Rescue of a telomere length defect of Nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit

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Nijmegen breakage syndrome (NBS) is a rare human disease displaying chromosome instability, radiosensitivity, cancer predisposition, immunodeficiency, and other defects [1, 2]. NBS is complexed with MRE11 and RAD50 in a DNA repair complex [3–5] and is localized to telomere ends in association with TRF proteins [6, 7]. We show that blood cells from NBS patients have shortened telomere DNA ends. Likewise, cultured NBS fibroblasts that exhibit a premature growth cessation were observed with correspondingly shortened telomeres. Introduction of the catalytic subunit of telomerase, TERT, was alone sufficient to increase the proliferative capacity of NBS fibroblasts. However, NBS, but not TERT, restores the capacity of NBS cells to survive γ irradiation damage. Strikingly, NBS promotes telomere elongation in conjunction with TERT in NBS fibroblasts. These results suggest that NBS is a required accessory protein for telomere extension. Since NBS patients have shortened telomeres, these defects may contribute to the chromosome instability and disease associated with NBS patients.

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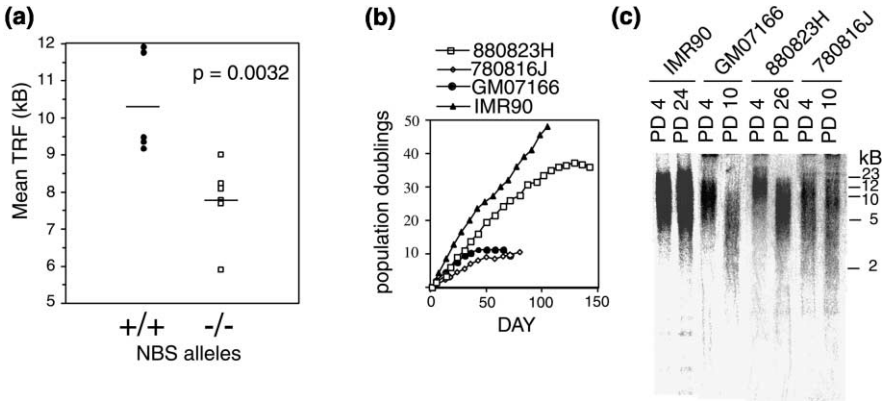
Results and discussion

We investigated whether the vulnerability of Nijmegen breakage syndrome (NBS) cells to chromosome instability could be associated with altered telomere dynamics. Blood mononuclear cell DNAs were isolated from affected patients and their family members for the typical 657del5 NBS frameshift mutation [8] and from unaffected families. These samples were analyzed by restriction enzyme digestion, fractionation by gel electrophoresis, and *in gel* hybridization to telomere repeats using a telomere-specific probe as previously described [9]. We examined samples from NBS patients with an age ranging from 7 to 18 years and unaffected individuals ranging from 9 to 46 years of age. Quantitation of the Terminal Restriction Fragment (TRF) differences indicated that the telomeric DNA for NBS patients was significantly shorter. NBS patients yielded a mean TRF of 7.77 kb compared with 10.32 kb for normal patients ($p = 0.0032$; Figure 1a). NBS heterozygotes were not significantly different from the TRF values for the unaffected patients; although, the data was insufficient to monitor TRF as a function of age (data not shown). These results are suggestive of a strong correspondence between reduced telomere length and the diverse clinical symptoms in NBS patients, including a profound rate of malignancies at an early age [10, 11]. Previous indications of errors in telomere metabolism have been observed with ataxia telangiectasia and Werner syndrome patients, also having chromosome anomalies and growth deficiencies [12, 13].

Evidence of shortened telomeres may be attributable to undue expansion of NBS peripheral lymphoid cells prompted by frequent infections or may be formed by an intrinsic defect in telomere regulation. To study potential dynamics of telomere length modulation per se, we investigated primary NBS fibroblasts obtained relatively early in passage history, (GM07166-Passage 8; 880823H-Passage 5 and 780816J-Passage 12). GM07166 and 780816J fibroblasts grew poorly and reached senescence after ~10 population doublings (PD) (Figure 1b). In keeping with the limited proliferative potential, significant telomere decay was already evident for GM07166 and 780816J cells relatively early in their passage history (Figure 1c). 880823H cells had longer telomeres and a correspondingly increased proliferative capacity. Yet, at >25 PD (population doubling), 880823H cells yielded shorter TRF and

Figure 1



Decreased telomere length and poor proliferation in Nijmegen breakage syndrome cells. **(a)** Peripheral blood mononuclear cell genomic DNA was isolated, and telomere lengths were assessed by *in gel* hybridization using a radiolabeled telomere repeat probe, $(C_3TA_2)_3$. A comparison of mean Terminal Restriction Fragment (TRF) from NBS patients and unaffected individuals ($NBS^{+}/^{+}$) is shown. **(b)** Primary fibroblasts from three NBS patients and an unaffected individual were compared for population doubling over time. The three NBS cultures (GM07166, 780816J, and 880823H) had poorer growth properties than the control culture (IMR90). **(c)** Cell cultures at early and late passage were compared for telomere TRF by *in gel* hybridization.

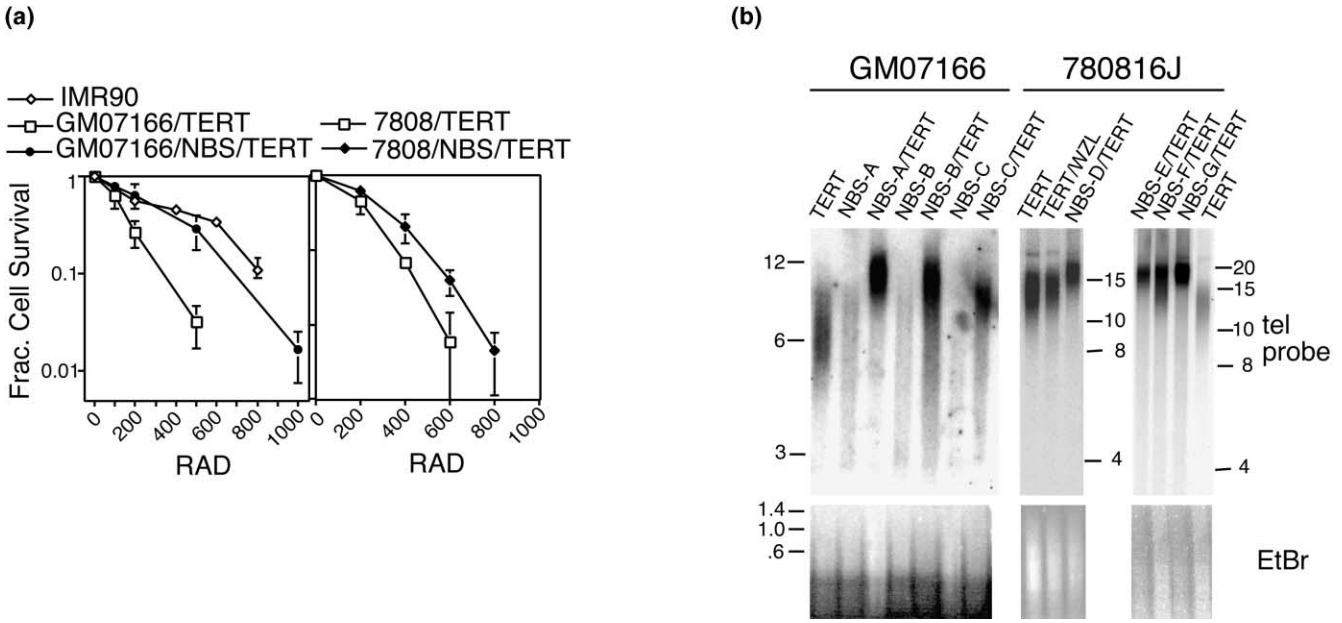
reduced the proliferative capacity, in contrast to wild-type fibroblasts, IMR90 (Figure 1b). Therefore, 880823H cells also reveal an NBS-related defect after extended passaging. For early passage of GM07166 and 880823H cultures, the doubling times were equivalent to that of IMR90 cells, indicating that the NBS cells had not undergone more cell divisions (data not shown). We conclude that NBS fibroblasts have evidence of premature telomere malfunction. Thus, it is possible that the observed reduction in telomeric DNA for NBS mononuclear blood cells may contribute to the poor proliferative immune responses of these patients [14].

The absence of NBS may hamper telomere metabolism in diverse ways. NBS-containing complexes and DNA repair subunits of the DNA-dependent protein kinase have been shown to localize to telomeres, and NBS is capable of interacting with telomere-asssociated TRF proteins [6, 7]. Likewise, patients with ataxia telangiectasia, ATM, exhibit telomeric repeat shortening in blood cells [15], and NBS is regulated by ATM protein kinase phosphorylation in DNA damage responses [16, 17]. As telomerase is likely to function coordinately with other proteins, NBS may positively facilitate the telomere-lengthening process in steps such as t-loop formation and function or cell cycle-specific associations [6]. Therefore, to study the role of NBS in telomere regulation, we introduced the NBS gene into NBS fibroblasts via retroviral transduction as previously described [17, 18] (see Supplementary material available with this article online). As these cells do not express telomerase activity, TERT was delivered on a second retroviral vector. Thus, the four resulting NBS cell lines with the following expression profiles could be compared ($NBS^{-}/TERT^{-}$, $NBS^{-}/TERT^{+}$, $NBS^{+}/TERT^{-}$, and $NBS^{+}/TERT^{+}$) at similar passage doublings. IMR90 cells were also transduced with TERT or a control virus (pBABEneo). The presence or absence of NBS did not alter in vitro telomerase activity scored with the TRAP assay (data not shown).

NBS cells are hypersensitive to gamma irradiation (γR) [1], reflective of DNA repair or DNA damage signaling defects. We observed that $NBS^{+}/TERT^{+}$ cells derived from GM07166 or 780816J fibroblasts had significantly elevated the γR cell survival properties (Figure 2a). In contrast, $TERT^{+}$ cells from either patient source remained radiosensitive. GM07166 $NBS^{+}/TERT^{-}$ cells were also found to reconstitute normal survival following γR, but 780816J $NBS^{+}/TERT^{-}$ cells could not be established (data not shown). Therefore, restoration of NBS expression reconstitutes the DNA repair and damage signaling defects of NBS cells.

NBS fibroblasts with added TERT and/or NBS were next evaluated for telomere extension. The presence of NBS or TERT alone had no restorative effect on telomere length (Figure 2b). Strikingly, the coexpression of NBS and TERT generated telomere lengths significantly greater than the parental values for three independent infections for the two cell lines (Figure 2b). For the GM07166 $TERT^{+}$ cells, TRF values were increased from 7.35 to 11.71 ($p = <0.015$) by the addition of NBS. Likewise, TRF values were as follows for 780816J (7.9 kb), 780816J $TERT^{+}$ (11.5 kb), 780816J $TERT^{+}$ WZL (11.9 kb), and 780816J $NBS^{+}/TERT^{+}$ (16.6 kb). NBS or WZL denotes the infection of 780816J $TERT^{+}$ with either NBS or control retrovirus (see Materials and methods). Differences between $TERT^{+}$ and $NBS^{+}/TERT^{+}$ cells were highly significant ($p = <0.0001$). Thus, NBS promotes telomere elongation in conjunction with telomerase. Equal loading of DNAs was shown by ethidium bromide staining. Quantitation of terminal repeats using a PNA/telomere repeat probe and Flow-FISH [19] (data not shown) indicated the development of longer telomeres

Figure 2



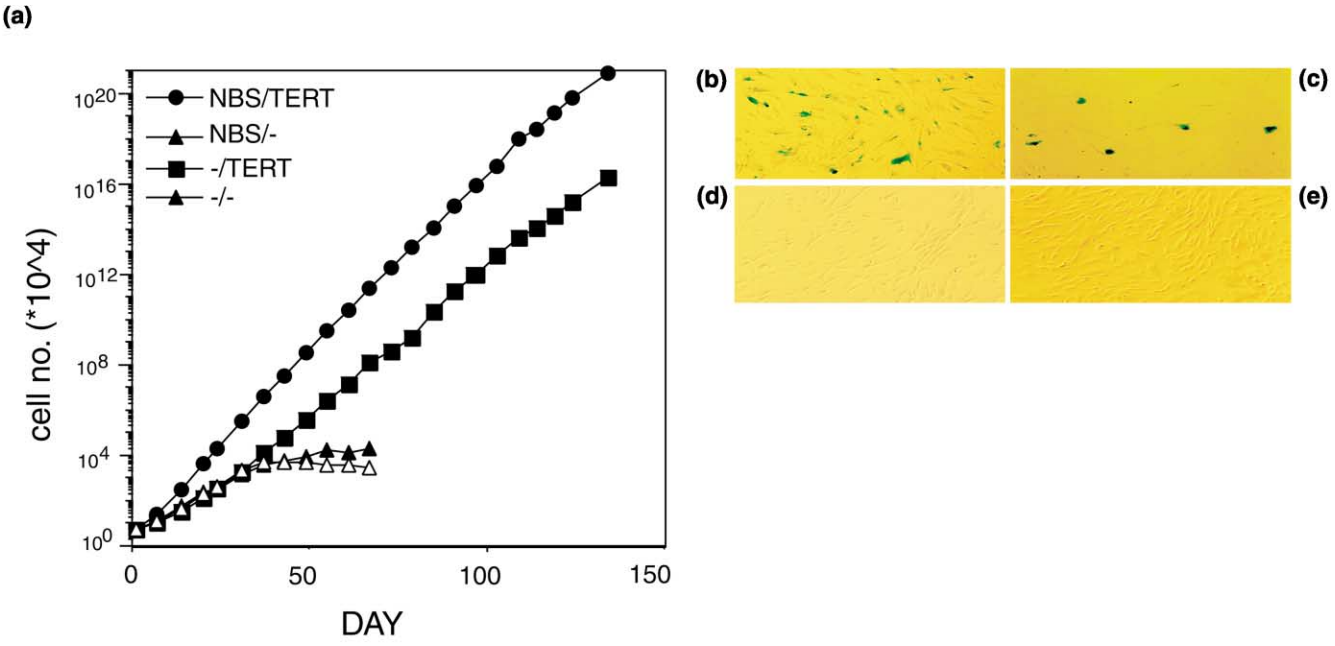
NBS is necessary for a telomere-lengthening function. **(a)** A clonogenic cell survival assay following γR was performed for GM07166 TERT$^+$, GM07166 NBS$^+$/TERT$^+$, 780816J TERT$^+$, 780816J NBS$^+$/TERT$^+$, and IMR90 cells. The fraction of cells surviving is calculated by comparison with the colony-forming efficiency of cells without irradiation. **(b)** NBS$^-$/TERT$^+$, NBS$^+$/TERT$^-$, and NBS$^+$/TERT$^+$ cells derived from NBS fibroblasts were analyzed for telomere length. NBS-A, -B, and -C denote NBS virus introduced into GM07166 TERT$^+$, and NBS-D, -E, and -F are three independent viral transductions into 780816J TERT$^+$, respectively. *In gel* hybridization with the telomere repeat probe and ethidium bromide staining of the gels prior to drying are shown.

in GM07166 NBS$^+$/TERT$^+$ cells (Relative Fluorescence Intensity, RFI, 13.3) compared to GM07166 (RFI, 5.8), GM07166 TERT$^+$, or GM07166 NBS$^+$ cells. Growth of the NBS$^+$/TERT$^+$ or TERT$^+$-only cells for extended passaging did not further alter telomere lengths, indicating an ability of TERT expression to stabilize telomere length, as noted for wild-type cells previously (data not shown). Taken together, these findings demonstrate that NBS contributes to telomere extension.

One control limiting the replicative potential of normal cells is telomere length, since the entry into senescence and/or induction of apoptosis is correlated with the erosion of telomere repeats [20]. Therefore, we investigated whether the introduction of NBS and/or TERT influences the replicative capacity of NBS fibroblasts. GM07166 NBS$^-$/TERT$^-$, NBS$^-$/TERT$^+$, NBS$^+$/TERT$^-$, and NBS$^+$/TERT$^+$ cells were cultured in parallel through repeated passages. The addition of NBS alone failed to extend the proliferative lifespan, as the division rate of NBS$^+$/TERT$^-$ cells was significantly slowed after limited passaging (Figure 3a). Similarly, NBS$^-$/TERT$^-$ cells arrested cell proliferation by day 50 in culture. The cells in these two cultures were heterogeneous, displaying a high proportion of enlarged and distended cells, suggestive of senescence. Furthermore, an induction of the senescence-associated acidic β-galactosidase activity [21] was readily evident for late passage GM07166 NBS$^-$/TERT$^-$ and NBS$^+$/TERT$^-$ cells (day 60; Figure 3b,c). In striking contrast, both NBS$^+$/TERT$^+$ and TERT$^+$ cells returned the parental NBS cells to normal growth and showed no senescence (Figure 3d,e). Also, these cells propagated continuously without evidence of an increase in a subpopulation that was undergoing apoptosis. Proliferation was greatest for NBS$^+$/TERT$^+$ compared with TERT$^+$ cells. Therefore, an ability to maintain telomere length, rather than an absolute requirement for NBS or telomerase, influences the senescence checkpoint in NBS cells.

Mammalian proteins that function positively and in tandem with telomerase have not previously been reported. Whereas deficiencies in the Ku double-strand break repair and DNA binding complex yield telomere shortening in yeast, the KU and telomerase (EST2) mutations are not epistatic [22, 23]. DNA-PK associates with mammalian telomeres [24, 25], but a functional role has yet to be defined. TRF1 and TRF2 negatively regulate telomere metabolism, and their dysregulation can lead to telomere errors and p53-dependent apoptosis [26–28]. These proteins bind to the telomeric repeats but are not known to directly regulate telomerase activity. NBS complexes may override negative controls to telomere length imparted by TRF proteins [6].

Figure 3



Replicative capacity and senescence is influenced by NBS and telomerase. **(a)** GM07166 fibroblasts transduced with NBS and/or TERT retroviruses were serially cultured at 2×10^4 cells per 60 mm well for a period of 2–4 months. A minus sign indicates the infection of control retroviral vectors, pBABEneo or pWZL. **(b–e)** Determination of senescent cells was assessed at day 60 in the passaging experiments of (a) by staining for acidic β-galactosidase activity [21]. The fraction of blue cells per culture were calculated: (b) GM07166 (0.4), (c) NBS/$^-$ (0.39), (d) $^-$/TERT ($<$0.01), and (e) NBS/TERT ($<$0.01).

NBS associates with MRE11 and RAD50 and controls the nuclease activity of MRE11 in vitro [3–5]. *Saccharomyces cerevisiae* mutations of these genes lead to cells with shortened telomeres [29], double-strand break repair deficiency, and meiotic defects, arguing that the effects we have observed with mammalian NBS reflect evolutionarily conserved pathways. NBS/MRE11/RAD50 complexes may facilitate telomerase by modifying telomere DNA ends, opening up the t-loop [6], or altering chromatin structure and need not be directly associated with a telomerase complex.

ATM and NBS patients have similar immunodeficiencies, predispositions to cancer, and cellular defects including radiosensitivity and chromosome instability [30]. The ATM protein kinase is activated by genotoxic agents [31, 32] and thus may be a transducer of DNA damage signals, possibly including signals at telomeres. Deficiency of ATM and the related yeast kinases, TEL1 and MEC1, show that telomere length decreases [12, 33, 34, 35]. It is possible that the requirement for ATM in telomere controls is also mediated through NBS. Alterations in telomere architecture induce an ATM- and p53-dependent apoptic response in tumor cell lines and primary lymphocytes [28]. The loss of NBS/MRE11/RAD50 cell proliferation properties may be a principal driving force in the increase of chromosome anomalies via telomere effects. Telomere metabolism errors may confer many of the pleotropic defects and cancer in NBS patients. As failure to allow telomere elongation may lead to an increased incidence of tumors in animal models [36, 37], this study defines a plausible role of NBS as a tumor suppressor manifested through a requirement in telomere extension.

Materials and methods

Cell lines and retroviral transduction

Low-passage NBS fibroblasts were obtained from Coriell Human Gene Mutant Cell Repository (GM07166) and Dr. Patrick Concannon (780816J, 880823H). Normal diploid fibroblasts (IMR90) were purchased from ATCC. Retroviral transductions were conducted following cloning of full-length NBS and TERT cDNAs into pWZL, modified with a blasticidin-resistance gene and/or pBABEneo, using pseudotyping with VSV-G envelope protein [17, 18, 38]. Drug selections were either with G418 (100 μg/ml) or blasticidin (2 μg/ml). Approximately 8–10 PD was required to establish a stably infected cell culture. Population doubling was adjusted to 0 following the establishment of a 1–100 mm plate for each viral transduced cell culture. To determine replicative potential, cells of different transduced genotypes were grown in parallel by passaging 2×10^4 cells/well of 6-well plates every 6 days, in duplicate. The passaging experiment was completed twice.

Supplementary material

Additional information about NBS protein expression and telomere length determinations is available at http://images.cellpress.com/supmat/supmatin.htm.

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