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Effect of *TERT* and *ATM* on Gene Expression Profiles in Human Fibroblasts

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Telomeres protect chromosomes from degradation, end-to-end fusion, and illegitimate recombination. Loss of telomeres may lead to cell death or senescence or may cause genomic instability, leading to tumor formation. Expression of human telomerase reverse transcriptase (*TERT*) in human fibroblast cells elongates their telomeres and extends their lifespan. Ataxia telangiectasia mutated (*ATM*) deficiency in A-T human fibroblasts results in accelerated telomere shortening, abnormal cell-cycle response to DNA damage, and early senescence. Gene expression profiling was performed by serial analysis of gene expression (SAGE) on BJ normal human skin fibroblasts, A-T cells, and BJ and A-T cells transduced with *TERT* cDNA and expressing telomerase activity. In the four SAGE libraries, 36,921 unique SAGE tags were detected. Pairwise comparisons between the libraries showed differential expression levels of 1%–8% of the tags. Transcripts affected by both *TERT* and *ATM* were identified according to expression patterns, making them good candidates for further studies of pathways affected by both *TERT* and *ATM*. These include *MT2A*, *P4HB*, *LGALS1*, *CFL1*, *LDHA*, *S100A10*, *EIF3S8*, *RANBP9*, and *SEC63*. These genes are involved in apoptosis or processes related to cell growth, and most have been found to be deregulated in cancer. Our results have provided further insight into the roles of *TERT* and *ATM* by identifying genes likely to be involved in their function. Supplementary material for this article can be found on the *Genes, Chromosomes and Cancer* website at http://www.interscience.wiley.com/jpages/1045-2257/suppmat/index.html. © 2004 Wiley-Liss, Inc.

INTRODUCTION

Telomeres are nucleoprotein complexes that protect chromosome ends from being recognized and processed as broken DNA. Human and other vertebrate telomeric DNA consists of tandem arrays of $(TTAGGG)_n$ repeats, with a 3′ G-rich single-stranded overhang at the extreme ends. Telomeres become capped by looping of this single-stranded overhang back into the duplex telomeric DNA tract (T-loop), which is maintained and regulated by various telomere-binding proteins. In a capped state, telomeres are protected from multiple molecular interactions, including fusion with another telomere by DNA repair systems, degradation by exonucleases, and recombination (McEachern et al., 2000; Blackburn, 2001). Because of incomplete end replication by DNA-dependent DNA polymerases, telomeres progressively shorten in most cells, losing about 50–200 bp of telomeric DNA with each cell division. Once telomeres become critically short, they are unable to assume the capped structure. Uncapped DNA ends elicit various responses, such as cell-cycle arrest, senescence, or apoptosis, all of which limit the replicative life span of cells. Telomeric fusions can also occur, leading to genome instability (McEachern et al., 2000; Blackburn, 2001; Collins and Mitchell, 2002).

Telomere length can be stabilized or increased by telomerase, an RNA-dependent DNA polymerase that contains the telomerase reverse transcriptase holoenzyme and an essential RNA template (*TERT* and *TERC*, respectively, in humans). *TERC* is widely expressed in all human cells; however, it appears that most normal human cells do not produce sufficient *TERT* to yield readily detectable telomerase levels, except early embryonic cells, reproductive and stem cells, lymphocytes, and a subset of highly proliferating epithelial cells (Shay et al., 2001; Collins and Mitchell, 2002; Cong et al.,

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2002; Masutomi et al., 2003). Telomerase deficiency because of defective processing of *TERC* has been implicated in the human disorders dyskeratosis congenita (DKC) and Hoyeraal-Hreidarsson syndrome. These share the characteristics of premature telomere shortening, growth retardation, neurologic abnormalities, defects in hematopoiesis, immunodeficiency, and reduced life span. DKC patients also have an increased predisposition to malignancies, presumably because of genome instability caused by telomere dysfunction (Collins and Mitchell, 2002).

Telomere function needs to be restored to allow long-term cancer cell survival, and 85% or more of human tumors produce active telomerase by expressing *TERT* (Shay et al., 2001; Hackett and Greider, 2002). Expression of *TERT* in cultured human fibroblasts and other cells also increases telomere length and leads to an extended life span (Bodnar et al., 1998; Steinert et al., 2000; Harley, 2002). However, telomerase expression alone does not result in malignant transformation, and *TERT*-immortalized cells do not exhibit aberrant growth control (Bodnar et al., 1998; Harley, 2002).

The cellular response to DNA double-strand breaks involves activation of the ataxia telangiectasia mutated (ATM) serine/threonine protein kinase that regulates a complex network of downstream signaling pathways. To date, more than 20 phosphorylation targets of ATM have been identified; these proteins have various roles in the implementation of cell-cycle checkpoints, execution of DNA repair processes, apoptosis, and changes in transcription (Kastan and Lim, 2000; Thompson and Schild, 2002; Shiloh, 2003). Mutations in *ATM* cause the autosomal recessive disorder ataxia telangiectasia (A-T). Affected individuals exhibit neuronal degeneration (leading to cerebellar ataxia), immunodeficiency, short stature, sterility, premature aging, clinical radiation sensitivity, and a highly increased risk of cancer, predominantly leukemias and lymphomas (Taylor et al., 1996; Kastan and Lim, 2000). The phenotype of A-T cells is characterized by hypersensitivity to ionizing radiation, cell-cycle checkpoint defects, dysfunctional apoptosis, increased chromosomal aberrations, and premature senescence (Kastan and Lim, 2000; Shiloh, 2003). Furthermore, *ATM* deficiency leads to defective telomere maintenance, resulting in accelerated telomere shortening, which is a likely contributor to the chromosomal abnormalities and reduced life span of A-T cells (Metcalfe et al., 1996; Vaziri et al., 1997; Tchirkov and Lansdorp, 2003). In mice carrying homozygous mutations in *Atm* and the telomerase RNA gene (*Terc*), there are a synergistic effect of telomere shortening and genome instability and a severe proliferation defect in all cells, leading to multiorgan system failure, accelerated aging, and a reduced life span (Wong et al., 2003). These findings strongly suggest a role for *ATM* in telomere maintenance; however, the precise mechanism has not yet been elucidated. Expression of *TERT* in A-T fibroblasts elongates telomeres and rescues the premature-senescence phenotype without inducing malignant transformation; however, other A-T phenotypes related to the DNA damage response still remain, such as radiosensitivity, cell-cycle checkpoint defects, and chromosomal abnormalities (Wood et al., 2001).

To elucidate further the roles of telomerase and ATM in telomere function, genome stability, and cell survival, we assessed global gene expression profiles of human BJ normal fibroblasts and A-T fibroblast cells, as well as their immortalized counterparts expressing *TERT*, using serial analysis of gene expression (SAGE).

MATERIALS AND METHODS

Cell Strains and Culture

The BJ neonatal human foreskin fibroblast cell strain was a kind gift from Dr. J. Smith (Houston, TX). The AG 04405 (male, 7 years) A-T homozygous human skin fibroblast strain was acquired from the National Institute of Aging Cell Culture Repository (Camden, NJ). The fibroblast cells were grown in Ham's F10 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged 1–2 times a week, and the culture medium was changed 2–3 times per week (including passages). Population doubling (PD) was calculated at each passage from the final cell number N_f and the initial seeded cell number N_0 as $PD = \log_2(N_f/N_0)$.

Retrovirus-Mediated *TERT* Transduction in Fibroblast Cells

To generate the *TERT* retroviral construct, the full-length coding sequence of *TERT* (courtesy of Dr. Robert Weinberg) was inserted into the polylinker of the LZRS-linker-internal ribosomal entry site green fluorescent protein as described previously (Hooijberg et al., 2000). This was used to produce retroviral supernatant by transfection into Phoenix amphotrophic (ΦNX-A) cells. Then PG13 cells were used to achieve large-scale virus production (Rufer et al., 2001), and retroviral supernatant was used for transducing BJ fibroblasts at

PD = 63, and AT fibroblasts at PD = 17. GFP-positive BJ and AT fibroblasts (from here on called BJ-TERT and AT-TERT) were sorted on a FACStar Plus, Vantage SE (Becton Dickinson, San Jose, CA) fluorescence-activated cell sorter. One clone of BJ-TERT fibroblasts and a bulk population of AT-TERT cells were isolated and cultured as described above. Telomerase expression was confirmed in the BJ-TERT cells at PD = 82 and 107 and in the AT-TERT cells at PD = 33 using the telomeric repeat amplification protocol (TRAP) assay as described previously (Kim and Wu, 1997; data not shown), with the following modifications. Elongation of the telomerase substrate (TS) primer (Kim and Wu, 1997) by telomerase was allowed for 30 min at room temperature, followed by 30 cycles of polymerase chain reaction (PCR) amplification of the products at 95°C for 60 sec, 50°C for 45 sec, and 72°C for 60 sec using the anchor return primer. The PCR products were then separated on a 15% polyacrylamide gel, stained with SYBR® green (Mandel, Guelph, Ontario, Canada), and visualized with a Storm 820 phosphoimaging system (Molecular Dynamics, Sunnyvale, CA).

SAGE Library Construction

The population doublings of fibroblast cells chosen for SAGE analysis were $PD_{BJ} = 37$, $PD_{BJ\text{-}TERT} = 110$, $PD_{AT} = 21$, and $PD_{AT\text{-}TERT} = 21$. For comparison, the maximum life spans of these cells are approximately $max PD_{BJ} \sim 85$, $max PD_{BJ\text{-}TERT} > 130$, $max PD_{AT} \sim 35$, and $max PD_{AT\text{-}TERT} > 60$.

From the fibroblast cells, 300-350 μg high-quality total RNA was prepared by use of Trizol reagent (Invitrogen, Burlington, Ontario, Canada). The cells were ~80% confluent at the time of RNA isolation. For removal of contaminating genomic DNA, the total RNA was treated with DNase I (Invitrogen). From the total RNA, polyA$^+$ RNA was isolated using the MessageMaker kit (Invitrogen) following the manufacturer's protocol. cDNA synthesis and the subsequent steps of SAGE library preparation were performed as described previously (Velculescu et al., 1995, 1997; a detailed protocol can be obtained from http://www.sagenet.org), using *Nla*III as the anchoring enzyme and *Bsm*FI as the tagging enzyme. In addition, Phase Lock Gel™ (Eppendorf, Hamburg, Germany) was used at every phenol–chloroform extraction step to increase the recovery and purity of DNA (Ye et al., 2000). During library construction, ditags were amplified by 27–29 cycles of PCR.

SAGE Library Sequencing

The library clones were sequenced on an ABI Prism 3700 DNA analyzer using BigDye primer cycle sequencing reagents (Applied Biosystems, Foster City, CA). Sequence reads were processed, and their quality was assessed by use of *Phred* (Ewing and Green 1998; Ewing et al., 1998). Vector sequences were then removed, and 14 base-pair tags were isolated. Linker sequences and duplicate ditags also were removed, and only tags of equal or more than 95% accuracy (derived from *Phred* quality scores) were kept for further analysis.

SAGE Data Analysis

Pairwise comparisons were made between SAGE libraries to assess differences in tag abundance by a statistical test of Audic and Claverie (1997). Significant differences were determined at 95% confidence ($P < 0.05$). Tag-to-gene mapping to the human expressed sequence tag (EST) database, UniGene (http://www.ncbi.nlm.nih.gov/UniGene), was performed with DISCOVERYspace SAGE analysis software (Zuyderduyn et al., in preparation), which uses two tag-to-gene-mapping methods: the National Center for Biotechnology Information's (NCBI) SAGEmap (Lash et al., 2000; http://www.ncbi.nlm.nih.gov/SAGE/), as of December 2002; and the Cancer Genome Anatomy Project's (CGAP) SAGE Genie (Boon et al., 2002; http://cgap.nci.nih.gov/SAGE), as of April 2003. Pairwise library distances, which were based on differences between normalized tag counts, were calculated as follows:

$$d_{xy} = \sqrt{\sum_{i=1}^{36921} [(x_i \times 10^4/x_{all}) - (y_i \times 10^4/y_{all})],^2}$$

where d is the distance between x and y libraries in a multidimensional space in which each tag defines one dimension, x_i and y_i are the counts of the i tag, and x_{all} and y_{all} are the total number of tags in libraries x and y, respectively. There are 36,921 unique tags among the four libraries in this study.

Assigning Molecular/Cellular Function

To the tags that uniquely match one UniGene cluster (using SAGEmap), gene ontology (GO) terms (http://www.geneontology.org/) were assigned where available. UniGene annotations and GO terms were then searched using specific keywords as regular expressions in the Perl computer program language; (Schwartz and Phoenix, 2001), and matches were assigned to functional catego-

ries. The categories and the keywords used for each category were: DNA metabolism/repair (replication, recombination, DNA polymerase, DNA helicase, DNA ligase, nuclease, repair, glycosylase); transcription/RNA processing (transcription, RNA processing, splicing); protein synthesis (translation, protein biosynthesis, protein synthesis, ribosome); protein folding/chaperone (protein folding, chaperone); protein degradation (proteasome, proteinase, protease, peptidase, proteolysis, peptidolysis, ubiquitin); signal transduction (signal transduction, signaling, phosphatase, kinase, growth factor, receptor, GTPase, RAS, SH2, SH3, interleukin, calcium); cell-cycle regulation (checkpoint, arrest, cycle, cyclin, proliferation, mitosis, cell growth); transport (transport, export, import, channel); metabolism (synthase, synthetase, synthesis, oxidase, oxygenase, hydrogenase, hydroxylase, esterase, saturase, lipase, transferase, ATPase, isomerase, reductase, carboxylase, glycolysis, aldolase, catabolism, glucosidase, aminidase, phosphorylase, sulfatase); mitochondria (mitochondr); structural/cytoskeletal (histone, matrix, cytoskeleton, membrane, actin, myosin, collagen); differentiation (differentiation); cell death/aging (mort, MORF, death, apoptosis, senescence, immortal, survive, aging); uncharacterized (EST, cDNA, hypothetical, open reading frame, *Homo sapiens*, +mRNA, expressed sequence, KIAA\d+ protein, KIAA\d+ gene product, DKFZP\w+ protein, PTD\d+ protein, HSPC\d+ protein, GW\d+ protein, P\d+ protein, \d+\s*$).

RESULTS

SAGE technology (Velculescu et al., 1995, 1997) enables the quantitative assessment of polyadenylated transcripts by generating 14-bp-long sequenced tags from near the 3′ ends. In most SAGE libraries, including those used in this study, the tags consist of 10 bp following the 3′-most *Nla*III restriction site in each transcript. The relative abundance of each tag among all tags in a library is proportional to the amount of corresponding mRNA within the original mRNA population. Thus, by using the SAGE method, expression patterns of thousands of genes can be evaluated by comparing the counts of corresponding tags (Velculescu et al., 1995, 1997).

In this study, four SAGE libraries were generated from human skin fibroblast cell lines (Fig. 1). The BJ normal fibroblasts and the AT fibroblasts derived from an A-T individual were primary cells. The BJ-TERT and AT-TERT cell lines were prepared from the BJ and AT cells, respectively, via

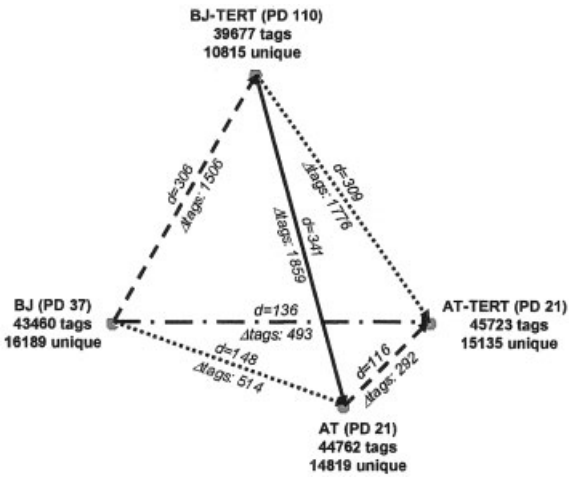

Figure 1. SAGE libraries and overall comparisons. The number of all observed tags and unique tags is shown for each library. PD represents population doubling of the cells at the time of RNA isolation. The label d, which also gives the length of the corresponding arrow, means the distance between two libraries calculated from the differences between normalized tag counts. A Δ indicates number of differentially expressed tags at $P < 0.05$. Different arrow styles represent changes in the states of functional *TERT* and *ATM* between libraries (dash: +*TERT*, dot: −ATM, long dash–dot: +*TERT-ATM*, solid: −*TERT-ATM*).

transduction with a 5′-*LTR*-ψ-*TERT-IRES-GFP-LTR*-3′ retroviral vector. Expression of *TERT* was confirmed by the TRAP assay (data not shown). SAGE library sequencing and processing resulted in more than 39,000 total tags and more than 10,000 unique tags from each sample (Fig. 1). Raw SAGE data are publicly available with Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) accession numbers GSM14916, GSM14917, GSM14918 and GSM14919, which correspond to the BJ, BJ-TERT, AT, and AT-TERT libraries, respectively. Combining the four libraries yielded 36,921 unique tags. The full list of these tags (without the initial CATG) and counts in each library are shown in Supplementary Table S1. (Supplementary material for this article can be found on the *Genes, Chromosomes and Cancer* website at http://www.interscience.wiley.com/jpages/1045-2257/suppmat/index.html.) The distribution of tag abundance was similar in the four libraries. Between 65% and 69% of the tags were observed only once (singletons), the majority, 94%–97%, were found less than 10 times, and a very small fraction, 0.2%–0.4%, were detected 100 times or more.

Differences among the four SAGE libraries were assessed through pairwise comparisons (Fig. 1). Library distances were calculated based on differences of normalized tag counts, taking every tag into account. Differentially expressed tags were determined by a statistical analysis developed by

Audic and Claverie (1997). The full lists of differentially expressed tags (up- and downregulated, $P < 0.05$) between the BJ and BJ-TERT, AT and AT-TERT, BJ and AT, and AT and AT-TERT libraries are shown in Supplementary Tables S2–S5, respectively. The proportion of up- and downregulated tags among the differentially expressed tags was between 45% and 55%. Based on library distances and numbers of differentially expressed tags ($P < 0.05$), the most similar libraries were from the AT and AT-TERT cells. The largest difference was between the BJ-TERT and AT libraries. In addition, the BJ-TERT library was most distant from the other three. Besides overexpression of *TERT* and functional *ATM*, this is probably because of the accumulation of mutations on the long-term cell culture of BJ-TERT cells (population doubling PD 110) compared to the others (PD 21–37). In addition, clone selection and the site of retroviral integration also may have influenced the gene expression profile of this cell line.

Genes or ESTs were assigned to SAGE tags by comparison of tag sequences to human UniGene clusters using SAGEmap of the NCBI (Lash et al., 2000; http://www.ncbi.nlm.nih.gov/SAGE/). The complete list of tags and corresponding UniGene clusters is shown in Supplementary Table S1. Table 1 shows the efficacy of this tag-to-gene mapping. In the four libraries, 69%–75% of tags had a reliable match to at least one UniGene cluster, and 25%–31% did not. As expected, the proportion of singleton tags was highest among those with no match, as the singleton category is enriched for sequencing and other artifacts, as well as low-abundance transcripts that may be more difficult to isolate and characterize. Among the ambiguous tags (29%–31%) were the lowest numbers of singletons. This again is not surprising, because tags belonging to more than one transcript are generally expected to yield higher counts.

Another publicly available tag-to-gene-mapping method is SAGE Genie of the CGAP (Boon et al., 2002; http://cgap.nci.nih.gov/SAGE). Supplementary Table S1 shows all the tags from our libraries together with matching UniGene clusters obtained with SAGE Genie. The efficacy of this tag-to-gene-mapping approach is shown in Table 1. Compared to SAGEmap, the SAGE Genie approach is more efficient in finding genes or ESTs for tags. As a result, the number of tags with no reliable UniGene match was reduced (16%–22% in the four libraries), and the fraction of ambiguous tags was increased (46%–50%). The main advantage of SAGE Genie tag-to-gene mapping, however, is the option of providing a best match by use of several other databases in addition to UniGene (Boon et al., 2002). The results shown in Figure 2, Supplementary Tables S2–S7, and Tables 2 and 3 utilized the SAGEmap approach; in addition, the genes shown in Tables 2 and 3 also were confirmed as the best match by SAGE Genie.

Checking the expression of *TERT* and *ATM* in our libraries is of particular interest. The 3′-most tag of endogenous *TERT* is TCCGGCTGAA. This tag was not detected in any of the four libraries, which is consistent with the idea that most normal somatic cells express low or undetectable levels of *TERT* (Shay et al., 2001; Collins and Mitchell, 2002; Cong et al., 2002; Masutomi et al., 2003). The 3′-most tag in the retroviral vector used to induce *TERT* expression is GAAAATACAT, located within the 3′ long terminal repeat (LTR). This tag was not found in the BJ and AT libraries but was present in the BJ-TERT and AT-TERT libraries, with counts of 30 and 23, respectively. This tag also maps to two other uncharacterized transcripts in UniGene. When its expression was looked for in other, publicly available SAGE libraries, it could be found in only three, with very low counts (1 or 2 in ~60,000 tags). Thus, the high counts in the BJ-TERT and AT-TERT libraries most likely originated from the retroviral vector. A-T individuals lack functional ATM proteins (Kastan and Lim, 2000; Shiloh, 2003). However, full-length *ATM* mRNA might still be present in AT cells. The 3′-most tag of *ATM* is CCATTGCACT. Unfortunately, this tag is highly repetitive, being the 3′-most tag in several other genes. Thus, expression levels for *ATM* cannot be inferred from the counts of this tag.

To help gain an insight into the roles of differentially expressed genes between the BJ and BJ-TERT, AT and AT-TERT, BJ and AT, and BJ-TERT and AT-TERT libraries, gene ontology molecular function terms were assigned to tags that matched one UniGene cluster where available (listed in Supplementary Tables S2–S5). These tags were then sorted into functional categories, which are shown in Figure 2. Because many genes are involved in multiple functions, some are listed more than once. Therefore, the number of tags from each functional category added together resulted in 131%–135% of the number of differentially expressed tags matching one UniGene cluster in these comparisons. Most well-characterized differentially expressed genes are involved in signal transduction or metabolism or have structural/cy-

TABLE 1. Efficacy of Tag-to-Gene Mapping

SAGEmap (NCBI)							
Library	Tags	Amb[a]	%	One[b]	%	No[c]	%
1 BJ	16189	4642	*29*	6697	*41*	4850	*30*
2 BJ-TERT	10815	3117	*29*	4335	*40*	3363	*31*
3 AT	14819	4544	*31*	6195	*42*	4080	*28*
4 AT-TERT	15135	4686	*31*	6643	*44*	3806	*25*
All[d]	36921	9459	*26*	14493	*39*	12969	*35*
Library	NS[e] tags	NS amb	%	NS one	%	NS no	%
1 BJ	5030	1931	*38*	2343	*47*	756	*15*
2 BJ-TERT	3678	1390	*38*	1654	*45*	634	*17*
3 AT	5007	1981	*40*	2301	*46*	725	*14*
4 AT-TERT	5325	2077	*39*	2547	*48*	701	*13*
All	10358	3770	*36*	4823	*47*	1765	*17*
Comparison	Tags	Amb	%	One	%	No	%
diff[f]_12	1506	652	*43*	651	*43*	203	*13*
diff_13	514	227	*44*	212	*41*	75	*15*
diff_14	493	221	*45*	204	*41*	68	*14*
diff_23	1859	782	*42*	834	*45*	243	*13*
diff_24	1776	786	*44*	781	*44*	209	*12*
diff_34	292	136	*47*	109	*37*	47	*16*
SAGE Genic (CGAP)							
Library	Tags	Amb	%	One	%	No	%
1 BJ	16189	7459	*46*	5541	*34*	3189	*20*
2 BJ-TERT	10815	5032	*47*	3448	*32*	2335	*22*
3 AT	14819	7196	*49*	4983	*34*	2640	*18*
4 AT-TERT	15135	7504	*50*	5141	*34*	2490	*16*
All	36921	15465	*42*	12471	*34*	8985	*24*
Library	NS tags	NS amb	%	NS one	%	NS no	%
1 BJ	5030	3004	*60*	1627	*32*	399	*7.9*
2 BJ-TERT	3678	2142	*58*	1162	*32*	374	*10*
3 AT	5007	3064	*61*	1592	*32*	351	*7*
4 AT-TERT	5325	3230	*61*	1756	*33*	339	*6.4*
All	10358	5848	*56*	3496	*34*	1014	*9.8*
Comparison	Tags	Amb	%	One	%	No	%
diff_12	1506	989	*66*	421	*28*	96	*6.4*
diff_13	514	353	*69*	136	*26*	25	*4.9*
diff_14	493	342	*69*	131	*27*	20	*4.1*
diff_23	1859	1211	*65*	534	*29*	114	*6.1*
diff_24	1776	1164	*66*	511	*29*	101	*5.7*
diff_34	292	199	*68*	71	*24*	22	*7.5*

[a]Amb: number of ambiguous tags that match more than one UniGene cluster.
[b]One: number of tags matching one UniGene cluster.
[c]No: number of tags that have no reliable match in UniGene.
[d]All: four libraries combined.
[e]NS: nonsingleton, count $>$ 1 in the library, or count $>$ 1 in at least one of four libraries when combined.
[f]diff_*xy*: number of differentially expressed tags between *x* and *y* libraries ($P < 0.05$).

toskeletal roles. There was a significant proportion of uncharacterized transcripts, at least 23%–29% of the tags in these four comparisons.

Differentially expressed tags between the BJ and BJ-TERT or AT and AT-TERT libraries defined genes whose expression is influenced by

TABLE 2. Differentially Expressed* Tags Between BJ Versus BJ-TERT and AT Versus AT-TERT

Upregulated in BJ-TERT versus BJ and AT-TERT versus AT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name[a]	Function
CTAGCCTCAC[b]	57	257	80	144	14376 actin, gamma 1	cytoskeleton
GATCCCAACT	75	131	8	39	118786 metallothionein 2A[c]	apoptosis
TTATGGGATC	31	76	34	55	5662 guanine nucleotide–binding protein (G protein), beta polypeptide 2–like 1	signal transduction
GACCAGGCCC	33	73	25	49	300772 tropomyosin 2 (beta)	structural
GGTTATTTTG	28	45	19	34	82085 serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	protease inhibitor
CCTGGAAGAG	17	41	5	16	75655 procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone–binding protein p55)	endoplasmic reticulum
GTGCGCTAGG	9	21	2	9	9408 hypothetical protein MGC49942	
GCAGTCGCTT	6	18	2	10	100002 dynein light chain 2A	cytoskeleton
GTCTGCGTGC	1	14	1	7	82159 proteasome (prosome, macropain) subunit, alpha type, 1	proteasome
ACTTGGAGCC	1	9	2	9	177656 calmodulin 1 (phosphorylase kinase, delta)	signal transduction
CTCCATCGGC	2	9	0	5	65238 ring finger protein 40	signal transduction
TCCAATACTG	1	7	1	9	84153 dynactin 2 (p50)	cell proliferation
TCTTTCCAGA	0	5	0	5	236963 protein phosphatase 2A, regulatory subunit B[c] (PR 53)	signal transduction

Downregulated in BJ-TERT versus BJ and AT-TERT versus AT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name	Function
GCCCCCAATA	257	43	481	260	227751 lectin, galactoside-binding, soluble, 1 (galectin 1)	apoptosis
GAAGCAGGAC	79	2	142	91	180370 cofilin 1 (nonmuscle)	cytoskeleton, signal transduction
TCTTGTGCAT	51	1	102	73	2795 lactate dehydrogenase A	metabolism
GATAGCACAG	28	1	7	0	180324 human insulinlike growth factor binding protein 5 (IGFBP5) mRNA	signal transduction
TGCATCTGGT	20	0	29	11	75410 heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	protein folding; chaperone
TGAAGTAACA	14	0	20	6	150580 putative translation initiation factor	protein synthesis
TTGACACTTT	11	0	32	14	26136 hypothetical protein MGC14156	
GGAGCTCTGT	11	0	7	1	227750 NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15 kDa	metabolism
AGCAGATCAG	9	0	94	24	119301 S100 calcium-binding protein A10 [annexin II ligand, calpactin I, light polypeptide (p11)]	signal transduction
CTACTGCACT	8	1	9	2	126133 *Homo sapiens* cDNA FLJ33630 fis, clone BRAMY2022525	

Upregulated in BJ-TERT versus BJ but downregulated in AT-TERT versus AT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name	Function
CGCCGCGGTG	31	51	15	6	4835 eukaryotic translation initiation factor 3, subunit 8, 110 kDa	protein synthesis
GGGGCTGCCC	9	47	17	7	195727 tumor endothelial marker 1 precursor	signal transduction

Downregulated in BJ-TERT versus BJ but upregulated in AT-TERT versus AT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name	Function
ACCAAAAACC	146	19	20	44	172928 collagen, type I, alpha 1	structural; differentiation
GGCTTTACCC	32	0	39	65	119140 eukaryotic translation initiation factor 5A	protein synthesis
TGTCATCACA	32	0	20	37	83354 lysyl oxidase–like 2	aging; cell adhesion
ACAAACTTAG	11	0	4	13	177656 calmodulin 1 (phosphorylase kinase, delta)	signal transduction
GTTTGTATAC	10	0	12	27	5978 LIM domain only 7	signal transduction
TTTGGTCTTT	8	0	3	11	109773 hypothetical protein FLJ20625	
TTTCTAGGGG	7	0	1	11	108969 PTD008 protein	
TCAACGGTGT	6	0	0	7	279886 RAN-binding protein 9	protein complex assembly
CAAAGGAAGC	5	0	0	5	31575 SEC63 protein	endoplasmic reticulum

*$P < 0.05$ in both pairwise comparisons.

[a]Only tags matching one UniGene cluster are shown. Tag-to-gene mapping for these tags was confirmed by both SAGEmap and SAGE Genie.

[b]The tags in each group are sorted by abundance.

[c]The highlighted tags show differential expression from both *TERT* and *ATM*.

TERT. Similarly, differentially expressed tags between BJ and AT or between BJ-TERT and AT-TERT should yield genes in which *ATM* affects expression. However, in these pairwise comparisons, some differential expression alternatively could result from random genetic or epigenetic changes during cell culture. To identify a subset of tags more likely to be influenced by *TERT* or *ATM*, we performed double comparisons between the BJ and BJ-TERT and between the AT and AT-

TABLE 3. Differentially Expressed* Tags Between BJ Versus AT and BJ-TERT Versus AT-TERT

Upregulated in AT versus BJ and AT-TERT versus BJ-TERT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name[a]	Function
GCCCCCAATA[b]	257	43	481	260	227751 lectin, galactoside-binding, soluble, 1 (galectin 1)[c]	apoptosis
GAAGCAGGAC	79	2	142	91	180370 cofilin 1 (nonmuscle)	cytoskeleton; signal transduction
ATCTTGTTAC	83	5	115	145	287820 fibronectin 1	cell motility; signal transduction
TCTTGTGCAT	51	1	102	73	2795 lactate dehydrogenase A	metabolism
AGCAGATCAG	9	0	94	24	119301 S100 calcium-binding protein A10 [annexin II ligand, calpactin I, light polypeptide (p11)]	signal transduction
AGCACCTCCA	30	0	65	65	75309 eukaryotic translation elongation factor 2	protein synthesis
GTCTGGGGCT	16	1	39	41	75725 transgelin 2	
TATGACTTAA	16	0	38	30	89230 potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	potassium transport
AAATGCCACA	15	2	35	25	65450 reticulon 4	
GAAACAAGAT	10	0	34	23	78771 phosphoglycerate kinase 1	metabolism
TTGACACTTT	11	0	32	14	26136 hypothetical protein MGC14156	
CCACTCCTCA	8	0	24	14	82890 defender against cell death 1	apoptosis
TAGATTTCAA	4	0	21	31	197540 hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	transcription
GCCATATTAT	6	0	18	26	19280 cysteine-rich motor neuron 1	signal transduction
TTCACAGATT	6	0	18	16	8107 G-protein gamma-12 subunit	signal transduction
CCAAGTTTTT	5	0	18	12	75914 coated vesicle membrane protein	protein transport
CCCCCGTGAA	6	0	17	11	182018 interleukin-1 receptor-associated kinase 1	signal transduction
TGCCATCTGT	6	0	16	21	23960 cyclin B1	cell cycle regulation
CAAAATATGT	4	0	15	16	79141 vascular endothelial growth factor C	cell proliferation; signal transduction
CTCATAGCAG	5	0	14	25	279860 tumor protein, translationally controlled 1	
AAAAGCTTGA	5	0	14	11	434296 *Homo sapiens,* clone IMAGE:5174044, mRNA	
TAATTACTCT	4	0	13	15	44163 13kDa differentiation-associated protein	
GGTTTGGCTT	2	1	13	10	73818 ubiquinol-cytochrome c reductase hinge protein	metabolism; mitochondrial
AATTTTATTT	0	1	11	9	2853 poly(rC)-binding protein 1	
TGCAGATATT	1	1	11	9	84113 cyclin-dependent kinase inhibitor 3 (CDK2-associated dual-specificity phosphatase)	cell cycle regulation; signal transduction
TGGTGACAGT	3	0	11	8	301005 histone H2A.F/Z variant	structural
GTCTTTCTTG	1	0	11	7	151536 RAB13, member RAS oncogene family	cell adhesion
CTCTCAATAT	1	0	10	5	279518 amyloid beta (A4) precursorlike protein 2	
CTCGAATAAA	1	0	9	8	34871 zinc finger homeobox 1b	transcription
GTTATAATAC	1	0	9	6	296323 serum/glucocorticoid-regulated kinase	signal transduction; sodium transport
CTCAGGAAAT	1	0	8	12	284292 ubiquinol–cytochrome c reductase complex (7.2 kD)	metabolism
ATTCCAATCT	1	0	7	11	178710 clathrin, heavy polypeptide	structural; protein transport
AATACTTTTG	0	0	7	7	165998 PAI-1 mRNA-binding protein	mRNA processing
GACGTCTTAA	1	0	7	6	251531 proteasome (prosome, macropain) subunit, alpha type, 4	proteasome
AGAGCAAGTA	0	0	5	8	78050 small acidic protein	
AGTTTAAGCA	0	0	5	5	7885 phosphatidylinositol-binding clathrin assembly protein	protein complex assembly

Downregulated in AT versus BJ and AT-TERT versus BJ-TERT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name	Function
CGCCGCCGGC	110	220	59	53	182825 ribosomal protein L35	protein synthesis
GCTGGTGCCT	78	112	41	31	125359 Thy-1 cell surface antigen	structural
GATCCCAACT	75	131	8	39	118786 metallothionein 2A	apoptosis
CGCCGCGGTG	31	51	15	6	4835 eukaryotic translation initiation factor 3, subunit 8, 110 kDa	protein synthesis
CCAGTGGCCC	28	42	10	18	180920 ribosomal protein S9	protein synthesis
GCACCTGTCG	26	47	13	9	1239 alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150)	protein degradation, signal transduction
GCCGGGTGGG	23	29	8	6	74631 basigin (OK blood group)	signal transduction
TTAGTGTCGT	20	30	3	4	111779 secreted protein, acidic, cysteine-rich (osteonectin)	structural
CCTGGAAGAG	17	41	5	16	75655 procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	endoplasmic reticulum
GCGGGGTGGA	15	11	0	2	85155 zinc finger protein 36, C3H type–like 1	transcription
CCAGGCACGC	12	21	4	6	54277 DNA segment on chromosome X (unique) 9928 expressed sequence	
CAGCTGGGGC	12	16	2	5	172550 polypyrimidine tract–binding protein 1	mRNA processing; mRNA splicing
ATGACGCTCA	10	19	2	1	8254 *Homo sapiens* cDNA FLJ36465 fis, clone THYMU2015321, moderately similar to *Homo sapiens* Diff33 protein homolog mRNA	
AGCCCGCCGC	10	16	0	1	154036 tumor-suppressing subtransferable candidate 3	apoptosis
GTGCGCTAGG	9	21	2	9	9408 hypothetical protein MGC49942	
GGTGCCCAGT	9	11	1	4	75607 myristoylated alanine-rich protein kinase C substrate	cytoskeleton; cell motility
GTGGCACGCG	8	8	1	2	350824 hypothetical protein FLJ31331	
GGGCTCGGGG	7	12	0	1	105927 stem cell growth factor; lymphocyte-secreted C-type lectin	cell proliferation, signal transduction
GCCGCCATCA	7	11	1	2	182429 protein disulfide isomerase–related protein	protein folding

TABLE 3

TABLE 3. Differentially Expressed* Tags Between BJ Versus AT and BJ-TERT Versus AT-TERT (Continued)

Upregulated in AT versus BJ but downregulated in AT-TERT versus BJ-TERT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name	Function
GGGGAAATCG	103	318	159	143	76293 thymosin, beta 10	cytoskeleton
TGAGTGGTAG	10	43	26	20	77886 lamin A/C	structural
CTGAGTCTCC	10	35	30	23	77269 guanine nucleotide–binding protein (G protein), alpha-inhibiting activity polypeptide 2	signal transduction
TGGGACTCCA	5	33	14	10	59384 hypothetical protein MGC3047	
AGGTCCTAGC	2	25	9	11	226795 glutathione S-transferase pi	metabolism
CCTCCACCTA	4	19	13	9	146354 peroxiredoxin 2	metabolism
CAGCCCAACC	1	16	8	5	28081 eukaryotic translation initiation factor 3, subunit 4 delta, 44 kDa	protein synthesis
CGCACCATTG	0	14	7	6	94672 GCN5 general control of amino-acid synthesis 5-like 1 (yeast)	
TGGAAGGACC	0	13	5	3	5086 hypothetical protein MGC10433	
CTCTTATCAC	0	10	5	3	100043 hypothetical protein BC013949	

Downregulated in AT versus BJ but upregulated in AT-TERT versus BJ-TERT

SAGE tag	BJ	BJ-TERT	AT	AT-TERT	UniGene ID and name	Function
TTTGGTTTTC	70	4	26	27	179573 collagen, type I, alpha 2	structural
AGGTCTTCAA	61	0	9	11	87409 thrombospondin 1	signal transduction
GCTCTGTCAC	26	0	6	10	168640 ankylosis, progressive homolog (mouse)	structural; phosphate transport
GCTAATAATG	14	0	4	10	70823 sulfatase FP	metabolism
TTTTGTGTGA	11	0	3	9	182698 mitochondrial ribosomal protein L20	protein synthesis; mitochondrial
TTTCTAGGGG	7	0	1	11	108969 PTD008 protein	
TCAACGGTGT	6	0	0	7	279886 RAN-binding protein 9	protein complex assembly
CAAAGGAAGC	5	0	0	5	31575 SEC63 protein	endoplasmic reticulum

*$P < 0.05$ in both pairwise comparisons.

[a]Only tags matching one UniGene cluster are shown. Tag-to-gene mapping for these tags was confirmed by both SAGEmap and SAGE Genie.

[b]The tags in each group are sorted by abundance.

[c]The highlighted tags show differential expression from both *TERT* and *ATM*.

TERT libraries, as well as between the BJ and AT and between the BJ-TERT and AT-TERT libraries. For example, if a tag was significantly up-regulated in both BJ-TERT versus BJ and AT-TERT versus AT, this was likely to result from *TERT* expression and much less likely to be a random change. However, when a transcript level was influenced by both *TERT* and *ATM*, the resulting expression pattern could be more complex. For example, transcription of a gene could be up-regulated in the presence of *TERT* and the absence of ATM, but downregulated in the presence of both *TERT* and ATM. The full list of differentially expressed tags ($P < 0.05$) between BJ versus BJ-TERT and AT versus AT-TERT is shown in Supplementary Table S6. The list of differentially expressed tags ($P < 0.05$) between BJ versus AT and BJ-TERT versus AT-TERT is in Supplementary Table S7. Tables 2 and 3 contain a subset of tags presented in Supplementary Tables S6 and S7, respectively. The tags shown in Tables 2 and 3 uniquely match one UniGene cluster. In addition, these tags map to the same UniGene cluster when both SAGEmap and SAGE Genie are used. Because the accuracy of tag-to-gene mapping relies on current databases, which are still not complete and are expected to change over time, it is important to use other methods to validate expression profiles of genes chosen for further studies.

Among the genes differentially expressed because of *TERT* (Table 2) and *ATM* (Table 3) status, there are 12 that seem to be affected by both (highlighted in Tables 2 and 3). Among these genes, 9 have been characterized: metallothionein 2A (*MT2A*); procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (*P4HB*); lectin, galactoside binding, soluble 1 (*LGALS1*); cofilin 1 (*CFL1*); lactate dehydrogenase A (*LDHA*); S100 calcium binding protein A10 (*S100A10*); eukaryotic translation initiation factor 3, subunit 8 (*EIF3S8*); RAN binding protein 9 (*RAN9*); and SEC63 protein (*SEC63*). Three are uncharacterized genes that code for hypothetical protein MGC49942, hypothetical protein MGC14156, and PTD008 protein. Interestingly, for all these genes, expression levels change in the opposite direction as an effect of *TERT* expression versus *ATM* deficiency. For example, *MT2A*, *P4HB*, and *MGC49942* are up-regulated in the *TERT*-expressing libraries but downregulated in the *ATM*-deficient libraries, compared to their non-*TERT*-expressing and *ATM*-proficient counterparts,

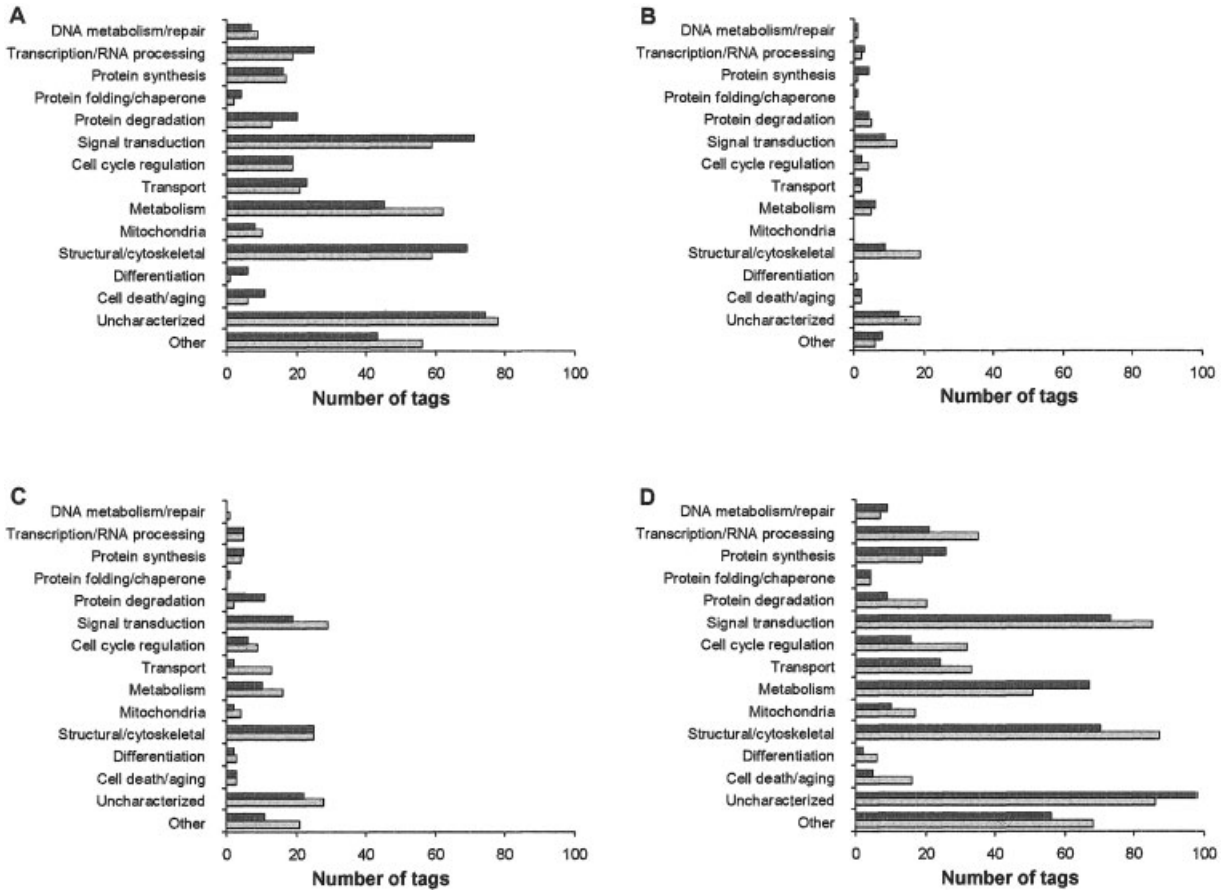

Figure 2. Numbers of differentially expressed tags ($P < 0.05$) between: (A) BJ and BJ-TERT, (B) AT and AT-TERT, (C) BJ and AT, and (D) BJ-TERT and AT-TERT libraries belonging to genes involved in various molecular/cellular functions. Tag-to-gene mapping was performed with SAGEmap, and all the tags shown here uniquely match one UniGene cluster. Black bars indicate up-regulated and gray bars indicate down-regulated genes in the (A) BJ-TERT, (B) AT-TERT, (C) AT, and (D) AT-TERT libraries. Genes in the "Other" category could not be assigned to any of the other 14 classes. Because several genes have multiple functions, 31%–35% of tags/genes, on average, appear in more than one functional category in these pairwise comparisons.

respectively. *LGALS1*, *CFL1*, *LDHA*, *S100A10*, and *MGC14156* show the opposite pattern: downregulation in the *TERT* libraries and up-regulation in the AT libraries. *EIF3S8*, *RANBP9*, *SEC63*, and *PTD008* show more complex expression patterns, for example, the direction of expression change in the *TERT* libraries depends on the state of *ATM*. These findings imply that for these genes, TERT may rescue the gene expression phenotype caused by lack of ATM.

The nine characterized genes described above have various roles in cell growth and cell death. *MT2A*, which is up-regulated in the presence of *TERT*, is protective against oxidative stress and apoptosis (Lu et al., 2002). *LGALS1*, which is downregulated in the *TERT* libraries, promotes apoptosis (Yang and Liu 2003). *EIF3S8*, *RANBP9*, and *SEC63* are involved in protein synthesis, protein complex assembly, and posttranslational protein transport (Meyer et al., 2000), respectively. *LDHA* codes for a metabolic enzyme, *CFL1* has a role in cell polarity and migration (Dawe et al., 2003), *S100A10* is involved in signal transduction, and *P4HB* is involved in ubiquitination. These genes have not been linked previously to telomerase or ATM function. However, deregulation of most of them has been observed in human tumors or cancer cell lines. Among the genes related to apoptosis, *MT2A* was shown to be up-regulated in renal cell carcinoma (Nguyen et al., 2000) and breast cancer cells (Barnes et al., 2000) and to be associated with cell proliferation in invasive breast cancer (Jin et al., 2002). *LGALS1* had increased expression in colon cancer (Hittelet et al., 2003), neuroblastoma and small cell lung carcinoma (Gabius et al., 2002), and hepatic (Shimonishi et al., 2001) and astrocytic (Camby et al., 2002) tumors. The genes involved in protein synthesis and trans-

port showed the following expression changes in cancers. *EIF3S8* was up-regulated in testicular seminoma (Rothe et al., 2000). The expression of *RANBP9* increased in breast cancer cell lines (Emberley et al., 2002). *SEC63* was found to be inactivated by frameshift mutations in colorectal (Mori et al., 2001) and gastric (Mori et al., 2002) cancers. The *LDHA* metabolic enzyme coding gene was deregulated in prostate cancer (Karan et al., 2002) and transcriptionally silenced in a retinoblastoma cell line (Maekawa et al., 2002). *CFL1*, which is involved in cell polarity and migration, was found to be up-regulated in ovarian cancer (Martoglio et al., 2000). The *S100A10* signal transduction gene was overexpressed in gastric cancer (El-Rifai et al., 2002) and renal cell carcinoma (Teratani et al., 2002).

The Protein families database (Pfam) (http://www.sanger.ac.uk/Software/Pfam) was searched using the (predicted) protein sequences from the three uncharacterized genes mentioned above, *MGC49942*, *MGC14156*, and *PTD008*. Alignments with other protein sequences revealed that the PTD008 and MGC14156 proteins belong to small families of short proteins of unknown functions. The MGC49942 hypothetical protein has no homology to any known protein.

DISCUSSION

Expression of *TERT* and *ATM* deficiency in dermal fibroblasts resulted in significant changes in the expression level of a large number of genes (1%–8%) involved in diverse cellular processes. *TERT*-immortalized fibroblast cells have a normal karyotype and exhibit apparently normal growth (Bodnar et al., 1998; Harley, 2002). Furthermore, expression of *TERT* rescues the premature senescence and accelerated telomere-shortening phenotypes in AT cells (Wood et al., 2001). However, our data demonstrate that immortalization by *TERT* does not preserve the gene expression phenotype of normal fibroblasts and does not revert the transcriptome of AT cells to normal.

To identify transcripts affected by *TERT* and *ATM* and to reduce the likelihood that these were random changes as a result of the cell culture, subsets of differentially expressed tags were selected between BJ versus BJ-TERT and AT versus AT-TERT libraries, as well as between the BJ versus AT and BJ-TERT versus AT-TERT libraries. Furthermore, the expression of many additional genes could be influenced by *TERT* and *ATM*, which were not significantly different (at $P < 0.05$) in these comparisons.

Twelve transcripts affected by both *TERT* and *ATM* that uniquely match one UniGene cluster were identified. Three of these are uncharacterized; the other nine have described roles in apoptosis or other processes related to cell growth and proliferation. These genes were selected based on their expression profile, not from previous knowledge about them; they had not been implicated previously in telomerase or *ATM* function. Interestingly, expression of most of these genes was found to be altered in various neoplasias. Because most cancers express *TERT* (Shay et al., 2001; Hackett and Greider, 2002), and *ATM* is often inactivated in tumors (Shiloh, 2003), it will be of interest to assess the expression of these genes in cancers with known telomerase and *ATM* status. Based on our SAGE results, these genes are good candidates for further biological characterization to elucidate their relation to *TERT*, *ATM*, and tumorigenesis.

TERT and ATM are not transcription factors; instead, they probably regulate gene expression indirectly. ATM induces a broad signaling network regulating cell-cycle checkpoints and DNA repair and promotes apoptosis or cell proliferation via phosphorylation of various proteins. Among the ATM target proteins, some are known transcription factors, such as p53, E2F1, and NF-κB (Thompson and Schild, 2002; Shiloh, 2003). [Interestingly, overexpression of *TP53* was reported to suppress *TERT*, although the exact mechanism is not known (Cong et al., 2002).] Thus, in our study, the differentially expressed transcripts from ATM status provided support for the idea that *ATM* is involved in diverse cellular processes. The role of *TERT* in gene expression is less clear because the main known role of telomerase is telomere maintenance. However, there is an emerging notion that telomerase may promote cell growth and survival independently of telomere lengthening (Blackburn, 2001; Blasco, 2002; Smith et al., 2003), and this was supported in this study by the effect of *TERT* on the transcriptome.

SAGE is a powerful technique for assessing the levels of a large number of transcripts without having prior sequence information. In our analysis, several differentially expressed tags affected by *TERT* and/or *ATM* were identified that did not map to known genes. Based on their expression pattern, these are likely to be linked to telomerase or ATM function, which encourages further investigation to identify and characterize these transcripts.

This analysis was the first in which SAGE was used to assess global gene expression profiles of *TERT*-expressing and/or *ATM*-deficient cells.

These data are a valuable resource, providing a baseline for further studies to elucidate the roles that these differentially expressed genes play in telomerase and ATM function, carcinogenesis, and tumor growth.

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