

Accumulation and altered localization of telomere-associated protein TRF2 in immortally transformed and tumor-derived human breast cells

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Abstract

We have used cultured human mammary epithelial cells (HMEC) and breast tumor-derived lines to gain information on defects that occur during breast cancer progression. HMEC immortalized by a variety of agents (the chemical carcinogen benzo(a)pyrene, oncogenes *c-myc* and *ZNF217*, and/or dominant negative p53 genetic suppressor element GSE22) displayed marked up-regulation (10-15 fold) of the telomere binding protein, TRF2. Up-regulation of TRF2 protein was apparently due to differences in post-transcriptional regulation, as mRNA levels remained comparable in finite life span and immortal HMEC. TRF2 protein was not up-regulated by the oncogenic agents alone in the absence of immortalization, nor by expression of exogenously introduced hTERT genes. We found TRF2 levels to be at least 2-fold higher than in control cells in 11/15 breast tumor cell lines, suggesting that elevated TRF2 levels are a frequent occurrence during the transformation of breast tumor cells *in vivo*. The dispersed distribution of TRF2 throughout the nuclei in some immortalized and tumor-derived cells indicated that not all the TRF2 was associated with telomeres in these cells. The process responsible for accumulation of TRF2 in immortalized HMEC and breast tumor-derived cell lines may promote tumorigenesis by contributing to the cells' ability to maintain an indefinite life span.

Telomeres, the nucleoprotein structures that cap the ends of the eukaryotic chromosomes, are critical for chromosomal integrity. Composed of TTAGGG DNA repeats bound by a complex of proteins, these specialized structures protect the chromosome ends from exonucleolytic attack and fusion. Due to the “end replication problem,” in the absence of telomerase - a specialized enzyme that maintains telomeric DNA, telomeres are eroded with successive cell divisions. When telomeres become critically eroded, the ensuing telomere dysfunction can produce genomic instability (Feldser et al., 2003), resulting in growth suppression or cell death (Kim et al., 2002; Maser & DePinho, 2002). Under normal circumstances, the finite life span conferred by telomerase repression in human cells limits the number of mutations that can accumulate in a single cell lineage, and serves as a stringent block to tumorigenesis. However, in rare circumstances of induced or inborn genetic errors, one or more mutations predisposing to immortality may arise in a single lineage prior to attaining critically short telomeres (Maser & DePinho, 2002; Yaswen & Stampfer, 2002). In such situations, the genomic instability induced by telomere dysfunction may promote transformation by producing further changes that complement the pre-existing mutations in conferring immortal potential.

Binding of TRF1 and TRF2 proteins and their interacting partners to the telomeric repeats is thought to reorganize the linear chromosome terminus into a protective t-loop structure, in which the G strand invades the duplex part of the telomere (Griffith et al., 1999). TRF2 binding near the 3'-overhang is considered crucial to the formation and stability of t-loops. Interference with TRF2 function by over-expression of a dominant negative form of TRF2 results in telomere dysfunction, genomic instability, and a proliferative growth arrest with features characteristic of senescence (Karlseder et al., 1999). On the other hand, artificially over-expressed TRF2 has been

reported to delay senescence (Karlseder et al., 2002). Little is known, however, about expression of endogenous TRF2 during human cell transformation.

Generation of immortally transformed human mammary epithelial cell (HMEC) lines

HMEC cultured from normal breast tissue display a finite life span, low or undetectable telomerase activity, and decreasing telomere length with passage (Stampfer & Yaswen, 2003). HMEC can spontaneously overcome a first RB-mediated, non-telomere length dependent proliferative arrest (stasis), associated with down-regulation of p16 expression (Brenner et al., 1998). The resultant p53(+), p16(-) post-selection HMEC cease net proliferation when their mean terminal restriction fragment (TRF) length is ~5 kb. As cells approach this second proliferative barrier, telomere dysfunction is evidenced by the presence of widespread chromosomal aberrations, particularly telomeric fusions, and mitotic failures (Romanov et al., 2001). In the p53(+) cultures, most cells remain viably arrested at all phases of the cell cycle, a growth arrest termed agonescence (Romanov et al., 2001). When p53 is inactivated, populations display the massive cell death typical of crisis (J. Garbe et al., submitted). Rare p53(+) and p53(-) immortal HMEC lines have been obtained following exposure to chemical carcinogens, over-expression of *c-myc* or *ZNF217* oncogenes, and/or a dominant negative p53 genetic suppressor element, GSE22 (Figure 1A) (Nonet et al., 2001; Stampfer & Bartley, 1985; Stampfer et al., 2003). Surprisingly, the newly immortal p53(+) lines initially show very low or undetectable telomerase activity and continue to divide with increasingly shortened mean TRF lengths. When the mean TRF length gets extremely short (<3 kb), growth becomes slow and heterogeneous. An extended process, termed conversion, ensues, during which telomerase activity and growth capacity gradually increase (Stampfer et al., 1997). In contrast, newly immortal p53(-) lines

quickly display telomerase activity (Stampfer et al., 2003). Recent studies have indicated that p53 is able to repress the expression of endogenous hTERT, the catalytic subunit of telomerase, in newly immortal lines; this repression is relieved during the process of conversion (Stampfer et al., 2003). Although telomerase activity remains very low until conversion, this low activity may be responsible for the observation that, unlike cells at agonescence, newly immortal p53(+) lines can continue to divide without exhibiting gross chromosomal instability (Walen & Stampfer, 1989).

TRF2 protein levels undergo large increases in immortally transformed HMEC

Protein lysates harvested from actively proliferating finite life span and immortal HMEC lines were examined for expression of telomere-associated proteins TRF2, TIN2, hRAP1, and TRF1. Immunoblots of total cellular protein probed with a specific monoclonal antibody to TRF2 indicated that four independently derived immortal HMEC lines (184A1, 184AA2, 184AA3, and 184AA4) displayed markedly increased levels of the 65/69 kD TRF2 doublet (Bilaud et al., 1997) compared to their carcinogen-treated extended life precursor strain, 184Aa (Figure 1B). A fifth immortal HMEC line, 184B5, derived from an independent carcinogen-treated extended life strain, 184Be, showed the same pattern. In contrast, levels of telomere-associated proteins, Tin2, hRap1, and TRF1 showed little differences in the same cultures (Figures 1B-D, and data not shown). The normalized levels of TRF2 protein observed in the immortal lines ranged from 10-15 times the levels present in the 184Aa precursor strain. Interestingly, the newly immortal, pre-conversion 184A1 line, with low or undetectable telomerase activity, displayed intermediate levels of TRF2 protein. The TRF2 levels were higher in the immortalized cells regardless of whether the cells were actively cycling or growth arrested in G0 by blockage of EGFR signal

transduction (Figure 1D) (Stampfer et al., 1993). Levels of TRF2 protein were approximately equivalent in independently derived finite life span strains 184 and 161, and in extended life strains 184Be and 184Aa (Figures 1D&E). A second TRF2 polyclonal antibody yielded identical results (data not shown).

DNA damage induced by irradiation and etoposide has been reported to induce the temporary accumulation of TRF2 mRNA in human promyelocytic HL60 cells (Klapper et al., 2003; Nakagami et al., 2002). To determine whether the increased levels of TRF2 protein observed in immortalized HMEC correlated with increased TRF2 transcript levels, total RNA from growing HMEC cultures was subjected to northern blot analysis. Unlike the large differences detected in TRF2 protein levels, differences in TRF2 mRNA levels were fairly small and did not correlate with the differences in protein levels (Figure 1F). The lack of correspondence between mRNA and protein differences suggests that variations in post-transcriptional regulation of TRF2 protein abundance exist among finite life span and immortalized HMEC. Inhibition of de novo protein synthesis using cycloheximide indicated that the half-life of TRF2 protein was greater than 12 hours in both finite life span 184 and fully immortal 184A1 HMEC (Supplementary Figure 1G). Although this experiment did not rule out differences in stability, it indicated that TRF2 levels are not regulated by rapid turnover, even under normal conditions. Thus, the difference in TRF2 protein accumulation is unlikely to be due to a simple change in stability, and is more likely to be due to changes in synthesis, modification, and/or compartmentalization.

Immortalizing factors or telomere dysfunction do not by themselves directly affect TRF2 levels

We next asked whether treatment with any of the immortalizing factors by themselves was sufficient to cause up-regulation of TRF2. We compared finite life span cells treated with four different immortalizing agents (the chemical carcinogen benzo(a)pyrene $\pm$ retroviral introduction of the dominant negative inhibitor of p53 function, GSE22 (Ossovskaya et al., 1996), the *c-myc* oncogene, or the *ZNF217* oncogene) with the immortal cell lines derived from these cultures following exposure to these agents. In all cases, the low level of TRF2 expression seen in unexposed finite life span HMEC was not significantly increased in the finite life span cultures that had been exposed to these agents (Figure 2A). In contrast, TRF2 protein levels were increased in the resulting immortally transformed lines. The level of TRF2 was also not significantly increased in cultures when they reached agonescence (Figure 2B). Since agonescence is associated with telomere dysfunction, end-to-end fusions, and genomic instability, these results indicate that TRF2 protein levels in HMEC are not stably elevated in response to telomere dysfunction alone.

Expression of exogenously introduced hTERT does not lead to increased TRF2 levels

During its conversion to full immortality, the immortal 184A1 line displayed slowly increasing expression of endogenous hTERT and telomerase activity (Stampfer et al., 1997, Stampfer et al., 2001, Stampfer et al. 2003). Transduction of exogenous hTERT into post-selection finite life span 184 HMEC or immortal 184A1 (before, during, and after conversion) produced rapid elevation of telomerase activity, telomere elongation, and acquisition of an indefinite life span (Stampfer et al., 2001). To determine directly whether increased TRF2 expression might be a

consequence of the expression of hTERT, the hTERT-transduced 184 and 184A1 cultures were assayed for TRF2 expression. TRF2 levels were not increased in 184 HMEC immortalized by hTERT transduction (Figure 2C). TRF2 levels also remained low in the early passage 184A1 line transduced with hTERT prior to conversion, a manipulation that circumvents the slow, heterogeneous growth phase associated with conversion (Stampfer et al., 2001). TRF2 levels in 184A1 cells transduced with hTERT during or after conversion to the fully immortal phenotype were consistent with the levels present at the time of transduction, and did not appear to be affected by the presence of added hTERT. Thus, over-expression of exogenously introduced hTERT did not influence TRF2 protein levels.

TRF2 levels are elevated in many breast tumor-derived cell lines

To start to determine the relevance of TRF2 elevation to human breast cancer, we compared TRF2 levels in common breast tumor cell lines with those in finite life span HMEC (Figures 1E and 2D). Protein lysates were prepared from randomly cycling cells and analyzed by immunoblotting. We found TRF2 levels to be at least 2-fold higher in breast tumor cell lines than in the control 184 cells in 11/15 lines examined, indicating that elevated TRF2 levels are a frequent occurrence in breast tumor cell lines. Levels of TRF2 protein in these tumor lines did not correlate with the relative levels of mRNA from the same lines (Figure 1F).

Exogenously introduced TRF2 affects the proliferative life span of post-selection HMEC

When artificially over-expressed in human diploid fibroblasts, TRF2 has been reported to bind ATM kinase and repress cellular responses to genome-wide DNA damage (Karlseder et al., 2004). To directly determine the consequences of increased TRF2 expression for growth and

immortalization of post-selection HMEC, additional copies of the TRF2 gene under control of the CMV promoter were retrovirally introduced into finite life span 184 HMEC alone, or with the dominant negative p53 element, GSE22. High expression levels of TRF2 protein were confirmed by immunoblotting (data not shown). The number of cumulative population doublings (PD) prior to agonescence was modestly increased compared to controls in post-selection 184 transduced with TRF2 (Figure 3A), similar to results previously reported for human diploid fibroblasts (Karlseder et al., 2002). We also obtained similar results using a second vector in which TRF2 expression was driven by a retroviral LTR instead of the CMV promoter in order to achieve lower TRF2 levels more consistent with endogenous levels observed in immortal cell lines (data not shown). Since TRF2 is a crucial stabilizing component of the protective t-loop structure (Griffith et al., 1999), up-regulated TRF2 may provide increased stability when the telomeres are relatively short. Over-expressed TRF2 may postpone telomere dysfunction by providing added protection to the telomeric ends (i.e., additional telomere erosion may be required to produce telomere dysfunction and the signal for p53-dependent growth arrest). In contrast, transduction of 184-GSE22 cells with TRF2 did not affect the number of cumulative PD achieved (Figure 3B). The inability of TRF2 over-expression to further increase the cumulative PD in cells with compromised p53 was also reported in fibroblasts (Karlseder et al., 2002). This data suggests that the increased telomere protection conferred by over-expressed TRF2 is short-lived, and does not interfere with p53-independent events that ultimately result in crisis.

TRF2 localization is abnormal in immortal HMEC and breast tumor cell lines

Indirect immunofluorescent studies with the anti-TRF2 antibodies revealed a punctate nuclear pattern in all interphase post-selection 184 HMEC, similar to that first reported in HeLa cells (Broccoli et al., 1997). However, in immortal HMEC, TRF2 immunofluorescence was heterogeneous both in abundance and localization (Figure 4). Both 184A1 and 184AA2 displayed gradations in nuclear size and TRF2 protein expression levels. Cells with smaller nuclei showed quantities and punctate distributions of TRF2 similar to those found in finite life span cells, where TRF2 co-localized with Tin2. However cells with larger nuclei had correspondingly high levels of TRF2 spread throughout the nuclei (although a portion of TRF2 remained co-localized with Tin2). A gradient of cells with intermediate characteristics was also observed. Tin2 levels and localization were similar in finite life span and immortal HMEC regardless of nuclei sizes or differences in TRF2 levels and localization. Tumor cell lines, T47D and BT474, with high levels of TRF2 on immunoblots, uniformly displayed TRF2 dispersed throughout the nuclei in essentially all cells, indicating a lack of dependence on cell cycle status. A contrasting tumor cell line MDA435, which displayed low levels of TRF2 by immunoblotting, uniformly displayed TRF2 in the punctate pattern typical of finite life span HMEC. Both TRF2 antibodies used in these studies yielded essentially the same results.

TRF2 protein abundance is increased in some aberrant breast tissues

Initial immunohistochemical experiments were performed using sectioned formalin-fixed, paraffin-embedded human breast tissues provided by the UCSF Cancer Center Tissue Core and the Breast Oncology Program. These experiments, performed with two different monoclonal anti-TRF2 antibody preparations, showed obvious positive staining in epithelial cell nuclei in

some areas of DCIS and invasive breast cancers (Figure 5). Staining of stromal and normal ductal epithelial cells in these sections was noticeably weaker. A comprehensive statistical analysis of TRF2 expression in clinical specimens is currently being implemented and will be fully described in a separate report.

The mechanism responsible for the up-regulation of endogenous TRF2 in immortalized HMEC remains to be determined, but may involve altered post-translational modifications and/or protein interactions since mRNA levels are unaffected. Blackburn has proposed a model in which telomeres exist in two interchangeable states, an open accessible form and a closed protected form (Blackburn, 2000). Evidence suggests that TRF2 binding to TTAGGG repeats promotes formation of the closed protected form. When eroded telomeres become critically shortened, the ends on one or more chromosomes may lack sufficient TTAGGG repeats to stably bind TRF2, thereby limiting formation of the protective t-loop structure and allowing loss/degradation of the 3' overhang. Proteins involved in DNA double strand break recognition and repair may participate in the cellular response to persistent unprotected telomeric structures. Normally, such structures may be resolved by progression of the associated repair pathways, including DNA ligase IV-dependent non-homologous end-joining of unprotected telomeres (Smogorzewska et al., 2002). TRF2 has been reported to bind to several proteins involved in double strand break recognition and repair, including the Rad50-MRE11-NBS1 complex (Zhu et al., 2000), ATM (Karlseder et al., 2004), as well as the RecQ helicases - WRN and BLM (Opresko et al., 2002). These proteins may respond to particular telomeric structures by interacting with and stabilizing or destabilizing TRF2 protein. However, in some cases, it is possible that molecular defects inhibit the resolution of the intermediates, causing accumulation of TRF2 protein. Alternatively,

molecular defects in HMEC undergoing immortalization may cause up-regulation of TRF2 protein independently of telomere dysfunction. The dispersed distribution of over-expressed TRF2 throughout the nuclei in some immortalized and tumor-derived cells indicates that not all the TRF2 is associated with telomeres in these cells. It will be of interest to determine what other proteins are associated with TRF2 in such cells, and whether association with TRF2 inhibits or augments their functions.

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Legends to Figures

Figure 1. TRF2 protein is significantly increased in immortally transformed HMEC. (A) Scheme showing generation of immortal HMEC lines. Primary cultures of 184 HMEC exposed to the chemical carcinogen benzo(a)pyrene [B(a)P] gave rise to extended life span cultures lacking p16 expression. Rare immortally transformed lines emerged from extended life span 184Aa or 184Be either spontaneously, following insertional mutagenesis, inactivation of p53 function, and/or transduction of breast cancer-associated oncogene *ZNF217*. Rare immortally transformed lines emerged from unexposed post-selection p16(-) 184 HMEC following transduction of breast cancer associated oncogene *c-myc*. See text and web site (www.lbl.gov/~mrgrs/mindex.html) for more details. (B) Representative immunoblot showing up-regulation of TRF2 protein in independently derived immortal HMEC lines. Total cell lysates were prepared from randomly cycling sub-confluent cultures of HMEC by lysing the cells with 2X SDS sample buffer. 50 μ g of protein samples were resolved on polyacrylamide gradient gels and electroblotted to nylon membranes and probed with antibodies to TRF2 (IMG-124; Imgenex, San Diego, CA or SC-9143; Santa Cruz Biotech., Santa Cruz, CA) or TIN2 (gift of J. Campisi, LBNL). Gel loading equivalence and blotting efficiency were determined by staining the blots with Ponceau S (Helena Labs, Beaumont, TX) and/or probing with an antibody to beta-actin. The asterisk associated with 184A1* indicates that these cells were harvested at an early pre-conversion passage which shows low or negligible telomerase activity. (C) Quantitation of the immunoblot shown in (B). Pixel densities for TRF2 and TIN2 bands were divided by those for the control bands, and plotted relative to the levels in 184Aa. (D) Immunoblot showing up-regulation of TRF2 protein in growth-arrested (G0) as well as actively cycling immortal 184A1 relative to finite life span post-selection 184 and carcinogen-treated extended life 184Aa HMEC. 184A1

14p is pre-conversion and grows well, while 184A1 19p has begun the conversion process and shows poor growth. Cells were growth-arrested as described (Stampfer et al., 1993). Relative levels of telomere-associated proteins TIN2 and RAP1 were analyzed using anti-hRAP (IMG-272; Imgenex), or anti-TIN2 antibodies and are shown for comparison. (E) Immunoblot showing relative levels of TRF2 protein in two finite life span post-selection (184 and 161), two carcinogen-treated extended life (184Be and 184Aa), and one fully immortal (184A1) HMEC, as well as two human breast tumor cell lines (MDA468, and T47D). Note that TRF2 can be detected in lysates of the extended life cultures in this longer exposure. (F) Northern blot showing relative levels of TRF2 mRNA in the HMEC described above as well as three human breast tumor cell lines (MDA436, MDA468, and Hs578T). Northern blots were prepared using 10 μ g of total RNA per sample as described previously (Nijjar et al., 1999). The blots were hybridized to a 32 P-labeled, 1200-bp EcoR1:Xho1 TRF2 cDNA probe. The TRF2 signal was measured using a phosphoimager and quantitative comparisons of TRF2-specific signals were performed using the ImageQuant software program. The ratios of signal intensities for the main TRF2 transcript divided by that of the ethidium bromide stained 18S rRNA are presented.

Figure 2. Inactivation of p53, or introduction of *c-myc*, *ZNF217*, or hTERT, or agonescence, do not by themselves directly affect TRF2 levels. TRF2 levels are elevated in a subset of breast tumor derived cell lines. (A) Immunoblots showing total TRF2 protein in post-selection 184, carcinogen-treated extended life 184Aa, and immortalized HMEC at indicated passages (p), after transduction with dominant negative p53 genetic suppressor element (GSE-22), oncogene *c-myc*, or oncogene *ZNF217*. Cells shown at passage levels ≤ 17 p are finite life span; cells at passage

levels $\geq 23p$ are immortal. The 184Aa 17p sample shown is from an agonescent culture. (B) Immunoblot showing total TRF2 protein in good growing post-selection 184 9p and during agonescence. 184 16p and 48RS 25p were agonescent. (C) Immunoblot showing total TRF2 protein in post-selection 184 and immortal 184A1 alone or after transduction with hTERT at indicated passage levels. Passage numbers in parenthesis refer to the passage at which the cells were retrovirally infected. (D) TRF2 levels are high in some breast tumor derived cell lines. Protein samples were analyzed by immunoblotting as described in the legend to Figure 1. Quantification of immunoblots is shown. Signal intensities for TRF2 bands were divided by those for the control bands, and plotted relative to the levels in 184.

Figure 3. Effect of exogenously introduced TRF2 genes on cumulative population doublings achieved prior to agonescence/crisis. To subclone TRF2 into the retroviral vector pBabe (Morgenstern & Land, 1990), for LTR driven expression, a 1500bp cDNA fragment encompassing the entire open reading frame was excised with BamH1:EcoR1 from the pLPC.TRF2 vector and subcloned into the BamH1 – EcoR1 site of pBabe.Pu (Morgenstern & Land, 1990). The derivation of other retroviruses has been described (Nonet et al., 2001; Ossovskaya et al., 1996; Stampfer et al., 2001). Post-selection 184 HMEC were transduced with retroviruses encoding TRF2 or empty vector (CON) (A) alone or (B) with a dominant negative inhibitor of p53 function (GSE) and selected in 0.5 μ g/ml puromycin. The transduced cells were grown in the presence of selective drugs to confluence, then replated in triplicate at a fixed density of 1×10^5 /60mm dish. The total number of cells harvested at every subculture was calculated and the number of accumulated population doublings (PD) per passage determined

using the equation, $PD=(A/B)/\log 2$, where A is the number of harvested cells, and B is the number of plated cells, not corrected for plating efficiency. Experiments were terminated when the cultures failed to achieve confluence within 3 weeks. Each experiment was repeated three times and in each case representative data from one experiment is shown.

Figure 4. TRF2 is dispersed throughout the nuclei in some immortal HMEC and breast tumor cell lines. Cells were grown on 4-well chamber slides and fixed with 4% formalin. The slides were incubated with TRF2 and TIN2 specific antibodies at 5 μ g/ml concentrations and then incubated with Texas Red conjugated anti-mouse IgG for TRF2 (red) and FITC conjugated anti-rabbit IgG antibodies for TIN2 (green). DNA was stained with DAPI (blue) in the merged images. Stained cells were visualized using an Olympus BX51 microscope equipped for epifluorescence. Representative interphase nuclei are shown for each culture. In cases where TRF2 immunofluorescence was heterogeneous (184A1 48p and 184AA2 44p), representative nuclei containing both diffuse and punctate TRF2 distributions are shown.

Figure 5. Immunohistochemical staining indicates that TRF2 protein abundance is increased in some aberrant breast tissues. A-C; Area of ductal carcinoma in situ (DCIS) stained with (A) non-immune IgG, (B) anti-TRF2, (C) DAPI. Note the positive staining in epithelial cells, but not in stromal cells. (D) Normal duct stained with anti-TRF2. (E) DCIS stained with anti-TRF2. (F) Higher magnification of individual duct shown in (E). Note the heterogeneous staining.