triple goal adjustment (27, 28). Following normalization, the variance between the replicates was stabilized using the local pooled error test (29). *P* values were adjusted to reflect the false discovery rate using the Benjamini-Hochberg method (30). Genes that differed in expression between MU and WT cells were identified based on significance ($P \leq 0.05$) and fold change (≥2.0-fold). Genes that responded to radiation differentially in MU and WT cells were identified based on difference in significance ($P \leq 0.05$ in one condition, $P \geq 0.20$ in the other condition). In addition, a small fold change cutoff (±33%) was applied. Another set of genes were identified based on an analysis of rank variance. Change in rank order was calculated by subtracting the expression rank values for the irradiated states from the unirradiated states. Genes that differed in change in rank order by >1,500 were also included, again with a 33% fold change cutoff. The complete data sets can be obtained at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) accession GSE2084.

Flow cytometry. The fluorescent indicator 5(6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate-acetyl ester (CM-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR) and was solubilized in DMSO to a concentration of 0.5 mg/mL and immediately diluted to a working concentration of 2 μg/mL with serum-free DMEM lacking phenol red (Invitrogen, San Diego, CA). Cultured fibroblasts, 2×10^5, were plated onto 10-cm dishes and allowed to attach for 8 hours. Cell were then washed twice with serum-free DMEM and were loaded with 2 μg/mL CM-H_2DCFDA in serum-free DMEM on the dish at 37°C for 30 minutes. Following dye loading, cells were washed with PBS, trypsinized, harvested by centrifugation at 700 × *g*, and analyzed immediately. For flow cytometry, cells were gated by forward/sidescatter and propidum iodide (20 μg/mL) exlusion using a Becton Dickinson FACSCalibur flow cytometer and analyzed using CellQuest software.

Reverse transcription-PCR. cDNA generated from primary fibroblasts and fribroblasts treated with ionizing radiation were amplified with PCR primers specific to β-actin (Ambion, Austin, TX) or primers specific to mouse Ifi202b and AMP-dependent protein kinase α2 (AMPKα2)/Prkaa2. The signal obtained from the actin standards was reduced to be comparable with that obtained from Ifi202b and AMPKα2 using actin competimer oligonucleotides (Ambion). Specific primer sequences used were MmIfi202bFOR, 5′-AACTGCCACTGTGTCAGAGGCT-3′; MmIfi202-bREV, 5′-AGCAAACTGTGATTTCCAGAGTGT-3′; MmTimp3FOR, 5′-AACTCCGACATCGTGATCCGGGCCAA-3′; MmTimp3REV, 5′-CAATTG-CAACCCAGGTGGTAGCGGTAAT-3′; MmGSTm2FOR, 5′-AGATCACCCAG AGCAATGCCATCCTG-3′; MmGSTm2REV, 5′-GAGTAGAGCTTCATCTTCT-CAGGGAGAC-3′; MmPrkaa2FOR, 5′-GGTGATCAGCACTCCGACAGACT-3′; MmPrkaa2REV, 5′-CAAAGTCAGCTATCTTAGCGTTC-3′; MmICAM-1FOR, 5′-CTCATGCCGCACAGAACTGGATCTCAGGC-3′; and MmICAM-1REV#2, 5′-CCTCTGCGTCTCCAGGATCTGGTCCGCT-3′.

Results

Abnormal gene expression in Oct-1-deficient fibroblasts. Microarrays were used to identify improperly regulated genes in Oct-1-deficient cells. Thirty-four thousand genes and expressed sequence tags were tested using primary mouse embryonic fibroblasts derived from Oct-1-deficient mice or wild-type littermates (MU or WT). Four replicates were done. Differentially regulated genes were identified using two independent methodologies that yielded similar results.

In the first method, genes were deemed significant based on a fold change of ≥2.0 and a Benjamini-Hochberg adjusted $P \leq 0.05$. Eighty-eight genes were differentially regulated between WT and MU cells (Supplementary Table S1). Only annotated transcripts are listed although a number of unannotated transcripts were also differentially expressed. The complete data set has been deposited (GSE2084) at the NCBI Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/).

The most significantly down-regulated gene in MU cells compared with WT was the X chromosome inactivation center–specific transcript (*XIST*; approximately −50-fold) and the most significantly up-regulated were the Y-linked genes DEAD box polypeptide 3 and eukaryotic translation initiation factor 2 subunit 3 (approximately +50-fold; Supplementary Table S1). These genes are differentially regulated between male and female cells, indicating that the primary mouse embryonic fibroblasts were derived from embryos of two different genders (the Oct-1 mutant cells being male).

The largest category of differentially regulated genes, including the next two most differentially expressed genes, was involved in cellular stress and oxidative response (Table 1), including IFN-activated gene 202b (*Ifi202b*), which was identified twice (up-regulated, 30- to 40-fold), peroxiredoxin 2 (*Prdx2*; up-regulated, ~10-fold), and growth arrest–specific 5 (*Gas5*; down-regulated, approximately −4.5-fold). Other differentially regulated stress response genes included *Gbp2*, an IFN-inducible antiviral GTPase (31), and *Timp3*, a regulator of extracellular matrix remodeling. *Timp3* is differentially regulated in cancer cells and after treatment of normal cells with agents that induce oxidative stress (32–34). In addition to Prdx2, a number of other antioxidant proteins were induced in the mutant cells, such as glutathione peroxidase 3 and glutathione *S*-transferase (GST) κ1. In addition, RAD52b and ataxia telangiectasia mutated, proteins involved in DNA damage repair and signaling, were up-regulated and down-regulated in mutant cells, respectively.

Expression of *Ifi202b* was verified by reverse transcription-PCR (RT-PCR; Fig. 1*A*). *Ifi202b* was heavily induced in MU cells whereas no expression was evident in WT cells (*lanes 1* and *3*). Expression was independent of exposure of the cells to ionizing radiation (*lanes 2* and *4*). Differential expression of *Timp3* was

Table 1. Stress/oxidative genes differentially expressed between Oct-1-deficient and wild-type primary mouse embryonic fibroblasts

Symbol; gene title*	Probe set†	Accession no.	Fold change	Adjusted *P*
Ifi202b; IFN-activated gene 202b	1457666_s_at	AV229143	39.99	<1e−09
Ifi202b; IFN activated gene 202b	1421551_s_at	NM_011940	29.99	<1e−09
Prdx2; peroxiredoxin 2	1430979_a_at	AK011963	10.23	<1e−09
Serping1; serine (or cysteine) proteinase inhibitor, clade G, member 1	1416625_at	NM_009776	4.68	<1e−09
Rad52b	1417661_at	NM_025654	3.56	<1e−09
Gpx3; glutathione peroxidase 3	1449106_at	NM_008161	2.62	3.51e−07
Cdo1; cysteine dioxygenase 1, cytosolic	1448842_at	NM_033037	2.55	8.98e−05
Timp3; tissue inhibitor of metalloproteinase 3	1419089_at	BI111620	2.53	<1e−09
Il-6; interleukin 6	1450297_at	NM_031168	2.48	6.48e−04
Timp3; tissue inhibitor of metalloproteinase 3	1419088_at	BI111620	2.33	5.64e−05
Gbp2; guanylate nucleotide binding protein 2	1418240_at	NM_010260	2.23	1.53e−04
Timp3; tissue inhibitor of metalloproteinase 3	1449334_at	BI111620	2.22	7.23e−06
Timp3; tissue inhibitor of metalloproteinase 3	1449335_at	BI111620	2.18	2.28e−08
Gstκ1; glutathione *S*-transferase κ1	1452823_at	AK002661	2.13	1.81e−02
Ndrg4; N-myc downstream regulated gene 4	1436188_a_at	AI837704	2.13	6.34e−06
Gas6; growth arrest–specific 6	1417399_at	NM_019521	2.04	1.21e−05
Atm; ataxia telangiectasia mutated	1421205_at	NM_007499	−2.27	8.74e−04
Gas5; growth arrest–specific 5	1436222_at	AW547050	−3.52	<1e−09
Gas5; growth arrest–specific 5	1424843_a_at	BC004622	−4.36	<1e−09

*Expressed sequence tags omitted.
†Probe sets of the form xxxxxxx_s_at omitted unless they were validated using RT-PCR or with a second probset.

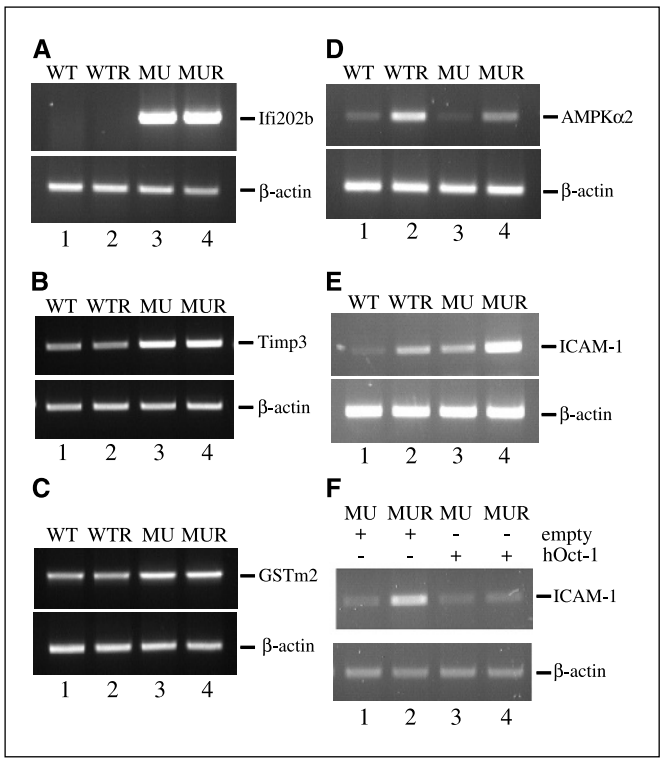

Figure 1. RT-PCR of differentially regulated genes. cDNA from primary mouse embryonic fibroblasts isolated from wild-type and Oct-1-deficient cells (*WT* and *MU*; *lanes 1* and *3*) and cells treated with 10-Gy ionizing radiation and incubated for 3 hours (*WTR* and *MUR*; *lanes 2* and *4*). β-Actin served as a control. *A, Ifi202b. B, Timp3. C, Gstμ2. D, AMPKα2. E, ICAM-1. F, ICAM-1* expression was scored in Oct-1-deficient mouse embryonic fibroblasts transduced with an empty MMLV (*lanes 1* and *2*) or a virus encoding human Oct-1 (*lanes 3* and *4*).

also verified (Fig. 1*B*). *Timp3* was elevated in mutant cells relative to the β-actin control and expression was not effected by ionizing radiation.

A separate method, gene set enrichment analysis (GSEA), was also used to verify the prior set of genes and to identify biological pathways altered by the loss of Oct-1. GSEA does not rely on fold changes or employ user-imposed cutoffs to filter groups of genes and can identify groups of coordinately regulated genes with common biological function (35). Applying GSEA, the three most robustly enriched pathways ($P \leq 10^{-3}$) were glutathione metabolism and reactive oxygen species (ROS), both of which were enriched in mutant cells, and fatty acid metabolism, which was enriched in wild-type cells. Many of the genes identified using GSEA were identical to those using fold change and *P* value cutoffs, verifying the earlier analysis. In addition, several new genes operating in the same pathway were identified (Table 2). One gene identified using GSEA that had not been identified using the previous method, *Gstm2*, was validated using RT-PCR (Fig. 1*C*). This gene was also more highly expressed in mutant cells relative to wild-type (*lanes 1* and *3*) and showed little change 3 hours following treatment with 10-Gy ionizing radiation (*lanes 2* and *4*).

Although present thrice on the array, we did not observe differential expression of Oct-1. The lack of change in steady-state RNA levels is attributable to the nature of the targeting strategy, which deleted a single Oct-1 exon, resulting in a frameshift mutation and a truncated protein. The targeted Oct-1 mRNA was still produced, indicating that the message was not subject to nonsense-mediated decay (18). The lack of this single exon should not affect message detection as probe sets on Affymetrix GeneChips are tiled across many exons. The POU domain transcription factors Oct-2, Oct-3/4, and Oct-6 were poorly expressed. Others, such as BRN4 and BRN5, were expressed to a

higher degree; however, no POU domain factor was significantly differentially regulated in Oct-1 mutant cells compared with wild-type.

Consistent with our earlier findings (18), the histone genes were not down-regulated in Oct-1-deficient cells. One histone gene (encoding H2A) was differentially regulated; however, this gene was ~2-fold more highly expressed in mutant cells relative to wild-type (Supplementary Table S1). Interestingly, two different genes from the growth arrest–specific cluster (*Gas5* and *Gas6*) were identified and were reciprocally regulated. These two genes have also been shown to be inversely regulated during preimplantation embryonic development (36).

Oct-1-deficient fibroblasts are sensitive to cytotoxic agents and harbor elevated oxidative levels. One interpretation of the above results is that Oct-1 directs a program of gene expression that allows cells to correctly respond to cellular stress. This idea predicts that Oct-1-deficient cells would behave abnormally after treatment with ionizing radiation or other stress inducing agents. Therefore, several experiments were done to determine whether Oct-1-deficient cells respond abnormally to cellular stress.

Gamma irradiation of cycling 3T3-immortalized mouse embryonic fibroblasts induced cell cycle arrest equally in both wild-type and Oct-1-deficient cells, with most cells arresting in G_2-M (not shown). Differences in long-term viability between normal and Oct-1-deficient cells were therefore determined. In Fig. 2*A*, WT 3T3 and MU 3T3 were given different doses of radiation (up to 8 Gy) and viability was assayed after 4 days by trypan blue exclusion. A reduction in viable cells was observed at all doses, indicating that the MU 3T3 cells were hypersensitive to γ radiation. For example, a dose of 8 Gy resulted in 34% viability in WT 3T3 cells in comparison with 13% in MU 3T3.

A similar difference in long-term viability was observed with doxorubicin treatment. WT 3T3 and MU 3T3 were treated with 0.2 μg/mL doxorubicin for 1 hour. The MU 3T3 cell line was also transduced with Moloney murine leukemia viruses (MMLV) containing either an empty cassette (MU empty) or the human Oct-1 cDNA (MU hOct-1). Following doxorubicin treatment, 10,000 viable cells were seeded into 24-well plates and incubated for 4 days. Relative amounts of cells were determined using crystal violet in three replicate experiments. Staining as quantified by spectrophotometry is shown as the fraction of the untreated cells (Fig. 2*B*). At this dose of doxorubicin, approximately half of the WT 3T3 cells were observed compared with the untreated controls. In contrast, only 15% of the untreated MU 3T3 cells were present. Transduction of the MU 3T3 line with a retrovirus encoding an empty cassette had no effect on viability whereas transduction with the Oct-1 cDNA partially restored clonal potential after treatment with doxorubicin.

The same cells were also treated with H_2O_2. At very high and very low doses, there was little difference in toxicity, with all cells dying or showing little response (not shown). However, at an intermediate dose of 2 mmol/L, there were stark differences. The results were most dramatic when the cells were first pretreated for

Table 2. Gene sets enriched between Oct-1-deficient and normal primary mouse embryonic fibroblasts using GSEA

Symbol; gene title	Probe set	Accession no.	Rank in gene list	Rank enrichment score*
Set 1. Glutathione metabolism (up-regulated in mutant); normalized enrichment score = 1.70, $P \leq 10^{-3}$				
Gpx3; glutathione peroxidase 3	1449106_at	NM_008161	59	0.1804
Gstm2; glutathione *S*-transferase μ2	1416411_at	NM_008183	397	0.2515
Gstm1; glutathione *S*-transferase μ1	1425626_at	J03952	530	0.3228
Gstm1; glutathione *S*-transferase μ1	1416416_x_at	NM_010358	961	0.3612
Gstm1; glutathione *S*-transferase μ1	1448330_at	NM_010358	1,100	0.4084
Gstm1; glutathione *S*-transferase μ1	1425627_x_at	J03952	1,163	0.4573
Gstt1; glutathione *S*-transferase θ1	1418186_at	BC012254	1,853	0.4670
Idh1; isocitrate dehydrogenase 1 (NADP+), soluble	1422433_s_at	NM_010497	2,322	0.4816
Gclm; glutamate-cysteine ligase, modifier subunit	1418627_at	NM_008129	2,493	0.5078
Gstm5; glutathione *S*-transferase μ5	1416842_at	NM_010360	2,496	0.5413
Set 2. Reactive oxygen species (up-regulated in mutant); normalized enrichment score = 1.66, $P \leq 10^{-3}$				
Prdx2; peroxiredoxin 2	1430979_a_at	AK011963	5	0.3619
Sepp1; selenoprotein P, plasma, 1	1452141_a_at	BC001991	58	0.4751
Gpx3; glutathione peroxidase 3	1449106_at	NM_008161	59	0.5906
Aox1; aldehyde oxidase	1419435_at	NM_009676	101	0.6827
Sod1; superoxide dismutase 1, soluble	1451124_at	BC002066	782	0.6926
Set 3. Fatty acid metabolism (down-regulated in mutant); normalized enrichment score = −1.57, $P \leq 10^{-3}$				
Decr1; 2,4-dienoyl CoA reductase 1, mitochondrial	1449443_at	NM_026172	22,581	0.0048
Acsl1; acyl-CoA synthetase long-chain family member 1	1422526_at	BI413218	22,202	−0.1781
D11Bwg0414e; DNA segment, Chr 11, Brigham and Women's Genetics 0414 expressed	1419642_at	BM220305	21,677	−0.2640
Decr1; 2,4-dienoyl CoA reductase 1, mitochondrial	1419367_at	NM_026172	21,422	−0.3294
Acsl1; acyl-CoA synthetase long-chain family member 1	1450643_s_at	BI413218	20,622	−0.3611

*Additional explanatory material is available at http://www.broad.mit.edu/gsea.

24 hours with 0.2 mmol/L, then subjected to 2 mmol/L for 24 hours (Fig. 3). WT 3T3 cells seemed to be largely normal (Fig. 3*A*) although they did not achieve the same density as the untreated cells (not shown). In contrast, MU 3T3 and MU empty were hypersensitive with very few cells remaining on the dish (Fig. 3*B* and *C*). Restoration of Oct-1 function in the MU Oct cell line partially restored the ability of the cells to survive treatment with H_2O_2 (Fig. 3*D*).

One common feature of treatment with ionizing radiation, doxorubicin, and hydrogen peroxide is oxidative damage. To determine whether loss of Oct-1 leads to increased oxidative levels within cells, early-passage primary mouse embryonic fibroblasts were cultured under normal conditions and incubated with the ROS-sensitive indicator CM-H_2DCFDA, which can detect reactive oxidative molecules in living cells. CM-H_2DCFDA passively diffuses into cells where oxidation results in the formation of fluorescent molecules. Concomitantly, cellular esterases cleave the acetate groups, resulting in charged molecules trapped within the cell. As shown in Fig. 3*E*, cells lacking Oct-1 (*dashed lines*) stained more intensely than did their wild-type counterparts. The mean fluorescence intensity was increased ~2-fold over wild-type levels (wild-type 14.8 versus Oct-1 33.9). An average of three independent experiments is shown in Fig. 2*F*. These experiments indicated that Oct-1 deficiency results in elevated ROS levels. Similar differences were observed with later-passage mouse embryonic fibroblasts (not shown). Restoration of Oct-1 function with a retrovirus encoding human Oct-1 resulted in a decrease in ROS levels (not shown). Treatment of mouse embryonic fibroblasts with 10-Gy ionizing radiation did not significantly alter ROS levels while treatment with 2 mmol/L H_2O_2 resulted in elevated ROS levels in WT and MU mouse embryonic fibroblasts. In former case, the difference in ROS levels between WT and MU was preserved whereas in the latter, the difference was narrowed (not shown).

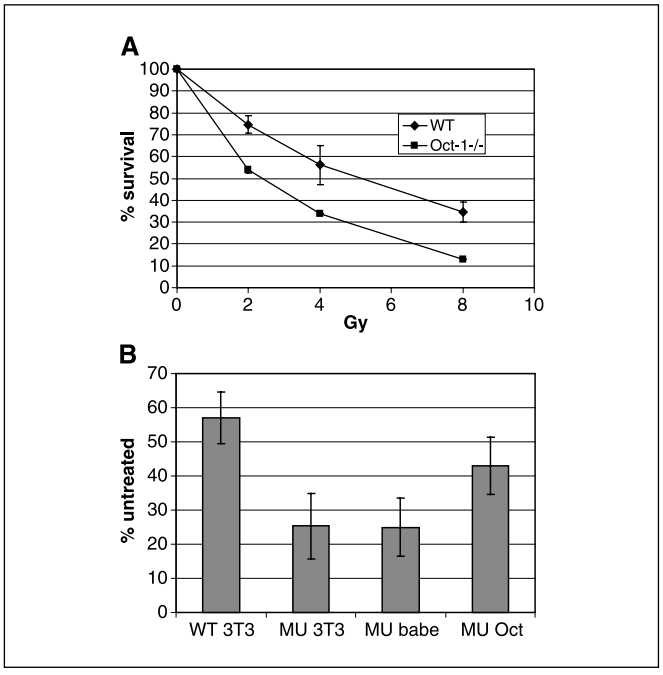

Figure 2. Oct-1-deficient fibroblasts are hypersensitive to ionizing radiation and doxorubicin. *A,* 3T3-immortalized mouse embryonic fibroblasts derived from wild-type (*WT*) or Oct-1 knockout mice (*MU*) were subjected to 0, 2, 4, or 8 Gy of ionizing radiation, returned to 37°C, and incubated for 4 days, at which time live cells were counted by trypan blue exclusion. Survival at each dose was calculated as the ratio between the number of cells scored in the irradiated and the unirradiated plates and expressed as a percentage. *Points,* average of triplicate counting of two independent experiments; *bars,* SD. Statistical significance was evaluated using a one-way ANOVA with a Student-Newman-Keuls posttest. The differences in viability at all radiation doses were found to be highly significant ($P \leq 0.001$). *B,* 3T3 mouse embryonic fibroblasts were treated with 0.2 mg/mL doxorubicin or PBS vehicle for 1 hour, following which 10,000 viable cells were plated into 24-well dishes. After 4 days, total cells attached to the dish were measured using crystal violet staining, elution, and spectrophotometry.

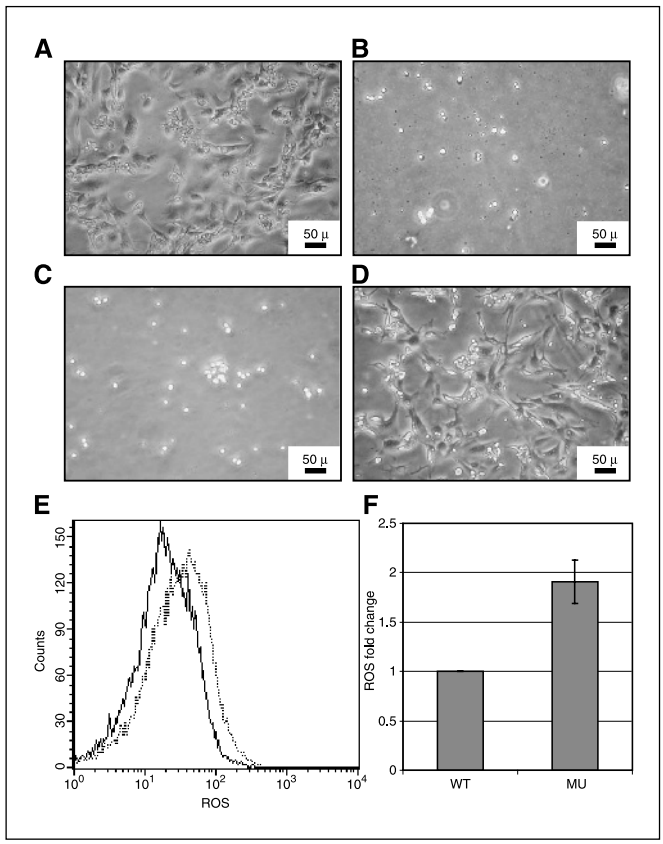

Figure 3. 3T3 fibroblasts lacking Oct-1 are hypersensitive to hydrogen peroxide and have elevated ROS levels. Low-magnification phase-contrast microscopic images of fibroblasts treated with peroxide for 24 hours. *A,* wild-type cells. *B,* Oct-1-deficient cells. *C,* Oct-1-deficient cells infected with an empty MMLV. *D,* Oct-1-deficient cells infected with a virus encoding the human Oct-1 cDNA. *E,* flow cytometry of early-passage primary mouse embryonic fibroblasts containing (*solid line*) or lacking (*dashed line*) Oct-1. ROS levels were measured by staining with CM-H_2DCFDA as described in Materials and Methods. Nonviable propidium iodide–positive cells were gated out. Thirty-thousand events for each cell type are shown. *F,* average of three independent experiments. For each experiment, the mean fluorescence of the MU cells was normalized to WT, which was taken as 1.0. *Bars,* SD.

Altered gene expression in Oct-1-deficient cells after treatment with ionizing radiation. Early-passage primary mouse embryonic fibroblasts were treated with 10 Gy of γ radiation (MUR and WTR). Three hours after treatment, gene expression levels were analyzed. Three hours was chosen because although at this early time point fold-change amplitudes may be relatively small, primary rather than secondary responses should dominate. Treatment of WT and MU cells with ionizing radiation leads to changes in a number of genes, including *p21/WAF1* and *Mdm2* (up-regulated) and *cdc25* and *cyclin B1* (down-regulated; data not shown). Of those genes that were modulated in

response to radiation, the fold changes in WTR versus WT and MUR versus MU correlated in the great majority of cases (not shown).

Two means were used to identify genes that respond abnormally to radiation in Oct-1-deficient cells. First, a set of genes was generated that responds significantly to stress in one case (e.g., WTR versus WT, $P \leq 0.05$) but fails to respond in the other case (e.g., MUR versus MU, $P \geq 0.20$). Because this process removes genes significantly altered after radiation in both wild-type and mutant, but of which expressions are different (e.g., genes strongly up-regulated in one case and down-regulated in the other), genes were also included based on change in rank of expression level relative to other genes on the array (see Materials and Methods). This operation identified many of the same genes found using *P* values; however, additional genes were identified with large changes in expression that had been previously missed [e.g., intercellular adhesion molecule 1 (*ICAM-1*)]. Together, these operations identified 98 differentially regulated genes (Supplementary Table S2).

A large group of genes involved in cellular stress response were identified (Table 3). Many of these genes were induced or repressed in WTR cells relative to WT, failed to change significantly in MUR versus MU, and showed little difference in expression between MU and WT in the unirradiated state. These genes included *Prkaa2/AMPKα2, Sdfr2*, and the p53 target gene *cyclin G1*. The AMPKα2 catalytic subunit was identified twice. The poor induction of this gene in response to ionizing radiation in MU cells was verified by RT-PCR (Fig. 1*D*). In the absence of radiation, very low expression of the AMPK α2-subunit was observed (*lanes 1* and *3*). These data are consistent with a previous report showing that α2 was poorly expressed in fibroblasts (37). α2 is strongly induced in WT cells in response to ionizing radiation (*lane 2*). This response is blunted in MU cells (*lane 4*).

Another group of genes, characterized by ICAM-1, tumor necrosis factor (TNF) receptor superfamily 14 (*Tnfrsf14*), *Tnfrsf10b/TRAILR2*, and prostaglandin E synthase (*Ptges*), increased more robustly in MUR cells relative to MU than in the wild-type situation. ICAM-1 is known to be induced by ischemia and UV, as well as by ionizing radiation (38, 39). TNFRSF14 is the cellular receptor for TNF superfamily 14 (LIGHT; ref. 40). We verified the expression of ICAM-1 by RT-PCR (Fig. 1*E*). ICAM-1 was induced much more robustly in mutant cells following irradiation compared with wild-type cells (compare *lanes 2* and *4*). Restoration of Oct-1 function using a retrovirus blunted the ability of MU cells to induce ICAM-1 expression following radiation (Fig. 1*F*). In addition, several genes involved in the cellular response to oxidative stress were identified. These genes included *sestrin 2* (*Sesn2*), nucleoredoxin (*Nxn*), and *Gstm4*.

GSEA was also applied to the irradiated states (MUR versus WTR). The two top enriched gene sets were "ROS" (up-regulated in MUR cells relative to WT, $P \leq 10^{-3}$), and "p53 down" (down-regulated in MUR cells relative to WT, $P = 0.028$). This set contains genes known to be coordinately down-regulated by p53 following cellular stress. No additional ROS genes were identified that had not been identified before; however, the p53 down set included a number of genes encoding protumorigenic and cell cycle proteins such as procollagen type XVIII and Cdc6, which are known to be normally down-regulated following cellular stress in cells with wild-type p53 (Supplementary Table S3). GSEA also identified "p53 up" as a gene set induced in ells relative to wild-type following ionizing radiation, however the change was less significant. These data indicate that following treatment with ionizing radiation, Oct-1-deficient cells activate and repress of a number of p53 targets more robustly than their wild-type counterparts.

Discussion

The absence of significant defects in the expression of immunoglobulin and other presumed target genes in cells lacking Oct-1 (18, 19) has led to an enigma about the function of this transcription factor. Here we show that a number of genes involved in stress response are altered in their expression in Oct-1-deficient cells, and that Oct-1-deficient cells respond abnormally to cellular stress.

Precedents for a pathway linking Oct-1 to cellular stress exist. Oct-1 protein levels become elevated in response to cellular stresses, including serum withdrawal and treatment with DNA damaging agents (41, 42). Oct-1 is also modified posttranslationally, for example through phosphorylation by DNA-dependent protein kinase, which is activated in response to DNA breakage (43). This phosphorylation has the ability to modulate the transcriptional regulatory activity of Oct-1 (42). Oct-1 interacts with poly(ADP-ribose) synthase, a chromatin-bound enzyme that transfers ADP-ribose moieties to proteins (44). ADP-ribosylation is known to occur in response to cellular stress and inflammation, and is associated with apoptosis (45). Finally, a recent study used microarrays to identify differentially expressed genes following induction of cellular stress with low pH. Strikingly, the octamer sequence was enriched in the regulatory regions of acutely and coordinately up-regulated genes (46).

In light of the data presented here, the regulation of known target genes by Oct-1 may take on an added significance related to stress. For example, herpes simplex virus, which is partially dependent on Oct-1 for early gene expression and completely dependent for viral replication (26), is induced to emerge from latency in response to fever and UV radiation (47). Another Oct-1 target, GADD45, is induced following UV radiation through p53-dependent and -independent response elements. The p53-independent site was mapped to a region ~100 nucleotides upstream of the transcription start site containing two octamer motifs that bind Oct-1. Although the octamer sites are important for UV induction, the intensity and composition of the Oct-1-containing complexes do not change in gel mobility shift assays following treatment with UV, indicating a possible role for Oct-1 posttranslational modification in this process (48, 49).

We identified a number of genes, involved in the cellular response to oxidative stress, that are differentially expressed in Oct-1-deficient cells. These include genes that are differentially regulated in the absence of any stimulus [*Prdx2*, glutathione peroxidase 3 (*Gpx3*), cysteine dioxygenase 1 (*Cdo1*), and glutathione *S*-transferase κ1 (*Gstκ1*)], as well as genes that are differentially regulated in response to γ radiation (*Sesn2, AMPKα2, Nxn, Ptges*, and *GSTm4*). *Sesn2* is a p53 target gene that harbors oxidoreductase activity and can regenerate overoxidized peroxiredoxins (50). Oct-1-deficient fibroblasts are hypersensitive to treatments with a number of agents such as γ radiation, doxorubicin, and hydrogen peroxide. Because Oct-1 is a transcription factor, these defects are likely due to the misexpression of one of more Oct-1 targets. Interestingly, the DNA binding activity of Oct-4, an Oct-1-related transcriptional regulator that is critical for embryonic stem cell pluripotency, is sensitive to oxidation (51).

Table 3. Stress/oxidative genes differentially expressed between Oct-1-deficient and wild-type primary mouse embryonic fibroblasts in response to 10 Gy γ radiation

Symbol; gene title*	Probe set †	Accession	Fold WTR/WT	Adjusted *P*
Sesn2; sestrin 2	1425139_at	AV308638	1.96	2.29e−03
Sesn2; sestrin 2	1451599_at	AV308638	1.89	1.75e−02
Sdfr2; stromal cell derived factor receptor 2	1423465_at	BB032852	1.79	4.86e−02
Ddit4; DNA damage–inducible transcript 4	1428306_at	AK017926	1.73	8.71e−03
Bbc3; Bcl-2 binding component 3	1423315_at	AW489168	1.72	2.85e−02
ICAM-1; intercellular adhesion molecule-1	1424067_at	BC008626	1.64	8.42e−05
Homer3; homer homologue 3	1424859_at	BC005773	1.57	0.26
Daf1; decay accelerating factor 1	1443906_at	BE686894	1.54	3.25e−02
Prkaa2; protein kinase, AMP-activated, α2 catalytic subunit	1434766_at	BQ175911	1.48	6.67e−08
Tnfrsf10b; tumor necrosis factor receptor superfamily, member 10b	1421296_at	NM_020275	1.46	0.99
Ccng1; cyclin G1	1420827_a_at	BG065754	1.45	3.60e−03
Prkaa2; protein kinase, AMP-activated, α2 catalytic subunit	1429463_at	BB612385	1.42	<1e−09
Nxn; nucleoredoxin	1422465_a_at	BB366804	1.29	0.76
Cox10; cytochrome *c* oxidase assembly protein 10, heme A farnesyltransferase	1429329_at	AK010385	1.22	0.99
Ptges; prostaglandin E synthase	1439747_at	BB730139	1.06	0.99
Gstm4; glutathione *S*-transferase μ4	1424835_at	AF464943	1.03	0.99
Tnfrsf14; tumor necrosis factor receptor superfamily, member 14	1452425_at	BC022125	−1.03	0.99
H2afx; H2A histone family, member X	1416746_at	NM_010436	−1.54	8.04e−03

*Expressed sequence tags omitted.
†Probe sets of the form xxxxxxx_s_at omitted unless they were validated using RT-PCR or were verified with a second probset.
‡Numerical rank based on average expression on MOE430A chip (1-22626) or B chip (1-22511) from four replicates, 1 being most highly expressed.

One common effect of ionizing radiation, doxorubicin, and hydrogen peroxide is oxidative stress and the generation of ROS. Oct-1-deficient cells display increased levels of ROS. Elevated ROS levels may be the basis for the observed sensitivity of Oct-1-deficient mouse embryonic fibroblasts to treatments associated with oxidative damage. However, it is unclear which of the genes identified using microarrays confer elevated ROS levels and which may be altered in their expression as a consequence of elevated ROS levels. Many genes elevated in mutant cells encode proteins that confer antioxidant properties, suggesting that in a number of cases the latter is true. A consequence of elevated ROS levels is DNA damage. We have observed that Oct-1-deficient cells display higher levels of γ-H2AX staining, indicating an increase in the average number of double-stranded DNA breaks.[5]

The promoter regions of the differentially expressed genes were searched for octamer sequences that might indicate a direct role for Oct-1 in their regulation. Inspection from 1,000 nucleotides upstream to 100 nucleotides downstream of annotated transcriptional start sites identified perfect octamer elements in 16 genes, including three genes with roles in the response to stress: *Rad52b, Sdfr2*, and *Ptges* (Supplementary Table S4). Three of these genes had conserved octamer sites in the same position and orientation in the orthologous human genes: *Prx1, Notch1*, and *endothelial lipase*. Thirty-six imperfect, but statistically significant, sites were identified from 500 nucleotides upstream to 100 nucleotides downstream of the transcription start site using MatInspector (52). Genes with near-perfect octamers included *Rgs4, Sdfr2, Sgol2, Ptges, Atm, Sesn2*, and *Prkaa2/AMPKα2* (Supplementary Table S5). *Rgs4, Sdfr2, Sgol2*, and *Ptges* also contain perfect octamer sites, and *Ptges, Atm, Sesn2*, and *Prkaa2/AMPKα2* have a known role in the stress response. Genes in which octamers were not identified could still be directly regulated by Oct-1. For example, Oct-1 could operate through octamer sites that do not meet the cutoff criteria or reside in unannotated promoter regions. Sequences only partially related to the canonical octamer are known to interact with Oct-1 (53). In addition, regions outside the nucleotides searched, such as further upstream, in the first intron or within distal enhancer sequences may contain functional Oct-1 binding sites. Oct-1 also has been shown to modulate gene expression through binding to cAMP-responsive element binding protein without direct Oct-1 DNA contact (54). Another possibility is that these genes are indirectly regulated by Oct-1 through the action of secondary transcriptional regulators that are in turn directly modulated by Oct-1. It is also possible that through the dysregulation of one or a few key Oct-1 targets, the cellular state (for example the redox level) is altered, resulting in differential expression of particular genes through an indirect mechanism. Distinguishing these possibilities is a major future task.

Two coordinately regulated sets of genes are modulated by p53 in cells treated with ionizing radiation—a group of growth suppressive genes that are induced and another group of growth promoting genes that are down-regulated. In the absence of Oct-1, several of these target genes are induced or repressed to a greater degree. These results suggest that wild-type Oct-1 may be capable of dampening the transcriptional response of p53, an effect that is potentially protumorigenic. This model predicts that increased Oct-1 function promotes tumor onset and/or progression and that

[5] Unpublished data.

Table 3. Stress/oxidative genes differentially expressed between Oct-1-deficient and wild-type primary mouse embryonic fibroblasts in response to 10 Gy γ radiation (Cont'd)

Fold MUR/MU	Adjusted *P*	Fold MUR/WTR	Adjusted *P*	Fold MU/WT	Adjusted *P*	Rank WT, WTR, MU, MUR[‡]
1.63	0.27	1.04	0.99	1.25	0.99	9433, 7042, 8664, 6933
1.37	0.99	−1.12	0.99	1.23	0.99	10113, 7936, 9436, 8378
1.09	0.99	−1.75	5.89e−02	−1.06	0.99	12658, 11138, 12863, 12615
1.48	0.52	1.11	0.99	1.30	0.59	4972, 3301, 4100, 3098
1.44	0.96	−1.18	0.99	1.01	0.99	8741, 6780, 8712, 7427
6.24	<1e−09	4.11	<1e−09	1.08	0.99	19383, 16809, 19072, 12583
1.76	1.40e−02	1.10	0.99	−1.03	0.99	8645, 7023, 8730, 6719
1.50	0.99	1.69	1.45e−02	1.73	3.48e−04	7091, 5401, 4959, 3865
1.09	0.99	−1.37	2.34e−05	−1.00	0.99	14476, 10813, 14580, 13900
2.03	4.75e−03	−1.21	0.99	−1.68	7.78e−03	14962,13936,17023,14476
1.23	0.99	−1.07	0.99	1.10	0.99	786, 528, 726, 576
1.10	0.24	−1.30	<1e−09	1.00	0.99	21376, 18687, 21380, 20908
1.42	4.63e−02	1.17	0.99	1.06	0.99	2272, 1839, 2180, 1619
−1.67	0.31	−1.81	1.60e−02	1.13	0.99	5262, 4656, 4823, 6787
1.34	3.03e−04	1.28	2.79e−04	1.01	0.99	16767, 16116, 16688, 13925
1.55	5.14e−02	1.76	2.78e−04	1.17	0.99	19755, 19589, 18937, 16661
1.40	1.23e−04	1.43	9.25e−06	−1.01	0.99	21060, 21204, 21166, 19309
−1.33	0.44	1.14	0.99	−1.01	0.99	1483, 2200, 1507, 1978

loss of Oct-1 function would be protective. This prediction is supported by transgenic mouse studies in which overexpression of a large fragment of Oct-1 under the control of a T-cell–specific promoter leads to T-cell malignancy (55). In addition, Oct-1 expression is increased in several human cancers. Using the ONCOMINE database, which contains publicly available gene expression sets generated from ~60 tumor panels covering 20 different human malignancies (56), several cancer types displayed a statistically significant ($P \leq 0.001$) increase in Oct-1 mRNA expression relative to control samples. These included increases in Oct-1 in small-cell lung carcinoma, lung adenocarcinoma, and lung carcinoid relative to normal lung and in acute lymphoblastic leukemia (ALL) induced by TEL-1/AML-1 translocation versus other forms of ALL. Oct-1 levels were also significantly decreased in diffuse large B-cell lymphoma versus normal germinal center B cells ($P = 0.089$; refs. 57–59). Although it is unclear whether Oct-1 is involved in the ontogeny of these cancers or whether differential expression is a consequence of stress *in situ* (e.g., from therapeutic treatment) or stress on the tumor sample, the association of Oct-1 may be of significance either in the onset or progression of disease or in the resistance of tumor cells to treatments such as radiotherapy or chemotherapy.

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