Folate Deficiency Induces Dysfunctional Long and Short Telomeres; Both States Are Associated with Hypomethylation and DNA Damage in Human WIL2-NS Cells

Caroline F. Bull, Graham Mayrhofer, Nathan J. O’Callaghan, Amy Y. Au, Hilda A. Pickett, Grace Kah Mun Low, Dimphy Zeegers, M. Prakash Hande, and Michael F. Fenech

Abstract

The essential role of dietary micronutrients for genome stability is well documented, yet the effect of folate deficiency or excess on telomeres is not known. Accordingly, human WIL2-NS cells were maintained in medium containing 30, 300, or 3,000 nmol/L folic acid (FA) for 42 days to test the hypothesis that chronic folate deficiency would cause telomere shortening and dysfunction. After 14 days, telomere length (TL) in FA-deficient (30 nmol/L) cultures was 26% longer than that of 3,000 nmol/L FA cultures; however, this was followed by rapid telomere attrition over the subsequent 28 days (P trend, P < 0.0001); both long and short telomere status was positively correlated with biomarkers of chromosome instability (P ≤ 0.003) and mitotic dysfunction (P = 0.01), measured by the cytokinesis-block micronucleus cytome (CBMN-cyt) assay. The early increase in TL was associated with FA-deficiency–induced global DNA hypomethylation (P = 0.05), with an effect size similar to that induced by the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine. Quantitative PCR analysis indicated a negative association between FA concentration and uracil incorporation into telomeric DNA (r = −0.47, P = 0.1), suggesting a possible plausible mechanism for uracil as a cause of folate deficiency–induced telomere dysfunction or deletion. Peptide nucleic acid-FISH (PNA-FISH) analysis showed that FA deficiency resulted in 60% of micronuclei containing acentric terminal fragments, an observation consistent with the 3-fold increase in terminal deletions (P = 0.0001). Together, these results demonstrate the impact of folate deficiency on biomarkers of telomere maintenance and integrity, and provide evidence that dysfunctional long telomeres may be as important as critically short telomeres as a cause of chromosomal instability. Cancer Prev Res; 7(1): 128–38. © 2013 AACR.
dTTP, forming thymidine for DNA synthesis and repair (14, 15). When folate is limiting, thymidine residues in DNA can be replaced by uracil, initiating a cycle of base excision repair, increased frequency of abasic sites, and heightened risk of double-strand breaks (DSB; ref. 16). In addition, compelling evidence has shown that repair responses can be restricted at telomeric DNA, suggesting that damage may be prolonged (17–19). The telomere, comprised of a repeating DNA hexamer (TTAGGG)n, is bound by proteins of the shelterin complex to form the "telosome," key components of which are "telomere repeat binding factors" (TRF1 and TRF2), the binding kinetics for which are highly sensitive to substrate changes (20, 21). It is plausible, therefore, that the thymidine-rich nature of telomeres makes them vulnerable to uracil misincorporation, incomplete excision repair of uracil, leading to abasic sites, unrepairred DNA strand breaks, and compromised telosome formation when folate is deficient. Accordingly, we hypothesized that telomeres would shorten at an accelerated rate under FA-deficient conditions, and tested this in an in vitro human cell line model.

Materials and Methods
Design
An in vitro model of chronic FA deficiency was established using human WIL2-NS cells. FA concentrations were determined based on pilot growth data, and on previous studies indicating that DNA damage occurs in WIL2-NS cells at 20 and 200 nmol/L FA (22). Cells were cultured for 42 days in medium either deficient (30 nmol/L), replete (300 nmol/L), or supraphysiological (control, 3,000 nmol/L) for FA. The "replete" concentration of 300 nmol/L is one which would only be achieved in serum of individuals supplementing with several milligrams of FA daily, whereas the "control" concentration of 3,000 nmol/L is not achievable in vivo in humans. We do, however, live in an era in which cells are increasingly taken out of our bodies, grown in culture, and returned to the body (e.g., stem cell, bone marrow, and skin graft transplantation). Such culture systems routinely use media with supraphysiological concentrations of FA and yet it is not known whether this is optimal for DNA damage prevention. The human WIL2-NS (B-lymphoblastoid) cell line was selected because folate deficiency has been identified as an important risk factor for B-cell lymphoma (23), and because its p53-deficient status has been identified as an important risk factor for B-cell lymphoma. WIL2-NS cells (American Type Culture Collection; CRL-8155) were cultured for 7 days in complete RPMI 1640 medium (Sigma) before seeding in treatment medium at day 0 of the experimental period. Cells were authenticated at the time of delivery (2002) to confirm p53-negative status, and in 2011 were confirmed as being karyotypically consistent with previous reports (25). Duplicate 90 mL cultures were maintained in 75 cm² vented-cap culture flasks (Becton Dickinson) at 37°C in a humidified atmosphere with 5% CO₂. Medium was replaced twice weekly. Complete RPMI medium (control, 3,000 nmol/L FA condition) was supplemented to contain 5% FBS (Thermo) and 1% penicillin/streptomycin (Sigma). L-Glutamine (1%; Sigma) was added immediately before use. Medium containing 30 or 300 nmol/L FA was prepared by dilution of complete RPMI medium with an appropriate volume of supplemented FA-free RPMI (Sigma). For 5-aza-2'-deoxycytidine (5azadC) studies, filter sterilized 5azadC (Sigma) stock solution (21.9 mmol/L in PBS) was added to RPMI medium (3,000 nmol/L FA) to final concentrations of either 0.2 or 1.0 µmol/L 5azadC. Control (0 µmol/L 5azadC) medium was prepared by adding equivalent volume of vehicle (1 × PBS).

Determination of TL
TL was measured in viable single cells at G1 in G1 using the flow cytometric method detailed previously (26). TL was expressed relative to a (long telomere) control cell line, 1301 European Collection of Cell Cultures (ECACC). Telomere-specific peptide nucleic acid-fluorescein isothiocyanate (PNA-FITC) labeling was conducted using kit K5327 (Dako). Doublets, debris (dead and dying cells), and cells at G2 were excluded based on cell cycle staining with propidium iodide. The coefficient of variation of replicate measurements was 13.5%.

CBMN-cyt assay
Chromosomal damage and cytostasis measures were determined using the CBMN-cyt assay, using the standard protocol detailed previously (27), with additional scoring criteria for fused nuclei (FUS) morphologies as described (9). See details in Supplementary Materials and Methods.

Measurement of uracil incorporation into telomeric DNA by quantitative real-time PCR
The amount of uracil present in telomeric repeat sequences was measured using a modification of the quantitative real-time PCR (qPCR) method used to measure TL, incorporating additional digestion steps. For details see Supplementary Materials and Methods. Detailed methods are provided in Supplementary Materials and Methods for cell growth, the CBMN-cyt assay, binucleated cell preparations for PNA-fluorescence in situ hybridisation (FISH), metaphase chromosome...
preparations, FISH with PNA probes, immunoaffinity purification-telomeric repeat amplification protocol (IP-TRAP), C-circle assay, terminal restriction fragment (TRF) analysis, DNA isolation and global DNA methylation, RNA isolation and expression analysis of hTERT, measurement of uracil incorporation into telomeric DNA by qPCR, and statistical analyses.

Results and Discussion

**Culture in folate-deficient medium is associated with rapid fluctuations in TL**

WIL2-NS cells were maintained in medium deficient (30 nmol/L), replete (300 nmol/L), or supraphysiological (3,000 nmol/L; control) for FA. The doubling time of the cells in culture varied considerably for each FA condition. The number of viable cells at each time point, indicating rate of cell division, was reduced in both the 30 and 300 nmol/L FA cultures, compared with the 3,000 nmol/L condition (Supplementary Fig. S1a). Cell viability and nuclear division index (NDI) were significantly lower at every time point in 30 nmol/L compared with that of 3,000 nmol/L FA ($P < 0.0001$), whereas the frequency of necrotic cells significantly increased at all time points in the 30 nmol/L condition ($P < 0.0001$; Supplementary Fig. S1b–S1d).

Pilot TL data from short-term (21 days) cultures indicated TL significantly increased in the short term (7–14 days) under FA-deficient conditions, followed by a modest decline between day 14 and day 21 (Supplementary Fig. S2). To examine the longer term chronic effects of FA deficiency, cells were then maintained in culture for 42 days. TL of samples at baseline, and weekly or fortnightly thereafter, were compared by flow cytometry and results expressed relative to the TL of reference cell line 1301. At day 14, TL in 30 nmol/L FA was $18.1 \pm 2.1$ (mean $\pm$ SD), 1.4-fold that of baseline ($12.7 \pm 3.4$), followed by rapid attrition ($P < 0.0001$). By day 42, TL had shortened to $12.6 \pm 1.2$, comparable with cells grown for the same length of time in 3,000 nmol/L FA ($13.5 \pm 2.7$; Fig. 1A). TL of control cultures remained stable throughout the 42-day period (mean, $13.7 \pm 0.6$). Mean area under the curves (AUC TL) for the 30, 300, and 3,000 nmol/L FA conditions was 638, 628, and 576, respectively. Despite the rapid TL shortening beyond day 14 in the 30 nmol/L condition, a negative correlation was recorded between [FA] and AUC TL, over the 42 days ($r = -0.47$; $P = 0.008$). These are new and intriguing observations, which do not support the original hypothesis that FA deficiency is associated with telomere shortening.

**TL is positively associated with biomarkers of CIN in the short term, but negatively associated in the longer term**

The relationships between [FA], TL, and CIN have not previously been examined. Biomarkers of CIN were scored in binucleated cells using the CBMN-cyt assay, specifically; micronuclei (MN; broken fragments or lagging chromosomes), nucleoplasmic bridges (NBP; indicative of dicentric chromosome (DC) formation due to...
Fusion of dysfunctional or critically short telomeres, or misrepair of DNA breaks), nuclear buds (NBud; gene amplification; ref. 27), and fused nuclei (FUS; suggesting failed chromatid separation and/or dysfunctional metaphase/anaphase transition; ref. 9). FA deficiency induced highly significant increases in the frequency of all biomarkers over 42 days ($P < 0.0001$–$P = 0.0009$; Fig. 1B–F).

Consistent with previous (shorter term) findings, micro-nuclei, NPBs, and FUS were positively correlated, yet frequencies of each were strongly, negatively associated with [FA] (Table 1; refs. 9, 10). Relationships between TL and CIN biomarkers at day 14 (the point at which the longest TL was observed) were positive and significant. By day 42, this had reversed, with correlations between CIN and TL being strongly negative (Table 1).

These data show that FA deficiency induces both shorter and longer telomeres, and that both states are strongly associated with CIN, indicating telomere dysfunction regardless of length.

**Possible mechanisms underlying the increase in TL.**

Questions that arise from the above findings are the following: (i) What mechanisms are responsible for the increase in TL in folate-deficient cultures? and (ii) Does a causal link exist between these and the evidence of CIN?

Telomere shortening is a pivotal event early in the pathogenesis of cancer; however, in established cancers telomerase activity (TA) can induce longer TL than that of adjacent normal tissue (28). An alternative TA-independent, recombination-driven telomere maintenance mechanism (TMM), called the alternative lengthening of telomeres (ALT), has been identified in approximately 10% of cancers (29, 30).

Hypomethylation of the subtelomeric promoter for “telomeric repeat-containing RNA” (TERRA) may influence the ALT phenotype, directly or indirectly (11, 31, 32). Accordingly, we considered whether the increased TL and CIN in WIL2-NS cells from FA-deficient cultures may be due to ALT-associated recombination, the latter potentially inducing formation of DC containing interstitial telomeric DNA, initiating telomere amplification via breakage–fusion–bridge (BFB) cycling.

To elucidate the mechanism of telomere elongation induced by FA deficiency, we explored (i) intrachromosomal (interstitial) telomeric DNA to determine if amplification may have occurred as a result of BFB cycling; (ii) the ALT or telomerase status of WIL2-NS cells; (iii) whether FA deficiency reduced global DNA methylation, and the association between DNA methylation and TL; and (iv) the impact of FA deficiency on expression of hTERT, the active subunit of telomerase.

**BFB cycling is not responsible for amplification of telomeric DNA.**

Numerous studies report increased incidence of anaphase bridges when TL and/or integrity is compromised (33–36); however, to our knowledge, evidence of telomeric DNA amplification through this mechanism has not been reported. DC formation is a known initiator of BFB cycling, gene amplification, and altered gene dosage (34, 36). We speculated that random breakage of DCs formed by fusion of dysfunctional telomeres may result in daughter cells receiving uncapped (fusigenic) chromosomes; one containing telomere sequences of both fused chromosomes (interstitially), and the other receiving a reduced telomere content.

| Table 1. Correlations between biomarkers of CIN, concentration of FA in culture medium, and TL |
|-----------------------------------------|----------------|----------------|----------------|----------------|-----------------|
| MN | NPB | NBud | FUS | DNA damage (all markers combined) | TL day 14 | TL day 42 |
| Log [FA] | | | | | | |
| $P$ | 0.0005 | 0.02 | 0.7 | 0.07 | 0.01 | 0.0003 | 0.065 |
| $r$ | -0.98 | -0.88 | 0.21 | -0.77 | -0.9 | -0.98 | 0.8 |
| MN | | | | | | |
| $P$ | 0.01 | 0.9 | -0.04 | 0.05 | 0.005 | 0.005 | 0.03 |
| $r$ | 0.81 | 0.94 | 0.94 | 0.81 | 0.94 | 0.85 |
| NPB | | | | | | |
| $P$ | 0.8 | 0.08 | 0.003 | 0.0002 | 0.004 | 0.002 |
| $r$ | 0.96 | 0.98 | 0.95 | 0.96 | 0.98 | 0.97 |
| NBud | | | | | | |
| $P$ | 0.58 | 0.77 | 0.02 | 0.64 |
| $r$ | 0.29 | 0.15 | 0.9 | -0.24 |
| FUS | | | | | | |
| $P$ | 0.003 | 0.01 | 0.01 |
| $r$ | 0.95 | 0.9 | -0.91 |
| DNA damage (all markers combined) | | | | | | |
| $P$ | 0.0002 | 0.003 |
| $r$ | 0.98 | -0.95 |

NOTE: Shaded values indicate statistically significant associations ($P < 0.05$), $r$ = Pearson correlation coefficient. DNA damage (all markers combined), frequency of binucleated cells displaying one or more MN, NPB, NBud, or FUS. All data are calculated using duplicate values at day 42, except TL data, which use mean TL values for each condition at two key time points: days 14 and 42.
To test the hypothesis that BFB cycling was responsible for telomere amplification, metaphase preparations from cells grown in control (3,000 nmol/L) or deficient (30 nmol/L) FA for 0, 7, 14, 21, 28, or 35 days were probed using PNA-FISH for the presence of interstitial telomeric DNA (i.e., telomeric sequences at nonterminal locations). Analysis showed no interstitial telomeric DNA in these metaphases (n = 40 metaphases per condition, per time point; data not shown), thus refuting this hypothesis.

WIL2-NS cells are ALT-negative and telomerase-positive

ALT− cells are characterized by highly heterogeneous TL distribution (potentially ranging from <3 kb to >50 kb), the presence of ALT-associated promyelocytic leukemia bodies (APB), and C-circles (29, 30). C-circles are a specific, quantifiable marker of ALT (29, 30). To test whether WIL2-NS cells are ALT+, we examined untreated (day 0) cells first by Southern blot to assess the degree of TL heterogeneity in TRF, and second for the presence of C-circles by the C-circle assay (30). TRF analysis showed a comparable spread of TL relative to HeLa cells (ALT+/TA+; Fig. 2B). WIL2-NS cells were then shown to be negative for C-circles relative to ALT+/TA+/HeLa reference cells (Fig. 2C).

Together, these results suggest that WIL2-NS are ALT−/TA−. The IP-TRAP assay showed WIL2-NS are positive for TA (Fig. 2D), consistent with an ALT−/TA− phenotype (29), and suggesting that the increase in TL observed at days 7 and 14 in the FA-deficient cultures may be mediated by telomerase.

Figure 2. Investigations into mechanisms underlying transient telomere elongation in low FA culture conditions. A, proposed model for telomeric DNA amplification via BFB cycling, wherein compromised (uncapped or broken) telomeres fuse to form a dicentric chromosome containing intrachromosomal telomeric DNA. Red squares, telomeric DNA; green circles, centromeres. B, southern (TRF) analysis of (day 0, untreated) WIL2-NS cells (reference cell lines; HeLa, ALT−/TA+, VA13, ALT−). C, detection of C-circles (CC) shows WIL2-NS cells to be CC-negative compared with ALT+/IIICF/c and ALT+/TA+/HeLa reference cells. D, WIL2-NS cells are positive (+) for TA, determined using the IP-TRAP assay. Sizes of molecular weight markers are indicated to the left of B and D. Buffer, buffer-only negative control; HCT116, telomerase-positive (TA+) reference cell line; GM847, ALT+/TA− reference cell line. E and F, global methylation status of WIL2-NS cells cultured for 14 days and 42 days (E) in medium containing 30 or 3,000 nmol/L FA (a, P = 0.046; b, P = 0.06); and for 4 days (F) in complete medium (CM; 3,000 nmol/L FA) containing 0, 0.2, or 1.0 mmol/L 5azadC (points not sharing the same letter differ significantly; P < 0.05). %5-meC; percentage of cytosine residues methylated at carbon-5, as a percentage of total DNA; mean ± SD; N = 3. G, TL of WIL2-NS cells cultured for 4 days in CM containing 0, 0.2, or 1.0 mmol/L 5azadC. Mean ± SD; N = 20 at day 0, n = 10 at day 4 (points not sharing the same letter differ significantly; P < 0.05).
FA deficiency results in global DNA hypomethylation

The relationship between DNA methylation status and TL is currently unclear. Telomeres lack CpG sites, the substrate for DNA methyltransferase (DNMT) enzymes, and, as a consequence, are unmethylated. In contrast, the subtelomere is normally heavily methylated, reduction in which (in gene knockout studies) has been associated with dramatically elongated telomeres, APBs, and telomeric sister chromatid exchange (TSCE; refs. 11, 32). Subtelomeric and pericentromeric DNA hypomethylation was also associated with increased TL and TSCE in a panel of neoplastic cells (12), suggesting a plausible association between global and subtelomeric methylation status, and telomere dysfunction.

The relationship between folate, DNA methylation, and TL has not previously been reported. We hypothesized that under FA-deficient conditions, TL and global DNA methylation status would be negatively associated. An ELISA-based assay was used to determine the percentage of 5-methylcytosine (%5me-C) in cells cultured in 30 nmol/L and 3,000 nmol/L FA at day 14 and day 42. Results showed global DNA methylation was lower in the 30 nmol/L compared with 3,000 nmol/L FA cultures, at both time points (Fig. 2E); 16% of variance was attributable to [FA] (ANOVA, \( P = 0.03 \)), 43% to time (\( P = 0.005 \)), and 11% to their interaction (\( P = 0.15 \)). Methylation status and [FA] were positively associated at day 14 (\( r = 0.8, P = 0.05 \)) and day 42 (\( r = 0.8, P = 0.06 \)). In agreement with our hypothesis, at day 14, the point at which TL was longest in the 30 nmol/L FA culture, TL was negatively and significantly associated with global methylation status (\( r = -0.8, P = 0.05 \)).

Global hypomethylation is associated with increased TL

To examine the impact of hypomethylation on TL, without the potentially confounding effect of reduced intracellular thymidine through FA deprivation, the protocol was repeated using WIL2-NS in complete medium (3,000 nmol/L FA) containing DNMT inhibitor, 5azadC. To minimize cytotoxic effects, the lowest possible (bioefficacious) concentrations (0.2 and 1.0 \( \mu \text{mol/L} \)) were used (37). Treatment could only be maintained for 4 days before cells became nonviable; however, as TL increased in cells in 30 nmol/L FA within 7 days, 4 days exposure to 5azadC was deemed sufficient to induce a comparable effect.

We verified that methylation status of WIL2-NS exposed to 0.2 and 1.0 \( \mu \text{mol/L} \) 5azadC was significantly lower than those from control (0 \( \mu \text{mol/L} \)) cultures; 21% of variance attributable to [5azadC] (ANOVA, \( P = 0.05 \); Fig. 2F). The correlation between [5azadC] and DNA methylation at day 4 was significant and negative (\( r = -0.8, P = 0.01 \)).

At day 4, TL was 29% and 50% greater, in the 0.2 and 1.0 \( \mu \text{mol/L} \) 5azadC, respectively, than in untreated controls (Fig. 2G). [5azadC] explained 27% of TL variance (\( P < 0.0001 \)), 39% due to time (\( P < 0.0001 \)) and 27% due to their interaction (\( P < 0.0001 \)). A significant, negative correlation was recorded between global DNA methylation and TL in these samples (\( r = -0.75, P = 0.02 \)). Relative to 3,000 nmol/L cultures, cells in 30 nmol/L FA had 74% less DNA methylation at day 14, and 26% longer TL. Similarly, the 30% reduction in methylation in the 0.2 \( \mu \text{mol/L} \) 5azadC culture had 30% longer TL, whereas the 355% reduction in methylation in the 1.0 \( \mu \text{mol/L} \) condition was associated with 50% longer TL, relative to control cultures. From these data, we concluded that DNA hypomethylation is a plausible candidate as the mechanism underlying increased TL observed after 7 to 14 days of suboptimal FA.

Expression of hTERT is unaffected by FA deficiency

hTERT, which is regulated at the transcriptional level (38). Animal models show that diets deficient for methyl-donors (including folate) increased DNMT expression, resulting in CpG island hypermethylation, in certain tissues (39), an effect that may plausibly impact hTERT expression. Accordingly, we determined whether hTERT expression was altered in WIL2-NS under FA-deficient conditions by quantifying RNA transcripts in cells following 14, 28, 35, and 42 days in 30, 300, or 3,000 nmol/L FA culture. qPCR results, expressed relative to baseline (day 0), normalized against housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (\textit{GAPDH}), showed no significant differences, with only 5.2% of variance attributable to [FA] (\( P = 0.77 \)), and 18.2% of variance due to time (\( P = 0.75 \); data not shown). Although telomerase is active in WIL2-NS, it seems that neither FA deficiency per se, nor an associated change in DNA methylation status, impacts transcription of \textit{hTERT}, thus increased TA is unlikely to have contributed to the longer TL observed.

Mechanisms for accelerated telomere loss

Uracil incorporation into genomic DNA under folate-deficient culture conditions is well documented (14), but whether uracil is incorporated into telomeres has not previously been examined. A functional telosome, key components of which are capping proteins TRF1 and TRF2, inhibits nonhomologous end joining and end fusions (40). The high specificity of capping proteins for their substrate was demonstrated in an elegant study showing substitution of a single telomeric guanine with 8-oxo-guanine (8oxoG) resulted in ~50% reduction in TRF1/2 binding, an abasic site within the telomere reduced binding 3-fold, and a lesion within each hexamer repeat reduced bound TRF1 and TRF2 to barely detectable levels (21). Accordingly, we hypothesized that uracil in telomeric DNA may impact telosome integrity in a similar manner. Furthermore, uracil glycosylase (UDG), part of the base excision repair (BER) mechanism, removes uracil from genomic DNA, resulting in a transient abasic site intermediate (15, 16). In low FA (low thymidine) conditions, uracil can be reincorporated at the “gap-filling” step, leading to futile cycling of BER and abasic site generation, potentially compromising TRF1/2 binding, and increasing the risk of DSB (15, 16). Recent findings indicate, however, that telomeric DNA damage is
not repaired (18, 19), resulting in a persistent damage response (phosphorylated (γ)-H2AX and/or ATM) at lesions, prolonged cell cycle arrest, and potentially replicative senescence.

To examine the factors underlying the telomere attrition observed in extended 30 nmol/L FA cultures, we tested (i) the relationship between [FA] and uracil within telomeres, (ii) the presence of telomeric DNA in biomarkers of DNA damage in cytokinesis-blocked binucleated cells, and (iii) “loss of telomere” events in metaphase preparations.

**Uracil incorporation into telomeric DNA is negatively associated with [FA] in medium**

Uracil content of telomeric DNA was measured in UDG-digested and -undigested DNA using an adaptation of a qPCR method developed in this laboratory to detect abnormal bases by digesting DNA with lesion-specific enzymes (41). Results showed that, following 42 days of culture in either 30 nmol/L or 300 nmol/L FA, the amount of uracil per kb of telomeric DNA was 2.9- and 3.4-fold greater, respectively than at baseline, whereas no uracil was detectable in telomeres of cells from 3,000 nmol/L FA cultures (n = 4, P = 0.3). The slightly greater amount of uracil observed in the 300 nmol/L culture (compared with 30 nmol/L) is consistent with previous findings in genomic DNA, an effect possibly due to the different rates of DNA synthesis and cell division between the midrange and lowest [FA], thus influencing the rate of uracil incorporation (14). As predicted, [FA] and uracil content in telomeric DNA were negatively associated (r = −0.47, P = 0.1) after 42 days in culture.

**FA deficiency is associated with DSBs, loss of terminal telomeric fragments, and mitotic dysfunction**

To examine the in situ location of telomeric and centromeric DNA in FA-deficient cultures, dual fluorochrome PNA-FISH was conducted on cytokinesis-blocked binucleated cells cultured in 30 nmol/L FA for 7, 14, 21, 28, or 35 days. Of 151 micronuclei scored (day 7, n = 37; day 14, n = 21; day 21, n = 41; day 28, n = 31; day 35, n = 21), 38% contained centromeric DNA (C+) and 79% contained telomeric DNA (T+; Fig. 3A–C). Consistent with previous data from 9-day FA-deficient cultures (42), only 25% of the 53 micronuclei containing ≥2 T signals were also C+.
indicating a lagging chromatid or chromosome), suggesting that the majority of T$^+$ micronuclei represent acentric fragments arising from DSB. Of 21 NPBs scored after days 7 ($n = 6$), 14 ($n = 1$), 21 ($n = 2$), 28 ($n = 8$), or 35 ($n = 4$) in 30 nmol/L FA (Fig. 3D–F), 38% were T$^+$, suggesting fusion between dysfunctional or compromised telomeres, 43% were C$^+$, and 24% contained both (C$^+$T$^+$), which together suggest mitotic disruption (9). The greatest proportion (43%) was for C$^+$T$^+$, suggesting that these NPBs arose from fusion between uncapped chromosomes, or DSB misrepair of breaks at non telomeric chromosome regions. Cells displaying FUS morphologies ($n = 196$) were scored following days 7 ($n = 37$), 14 ($n = 21$), 21 ($n = 40$), 28 ($n = 30$), or 35 ($n = 21$) in 30 nmol/L FA (Fig. 3G–I). The majority (78%) of fusion regions were C$^+$, and/or T$^+$ (74%), with 61% exhibiting both (C$^+$T$^+$) and only 8% displaying neither (C$^+$T$^+$). These results suggest that telomere end fusions or failure of sister chromatid separation could cause FUS morphologies supporting our previous observations (9).

FA deficiency causes telomere loss and chromosome aberrations

“Loss of telomere” (LOT) events were scored in metaphase spreads from cells cultured in 30 or 3,000 nmol/L FA for days 0, 7, 14, 21, 28, or 35 (20 metaphases/slide, $n = 2$; Fig. 4A and B). Data were expressed relative to that of baseline cultures (set arbitrarily at 1.0). Confirming indications from the above data, metaphases from 30 nmol/L FA cultures had an approximately 3-fold increase in LOT from day 14 to day 35, compared with cells maintained in 3,000 nmol/L FA. Of the variance in LOT, 43% was attributable to [FA] ($P = 0.0001$), 22% to time ($P = 0.04$), and 18% to their interaction ($P = 0.08$). At day 35, LOT was negatively correlated with log[FA] ($r = -0.96, P = 0.03$), and positively correlated with binucleated cells containing ≥1 micronuclei ($r = 0.96, P = 0.04$).

These same spreads were analyzed for aberrations (DC, acentric fragments, double minutes, chromosome or chromatid breaks, ring chromosomes, q or p arm fusions). The
presence of DC was of particular interest, as they represent end fusions from telomere loss or dysfunction, or DSB misrepair. Only one aberration, a DC at day 21, was observed in 3,000 nmol/L FA spreads (Fig. 4C). In contrast, 21 aberrations were recorded from 30 nmol/L FA cultures, 10 of which were DC (2 at day 14, 1 at day 21, 2 at day 28, 5 at day 35; Fig. 4D–P). No aberrations were recorded at day 0 or day 7, from 30 or 3,000 nmol/L FA cultures.

Together, these data show long-term FA deficiency is associated with micronuclei containing acentric fragments and lagging chromosomes, terminal chromosome loss, fused chromosomes (including DC), apparent dysfunctional chromatid separation, and uracil in telomeric DNA. These findings are consistent with those that may be expected from uracil incorporation into genomic and telomeric DNA, and support our original hypothesis that FA deficiency could cause accelerated telomere erosion.

Conclusions
The novel data herein demonstrate that FA deficiency compromises telomere homeostasis, evidenced by substantial fluctuations in TL, increased chromosome fusions (NPBs and DCs), and terminal deletions. These effects coincide with global DNA hypomethylation, increased presence of uracil in telomeres, and increased CIN. We propose a model whereby FA deficiency results in short-term net gain in telomere content arising from hypomethylation, reduced capping and enhanced access by telomerase to its substrate. In the longer term, however, as telomere maintenance and DNA repair mechanisms become overwhelmed (due to increasing uracil incorporation, BER processes, abasic sites, and DSB), homeostatic balance is lost resulting in increasing telomere attrition with time. Furthermore, we provide evidence that the rapid telomere loss observed beyond day 14 in the 30 nmol/L FA condition coincides with an increase in terminal deletions, leaving exposed chromosome ends to which telomerase can no longer bind, and is thus unable to add telomeric repeats. A diagrammatic summary of proposed pathways underlying FA-deficiency–induced telomeric and genomic instability is provided in Fig. 5.

Increased TL under hypomethylating conditions has only been reported previously in mouse knockout models (11, 32), or in cell lines exposed to cancer drugs such as 5azadC (12). The present data, however, show telomere lengthening and DNA hypomethylation induced by FA deficiency to a similar extent as that caused by 5azadC. As epigenetic modifications are strongly associated with cancer initiation, these findings potentially have wide-reaching implications for disease risk minimization in populations exposed to deficiency of folate, vitamins required for folate metabolism (such as vitamin B12), or essential factors in
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

10. Fenech M. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. Mutat Res 2012;733:21–33.

www.aacrjournals.org Cancer Prev Res; 7(1) January 2014 137

OnlineFirst November 19, 2013; DOI: 10.1158/1940-6207.CAPR-13-0264

Folate Deficiency Induces Dysfunctional Telomeres

Authors' Contributions
Conception and design: C.F. Bull, G. Mayrhofer, M.F. Fenech
Development of methodology: C.F. Bull, N.J. O’Callaghan, M.F. Fenech
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.F. Bull, A.V. Au, H.A. Pickett, M.P. Hande
Writing, review, and/or revision of the manuscript: C.F. Bull, G. Mayrhofer, M.F. Fenech

Acknowledgments
The authors thank M. Hor for technical assistance for the SazadC work, and S. Mitchell [the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Sydney, Australia] and V. Dhillon (CSIRO, Adelaide, Australia) for generating preliminary methylation status data for these samples.

Grant Support
C.F. Bull was supported by an Australian Postgraduate Award (APA) Scholarship through the University of Adelaide, CSIRO’s Preventative Health Flagship, and a Science Industry Endowment Fund (SIEF) John Stocker Postdoctoral Research Fellowship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 14, 2013; revised October 30, 2013; accepted November 1, 2013; published OnlineFirst November 19, 2013.


Folate Deficiency Induces Dysfunctional Long and Short Telomeres; Both States Are Associated with Hypomethylation and DNA Damage in Human WIL2-NS Cells


Updated version Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-13-0264

Supplementary Material Access the most recent supplemental material at: http://cancerpreventionresearch.aacrjournals.org/content/suppl/2013/11/20/1940-6207.CAPR-13-0264.DC_1

Cited articles This article cites 44 articles, 8 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/7/1/128.full#ref-list-1

Citing articles This article has been cited by 4 HighWire-hosted articles. Access the articles at: http://cancerpreventionresearch.aacrjournals.org/content/7/1/128.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/7/1/128. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.