

## MOLECULAR TARGETS FOR THERAPY (MTT)



## Telomerase is limiting the growth of acute myeloid leukemia cells

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**Telomeres play an important role in the proliferation and senescence of normal and malignant cells. To test the role of telomerase in acute myeloid leukemia (AML), we expressed the telomerase reverse transcriptase (hTERT) gene, a dominant-negative hTERT (DN-hTERT) (D868A, D869A) gene, or a gene encoding green fluorescence protein (GFP) in the leukemia cell line K562 and in primary AML cells from different patients, using retroviral vectors. Cells transduced with hTERT exhibited elevated levels of telomerase activity compared to GFP controls, whereas cells expressing DN-hTERT had decreased telomerase activity. K562 populations transduced with DN-hTERT showed reduced clonogenicity, telomere dysfunction and increased numbers of apoptotic cells compared to GFP- or hTERT-transduced cells. Two of four clones transduced with DN-hTERT died after 30 and 53 population doublings, respectively. Transduced AML cells were tested in primary colony-forming unit (CFU) and suspension culture assays. Relative to hTERT- and GFP-transduced controls, AML cells transfected with DN-hTERT produced fewer CFU and showed lower engraftment after transplantation into sublethally irradiated  $\beta_2\text{-m}^{-/-}$  nonobese diabetic/severe combined immunodeficient mice. We conclude that telomerase is limiting the growth of the leukemic cell line K562 and primary AML progenitor cells. Our data warrant further studies of the therapeutic use of telomerase inhibitors in AML.**

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## Introduction

Telomeres in human cells consist of 2–15 kb of noncoding double-stranded T<sub>2</sub>AG<sub>3</sub> repeats and 50- to 150-nucleotide overhangs of single-stranded repeats<sup>1</sup> and associated proteins.<sup>2</sup> Telomeres protect the ends of chromosomes from fusion, exonucleolytic degradation, aberrant chromosomal recombination, and functional telomeres are required for proper segregation of chromosomes during mitosis.<sup>3–6</sup>

The human telomerase complex, a ribonucleoprotein polymerase, is able to synthesize terminal (T<sub>2</sub>AG<sub>3</sub>)<sub>n</sub> telomeric repeats *de novo* and can compensate for the loss of telomeric DNA.<sup>7</sup> It consists of a catalytic subunit with reverse transcriptase activity encoded by the telomerase reverse transcriptase (hTERT) gene, a RNA template encoded by the hTERC gene and associated proteins.<sup>8,9</sup> In general, normal human somatic cells have only low or undetectable telomerase activity with

hematopoietic progenitor cells and activated T- and B-lymphocytes being notable exceptions. Telomerase is required to compensate for the loss of telomeres resulting from the end replication problem, exonuclease processing of 5' ends and lesions induced by radiation or oxidative stress.<sup>10–14</sup> Cells from many tissues show progressive shortening *in vivo* and *in vitro*, which could result in their replicative senescence and selection of abnormal cells.<sup>15–18</sup>

Numerous types of cancer, including acute myeloid leukemia (AML), use telomerase activity to maintain telomeres and prevent replicative senescence or apoptosis. Variable levels of telomerase have been detected in up to 85% of all AMLs.<sup>19–21</sup> AML is a highly aggressive disease in which an uncontrolled proliferation and maturation of myeloid progenitors is observed.<sup>22</sup> AML is thought to arise from genetic changes in a primitive hematopoietic progenitor or stem cell.<sup>23</sup> As in normal hematopoiesis, heterogeneity in the proliferative and self-renewal abilities of AML cells exists, suggesting that AML cell populations within individual patients are organized in a hierarchy.<sup>24–26</sup> AML progenitors and stem cells can be detected using *in vitro* and *in vivo* assays.<sup>27</sup> While a considerable number of AML blasts is capable of forming colonies in colony-forming assays,<sup>25,28,29</sup> only a minority of AML cells have the ability to initiate and sustain long-term proliferation *in vitro* or to engraft nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice.<sup>25,29</sup> Cells with long-term *in vitro* proliferative capacity and cells capable of engrafting immune-deficient mice are thought to be uniquely capable of maintaining the disease. These assays are therefore of great interest to determine the efficacy of treatment regimens aimed to eradicate AML stem and progenitor cells.

Telomerase is expressed in most AML cells and telomerase inhibition could potentially lead to eradication of leukemic stem cells. Several point mutants in the reverse transcriptase motifs of the catalytic subunit of hTERT have been identified and shown to reduce markedly telomerase activity upon overexpression in telomerase-positive cells.<sup>30,31</sup> Most likely, dominant-negative (DN) constructs disrupt telomerase activity by binding to limiting levels of the essential telomerase RNA template, resulting in inactive telomerase complexes. In order to study the role of telomerase in AML, we have examined the effect of overexpression and disruption of telomerase activity in the human leukemia cell line K562 and in primary AML progenitors. Our results show that telomerase is a promising target to inhibit the proliferation of AML cells.

## Materials and methods

## Patient cells

Peripheral blood (PB) cells from 12 AML patients at diagnosis were obtained with informed consent and approval of the

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**Table 1** Characteristics of AML samples used in this study

No.	ID	Age (years)	Sex	Source	FAB subtype	Cytogenetics	% of blasts	TRAP
1	109-14	22	M	PB	M1	Normal	90	++
2	110-36	37	F	PB	M1	Normal	78	+
3	NV253	78	F	PB	M1	NA	93	+
4	111-41	25	M	PB	M2	+4; t(8,21)	94	+/-
5	IT369	52	M	PB	M2/MDS	Normal	17	+/-
6	105-46	74	F	PB	M2	Normal	30	++
7	GHN323	50	F	PB	M4 eo	Inv 16; +22	55	+/-
8	DK367	53	M	PB	M4	-4; -12; -17; del5(q12, q23), etc	61	+/-
9	108-24	58	M	PB	M4	Normal	96	++
10	114-04	60	F	PB	M4	Normal	26	+
11	BC212	65	F	PB	M4	Normal	75	+/-
12	VW372	65	F	PB	M4	Normal	89	+/-

NA, not applicable.

Clinical Research Ethics Board of the University of British Columbia. Patients with FAB M3 type were excluded because of the unique characteristics of this AML subtype. Blood cells were subjected to density separation using Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden). Mononuclear cells were frozen in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc., Vancouver, Canada) with 50% fetal calf serum (FCS) (StemCell Technologies Inc.) and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, Oakville, Ontario, Canada), and stored at  $-135^{\circ}\text{C}$ . The patient characteristics of the samples used in this study are shown in Table 1.

### Retrovirus production

Gene transfer was achieved using retrovirus-mediated gene transfection. We constructed murine stem cell virus (MSCV)-based retroviral vectors<sup>32</sup> containing the gene for enhanced green fluorescent protein (GFP; Clontech, Palo Alto, CA, USA) under the control of the phosphoglycerate kinase promoter with or without the full-length hTERT cDNA (kindly provided by Dr Robert Weinberg, Massachusetts Institute of Technology, Boston, MA, USA) or DN-hTERT (D868A, D869A) cDNA (kindly provided by Dr Lea Harrington, University of Toronto, Toronto, Canada). Helper-free retrovirus pseudotyped with the gibbon ape leukemia virus envelope for efficient infection of human cells was generated, using PG13 packaging cells.<sup>33</sup>

### Retrovirus-mediated transfection of K562

K562 cells were cultured in DMEM with 10% FCS and 2 mM L-glutamine (StemCell Technologies Inc.). Cells were harvested, washed once in phosphate-buffered saline (PBS) (StemCell Technologies Inc.) and resuspended at a density of  $1 \times 10^6$  cells/ml in retrovirus-containing supernatant along with 4  $\mu\text{g}/\text{ml}$  hexadimethrine bromide (Polybrene) (Sigma-Aldrich). K562 cells were cultured overnight, washed once with PBS and further expanded for 5–6 days prior to subsequent experiments.

### Retrovirus-mediated transfection of AML cells

Primary AML cells were thawed and cultured in Iscove's modified Dulbecco's medium (IMDM) (StemCell Technologies Inc.), containing 20% FCS, 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory, Vancouver, Canada) and 10 ng/ml rhIL-3 (Novartis, Basel, Switzerland). At day 2 or 3, cells were

harvested and counted. A total of  $1-5 \times 10^6$  cells were then plated in this medium onto nontissue culture six-well plates (Falcon, Becton Dickinson, Bedford, MA, USA) coated with 6–10  $\mu\text{g}/\text{cm}^2$  RetroNectin™ (Takara Shuzo Ltd, Otsu, Japan) preloaded with 4 ml of retrovirus-containing supernatant. Plates were coated either at  $4^{\circ}\text{C}$  overnight or at  $37^{\circ}\text{C}$  for 2 h (h), subsequently blocked with 2% bovine serum albumin (BSA, Calbiochem-Novabiochem, San Diego, CA, USA) for 30 min at  $37^{\circ}\text{C}$ , and then washed once with PBS prior to use. After 4 h, cells were harvested from the wells by vigorous pipetting and washed once with PBS. Cells were resuspended in IMDM, containing 20% FCS, 50 ng/ml SF and 10 ng/ml rhIL-3, and kept in six-well plates for tissue culture (Falcon) overnight. The transfection procedure was repeated on two consecutive days. After transfection, cells were further expanded for 5–6 days and then sorted for GFP expression. The efficiency of transfection was estimated from the percentage of cells expressing GFP at around 0.82–34.3%.

### Flow cytometry

Viable GFP<sup>+</sup> K562 cells or GFP<sup>+</sup> primary AML cells (PI<sup>-</sup>) were isolated at a purity of >99%, using an FACStar<sup>plus</sup> (Becton Dickinson, San Jose, CA, USA), as described previously.<sup>34</sup> Cells were sorted and known cell numbers were used to initiate proliferation assays, colony-forming assays (CFU), suspension culture (SC) assays and transplantation assays. Both single-cell clones and bulk populations were obtained from the K562 cells and used in proliferation assays.

### Proliferation assays

A known number of sorted K562 clones and bulk cells were plated in media. Once a week, cell counts were performed using trypan blue. The number of population doublings (PDs) was calculated from the average cell count, using the following equation:  $\text{PD} = {}^{10}\log(\text{number of cell counted after expansion}) - {}^{10}\log(\text{number of cells seeded}) / {}^{10}\log 2$ .

### Primary CFU assays

Immediately after FACS sorting, a known number of sorted primary AML cells were plated in methylcellulose culture medium (Methocult H4330, StemCell Technologies Inc.), containing 20 ng/ml rhIL-3 (Novartis), 20 ng/ml rhIL-6 (Terry

Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario, Canada), 20 ng/ml rhGM-CSF (Novartis) and 50 ng/ml rhSF (Terry Fox Laboratory). After 10 days of incubation at 37°C in a 5% CO<sub>2</sub> humidified incubator, AML blast clusters (10–20 cells) or colonies (>20 cells) were counted and the numbers were pooled to determine the primary CFU frequency/10<sup>5</sup> cells calculated as follows: (the number of colonies/the number of cells plated) × 10<sup>5</sup>.

### SC assays

SCs were initiated with a known number of transduced AML PB cells sorted for their GFP expression and maintained generally, as described previously,<sup>29,35</sup> in 1.0 ml of serum-free medium, containing 20 ng/ml rhIL-3 (Sandoz, Basel, Switzerland), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Sandoz) and 50 ng/ml rhSF (Terry Fox Laboratory). Cultures were maintained at 37°C in a 5% CO<sub>2</sub> humidified incubator and 0.5 ml of fresh medium with growth factors was added weekly. Every second week, half the cells and volume of the SC were removed, the cells were washed in DMEM with 10% FCS and cultured in methylcellulose to determine the CFU content of the SC. SC were maintained for 8 weeks, then the entire contents of the wells were harvested and assessed for CFU content. To allow comparisons between experiments, the frequency of progenitors/10<sup>5</sup> cells at each time point was calculated from the number of colonies obtained by plating a given number of cells. No feeder layers were formed in SC initiated with PB cells of the AML patients.

### Transplantation of transduced AML cells into $\beta_2\text{-m}^{-/-}$ NOD/SCID mice

$\beta_2$ -Microglobulin-deficient nonobese diabetic/severe combined immunodeficient ( $\beta_2\text{-m}^{-/-}$  NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained in the animal facility of the British Columbia Cancer Research Center (Vancouver, Canada) under sterile conditions in sterile microisolator cages. The animals were provided exclusively with autoclaved food and water containing 100 mg/l ciprofloxacin and HCl. At 24 h prior to transplantation, mice were irradiated with 3.5 Gy  $\gamma$ -irradiation from a <sup>137</sup>Cs source at a dose rate of 1.22 cGy/min. A known number of sorted AML PB cells transduced with the MSCV vector alone or with the MSCV vector containing hTERT or DN-hTERT were suspended in 0.3 ml Alpha minimal essential medium (Alpha MEM) (StemCell Technologies Inc.) with 5% FCS and injected intravenously into the lateral tail vein of 6- to 8-week-old  $\beta_2\text{-m}^{-/-}$  NOD/SCID. BM aspirations were performed every 4 weeks on anesthetized mice as described elsewhere.<sup>36</sup> Eight or 12 weeks postinjection, the mice were killed by CO<sub>2</sub> inhalation. BM was removed from the femurs by flushing with Alpha MEM with 5% FCS.

### Flow cytometry analysis of murine tissues

Cell suspensions from femoral BM were lysed in ammonium chloride (StemCell Technologies Inc.) for 20 min and then washed in Hanks' medium (StemCell Technologies Inc.) with 5% human serum, for blocking of human Fc receptors, and prepared for flow cytometric analysis as described.<sup>29,35</sup> Briefly, cells were then stained with a Cy5-labeled anti-human CD45 antibody for 30 min on ice. Unlabeled cells were used as

control. Samples were analyzed using an FACScan (Becton Dickinson) to determine the percentage of cells expressing CD45 using gate settings that excluded  $\geq 99.9\%$  of the events in the unlabeled control cells. Human engraftment was defined as the expression of  $\geq 0.1\%$  CD45<sup>+</sup> cells in a sample.

### Telomerase repeat amplification protocol assay

Telomerase activity was measured by telomeric repeat amplification protocol (TRAP) assay using an end-labeled telomerase substrate (TS) primer as described.<sup>37</sup> Cell extracts were obtained from a positive control cell line (K562). Extension of the TS primer by telomerase was performed for 30 min at room temperature, and the products generated were amplified by 30 cycles of polymerase chain reaction at 95°C for 60 s, 50°C for 45 s and 72°C for 60 s, using the ACX-anchored return primer. Half of the amplified products were resolved on a 12% polyacrylamide gel and visualized by a phosphoimaging system (Storm 820, Molecular Dynamics Inc., Sunnyvale, CA, USA). The results of the semiquantitative TRAP assay were used to rank telomerase activity in primary AML cells relative to the activity measured in K562 cells. High telomerase levels were defined as levels comparable to the telomerase level in K562 cells. Intermediate levels (+) are defined as readily detectable levels but lower compared to K562. The definition of low levels (+/-) of telomerase activity is telomerase activity that is just detectable using this assay.

### Telomere length analysis

The telomere length of cells before and after transfection and selection was measured using *in situ* hybridization and flow cytometry (flow FISH) as described.<sup>38</sup>

### Cytospin preparation

Cells were counted and 2–4 × 10<sup>4</sup> cells were resuspended in 100  $\mu$ l medium containing 10% BSA (Calbiochem-Novabiochem) and spun at 1250 rpm for 1 min with high acceleration in a cytocentrifuge (Cytospin-2, Thermo Shandon). The slides were air-dried overnight, stained with Wright–Giemsa stain (Hematek 2000, Bayer Diagnostics, Tarrytown, NY, USA) and analyzed.

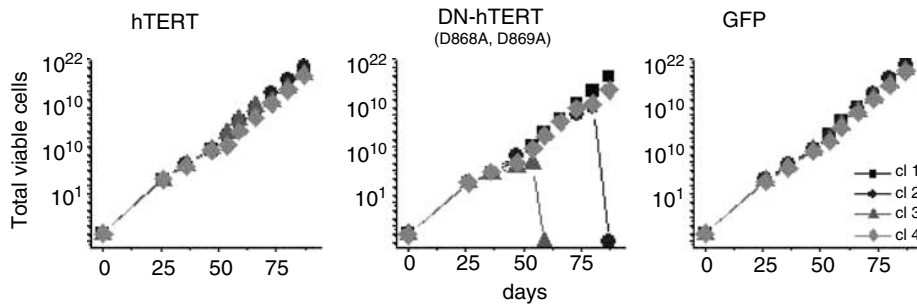
### Calculations and statistical analysis

The Friedman ranks test was used to study the correlation between the telomere length of K562 cells transduced with GFP, hTERT and DN-hTERT ( $n=8$ ). For a correlation between GFP- and DN-hTERT-transduced primary AML cells the nonparametric Wilcoxon's signed-rank test was used ( $n=7$ ). For both tests, a significant level of 0.05 was chosen.

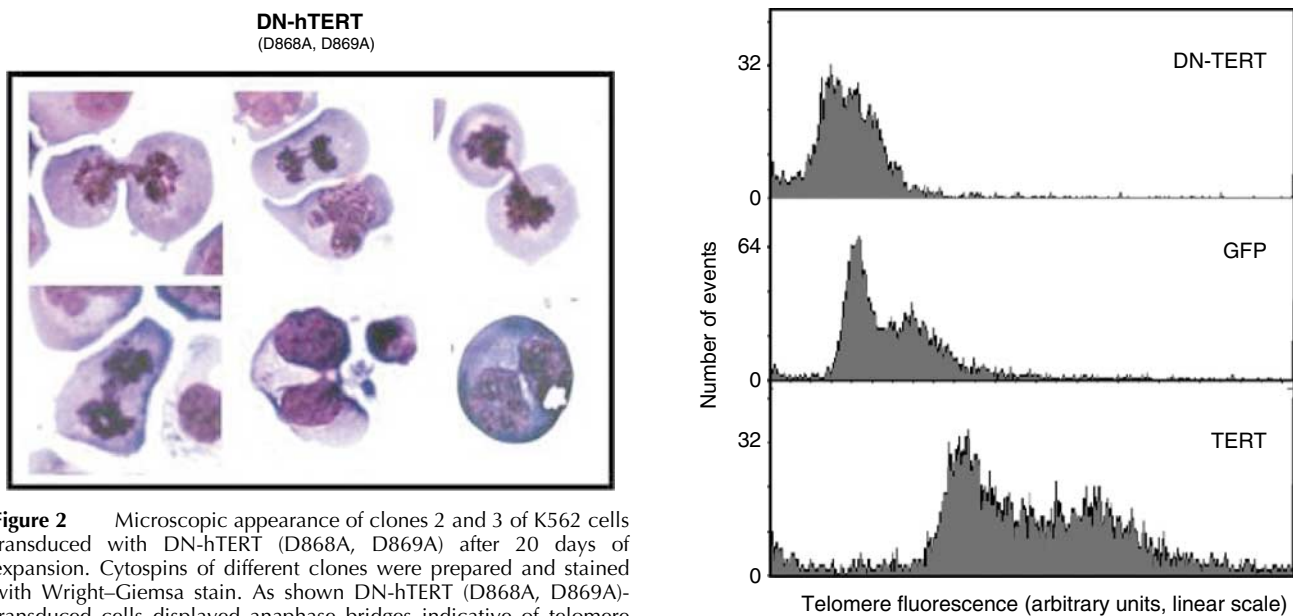
## Results

### Disruption of telomerase activity in K562 leads to telomere dysfunction and cell death

To study the role of telomerase in leukemia, we first transduced the leukemia cell line K562 with retroviral vectors encoding hTERT, DN-hTERT (D868A, D869A) and GFP. Transduced cells were sorted on the basis of GFP expression. Cells transduced



**Figure 1** Proliferation of K562 cells transduced with hTERT, DN-hTERT (D868A, D869A) and a GFP-control vector. After transfection cells were sorted and cloned. For each vector four clones were expanded up to 4 months and every week cell counts were performed. In two of the four clones transduced with the DN-hTERT vector (squares and triangles), GFP expression could not be detected after several weeks in culture, suggesting that either the transgene was lost or silenced. The proliferation of these two clones was comparable to the proliferation of the GFP control. Two of the four clones (triangles and circles) transduced with the DN-hTERT died after 60 days (30 PDs) and 90 days (53 PDs). One clone (diamonds) exhibited a reduced proliferation while expressing a low level of GFP. In clone 1 (squares), GFP expression could not be detected after several weeks in culture, suggesting that either the transgene was lost or silenced.



**Figure 2** Microscopic appearance of clones 2 and 3 of K562 cells transduced with DN-hTERT (D868A, D869A) after 20 days of expansion. Cytopins of different clones were prepared and stained with Wright–Giemsa stain. As shown DN-hTERT (D868A, D869A)-transduced cells displayed anaphase bridges indicative of telomere dysfunction and apoptotic morphology. No changes relative to untransduced K562 cells were seen for GFP-control- or hTERT-transduced cells.

**Figure 3** Loss of telomere repeats in K562 cells transduced with DN-hTERT. K562 cells transduced with hTERT, DN-hTERT (D868A, D869A) and a GFP-control vector were sorted and cloned. The telomere fluorescence histograms of representative clones following flow FISH<sup>38</sup> are shown. Note, compared to the GFP-control, that the telomere fluorescence in DN-hTERT-transduced cells was lower, whereas the telomere fluorescence in hTERT-transduced cells was higher.

with DN-hTERT showed a reduced clonogenicity (frequency of proliferating clones from transduced cells immediately after sorting) compared to the GFP-control- or hTERT-transduced cells (DN-hTERT,  $20.9 \pm 4.2\%$ ; GFP,  $28.9 \pm 2.6\%$ ; hTERT,  $27.3 \pm 1.9\%$ ; mean  $\pm$  s.d. from two separate experiments). When DN-hTERT cells were sorted for high and low GFP expression, we observed that clones expressing high levels of GFP showed a reduced proliferation rate. No such effect was seen for clones with low GFP expression. These results suggest that GFP expression in cells transduced with the DN-hTERT gene corresponds to the level of transgene expression and suppression of telomerase activity. Two of four DN-hTERT-transduced clones (clone 2 and 3) died after, respectively, 90 days (53 PDs) and 60 days (30 PDs) in culture. Another clone showed reduced proliferation while still expressing a low level of GFP and another clone lost GFP expression altogether and continued to proliferate like the GFP control. Overexpression of hTERT in four K562 clones did not alter the proliferation compared to controls (Figure 1).

Morphological analysis of the DN-hTERT clones at days 14–20 after cloning revealed multiple anaphase bridges indicative of telomere dysfunction (Figure 2).<sup>31,39</sup> Furthermore, many cells showed morphological features typical of apoptosis, such as nuclear chromatin condensation, cell shrinkage and cell fragmentation into apoptotic bodies. GFP-control- and hTERT-transduced cells exhibited similar morphology as untransduced K562 cells.

To determine the telomerase activity in the transduced clones after 14 days in culture, we performed telomerase (TRAP) assays. Telomerase activity in DN-hTERT clones that later died in culture was severely reduced. Both the hTERT and the GFP-control clones contained high levels of telomerase activity. Telomere length analysis by flow-FISH revealed shorter telo-

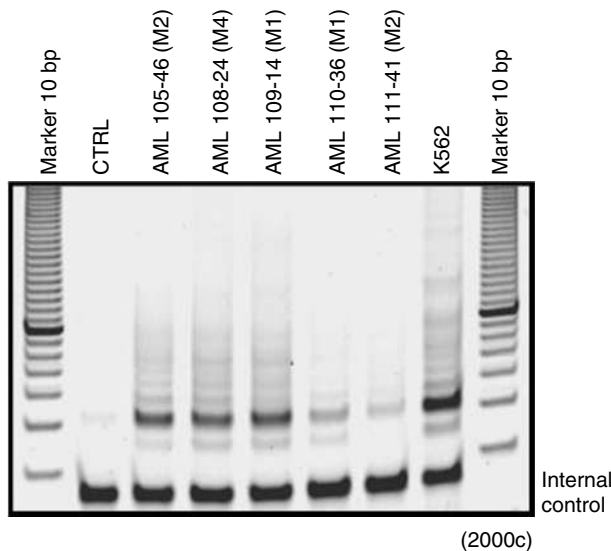
mere length in DN-hTERT clones compared to the GFP control (Figure 3). Interestingly, although telomerase activity appeared high in both the GFP-control and the hTERT clones, telomere length in the hTERT clones was significantly longer compared to the GFP-control clones ( $P=0.002$ ).

#### Transduction of primary AML cells with hTERT, DN-hTERT and GFP

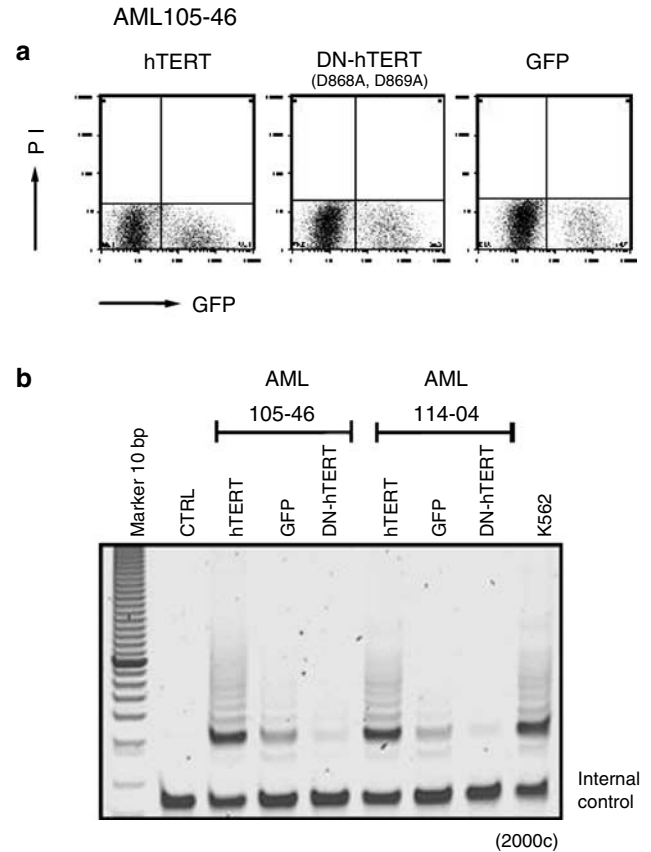
To determine the role of telomerase in primary AML cells, we determined telomerase activity in samples from 12 patients with various AML subtypes and cytogenetic abnormalities (Table 1). In all 12 samples, telomerase activity could be readily detected, although at variable levels (Figure 4). The primary AML cells from all patients were transduced with DN-hTERT and GFP control, and in eight cases also with the hTERT vector using a modification of our transfection protocol for human T-lymphocytes.<sup>40</sup> Using this strategy, gene transfer was obtained in all cases be it at variable efficiency ranging from 0.8 to 34.3%. Examples of results are shown in Figure 5a. No major differences in transduction efficiency between the three vectors were observed ( $10.7 \pm 9.0\%$  for the DN-hTERT,  $13.1 \pm 10.9\%$  for the hTERT and  $8.4 \pm 6.9\%$  the GFP control). After 5–6 days in culture, the transduced AML cells were sorted for GFP expression and a TRAP assay was performed to determine telomerase activity and the efficiency of gene transfer. High telomerase activity was detected in hTERT-transduced cells compared to GFP controls. In contrast, telomerase activity in DN-hTERT cells appeared to be reduced compared to the GFP controls (Figure 5b).

#### Telomerase activity in AML cell influences mean CFU frequency

To study the function of telomerase in AML progenitor cells, a known number of DN-hTERT or GFP-control cells were plated



**Figure 4** Telomerase activity in primary AML cells. Telomerase activity in  $2 \times 10^3$  AML blast after 2 days in culture was analyzed by TRAP assay. Negative controls using DEPC water and positive control extracts obtained from the cell line K562 were used in each experiment. All primary AML samples showed various degrees of telomerase activity.



**Figure 5** Effective transduction of primary AML cells. After transduction with retroviral vectors, AML cells were expanded for 10 days and analyzed for GFP expression. (a) GFP expression in AML cells from sample 105-46 transduced with hTERT, DN-hTERT and GFP. (b) AML samples 105-46 and 114-04 transduced with hTERT, GFP control and DN-hTERT (D868A, D869A) were analyzed for telomerase expression by TRAP assay ( $2 \times 10^3$  cells). Negative control using DEPC water and positive control extracts obtained from the cell line K562 were used.

into primary CFU and SC assays to determine the CFU and SC initiating cell content (SC-IC). In the primary CFU assays, colonies could be observed in all seven samples and no differences in CFU frequency between DN-hTERT- and GFP-control-transduced cells were observed (mean CFU frequency  $4.0 \times 10^3 \pm 4.3 \times 10^3$  vs  $2.8 \times 10^3 \pm 1.9 \times 10^3$  CFU/ $10^5$  cells, respectively). However, after 4 weeks in SC, colonies were observed in only two of seven samples transduced with DN-hTERT, while growth was observed in five of seven of GFP-control samples. In the two DN-hTERT samples (BC212 and NV253) in which growth was observed, the frequency of SC-IC was reduced in sample BC212, while in sample NV253 it was comparable to the GFP control (Figure 6). In two samples (GHN323 and IT369), no growth in either GFP-control- or DN-hTERT-transduced cells was observed. Overall, the CFU frequency after 4 weeks of suspension culture was  $7.9 \times 10^2 \pm 1.8 \times 10^3$  CFU/ $10^5$  cells in DN-hTERT-transduced cells compared to  $1.1 \times 10^4 \pm 2.7 \times 10^4$  CFU/ $10^5$  cells for the GFP control.

One AML sample (110-36) was transduced with hTERT, DN-hTERT and GFP control (Figure 6). The primary CFU assay again revealed no differences in primary CFU frequency for all three populations. After 2 weeks in SC, the CFU content of the DN-hTERT-transduced cells was reduced, while no colonies were

observed after 4 weeks in SC. In contrast, colonies could be observed following SC for up to 6 weeks in the hTERT cells and up to 4 weeks in the GFP-control cells.

*hTERT-transduced AML cells show high engraftment in  $\beta_2\text{-m}^{-/-}$  NOD/SCID mice*

Two primary AML samples (105-46 and 108-24) transduced with hTERT, DN-hTERT and GFP-control vectors were sorted for

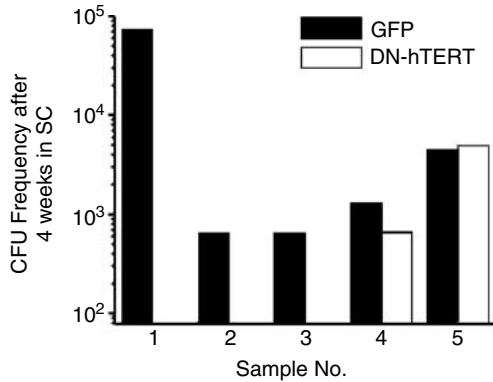
GFP expression, and similar cell numbers were transplanted into sublethally irradiated  $\beta_2\text{-m}^{-/-}$  NOD/SCID mice in duplicate (Table 2). BM was aspirated after 4 weeks and analyzed for engraftment of GFP<sup>+</sup> and CD45<sup>+</sup> cells. In general, higher engraftment levels were obtained using the hTERT-transduced AML cells compared to GFP-transduced cells, while AML cells transduced with DN-hTERT engrafted at lower levels compared to the GFP-control (Table 2). After 8 weeks, no human cells were observed in any of the mice except in one animal injected with hTERT-transduced cells from sample 105-46 (0.5%).

**Discussion and conclusions**

Activation or upregulation of telomerase is believed to play an important step in the progression of most human malignancies.<sup>41</sup> To study the effect and significance of telomerase activity in AML, we disrupted and overexpressed telomerase activity in the human leukemia cell line K562 as well as in various primary leukemia samples from different patients using retroviral vectors encoding hTERT, DN-hTERT (D868A, D869A) or GFP genes.

While telomerase levels in K562 and primary leukemia cells transduced with DN-hTERT were reduced, no direct obvious effect on the proliferation of these cells was observed. However, delayed effects were observed that appeared to be triggered by telomere dysfunction as cells exhibited anaphase bridges, apoptotic cell death and a reduced colony-forming ability several weeks after transfection and expansion.<sup>31</sup> The effect was specific to the inhibition of telomerase activity as the overexpression of wild-type hTERT, which differs from the mutant DN-hTERT (D868A, D869A) by two amino acids, did not affect viability or proliferation. In contrast, we observed an increase in the CFU content in hTERT-transduced AML cells as well as a higher engraftment into sublethally irradiated  $\beta_2\text{-m}^{-/-}$  NOD/SCID mice. Our results support the idea that telomerase activity is required for telomere maintenance and proliferation in leukemic cells.<sup>42</sup>

Interestingly, two of four K562 clones transduced with the DN-hTERT developed resistance to the effect of the DN-hTERT expression. One clone remained GFP<sup>+</sup> with a very low level of GFP expression, indicative of a very low expression of the transgene. Another clone completely lost GFP expression, possibly through gene silencing or loss of the transgene. These results suggest that inhibition of telomerase acts as strong selective disadvantage for telomerase-positive malignant cells. In view of the difficulty to inhibit telomerase activity using DN-hTERT and retroviral transduction strategies completely, our results may have underestimated the effect of telomerase inhibitors. More effective telomerase inhibition most likely will result in increased genomic instability due to telomere



Sample No.	Patient ID	Week0			Week4		
		GFP	DN-hTERT	hTERT	GFP	DN-hTERT	hTERT
1	DK367	4.8x10 <sup>3</sup>	5.2x10 <sup>3</sup>	ND	7.3x10 <sup>2</sup>	0	ND
2	110-36	8.3x10 <sup>1</sup>	6.7x10 <sup>1</sup>	8.8x10 <sup>1</sup>	6.5x10 <sup>2</sup>	0	2.5x10 <sup>3</sup>
3	VW372	1.9x10 <sup>3</sup>	2.4x10 <sup>3</sup>	ND	6.4x10 <sup>2</sup>	0	ND
4	BC212	4.4x10 <sup>3</sup>	3.8x10 <sup>1</sup>	ND	1.3x10 <sup>3</sup>	6.5x10 <sup>2</sup>	ND
5	NV253	3.0x10 <sup>3</sup>	2.8x10 <sup>3</sup>	ND	4.4x10 <sup>3</sup>	4.9x10 <sup>3</sup>	ND
6	GHN323	1.9x10 <sup>1</sup>	3.0x10 <sup>1</sup>	ND	0	0	ND
7	II360	4.5x10 <sup>3</sup>	1.3x10 <sup>1</sup>	ND	0	0	ND
Mean		2.8x10 <sup>3</sup>	4.0x10 <sup>3</sup>	8.8x10 <sup>1</sup>	1.1x10 <sup>4</sup>	7.9x10 <sup>2</sup>	2.5x10 <sup>3</sup>
SD		1.9x10 <sup>3</sup>	4.3x10 <sup>3</sup>		2.7x10 <sup>4</sup>	1.8x10 <sup>3</sup>	

**Figure 6** DN-hTERT-transduced AML cells show reduced CFU frequencies 4 weeks after SC. Cells were sorted for GFP expression and a known number of GFP-positive cells were subjected to primary CFU assays (week 0) and SC assays. Every 2 weeks, half of the cells were plated in methylcellulose and colonies were counted at day 10 to calculate the progenitor frequency/10<sup>5</sup> cells. The CFU frequency of the AML cells from the five patients in which growth was observed are shown. The table shows the progenitor frequencies of the primary CFU assay and the progenitor frequencies after 4 weeks in SC. Cells from one patient (110-36) were transduced with hTERT in addition to GFP and DN-hTERT. The hTERT-transduced showed a higher progenitor frequency compared to the GFP control.

**Table 2** NOD/SCID assay using two AML samples

Patient sample	Numbers of cells injected ( × 10 <sup>5</sup> )	% of CD45 expression at week 4		
		GFP	hTERT	DN-hTERT
105-46	50	5.6	11.3	2.6
	50		9.6	1.4
	36	5.3		
108-24	1.5	10.5		
	1.25	7.4	12	3.6
	0.7	2.9	2.6	

dysfunction and may possibly favor selection of cells that can bypass the effect of telomerase inhibition.<sup>43</sup>

The transfection of primary AML progenitor cells with retroviral vectors has been problematic.<sup>44,45</sup> This is the first report of effective transfection of AML progenitor cells with retroviral vectors for hTERT and DN-hTERT (D868A, D869A). The protocol described in this study may find application to mark genetically AML cells and to modify leukemia cells to test novel therapeutic approaches and generate tumor vaccines.<sup>44–49</sup> Further improvements in transfection efficiency could probably be obtained by targeting noncycling cell populations using lentiviral vectors.<sup>50</sup>

Our results support the notion that telomerase activity in AML is associated with disease progression and that high levels of telomerase activity indicate a poor prognosis.<sup>19,20,51–53</sup> As telomerase activity appears largely restricted to malignant cells, telomerase activity measurements may help with the prognosis and the evaluation of antileukemic treatments and disease progression. The value of telomerase inhibition in leukemia treatment needs to be established in clinical trials in combination with standard antileukemic agents.<sup>42,54,55</sup>

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