A Novel Transgenic Mouse Model Reveals Humanlike Regulation of an 8-kbp Human TERT Gene Promoter Fragment in Normal and Tumor Tissues

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Abstract

Telomerase activity is repressed in most human somatic tissues during differentiation processes but strongly up-regulated in most human tumors. Regulation of human telomerase activity primarily occurs at the level of transcriptional initiation of the TERT gene, which encodes the catalytic subunit of telomerase. We have generated a novel transgenic mouse model to study the regulation of the human TERT gene promoter in an in vivo system. For this purpose, we have cloned an 8.0-kbp human TERT promoter fragment in front of the bacterial lacZ reporter gene (hTERTp-lacZ), which encodes the β-galactosidase enzyme. Expression of the reporter gene was monitored by reverse transcription–PCR analysis, β-galactosidase staining of whole mount preparations, and histologic sections. We find that the activity of the human TERT promoter in most normal mouse tissues recapitulates the expression of the hTERT gene in normal human tissues and is under tighter control when compared with the endogenous mouse TERT gene expression. In testis, where highest lacZ expression was observed, the expression of the reporter gene was restricted to the spermatogonial stem cells and the spermatocytes. Intriguingly, we find increased levels of lacZ expression in mammary tumors of hTERTp-lacZ × p53−/− bitransgenic mouse mammary tumor model. Thus, this transgenic mouse model provides a suitable in vivo system to analyze the expression of the human TERT gene under physiologic conditions and during tumorigenesis. (Cancer Res 2005; 65(4): 1187-96)

Introduction

The activity of telomerase, a cellular reverse transcriptase that is capable of synthesizing telomeric DNA de novo, is necessary to overcome the end replication problem. Telomerase activity can be detected in early human development but is absent in most normal somatic cells except germ line cells and cells with proliferative capacity such as stem cells or cells of the mucosal lining of the gut (1, 2). On the other hand, high telomerase activity can be detected in >85% to 90% of human tumors and in vitro immortalized cells (3). Tumors that lack telomerase activity maintain their telomere length via a recombination-based mechanism (alternative lengthening of telomeres; ref. 4). Thus, telomerase or another mechanism for telomere maintenance is required for continuous cell proliferation.

The human telomerase enzyme is composed of two essential components, the RNA component [telomerase RNA component (TERC)], which acts as a template for reverse transcription (5), and the catalytic subunit telomerase reverse transcriptase (TERT) with the reverse transcriptase activity (6). In human somatic cells and tumor cell lines, the RNA component, human TERC (hTERC), is constitutively expressed independent of telomerase activity (7). In contrast, the expression of the human catalytic subunit, human TERT (hTERT), correlates very well with telomerase activity: hTERT gene expression is generally repressed in normal human cells and up-regulated in tumor cells (6, 8). Moreover, ectopic expression of hTERT in combination with activated ras oncogene and the genes encoded by the SV40-early region results in tumorigenic conversion of normal human cells (9). Accordingly, ectopic expression of a dominant-negative hTERT protein in tumor cell lines results in apoptotic cell death of these cells (10). These findings indicate that hTERT is the crucial component for telomerase activity and reactivation of hTERT gene expression is one of the key events during tumorigenesis. Therefore, understanding the mechanisms of hTERT gene regulation is essential for the development of diagnostic and therapeutic strategies for human diseases.

There is considerable evidence that the regulation of hTERT gene expression primarily occurs at the level of transcriptional initiation of this gene (3, 11, 12), although posttranscriptional mechanisms may contribute to regulate telomerase activity (13–15). Several transcription factors responsible for the regulation of the human and mouse TERT gene expression have been described, including c-myc, mad1, estrogen receptor, and others (11, 16–18). The regulatory function of c-myc may be modulated by bmi-1 (19) or by signaling pathways such as transforming growth factor-β1 (20) or by viral oncoproteins, such as the human papilloma virus type 16 oncoprotein E6 (21). Moreover, oncoproteins encoded by the early region of the SV40 tumor virus influence hTERT gene expression in a cell type–specific manner in human mesothelial cells but not in human fibroblasts or epithelial cells in vitro (9, 22). Accumulating data support the idea that different signal transduction pathways are involved in regulation of telomerase activity and TERT gene expression (20, 23). In fact, at least four regulatory pathways contribute to the regulation of human TERT gene expression (23).

Sequence analysis has revealed putative binding sites for several transcriptional activators as well as repressors in the promoters of human and mouse TERT genes (Fig. 1A), respectively. Whereas some of these binding sites are conserved between these species, the overall promoter homology is rather low (24). Accordingly, similarities and differences in the expression pattern of hTERT and...
mTERT have been reported. Generally, in normal somatic tissues, human TERT gene expression is more tightly regulated as compared with mTERT gene expression in the corresponding mouse tissues. It is remarkable, however, that increased levels of TERT mRNA and telomerase activity are found in both human and mouse tumors, emphasizing the role of telomerase activity during tumorigenesis. These observations suggest that regulatory mechanisms contributing to TERT gene expression may be conserved in both species and the apparent differences in several tissues may then be resulting from different promoter elements. Thus, a transgenic mouse model could be useful to explore the physiologic regulatory mechanisms involved in hTERT gene regulation in vivo and to better understand the role of telomerase in tumorigenesis and its potential for tumor therapy. For this purpose, we have generated transgenic mice with an 8.0-kbp human TERT promoter fragment driving expression of the bacterial lacZ gene.

We find that hTERT promoter activity in mouse tissues is regulated more stringently when compared with the endogenous mTERT promoter activity, as determined by reverse transcription–PCR (RT-PCR), although several tissues exhibit similarities in TERT regulation. In testsis, where strong mTERT and hTERT expression had been reported, highest lacZ expression was detected. By immunohistochemistry, we could show that the expression of the reporter gene was restricted to the spermatogonial stem cells and the spermatocytes. Importantly, human TERT promoter activity was up-regulated during mouse mammary tumorigenesis, as also was the case for the mouse TERT gene expression and telomerase activity.

Materials and Methods

Generation of hTERTp-lacZ Construct. The pSKT-NLS-lacZ vector containing the lacZ gene with a nuclear localization signal at the NH$_2$ terminus (2S) was used for the β-galactosidase (β-gal) reporter activity. For the cloning of the 8.0-kbp human TERT gene promoter, first, a 2.5-kbp XhoI-EcoRI human TERT promoter fragment excised from pGRN261 (Geron Corporation, Menlo Park, CA) was cloned in front of the lacZ gene. In a second cloning procedure, the hTERTp-lacZ reporter gene was generated with a KpnI-BstEMI fragment from pGRN310 (Geron Corporation) to provide the 8.0-kbp human TERT gene promoter lacZ reporter gene fusion vector (pSKT-hTERTp-lacZ). The resulting reporter plasmid was sequenced to prove the correct cloning procedure.

Generation of Transgenic Mice. An 11.3-kbp fragment from pSKT-hTERTp-lacZ vector was released by BglII-NotI digestion and used to generate fertilized mouse oocytes according to standard methods (26). Fertilized oocytes were isolated from superovulated F2 intercrosses of C57BL/6j and B6D2F2 mice, microinjected, and reimplanted into pseudopregnant females of the same hybrid mouse strain. Offspring were screened for the presence of the transgene by PCR analysis of genomic tail DNA using lacZ-specific primer pairs (see below). Genomic tail DNA from these PCR-identified founder animals was subjected to Southern blot hybridization using a 1.1-kbp radiolabeled lacZ probe to confirm the results. Eleven founders out of 85 did contain the transgene construct, both by PCR and Southern blot analysis. Transgene expression was determined by RT-PCR and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of the embryos where 4 of these 11 were found to express the transgene.

To analyze the hTERT promoter activity in a pure mouse background, transgenic animals showing lacZ gene expression were back-crossed for 10 generations with wild-type C57BL/6j mice. All further analyses were done with these C57BL/6j transgenic mice, except for the tumorigenesis studies. For the tumorigenesis studies, hTERTp-lacZ transgenic mice were backcrossed with wild-type BALB/c mice for four generations before they were mated with BALB/c p53–/– mice to generate hTERTp-lacZ × p53–/– mice.

X-Gal Staining. X-gal staining of embryos was done following standard procedures (27), except that PBS was used at pH 8.0 to reduce background staining. Adult mouse testes were incubated in 4% paraformaldehyde for 2 to 4 hours at room temperature for fixation. The testes were washed thrice in PBS, embedded in gelatin/bovine serum albumin and sectioned at 100- to 200-μm thickness with a vibratome (Leica, Bensheim, Germany). The sections were stained for 1 hour at 37°C, briefly rinsed in PBS, and photographed.

Immunohistochemistry. Tissues were routinely fixed in 4% buffered formalin and paraffin embedded. After deparaffinization, β-gal immunostaining was done using the ChemMate Envision detection kit (DakoCytomation, Glostrup, Denmark). Briefly, after a 20-minute microwave pretreatment at 95°C in target unmasking fluid (TUF, PanPath, Amsterdam, Netherlands) and blocking of endogenous peroxidase with a peroxidase blocking solution (DakoCytomation) for 5 minutes, tissue sections were incubated with the purified mouse anti–Escherichia coli β-gal monoclonal antibody (Promega, Mannheim, Germany) at a concentration of 4 μg/mL (for testis sections) or 20 μg/mL for tumor sections overnight at 4°C. Application of the primary antibody was followed by incubation with the Envision detection reagent consisting of a dextran backbone to which a large number of peroxidase molecules and goat anti-mouse/anti-rabbit secondary antibody molecules have been coupled. 3,3’-Diaminobenzidine chromogen solution and a substrate buffer containing hydrogen peroxide.
served as substrate system. Tissue culture supernatant containing monoclonal mouse IgG2a antibodies to Aspergillus niger (DakoCytomation) was used as negative control at appropriate dilution. Tissue sections were counterstained by hematoxylin and permanently mounted.

RNA Isolation, Radioactive RT-PCR. For RNA isolation from mouse and human tissues, 20 to 50 mg tissue were homogenized on ice in peqGOLD RNA PURE solution (peqlab, Erlangen, Germany) with Polytron (Kinematica AG, Littau-Luzern, Switzerland) and RNA was prepared according to the RNA purification protocol of the supplier. First-strand cDNA synthesis was done using 1 μg total RNA with ThermoScript reverse transcriptase kit (Invitrogen, Carlsbad, CA) in the presence of 100 ng oligo(dT)20 in a volume of 20 μL at the following conditions: 1 hour at 50°C followed by 15 minutes at 75°C followed by 20 minutes at 37°C in the presence of 2 units of RNase H to digest the RNA. Two microliters of the cDNA were used for PCR analysis in a total volume of 50 μL containing 0.2 μmol/L specific primers, 10% DMSO, 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxyribonucleotide triphosphate and 1 unit of Taq polymerase (Eppendorf, Hamburg, Germany). For radioactive PCR analysis, 2.5 μCi of [α-³²P]dCTP (3,000 Ci/mmol, Hartmann Analytik GmbH, Braunschweig, Germany) was added to the reaction. Amplification products were analyzed on 4% (GAPDH, hTERT) or 6% (mTERT, lacZ) non-denaturing polyacrylamide gels.

For radioactive PCR, the linear range of amplification was determined previously and amplified (94°C, 30 seconds; 55°C, 30 seconds; 72°C, 1 minute) was done for 19 cycles for GAPDH, 25 cycles for lacZ, and 30 cycles for mTERT and hTERT. The primers and the length of the PCR products were (GAPDH)-FOR (5'-ACCACAGTCTCATGTCCATC-3') and GAPDH-REV (5'-TCCACACCCCCTGGTCTCTGA-3') for GAPDH (452 bp); lacZ-FOR (5'-ATCAGGATTTTCATGTTGCC-3') and lacZ-REV (5'-AGACCATTTTCAATCCGCACC-3') for lacZ (358 bp); mTERT-FOR (5'-ATGCCGCTTCTAGTATGGTGC-3') and mTERT-REV (5'-CTCTGAAAGCGACCGAGCG-3') for mTERT (279 bp) as well as hTERT-FOR (5'-CTCGCTAGTCCGAGGTAAACGCC-3') and hTERT-REV (5'-GGTGTCATGCATCGAGATGG-3') for hTERT (451 bp).

Extract Preparation and Telomeric Repeat Amplification Protocol Assay. To determine the endogenous enzymatic activity of telomerase in mouse or human tissue extracts, we used the telomeric repeat amplification protocol (TRAP) with the TRAPEze kit (Serologicals Corporation, Norcross, GA) according to the recommendations of the supplier.

Results

Expression of lacZ Reporter Gene under the Control of an 8.0-kbp Human TERT Promoter in Transgenic Mice. We have cloned an 8.0-kbp fragment of the human TERT gene promoter in front of the bacterial lacZ gene that encodes the β-gal enzyme (Materials and Methods) to investigate the regulation of the human TERT gene promoter under physiologic conditions in vivo (Fig. 1B). To determine human TERT promoter activity in transgenic mice we isolated total RNA from mouse tissues and measured lacZ gene expression in three transgenic mouse lines (6340.3, 6335.5, and 6323.6, respectively). We used the very sensitive radioactive RT-PCR method, which is suitable to detect low abundant mRNA species and allows quantification of the reporter gene. In parallel, we measured the expression of the GAPDH house keeping gene as a normalization control.

The activity of the human TERT promoter was at highest levels in the testes of all transgenic mice, repeatedly (Fig. 2). In fact, the reporter gene was detectable exclusively in the testes of two transgenic mouse lines, 6335.5 and 6323.6 (Fig. 2B and C, respectively). In the transgenic mouse line 6340.3, lacZ gene expression could be detected in the spleen, kidney, small and large intestine, mammary glands, lymphocytes, and the brain, although at low levels (Fig. 2A). The apparently strong signal in the brain sample (Fig. 2A) and the weak signals in the heart and muscle

Figure 2. Activity of the human TERT gene promoter in hTERTp-lacZ transgenic mice. The activity of the human TERT gene promoter was determined by means of the reporter gene (lacZ) mRNA levels. Total RNA was isolated from various mouse tissues and reverse transcribed, as described in Materials and Methods. To better quantify the signals we have used radioactive PCR method. Amplification of the cDNAs was done in the presence of trace amounts of [α-³²P]dCTP in addition to nonradioactive deoxyribonucleotide triphosphate in the reaction mixture. The linear range of amplification was determined beforehand (Material and Methods). LacZ gene mRNA levels in the hTERTp-lacZ transgenic mouse lines (A) 6340.3, (B) 6335.5, and (C) 6323.6. Representative RT-PCR results with total RNA from indicated tissues. M, 100-bp ladder. One microgram of the 100-bp ladder was end labeled according to standard procedures and ~1,000 cps were loaded per lane. The apparent difference in 100-bp resolution is due to different running times and polyacrylamide gel concentrations. Signals from RT-PCR results with RNA from 6340.3 transgenic mouse were quantified with TINA 2.0 program (Raytest, Straubenhardt, Germany) and normalized to the expression pattern of the ubiquitous housekeeping GAPDH gene. LacZ gene expression in the testis was set as 1, arbitrarily.
samples (Fig. 2A) were due to RNA loading, as was found by normalizing the signals to GAPDH mRNA levels.

Expression of Endogenous Human and Mouse TERT Genes Differ in Many Tissues. Differences in the tissue expression pattern between mouse and human TERT genes have been described (ref. 28 and references therein). Most of these analyses compared TERT gene expression in only few tissues at the same time in both organisms making it difficult to directly compare the results. For this reason, we measured endogenous mouse and human TERT gene mRNA levels in a broader array of tissues under the same conditions by radioactive RT-PCR (Fig. 3).

To define similarities and differences between endogenous human and mouse TERT gene expression we first tested endogenous TERT gene expression in tissues of adult wild-type mouse. In accordance with previously published data, TERT mRNA was detectable in several mouse tissues with the strongest expression found in testis, ovary/oviduct, and liver and moderate expression in spleen and small and large intestine (Fig. 3A).

Figure 3. TERT gene expression in mouse and human tissues. Expression of TERT gene was determined by radioactive RT-PCR (for details, see Materials and Methods and legend to Fig. 2). A, endogenous TERT gene expression in mouse tissues. Total RNA was isolated from indicated tissues from a wild-type mouse and RT-PCR was done as described. B, endogenous TERT gene expression in human tissues. Human tissue total RNA was either obtained commercially (left, Human Total RNA Master Panel II, BD Biosciences, Palo Alto, CA) or was prepared from freshly frozen tissue samples (right) as described. C, endogenous TERT mRNA is absent from normal human ovaries but up-regulated in ovarian cancer. Total RNA was either prepared from freshly frozen tissue samples (lanes 1 and 2) or obtained commercially (BioCat GmbH, Heidelberg, Germany; lanes 3 and 4). – and +, negative and positive PCR controls, respectively.
No mTERT mRNA could be detected in the samples obtained from skeletal muscle, skin, and heart, whereas weak mTERT expression was found in kidney, brain, lung, and mammary tissues (Fig. 3A).

Generally, telomerase activity correlates with mTERT gene expression. In some mouse tissues, TERT mRNA was found without detectable telomerase activity (29). We used the TRAP assay to determine whether telomerase activity correlates with TERT gene expression in C57BL/6j mice. Mouse tissues with high and moderate TERT mRNA levels (e.g., testis, liver, spleen, ovary/oviduct, and intestine; Fig. 3A) exhibited telomerase activity as was expected from RT-PCR expression results for mTERT (data not shown). It is worth noting that endogenous mTERT expression and telomerase activity in all transgenic mouse lines (6340.3, 6335.5, and 6323.6) coincided completely with wild-type control animals (data not shown).

Examination of TERT mRNA levels in normal human tissues revealed strong TERT gene expression in testis and moderate and weak expression in the mucosal lining of the colon and in the small intestine, respectively (Fig. 3B). TERT expression was very weak or undetectable in all the other normal human tissues tested (Fig. 3B and C).

Reports on hTERT gene expression in normal human ovary were ambiguous. In one report (17), low or undetectable TERT gene expression has been found in primary human ovarian surface epithelial cells, and TERT gene expression was up-regulated upon estrogen-treatment. On the other hand, Ulaner et al. (30) described absence of telomerase activity in normal human ovaries despite TERT gene expression. These authors found several alternatively spliced variants in ovarian and uterine tissues and suggested that regulation of telomerase activity may be regulated at multiple levels including TERT gene expression and alternative splicing of TERT transcripts in these tissues. We have determined hTERT mRNA levels in normal human ovaries from two different sources to test and to correlate endogenous hTERT expression to endogenous mTERT expression (Fig. 3A), and to human TERT promoter activity in our transgenic mice (Fig. 2). We could not detect hTERT mRNA in normal human ovary but in a human ovarian tumor sample (Fig. 3C).

**Expression of the lacZ Gene under the Control of the 8.0-kbp Human TERT Promoter Fragment Resembles Endogenous Human TERT Gene Expression.** Taken together, we find substantial differences in endogenous TERT mRNA levels between several human and mouse tissues (Fig. 3, Table 1). Strong or moderate TERT gene expression was found in testis and in small and large intestine from human and mouse, respectively. In the same line, weak or no endogenous TERT mRNA was detectable in several human and mouse tissues (kidney, heart, muscle, and skin) indicating that TERT gene regulation is conserved in some organs between these two species. The differences in human and mouse TERT gene expression were observed in the liver, ovary/oviduct, brain, spleen, and the lung. Whereas no or very weak hTERT expression could be detected in these tissues, strong (liver, ovary/oviduct, spleen) or significant (lung and brain) mTERT expression was detectable repeatedly.

Comparison of the 8.0-kbp human TERT promoter activities, as measured by lacZ reporter gene expression in transgenic mouse tissues, with the endogenous TERT gene expression in human and mouse tissues reveals a stringent regulation of this promoter fragment (Table 1). In the testis, reporter gene expression under the control of the human promoter fragment was the strongest in all transgenic mouse tissues tested.

**In Testis, Expression of the lacZ Reporter Gene Is Restricted to Spermatogonias and Spermatocytes.** Expression of human and mouse TERT genes in testis had been reported (31–33). To determine cell type–specific hTERT promoter activity, we chose the testsis because of the abundant reporter gene expression in this tissue. First, we applied the X-Gal staining method on vibratome sections of the testes to detect β-gal enzymatic activity. Enzymatic β-gal activity was detectable only in the seminiferous tubuli of the testes of the hTERTp-lacZ transgenic mice (Fig. 4A), but not in the testes of the wild-type mice (Fig. 4B). For a more detailed analysis of the cell type–specific expression of the β-gal protein, we applied immunohistochemistry on paraffin-embedded testis sections (Materials and Methods). Whereas wild-type testis sections did not show any staining with the anti-β-gal (Fig. 4D), strong β-gal immunoreactivity was observed in the primordial spermatogonia and primary spermatocytes within the tubuli seminiferi of the hTERTp-lacZ mice (Fig. 4C). No background staining was observed with control antibodies (data not shown). Furthermore, histologic analyzes (H&E staining) clearly showed that Sertoli and Leydig cells completely lacked β-gal immunostaining (data not shown), indicating the cell type–specific and appropriate activity of the human TERT promoter in the germ cells. Experiments presented in Fig. 4 were obtained with testes from the 6340.3 transgenic line. Both, X-Gal staining and immunohistochemical detection led to the same results with testes from the 6335.5 transgenic mouse line (data not shown). The third 6323.6 transgenic mouse line was not tested in these experimental settings.

**Human TERT Promoter Activity in Mouse Embryonic Tissues.** Telomerase activity can be detected at early embryonic stages during human gestation (1). To determine human TERT promoter activity during embryogenesis in our transgenic mouse lines, we did whole mount X-Gal staining on mid- and late-stage embryos. Predominant β-gal activity was detectable in the olfactory epithelium from E10 dpc, which was sustained until E17.5 dpc embryonic stage in both 6340.3 and 6335.5 hTERTp-lacZ transgenic mice (data not shown). From the third line (6323.6) only E11.5 to E14.5 dpc embryos were analyzed showing strong X-Gal staining in the olfactory epithelium (data not shown). Figure 5 shows a representative embryo at E15.5 with strong staining in the olfactory epithelium, the midbrain, along the dorsal root ganglion, and the neural layer of the retina, all indicative for TERT promoter activity in highly proliferating cells during embryogenesis.

Although we have not analyzed adult mouse brain for TERT promoter activity and the endogenous mTERT as well as endogenous hTERT expression in the olfactory epithelium of adult mouse and human, it is striking that the olfactory epithelial cells retain regenerative capacity throughout life and may require telomerase activity for continuous proliferation. We also find it remarkable that about 30% of mTERC−/− embryos from the fifth generation did not develop normally and failed to close the neural tube, preferentially at the forebrain and midbrain (34), emphasizing an essential role of telomerase in brain development and neuronal differentiation. Additional studies will prove the relevance of this expression pattern for human and/or mouse TERT gene expression in embryonic and adult mouse brain.

**Human TERT Promoter Activity Is Up-regulated in the p53+/− Mouse Mammary Tumor Model.** Because telomerase activity is found in more than 90% of human breast cancers (35), it was tempting to investigate the human TERT promoter activity in a mouse mammary tumor model. For this purpose, hTERTp-lacZ transgenic mice were back-crossed with the p53 heterozygous mice.
into a BALB/c (p53+/− BALB/c) background to generate bitransgenic mice. Alterations of p53 tumor suppressor gene or protein are found in more than 50% of human tumors, including breast malignancies. Kupperwasser et al. (36) have recently described that spontaneous mammary carcinomas, among other tumors, develop in p53+/− mice in BALB/c background. To detect human TERT promoter activity during tumorigenesis we have analyzed reporter gene expression in mammary tumors in this bitransgenic (p53+/− × hTERTp-lacZ) mouse model. Thus far, tumors have arisen in two bitransgenic mice. In one case, one mouse developed mammary tumors in a cervical mammary gland at the age of 8 months (the other mammary glands of this mouse had no obvious phenotypic alterations). Intriguingly, this hTERTp-lacZ mouse was from the 6335.5 line, which did not show lacZ signal in any normal tissue of adult mice, except in the testis of adult male mouse (Fig. 2; Table 1). When we tested reporter gene expression by radioactive RT-PCR, lacZ mRNA was clearly detectable in the mammary gland within the tumor but not in the nontumor mammary glands from the same mouse (Fig. 6A, right). No lacZ expression was detectable in other tissues of this mouse, as expected. In the second case, tumor was observed in a thoracic mammary gland of the other bitransgenic line (6340.3 × p53+/−) at the age of 13 months. RT-PCR analysis revealed an up-regulation of reporter gene expression in the tumor (thoracal) mammary, indicating increased human TERT promoter activity (Fig. 6A, left). Moreover, by immunohistochemistry on paraffin-embedded tissue sections, we also detected β-gal protein in tumor cells but not in the surrounding stroma cells (Fig. 6C). This result clearly confirms the RT-PCR data (Fig. 6A).

We also find increased endogenous mTERT mRNA levels and telomerase activity in the tumor samples (Fig. 6A and B). As expected, strong endogenous mTERT expression in the liver did correlate with telomerase activity (Fig. 3A). Although our results with respect to the hTERT promoter activity during tumorigenesis are preliminary at the moment, the marked expression of lacZ in the mammary tumors of the hTERTp-lacZ × p53+/− bitransgenic mice, especially in the 6335.5 × p53+/− bitransgenic line, argues for the reactivation hypothesis of telomerase during tumorigenesis.

### Discussion

We report the generation and analysis of a novel transgenic mouse model to investigate human TERT gene promoter activity in vivo. We find that the expression pattern of the lacZ reporter under the control of an 8.0-kbp human TERT promoter fragment mostly recapitulates the expression pattern of the TERT gene found in normal human tissues and is stringently regulated, unlike the broader expression pattern of the endogenous mouse TERT gene. Moreover, human TERT promoter activity is up-regulated in a bitransgenic mouse mammary tumor model. Importantly, up-regulation of hTERT promoter in the tumor tissue in BALB/c p53+/− × hTERTp-lacZ (6335.5) bitransgenic mouse, in which no promoter activity was detectable in normal adult tissues except testis (Figs. 2 and 6A), is in favor of the reactivation hypothesis of human TERT gene during tumorigenesis. Our findings that human TERT promoter activity and endogenous mTERT expression as well as telomerase activity are increased in the hTERTp-lacZ × p53+/− bitransgenic mouse mammary tumors and are compatible with the elevated telomerase activity in human and mouse mammary tumors (35, 37–39).

The liver tissue is the most remarkable among the adult human and mouse normal tissues with respect to TERT gene expression: despite its regenerative capacity in both organisms, hTERT expression is absent in normal human liver, whereas strong mTERT expression and telomerase activity was found in livers of all mice strains thus far analyzed (1, 24, 29, 40), including this study. Thus, absence of hTERT promoter activity in livers of our monotransgenic mice reflects humanlike regulation of the 8.0-kbp hTERT promoter fragment in this organ. Similarly, lacZ expression (i.e., human TERT promoter activity) was absent in the ovaries of the transgenic mice

Table 1. Endogenous TERT mRNA levels in adult human and mouse tissues in comparison with the lacZ reporter gene mRNA levels in the hTERTp-lacZ mice

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as also was endogenous TERT expression in human ovaries, in contrast to the strong endogenous mouse TERT expression in this tissue. Reports about hTERT expression in the ovary and its relevance to telomerase activity are nonconclusive. In one report (17), similar to our results, low or undetectable TERT gene expression has been found in primary human ovarian surface epithelial cells and TERT gene expression was up-regulated upon estrogen treatment and correlated with up-regulation of telomerase activity. On the other hand, Ulaner et al. (30) described absence of telomerase activity in normal human ovaries despite TERT gene expression. These authors found several alternatively spliced variants in ovarian and uterine tissues and suggested that regulation of telomerase activity may be regulated at multiple levels including TERT gene expression and alternative splicing of TERT transcripts in these tissues. Although the primers we have used in this study for endogenous hTERT amplification are suited to detect all hTERT transcripts (including the splice variants) we could not observe any TERT PCR products in normal ovaries but in ovarian cancer. Currently, we have no satisfactory explanation for this apparent discrepancy. In contrast to the different regulation of TERT gene expression and thus telomerase activity in human and mouse livers and ovaries, TERT gene expression/telomerase activity is present in both human and rodent testes. Our results that the hTERT promoter activity located to spermatogonia and spermatocytes in the testes of the transgenic mice are in good agreement with reports showing that telomerase activity is present in spermatogonia and spermatocytes but is absent from Sertoli cells in both humans (32, 41) and rodents (31, 33).

Figure 4. hTERT promoter activity in mouse testis as determined by X-Gal staining and β-gal immunostaining. X-gal stainings: 150-μm vibratome sections of the testes of hTERTp-lacZ transgenic mice (A) and wild-type mice (B) were prepared and X-gal stainings were done as described in Materials and Methods. Photographs were taken with a digital camera (Canon Powershot G4, Tokyo, Japan) on a stereomicroscope (Leica). Magnification ×10. β-gal immunostainings: 2-μm paraffin sections of the testes of hTERTp-lacZ transgenic mice (C) and wild-type mice (D) were subjected to immunohistochemical staining with β-gal-specific antibodies (C and D) or IgG2a isotype control antibodies (data not shown). Specific β-gal immunoreactivity was detected in spermatogonia (red arrows) and spermatocytes (black arrows) within mouse testicular tubuli.

Figure 5. hTERT promoter activity in E15.5 dpc mouse embryos. A, lateral view of an E15.5 dpc hTERTp-lacZ (6340.3) mouse embryo. hTERT promoter activity was monitored by whole mount X-Gal staining. 1, 2, and 3, layers of vibratome sections that are shown in detail in B to D. Transverse vibratome sections (100 μm) of gelatin-embedded embryos were prepared and X-gal stainings were done as described in Materials and Methods. Areas of β-gal activities are indicated.

In two of the three hTERTp-lacZ transgenic mouse lines (6335.5 and 6323.6), reporter gene expression was detectable in testes only, despite the use of the very sensitive radioactive RT-PCR method. This may be due to the integration of the reporter construct in a
region of transcriptionally inactive chromosome. Another and a more likely reason may be the integration into the vicinity of a silencer, because promoter activity was readily detectable in testes of both transgenic mouse lines and located to the seminiferous tubuli in the 6335.5 transgenic line (transgenic mouse line 6323.6 was not analyzed in that detail). It is worth mentioning that during embryogenesis, expression of the reporter gene was found in all hTERTp-lacZ transgenic mice presented in this study (data not shown). Remarkably, we find reactivation of the lacZ reporter gene expression in the BALB/c p53−/− × hTERTp-lacZ (6335.5) bitransgenic mouse with a tumor in the thoracic mammary gland; right hTERTp-lacZ (6335.5) × p53−/− bitransgenic mouse with a tumor in the cervical mammary gland. RT-PCR and TRAP assays were done as described. −, negative PCR control: 2 μL H2O; +, positive PCR control: 2 pg pSKT-lacZ or pBABE-mTERT. C, β-gal immunostaining. 2-μm paraffin sections of the tumor tissue of hTERTp-lacZ (6340.3) × p53−/− bitransgenic mouse were subjected to immunohistochemical staining with β-gal-specific antibodies (left) or IgG2a isotype control antibodies (right). Specific β-gal immunoreactivity was detected in tumor cells (red arrows) but not in the surrounding stroma cells (black arrows).

Despite the differences in TERT gene regulation and in telomere length, telomerase activity and telomere length regulation are subject to similar control mechanisms in both mouse and human: (a) expression of the TERT gene is regulated at the transcriptional level in mouse and human cells (3, 11, 42); (b) essentially, all factors involved in telomere biology are conserved in both species; (c) late-generation telomerase null mice show defects in proliferative organs (43); (d) telomerase activity is up-regulated not only in 85% to 90% of human but also in mouse tumors (24, 35, 44); and (e) overexpression of TERT induces tumor formation in mice despite their long telomeres (39, 45). In this regard, it may seem astonishing, at first view, that mTERC−/− mice can give rise to tumors despite the absence of telomerase. There is good evidence, however, that
tumors that arise in telomerase null mice use the alternative lengthening of telomeres mechanism for telomere maintenance (46, 47).

Although we cannot exclude the contribution of distal enhancer or silencer sequences on hTERT gene expression, the 8-kbp hTERT promoter fragment used here seems to harbor most of the regulatory regions responsible for its proper regulation in normal and tumor tissues of mice. We used the 8-kbp hTERT promoter fragment for two reasons. First, in a separate experimental setting with stably transfected U937 cell line, we have found that luciferase gene expression under the control of the 8-kbp promoter fragment (pGRN310) was down-regulated during retinoic acid-induced or the phorbol ester (12-O-tetradecanoylphorbol-13-acetate)-induced differentiation of this cell line, similar to the endogenous TERT gene expression,5 suggesting that this promoter fragment may be suitable to study human TERT gene expression. Moreover, by stably transfecting HeLa cells, Lin and Elledge provided evidence that a 2.0-kbp upstream regulatory sequence of the human TERT gene promoter may suffice for proper regulation of the hTERT gene (23). Second, position-independent expression of a gene in transgenic mice is observed only when large promoter fragments are used (48).

Our findings suggest that the regulatory mechanisms contributing to TERT gene expression are well conserved between humans and mice. Evolutionary conservation of regulatory mechanisms has been shown in transgenic mice by using promoter fragments from different species: expression of an 8.0-kbp fragment of the chicken lysozyme promoter in transgenic mice was essentially the same as in chicken macrophages, whereas endogenous mouse lysozyme gene is regulated differently (47). Studies with the human β-globin locus in transgenic mice have showed that the developmental stages of activation of the various globin genes observed in humans are faithfully reproduced in mice (49). Because general regulatory mechanisms and most transcription factors driving these mechanisms are conserved during evolution, it has been suggested that differences in the species-specific expression pattern may result from differences of the cis regulatory elements in the respective promoter regions (44). This may hold true in the case of human and mouse TERT gene regulation as well.

Thus, the hTERTp-lacZ transgenic mice provide a suitable model to address the regulatory mechanisms involved in hTERT gene expression under physiologic conditions in vivo. By generating bitsransgenic mice, contributions of specific transcription factors could be directly tested in a tissue and developmental specific manner. Furthermore, a more detailed analysis of hTERT promoter activity during tumor initiation and progression may provide a basis for a hTERT promoter-based tumor therapy.

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