The impact of folate on telomere length

and chromosome stability in

human WIL2-NS cells and lymphocytes

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CHAPTER 4:
THE IMPACT OF FOLIC ACID CONCENTRATION ON TELOMERE LENGTH AND CHROMOSOMAL DAMAGE IN WIL2-NS CELLS *IN VITRO*

4   THE IMPACT OF FOLIC ACID CONCENTRATION ON TELOMERE LENGTH AND CHROMOSOMAL DAMAGE IN WIL2-NS CELLS IN VITRO

4.1 INTRODUCTION

Extensive evidence exists of the impact of dietary and environmental factors on chromosome stability[1,35,115-118,317]. However, knowledge of the impact of nutrition on the length and structural integrity of telomeres is limited. Mitotic disturbances instigated by loss of telomere integrity include chromosomal instability (CIN), events such as structural rearrangement of chromosomes, and formation of nucleoplasmic bridges (NPBs) arising from chromosome end fusions[13,14]. NPBs can lead to breakage-fusion-bridge (BFB) cycles where breakage leaves chromosome ends unprotected and prone to further end fusions. A cycle of fusions and subsequent breakages are established, resulting in gene amplification, altered gene dosage and a compounding effect of CIN (see Figure 1.2 "*Gene amplification by breakage-fusion-bridge (BFB) cycles*"[12,281]. Given the pivotal role that telomeres play in maintaining genome stability, knowledge of how external factors can affect telomere length (TL) and function may prove to be an effective strategy for preventing degenerative diseases of ageing, immune dysfunctions and cancers *in vivo* caused by chromosomal damage.

One dietary factor known to be critical for maintaining genome stability is folate (vitamin B9), which is used in supplements in its synthetic (unreduced and monoglutamated) form, known as folic acid (FA). Moderate FA deficiency has been shown to result in chromosomal damage comparable in extent to that induced by carcinogenic doses of radiation exposure[318]. FA acts via two distinct, but interconnected, biochemical pathways. One pathway is that of maintenance methylation of cytosine in DNA. This is necessary for maintaining and controlling gene expression patterns and chromosomal structural integrity, particularly at regions with tandem repeat sequences that are normally hypermethylated, such as the centromere and subtelomere[148,319]. The second pathway is the essential role of FA as the methyl donor that facilitates the conversion of dUMP to dTTP, which is essential for DNA synthesis and repair (see Figure 1.4 "*Folate pathway and genome maintenance*"[74]. Under conditions of low FA, the cell is unable to produce sufficient dTTP, resulting in the incorporation of uracil into DNA[74,99]. Subsequent excision of this aberrant base by uracil glycosylases results in abasic sites, leading potentially to strand breaks due to the endonuclease

cleavage step in excision repair, resulting in acentric chromosome fragments and increased micronucleus (MN) formation[75,318]. Previous evidence has shown that the presence of abasic sites (generated by excision of uracil) within twelve bases of each other, on opposing strands of the DNA molecule, results in a double DNA strand break[75]. Because of the thymidine-rich nature of the repeating sequence of the telomere, it is anticipated that the structural integrity of the telomere may be particularly vulnerable to the impact of low FA.

The purpose of the experiments presented in this chapter was to explore the impact of FA levels on TL and CIN using WIL2-NS cells *in vitro* as the model. The specific hypotheses and aims are provided below.

4.1.1   Aims

1. To determine whether FA deficient conditions result in TL shortening and increased chromosome instability (nucleoplasmic bridges, nuclear buds and/or micronuclei) in WIL2-NS cells (cell line) *in vitro*.

2. To determine the optimal folic acid (FA) requirement for maintenance of telomere length (TL) in WIL2-NS cells *in vitro*.

4.1.2   Hypotheses

4. FA deficiency causes telomere shortening and an increase in chromosomal instability.

5. Telomere shortening caused by FA deficiency is associated with increased chromosomal instability.

4.2 EXPERIMENTAL DESIGN

WIL2-NS cells were grown in bulk cultures at different FA concentrations and multiple samples were collected at different time-points for biomarker and cell count measurements (Figure 4.1). Two separate experiments were conducted in which WIL2-NS cells were maintained in culture for either (i) short term (21 day) or (ii) long term (42 day) study. Complete medium was prepared containing either 3000 (replete), 300 or 30nM FA, as described previously (Chapter 3.2.1). In the short-term study, cells were sampled at day 0, 7, 14 and 21, while in the long-term study, samples were taken at days 0, 14, 28, 35 and 42.

In setting up the experiments, cells were thawed from liquid nitrogen storage, washed twice in RT PBS (Chapter 3.2.5) and grown in complete medium that was replete in FA (3000nM) for seven days prior to splitting at day 0 into the three different FA treatments. Each culture was maintained at 90ml total volume in a $75cm^2$ vented-cap culture flask (BD Falcon, Bedford, USA) and subcultured with fresh media twice weekly. It was necessary to vary the seeding concentration at each subculture, depending on the treatment condition, to achieve the required numbers of viable cells (40×10^6) for the following sample point and to prevent overcrowding. At each sample point, cell numbers and viability were assessed (Coulter Counter and Trypan blue exclusion, Chapters 3.2.2x and 3.2.3), telomere length (TL) was measured and markers of chromosomal damage were examined. Total growth in each condition was calculated by counting the numbers of viable cells in the culture at the time of harvesting and extrapolating to the numbers that would have been expected if all cells had been re-seeded at each split. TL was measured by flow cytometry (Chapter 3.4) and chromosomal damage was assessed using the Cytokinesis Block Micronucleus Cytome (CBMN Cyt) assay (Chapter 3.3). In the CBMN Cyt assay cells were harvested 24 hours following the addition of Cyto-B, for example day 7 samples received Cyto-B on day 7, but were harvested after 24 hours in culture, *ie.* on day 8.

Homocysteine (Hcy) concentration was measured in fresh medium at Day 0 and in spent medium at day 42 of the long-term study (Chapter 3.7.1). Expression of *hTERT* (encoding the catalytic subunit of the telomerase enzyme) was assessed at each time point during the long-term experiment. RNA was isolated from cell samples by Dr Nathan O'Callaghan (Chapter 3.5.5.1), converted to cDNA using Invitrogen "SuperScript III first-Strand Synthesis System for RT-PCR" (Chapter 3.5.5.2) and amplified by RTm-PCR (Chapter 3.5.5.3). The results were normalised against GAPDH and expressed relative to the levels of *hTERT* transcripts at day 0 (Chapter 3.5.5.3).

4.2.1   Folic acid concentrations and cell growth estimates

It was necessary to select concentrations of FA that were adequate to maintain growth of WIL2-NS cells for the full 3- and 6-week study periods, whilst also being within a range that might be expected to produce measurable differences in TL and CIN.   Three concentrations were selected, ranging from 30nM to 3000nM (standard medium), based on previous studies in this laboratory[36,290].   Pilot studies on WIL2-NS cells indicated a population doubling time of approximately one day in FA-replete medium.   In contrast, the growth rate of cells in medium containing 300nM folate was approximately half that in FA-replete medium, while in 30nM FA, growth was reduced to approximately 25% of that in FA-replete medium.   Cell numbers required for each assay were calculated, and a seeding plan was prepared to ensure that adequate numbers of cells would be available for all assays through to the final sample in each of the experiments.
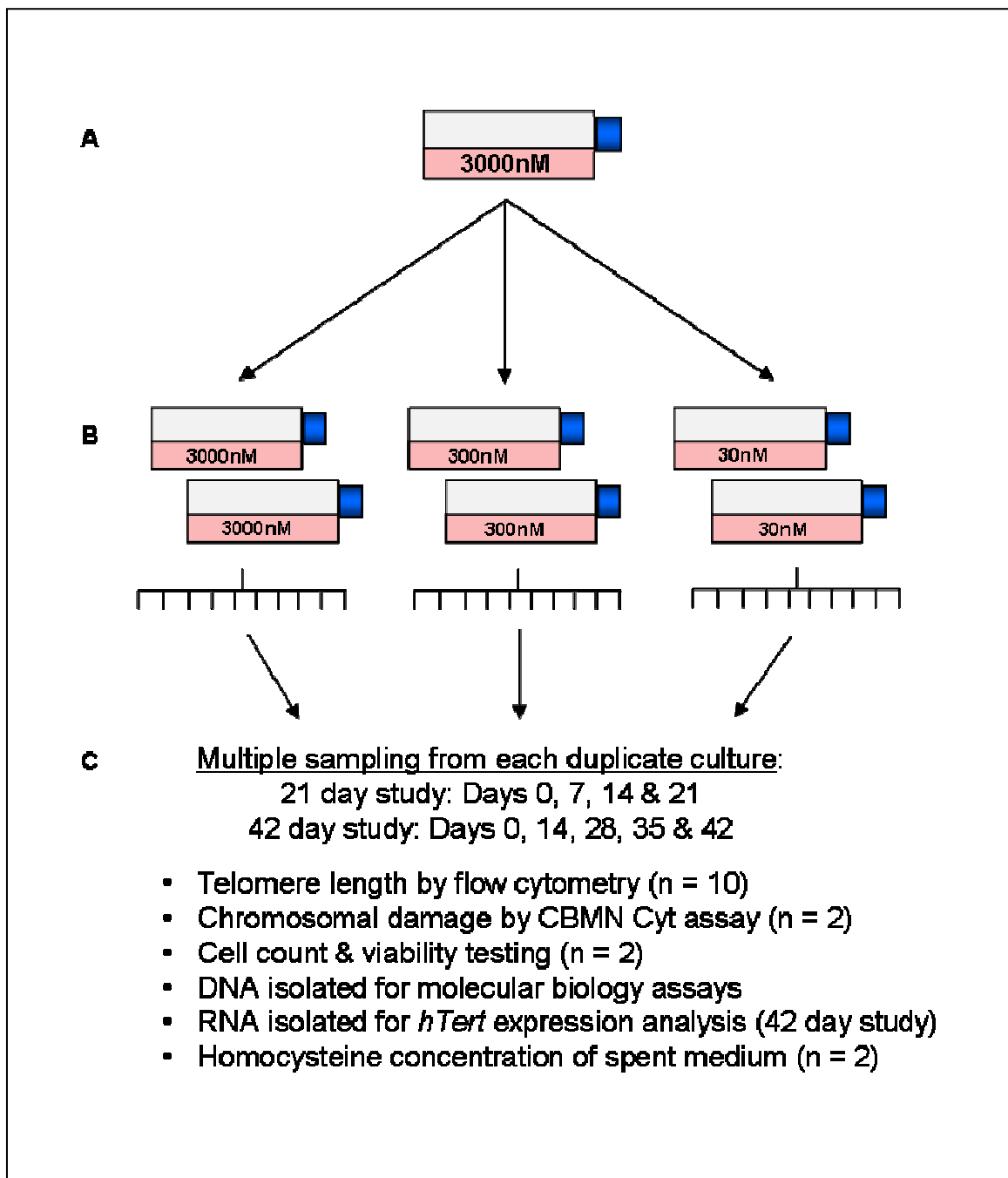
Figure 4.1 Overview of experimental design to study the response of WIL2-NS cells to FA deficiency. (A) Cells were thawed from liquid nitrogen storage, washed twice in PBS and cultured in FA-replete (3000nM) complete medium for 7 days prior to (B) splitting into duplicate cultures with complete medium containing either 3000, 300 or 30nM FA. (C) Multiple samplings were conducted from each culture and assays conducted as specified.

4.3   RESULTS

4.3.1   Cell growth & viability, necrosis and nuclear division index (NDI)

4.3.1.1 Short term (21-day) study

The increase in cell numbers under each growth condition was approximately linear. Two-way ANOVA indicated that 9.4% of growth variance was attributable to FA concentration (p = 0.007), while 32.8% was due to time (p = 0.0002).   42.2% of variance was due to the interaction of FA concentration and time (p = 0.001) (Figure 4.2A).   Bonferroni post-test indicated significant differences in growth at day 21 between 3000nM and 300nM FA (p < 0.01), and between 3000nM and 30nM FA (p < 0.01).

When analysed by two-way ANOVA, approximately 16.7% of the observed variance in viability of cells grown under each condition was found to be due to FA concentration (p = 0.03). The impact of time was responsible for 25.4% of variance (p = 0.02), and 36.4% of variance was attributable to the interaction of FA with time (p = 0.04). The cells cultured in 3000nM FA retained a steady viability of 75-85% for the full 21 days, while viability of cells in 300nM FA fell from 82% to 66% over the first 7 days. Viability of cells in 30nM FA decreased more rapidly, from 82% at day 7 to 64% at day 21 (Figure 4.2B).

Numbers of cells with morphological evidence of necrosis showed an inverse relationship to FA concentration, with 28.1% of the variance due to interaction of FA and time (p = 0.02). FA alone was responsible for 2.0% of variance (p = 0.45), and time alone for 55.8% of variance (p = 0.0002) (Figure 4.2C).

Nuclear division index (NDI) was also affected significantly by the interaction of FA treatment and time (10.3% of variance, p = 0.007). FA alone was responsible for 1.5% of variance (p = 0.14), while 84.3% of variance was attributable to time (p < 0.0001) (Figure 4.2D).

A

Source of variation	% of total variation	P value
Interaction	42.15	0.001
FA	9.43	0.007
Time	32.8	0.0002

B

Source of variation	% of total variation	P value
Interaction	36.26	0.0353
FA	16.68	0.0324
Time	25.43	0.0215

C

Source of variation	% of total variation	P value
Interaction	28.09	0.0202
FA	1.99	0.4538
Time	55.77	0.0002

D

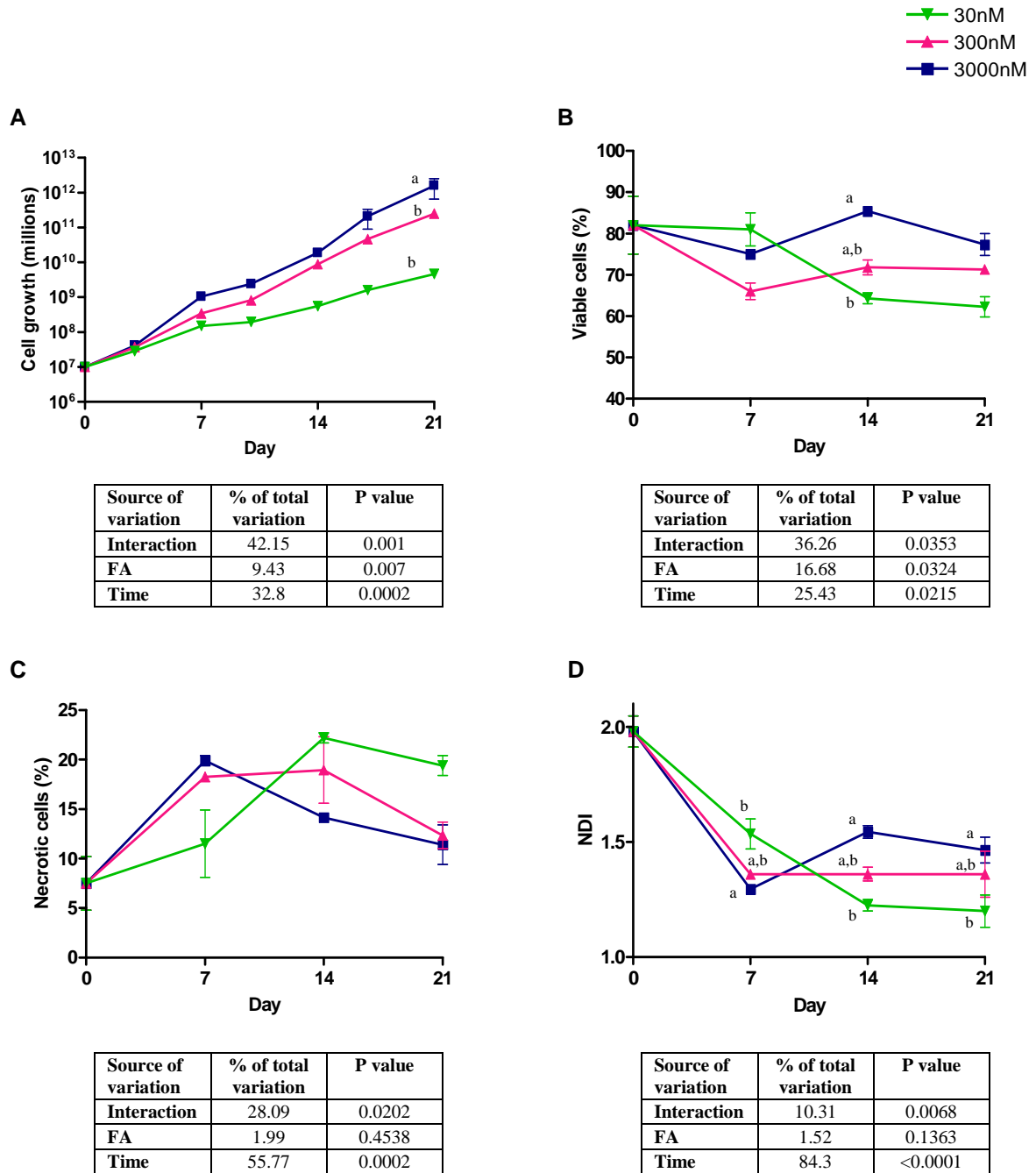| Source of variation	% of total variation	P value
Interaction	10.31	0.0068
FA	1.52	0.1363
Time	84.3	<0.0001

Figure 4.2 Effect of FA concentration on WIL2-NS cells grown in medium containing 30, 300 or 3000nM folic acid (FA) over 21 days. (A) Cell growth (calculated as described in 4.2); (B) Cell viability; (C) Percentage of necrotic cells; (D) Nuclear division index (NDI). (Points not sharing the same letter at each time point differ significantly, as measured by the Bonferroni post test. N = 2 for each FA concentration at each time point. Error bars indicate SD).

4.3.1.2 Long term (42 day) study

There was an approximately linear increase in numbers of viable cells over the 42 days of culture in medium containing 3000nM and 300nM FA. However, there was little net increase in viable cells beyond day 21 days in culture medium containing 30nM FA. The viable cell count with time area under the curve (AUC) measurements for cells grown in 3000nM, 300nM and 30nM FA were 1.38×10^{21}, 4.1×10^{18}, and 1.68×10^{12} respectively. However, two way ANOVA indicated that the differences were not statistically significant with 19.4% of the variance attributable to time (p = 0.13), 4% to FA concentration (p = 0.19), and 38.4% to the interaction of both factors (p = 0.1) (Figure 4.3A).

The viability of cells cultured in 3000nM FA remained between 80-90% throughout the observation period, with a mean of 87.3%. Viability was also steady in cultures grown in 300nM FA, with a mean of 79.4%. However, viability of cells decreased rapidly with time in cultures grown in 30nM FA, the lowest FA (30nM), declining from 72% at day 0 to 38% at Day 42, with a mean viability of 49.6% over 42 days. Two-way ANOVA analysis showed that 72.7% of the variance in viability was attributable to FA treatment (p < 0.0001), 4.6% to time (p = 0.0015) and 20.1% to the interaction of FA with time (p < 0.0001) (Figure 4.3B).

Proportions of morphologically necrotic cells showed an inverse relationship to viability. Proportions of necrotic cells were relatively constant in the cultures containing 3000nM and 300nM FA, but increased with time in those containing 30nM FA. Analysis by ANOVA showed 26% of the variance was due to the interaction of FA treatment with time (p = 0.0017) and 61.4% and was due to FA treatment (p < 0.0001) (Figure 4.3C).

NDI was sustained at approximately control levels until at least day 35 in cultures containing 3000nM and 300nM FA. However, NDI fell during the first 14 days of culture in medium containing 30nM FA, and remained low for the rest of the period of observation. Analysis by ANOVA showed that 48% of the variance observed was attributable to FA concentration in media (p < 0.0001), 19.1% due to time (p < 0.0001) and 27.6% to the interaction of FA with time (p = 0.0001) (Figure 4.3D).
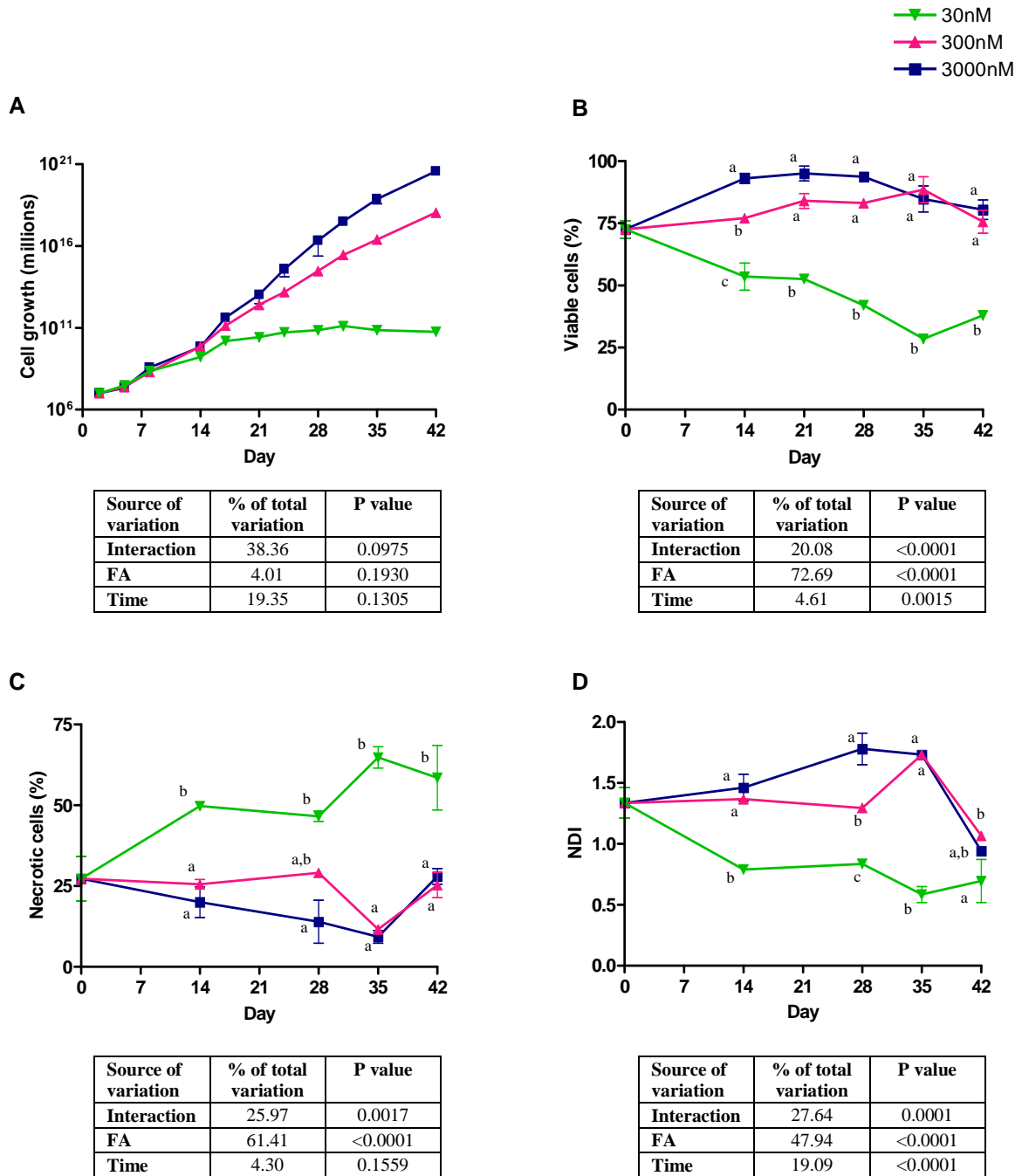
Figure 4.3 The effects of FA deficiency on WIL2-NS cells grown in medium containing 30, 300 or 3000nM folic acid (FA) over 42 days. (A) Cell growth (calculated as described in 4.2); (B) Cell viability; (C) Percentage of necrotic cells; (D) Nuclear division index (NDI). (Points not sharing the same letter at each time point differ significantly, as measured by the Bonferroni post test. N = 2 for each of these measures for each FA concentration and time point. Error bars indicate SD).

4.3.2   Assessment of telomere length by flow cytometry

4.3.2.1 Short term (21 day) study

Cells grown in the lowest concentration of FA (30nM) exhibited a 210% increase in TL by day 7 (24.8 ± 2.9) relative to that of Day 0 (11.8 ± 5.0), and a 147% increase relative to cells that were maintained in 3000nM FA for the same period (16.8 ± 2.1). TL remained elevated through to day 14, followed by a downward trend at day 21 (Figure 4.4 & Table 4.1A). TL of cells grown in 300nM FA also increased, but this effect was delayed and less pronounced (relative to 30nM FA), peaking at day 14 (20.9 ± 2.2). At this time, TL was increased by 125% relative to cells maintained for the same period in 3000nM FA (16.7 ± 1.1). In the cells grown in FA-replete medium (3000nM) throughout, TL increased slightly from a Day 0 value of 11.8 ± 5.0 to 16.8 ± 2.1 at day 7 and then remained constant through to day 21. Analysis by two-way ANOVA showed statistically significant effects for time ($p < 0.0001$), FA concentration ($p < 0.0001$), and interaction of these two factors ($p = 0.004$), explaining 42.6%, 14.4% and 8.6% of the variance, respectively (Figure 4.4).

Bonferroni post test analysis of the results obtained at day 7 showed significant differences in TL between cells grown in 30nM and 300nM FA ($p < 0.001$), and between cells grown in 30 and 3000nM ($p < 0.001$) (Figure 4.4 & Table 4.1A).

A strong, negative relationship was observed between concentration of FA in medium and areas under the curves (AUC) shown in Figure 4.4 (AUC TL) (Figure 4.6A), ($r = -0.96$, $p = 0.002$).

4.3.2.2 Long term (42 day) study

To examine whether the decline in TL after 21 days of culture in 30nM FA was the start of a continuing trend, culture was extended to 42 days. Confirming the observation in the short term culture, cells cultured in 30nM FA showed an increase in TL from 12.7 ± 3.4 at commencement of culture to 18.1 ± 2.1 at the common data point of 14 days, an increase of 142% (Figure 4.5 & Table 4.1B). Beyond this point, TL declined rapidly and consistently to 12.6 ± 1.2 at day 42 (p-trend $p < 0.0001$), returning to approximately the same length as at the commencement of culture and lengths similar to those of cells grown in either 300nM or 3000nM throughout the culture period. TL of cells grown in 300nM FA (15.3 ± 1.0) increased by 120% from commencement of culture, before declining to approximately pre-culture levels beyond day 35. The TL of cells grown in 3000nM FA remained approximately constant throughout the 42 day period of study, with a mean value across all sample points of 13.7 ± 0.6 (Figure 4.5 & Table 4.1B). Two-way ANOVA analysis indicated 8.2% of variance was attributable to the interaction of FA concentration and time ($p < 0.02$). Variance due to FA concentration alone was not significant, probably as a result of the large increase, followed by equally sharp decrease, in TL cells grown in 30nM FA (Figure 4.5).

A strong, negative relationship was observed between concentration of FA in medium and TL (Figure 4.6B), when comparisons were made of areas under the curves (AUC) shown in Figure 4.5 ($r = -0.93$, $p = 0.007$).
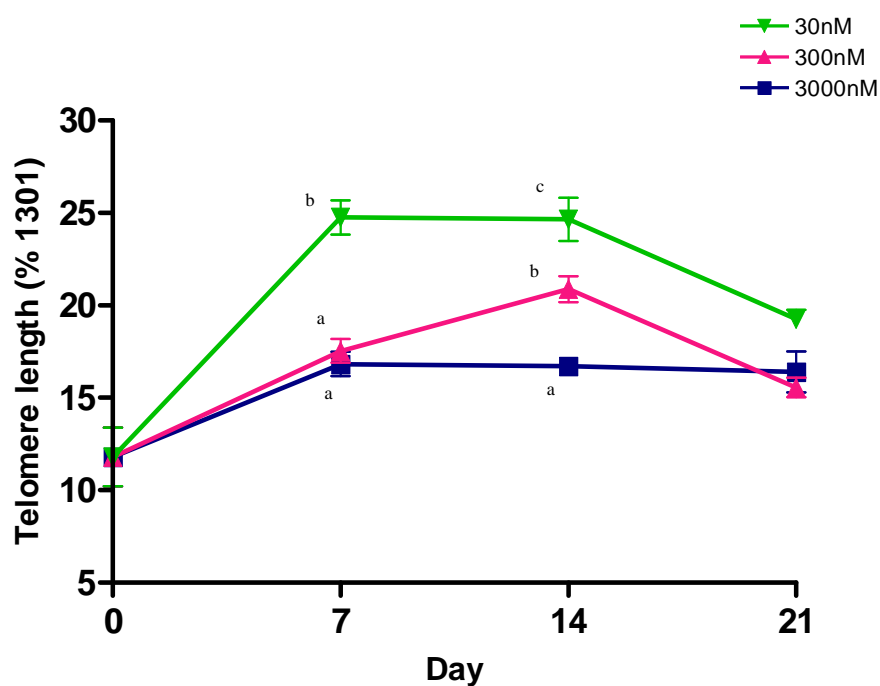
Source of variation	% of total variation	P value
Interaction	8.59	0.0004
FA	14.39	p <0.0001
Time	42.65	p <0.0001

Figure 4.4 Telomere length measured by flow cytometry in WIL2-NS cells grown in medium containing 30, 300 or 3000nM folic acid (FA) over 21 days. The TL of each sample was calculated relative to the TL of the reference standard 1301 cell line. N = 10. Error bars indicate SD.   Points not sharing the same letter at each time point differ significantly, as measured by Bonferroni post test. Data tables represent the results of two-way ANOVA analysis.
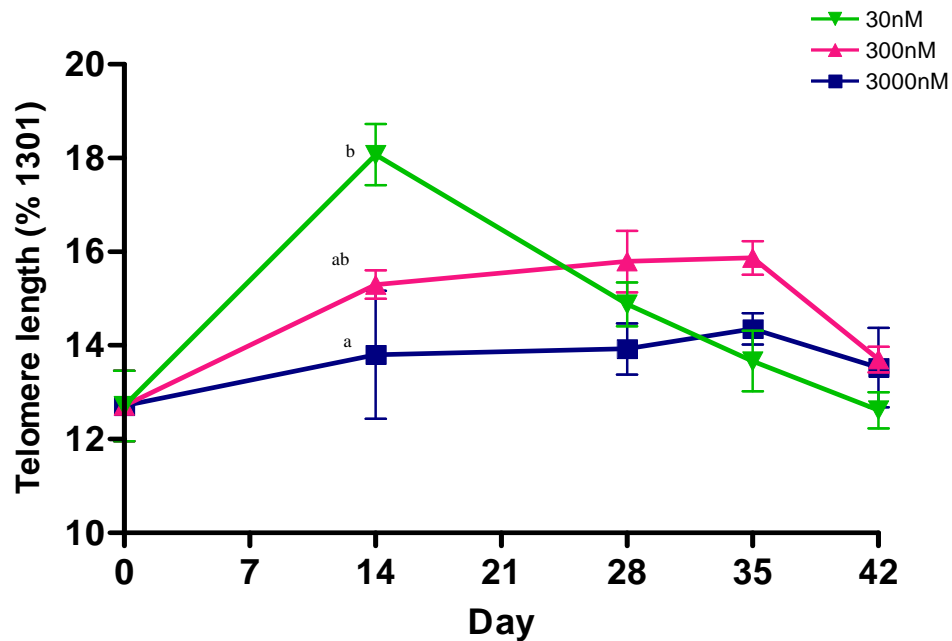
Source of variation	% of total variation	P value
Interaction	8.23	0.0235
FA	2.05	0.1051
Time	16.28	< 0.0001

Figure 4.5 Telomere length measured by flow cytometry in WIL2-NS cells grown in medium containing 30, 300 or 3000nM folic acid (FA) over 42 days. The TL of each sample was calculated relative to the TL of the reference standard 1301 cell line. N = 20 at day 0, n = 10 at all times thereafter. Error bars indicate SD. Points not sharing the same letter at each time point differ significantly, as measured by Bonferroni post test. Data tables represent the results of two-way ANOVA analysis.

Table 4.1 Telomere length of WIL2-NS cells grown in medium containing 30, 300 or 3000nM folic acid (FA) measured by flow cytometry. Cells cultured for (Table A) 21 days (n = 10) or (Table B) 42 days (n = 20 at day 0, n = 10 at all times thereafter). Data represent mean ± SD for each treatment at each time point. P values are shown for comparisons between treatments by one-way ANOVA at each time point. Points not sharing the same letter at each time point differ significantly, as measured by Tukey's post test. Also shown is area under the curve (AUC) calculated for TL versus time for each treatment.

A

Day	30nM	300nM	3000nM	One-way ANOVA P value
0	11.8 ± 5.00	11.8 ± 5.00	11.8 ± 5.00	-
7	24.75 ± 2.90^a	17.54 ± 2.02^b	16.83 ± 2.07^b	*< 0.0001*
14	24.65 ± 3.69^a	20.88 ± 2.19^b	16.71 ± 1.11^c	*< 0.0001*
21	19.27 ± 1.57^a	15.56 ± 1.67^b	16.41 ± 3.49^b	*0.005*
AUC	*454.5*	*364.7*	*333.5*	

B

Day	30nM	300nM	3000nM	One-way ANOVA P value
0	12.71 ± 3.36	12.71 ± 3.36	12.71 ± 3.36	-
14	18.07 ± 2.07^a	15.30 ± 0.96^{ab}	13.81 ± 4.32^b	*0.0076*
28	14.88 ± 1.48	15.79 ± 2.07	13.92 ± 1.73	*0.0805*
35	13.67 ± 2.04^a	15.87 ± 1.14^b	14.35 ± 1.05^{ab}	*0.0080*
42	12.62 ± 1.22	13.70 ± 0.85	13.53 ± 2.68	*0.3548*
AUC	*638.1*	*628.0*	*576.3*	

A

$r = -0.963$
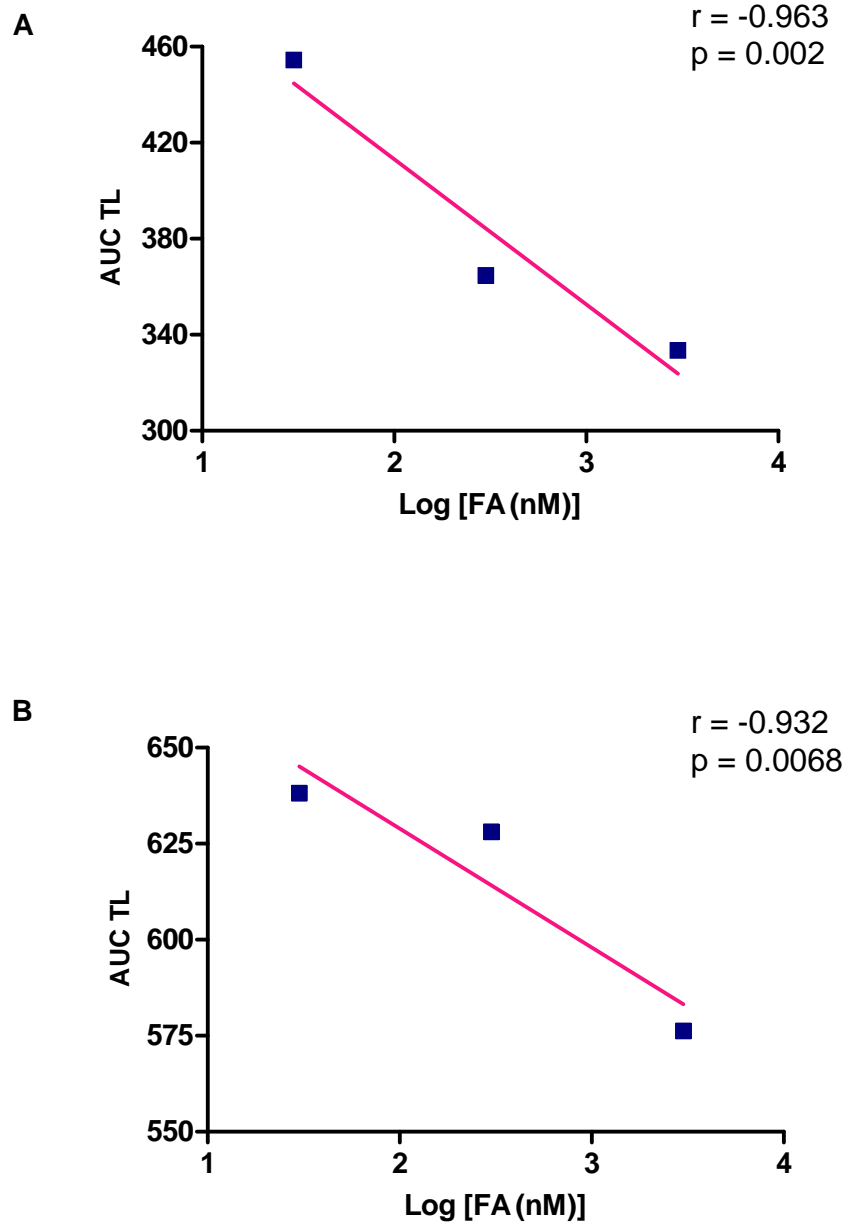$p = 0.002$

B

$r = -0.932$
$p = 0.0068$

Figure 4.6 Correlation between FA concentration in medium and telomere length of WIL2-NS cells cultured over (A) short term (21 days), and (B) long term (42 days). Data shown is for areas under the curves (from Figures 4.4 and 4.5, respectively) for telomere length with time (AUC TL) versus log of FA concentration in medium (nM).

4.3.3 Chromosomal damage

4.3.3.1 Frequency of BN cells displaying one or more DNA damage biomarker (21 day study)

The results in this section, and in Table 4.2, show the frequency of BN cells that display one or more of the biomarkers of DNA damage examined in the CBMN Cyt assay (MNi, NPB, NBuds), per 1000 BN cells. Each BN cell containing a damage event, irrespective as to whether it contains one or multiple DNA damage biomarkers, is recorded as a single event in this data.

Analysis by two-way ANOVA indicated that FA concentration in medium was responsible for the significant increases observed in the frequency of BN cells displaying one or more MNi ($p < 0.0001$), one or more NPB ($p = 0.0001$) and one or more NBud ($p = 0.03$) (Table 4.2).

The frequency of cells containing one or more MNi doubled over 21 days from 35.5 ± 3.5 (mean $\pm$ SD) at day 0 to 71.0 ± 4.2 in 30nM FA. Over the same time period MNi in cells in 300nM FA increased 134% to 47.5 ± 10.6, while the frequency of cells cultured in FA-replete medium (3000nM) containing MNi decreased to 22.0 ± 4.2 (Table 4.2A).

BN cells displaying one or more NPB increased from 14.0 ± 5.7 at day 0 to 62.0 ± 12.7, 52.5 ± 12 and 14.5 ± 6.4 at day 21 for the 30, 300 and 3000nM cultures, respectively. These values represent an increase of 442% in the 30nM culture, 375% in the 300nM culture and 103% in cells containing one or more NPB in the 3000nM condition (Table 4.2B).

The frequency of cells containing one or more NBud increased in the 30nM culture from 2.0 ± 1.4 at day 0, to 13.5 ± 12.0 at day 21, an increase of 675%. The frequency in the 300nM FA culture doubled from 2.0 ± 1.4 to 4.0 ± 1.4 after 21 days, while in the 3000nM FA culture the frequency of cells with one or more NBud decreased to 0.5 ± 0.7 at day 21 (Table 4.2C).

Analysis by two-way ANOVA indicated 47.8% of observed variance in the frequency of cells exhibiting one or more of any of the DNA damage biomarkers (MNi or NPB or NBuds) was attributable to FA treatment ($p < 0.0001$), 26.6% to time ($p < 0.0001$), and 23.2% to the interaction of FA and time ($p < 0.0001$) (Table 4.2D).

Table 4.2 Frequency, in the binucleated (BN) subset, of WIL2-NS cells displaying one or more of the DNA damage biomarkers scored in the CBMN Cyt assay following culture in medium containing 30, 300 or 3000nM folic acid (FA) over 21 days. Data is shown for each FA concentration and for each time point. Frequency per 1000 BN cells containing (A) one or more micronuclei (MNi), (B) one or more nucleoplasmic bridge (NPB), (C) one or more nuclear bud (NBud), and (D) one or more of any DNA damage biomarker (MN or NPB or NBud).   (N = 2. Mean ± SD. P values represent analysis by two-way ANOVA. Data not sharing the same superscript letter within each time point differ significantly from each other, as measured by Bonferroni post test).

		[Folic acid] in medium (nmol/L)			
	Time	30	300	3000	
(A) **Frequency of BN with** **1 or more MNi**	Day 0	35.5 ± 3.5	35.5 ± 3.5	35.5 ± 3.5	
	Day 7	64.5 ± 6.4^a	42.5 ± 7.8^b	27.5 ± 3.5^b	Effect of time
	Day 14	92.0 ± 2.8^a	70.5 ± 7.8^b	29.0 ± 2.8^c	$p < 0.0001$
	Day 21	71.0 ± 4.2^a	47.5 ± 10.6^b	22.0 ± 4.2^c	
		Effect of FA, $p < 0.0001$			Interaction, $p = 0.0002$
(B) **Frequency of BN with** **1 or more NPB**	Day 0	14.0 ± 5.7	14.0 ± 5.7	14.0 ± 5.7	
	Day 7	38.0 ± 14.1	21.5 ± 3.5	28.0 ± 4.2	Effect of time
	Day 14	64.5 ± 0.7^a	34.0 ± 0.0^b	19.5 ± 7.8^b	$p = 0.0001$
	Day 21	62.0 ± 12.7^a	52.5 ± 12.0^a	14.5 ± 6.4^b	
		Effect of FA, $p = 0.0001$			Interaction, $p = 0.0037$
(C) **Frequency of BN with** **1 or more NBud**	Day 0	2.0 ± 1.4	2.0 ± 1.4	2.0 ± 1.4	
	Day 7	12.0 ± 4.2	4.5 ± 3.5	3.0 ± 1.4	Effect of time
	Day 14	18.0 ± 18.4	4.0 ± 2.8	2.0 ± 0.0	$p = 0.5746$
	Day 21	13.5 ± 12.0	4.0 ± 1.4	0.5 ± 0.7	
		Effect of FA, $p = 0.0326$			Interaction, $p = 0.7002$
(D) **Frequency of BN with** **1 or more DNA damage** **biomarker**	Day 0	51.5 ± 10.6	51.5 ± 10.6	51.5 ± 10.6	
	Day 7	114.5 ± 16.3^a	68.5 ± 0.7^b	58.5 ± 2.1^b	Effect of time
	Day 14	174.5 ± 14.8^a	108.5 ± 10.6^b	50.5 ± 4.9^c	$p < 0.0001$
	Day 21	146.5 ± 3.5^a	104.0 ± 2.8^b	37.0 ± 9.9^c	
		Effect of FA, $p < 0.0001$			Interaction, $p < 0.0001$

4.3.3.2 Total number of DNA damage biomarkers per 1000 BN cells (21 day study)

The results presented in the previous section (4.3.3.1) show the frequency of BN cells with one or more of the biomarkers of DNA damage examined in the CBMN Cyt assay (MNi, NPB, NBuds), per 1000 BN cells. The total number of each of these biomarkers, however, is typically higher than the data presented in 4.3.3.1 because an individual BN cell may display multiple DNA damage events (see example in Figure 3.5). Accordingly, the actual number of DNA damage biomarkers present per 1000 BN is presented in this section, and in Figure 4.7. The data presented here shows the total number of each biomarker (MNi, NPBs or NBuds, individually, and collectively) recorded per 1000 BN cells, at each time point for each FA treatment over 21 days.

The total number of MNi per 1000 BN cells increased in cells grown in low (30nM or 300nM) FA medium. ANOVA analysis indicated that FA deficiency was responsible for 54.2% of variance ($p < 0.0001$), 19.3% was due to time ($p < 0.0001$), and 23.2% of observed variance was due to interaction of FA and time ($p < 0.0001$) (Figure 4.7A).

The total number of NPB present in 1000 BN cells also increased, with 34.1% of the overall variance due to FA concentration ($p < 0.0001$), 34.1% being attributable to time ($p = 0.0002$), and 23.2% to the interaction of both factors ($p = 0.0064$) (Figure 4.7B). The total number of NBuds increased only in the cells grown in 30nM FA, with FA concentration being responsible for 33.3% of the variance for this biomarker ($p = 0.03$) (Figure 4.7C).

Analysis of all damage events combined (total MNi plus NPB plus NBuds) showed that 51.1% of variance was attributable to FA concentration ($p < 0.0001$), 23% to time ($p < 0.0001$) and 23.5% to the interaction of the two ($p < 0.0001$) (Figure 4.7D).

A strong positive correlation was observed when comparisons of areas under the curves were made between telomere length with time (AUC TL, from Figure 4.4), and AUC total DNA damage biomarkers (based on Figure 4.7D) ($r = 0.98$, $p = 0.0008$) (Figure 4.8 & Table 4.3).

A strong negative relationship was observed between FA concentration in medium and AUC total DNA damage biomarkers ($r = -0.99$, $p < 0.0001$) (Figure 4.9 & Table 4.4).

Overall, these results indicated that MNi, NPB and NBuds increased in cells cultured in conditions of FA deficiency, and are paralleled by increases in TL.
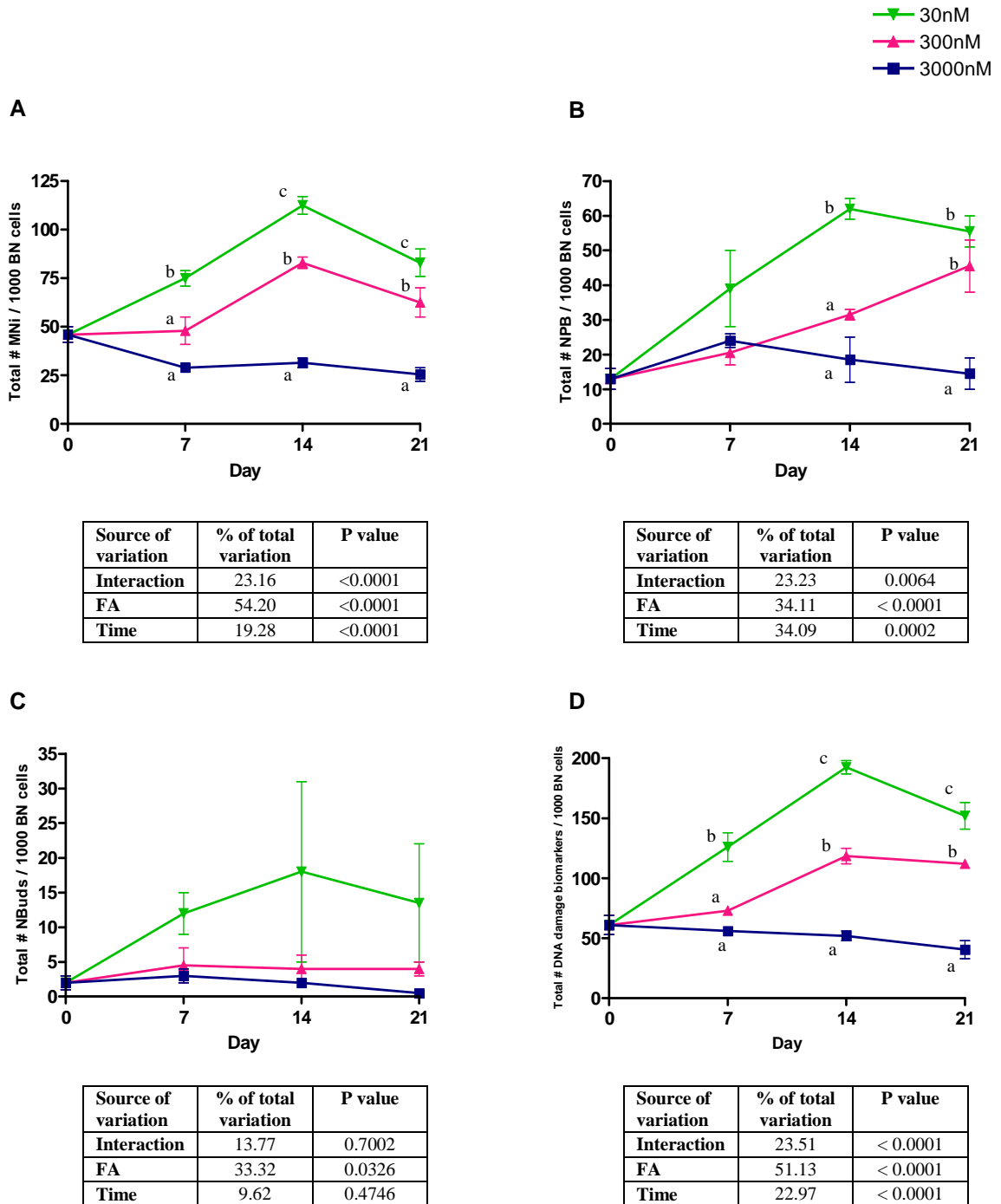
A

Source of variation	% of total variation	P value
Interaction	23.16	<0.0001
FA	54.20	<0.0001
Time	19.28	<0.0001

B

Source of variation	% of total variation	P value
Interaction	23.23	0.0064
FA	34.11	< 0.0001
Time	34.09	0.0002

C

Source of variation	% of total variation	P value
Interaction	13.77	0.7002
FA	33.32	0.0326
Time	9.62	0.4746

D

Source of variation	% of total variation	P value
Interaction	23.51	< 0.0001
FA	51.13	< 0.0001
Time	22.97	< 0.0001

Figure 4.7 Total number of DNA damage biomarkers in WIL2-NS grown in medium containing either 30, 300 or 3000nM folic acid (FA) for 21 days, measured by the CBMN Cytome assay. Data represent the total number of DNA damage biomarker events present per 1000 BN cells. (A) Total micronuclei (MN); (B) Total nucleoplasmic bridges (NPB); (C) Total nuclear buds (NBud); and (D) Total number of damage events in BN cells (MN, NPB and NBuds combined). (N = 2. Groups not sharing the same letter at each time point differ significantly from each other, as measured by Bonferroni post-test. Error bars indicate SD).
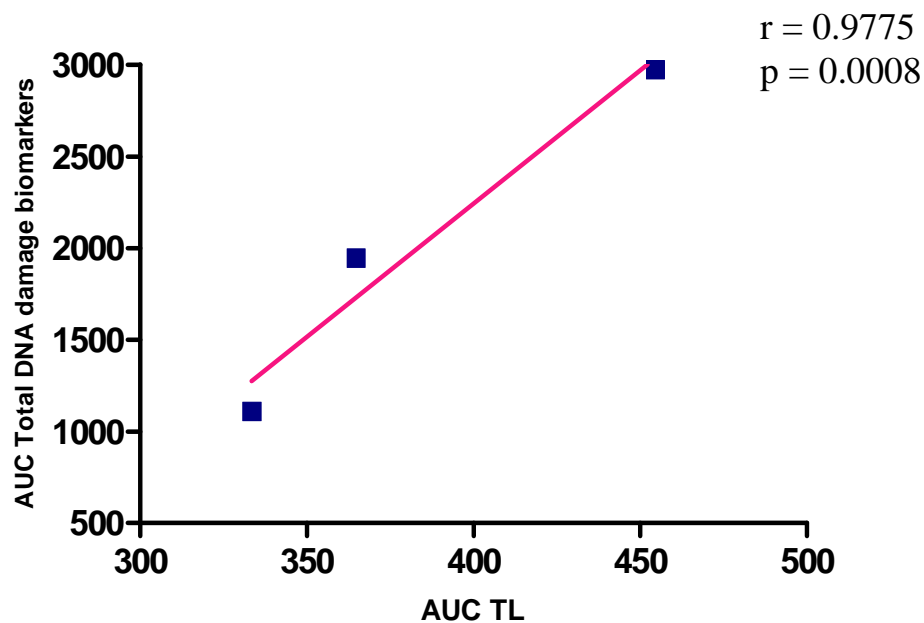
Figure 4.8 Correlation between area under the curve (AUC) of telomere length with time (AUC TL, obtained from Figure 4.4) and AUC total DNA damage biomarkers (obtained from Figure 4.7D) in WIL2-NS cells following 21 day culture in 30, 300 or 3000nM FA.

Table 4.3 Correlations between AUC TL and AUC MNi, AUC NPB and AUC NBuds (individually and combined) in WIL2-NS cells following 21 day culture in 30, 300 or 3000nM FA.

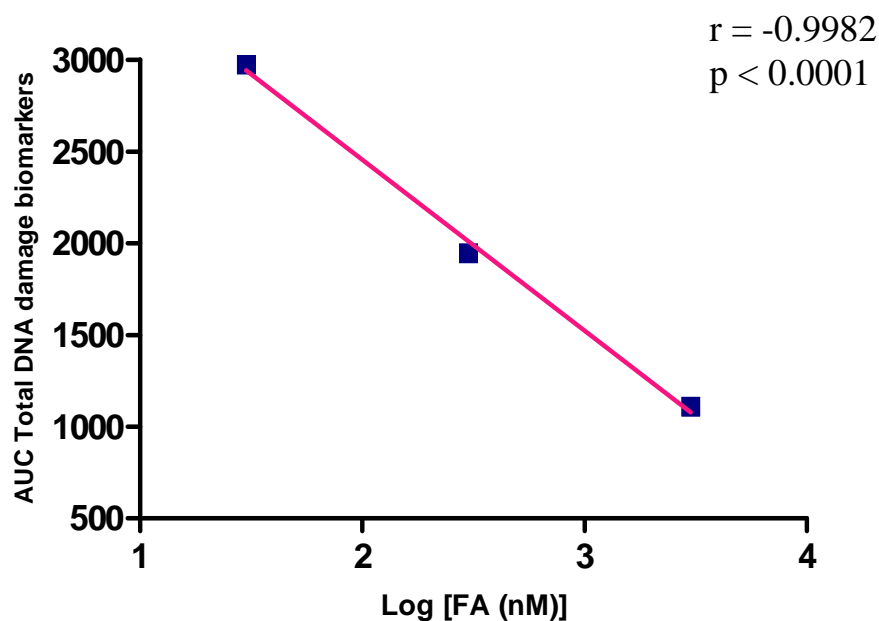|	Pearson's r	P value
Total MNi	0.9376	0.0057
Total NPB	0.9980	< 0.0001
Total NBuds	0.9955	< 0.0001
Total DNA damage biomarkers	0.9975	0.0008

Figure 4.9 Correlation between FA concentration in medium (nM) and areas under the curves (AUC) for total DNA damage biomarkers with time (obtained from Figure 4.7D) in WIL2-NS cells following 21 day culture in 30, 300 or 3000nM FA.

Table 4.4 Correlations between FA concentration in medium (nM) and AUC MNi, NPB and NBuds (individually and combined, per 1000 BN cells) in WIL2-NS cells following 21 day culture in 30, 300 or 3000nM FA.

	Pearson's r	P value
Total MNi	-0.9966	< 0.0001
Total NPB	-0.9783	0.0007
Total NBuds	-0.9332	0.0065
Total DNA damage biomarkers	-0.9982	< 0.0001

4.3.3.3 Frequency of BN cells displaying one or more DNA damage biomarker (42 day study)

The results in this section, and in Table 4.5, show the frequency of BN cells that display one or more of the biomarkers of DNA damage examined in the CBMN Cyt assay (MNi, NPB, NBuds), per 1000 BN cells. Each BN cell containing a damage event, irrespective as to whether it contains one or multiple DNA damage biomarkers, is recorded as a single event in this data. Representative photomicrographs of the DNA damage biomarkers scored in this study are presented in Figure 4.10 below. In addition to the progressive increase in DNA damage, these images also show the increasingly abnormal cellular and nuclear morphologies observed in WIL2-NS cells grown in medium containing 30, 300 and 3000nM FA over 42 days (Figure 4.10).

Analysis by two-way ANOVA indicated that FA concentration in medium was responsible for the significant increases observed in the frequency of BN cells displaying one or more MNi ($p < 0.0001$), one or more NPB ($p < 0.0001$) and one or more NBud ($p = 0.009$) (Table 4.5).

The frequency of cells containing one or more MNi increased by over 400% from day 0 (29.0 ± 8.5) to day 42 (120.5 ± 0.7) in 30nM FA. Over the same time period MNi in cells in 300nM FA increased 290% to 85.5 ± 0.7, while the frequency of cells cultured in FA-replete medium (3000nM) containing MNi remained constant at around 24 micronucleated BN cells per 1000 until day 35, followed by an increase to 63.5 ± 7.8 at day 42 (Table 4.5A).

BN cells displaying one or more NPB increased from 12.5 ± 4.9 at day 0 to 92 ± 9.9, 30.5 ± 2.1 and 26.5 ± 7.8 at day 42 for the 30, 300 and 3000nM cultures, respectively. These values represent an increase of 765% in the 30nM culture, 250% in the 300nM culture and 215% in cells containing one or more NPB in the 3000nM condition (Table 4.5B).

The frequency of cells containing one or more NBud increased in the 30nM culture from zero at day 0, to 5.0 ± 4.2 at day 42, however the frequency peaked in all conditions at day 14 where 17 ± 7.1 NBuds were recorded in 30nM FA, 4.5 ± 3.5 in 300nM and 1.0 NBud was scored in cells cultured in 3000nM FA (Table 4.5C).

Analysis by two-way ANOVA indicated 55.2% of observed variance in the frequency of cells exhibiting one or more of any of the DNA damage biomarkers (MNi or NPB or NBuds) was attributable to FA treatment ($p < 0.0001$), 23.8% to time ($p < 0.0001$), and 18.4% to the interaction of FA and time ($p < 0.0001$) (Table 4.2D).
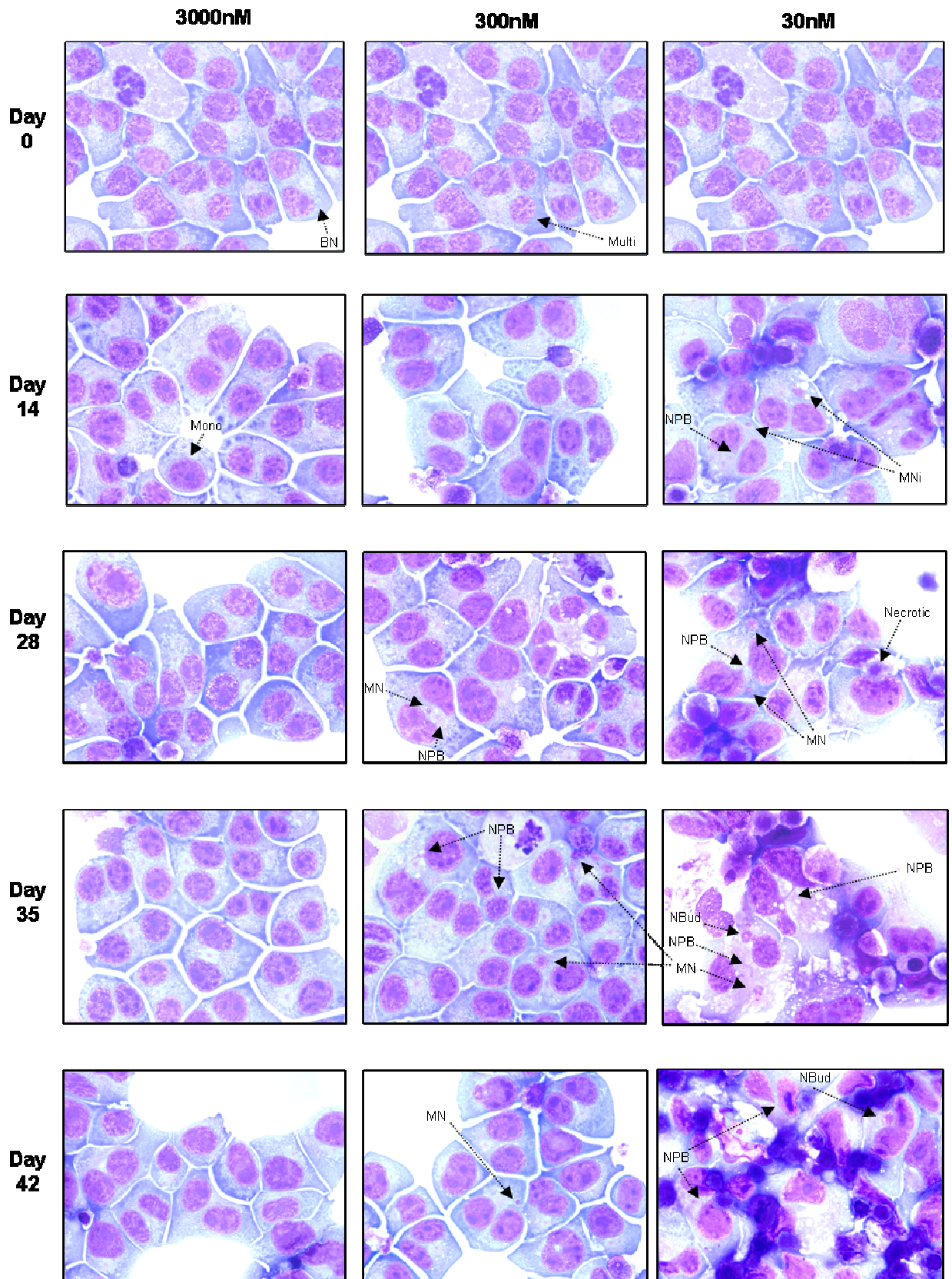
Figure 4.10   Representative photomicrographs indicating the progressive increase in chromosomal damage and abnormal cellular and nuclear morphology in WIL2-NS cells grown in medium containing 30, 300 or 3000nM FA over 42 days. Arrows indicate damage scored as part of the CBMN Cytome assay. (BN, binucleated cell; Multi, multinucleated cell; MN/MNi, micronucleus/nuclei; NBud, nuclear bud; NPB, nucleoplasmic bridge). (1000x Magnification).

Table 4.5 Frequency, in the binucleated (BN) subset, of WIL2-NS cells displaying one or more of the chromosomal damage biomarkers scored in the CBMN Cyt assay following culture in medium containing 30, 300 or 3000nM folic acid (FA) over 42 days. Data is shown for each FA concentration and for each time point. Frequency per 1000 BN cells containing (A) one or more micronuclei (MNi), (B) one or more nucleoplasmic bridge (NPB), (C) one or more nuclear bud (NBud), and (D) one or more of any damage biomarker (MN or NPB or NBud).   (N = 2. Mean ± SD. P values represent analysis by two-way ANOVA. Data not sharing the same superscript letter within each time point differ significantly from each other, as measured by Bonferroni post test).

| | Time | [Folic acid] in medium (nmol/L) | | | |
		30	300	3000	
	Day 0	29.0 ± 8.5	29.0 ± 8.5	29.0 ± 8.5	
	Day 14	114.5 ± 17.7^a	77.5 ± 7.8^b	24.5 ± 4.9^c	Effect of time
Frequency of BN with	Day 28	96.5 ± 6.4^a	55.0 ± 19.8^b	23.0 ± 0.0^c	$p < 0.0001$
1 or more MNi	Day 35	130.0 ± 9.9^a	46.5 ± 6.4^b	23.0 ± 4.2^b	
	Day 42	120.5 ± 0.7^a	85.5 ± 0.7^b	63.5 ± 7.8^b	
		Effect of FA, $p < 0.0001$			Interaction, $p < 0.0001$
	Day 0	12.5 ± 4.9	12.5 ± 4.9	12.5 ± 4.9	
	Day 14	53.0 ± 8.5^a	19.5 ± 7.8^{ab}	11.0 ± 5.7^b	Effect of time
Frequency of BN with	Day 28	63.5 ± 10.6^a	18.5 ± 4.9^b	5.5 ± 2.1^b	$p = 0.0001$
1 or more NPB	Day 35	97.0 ± 29.7^a	17.0 ± 7.1^b	13.0 ± 2.8^b	
	Day 42	92.0 ± 9.9^a	30.5 ± 2.1^b	26.5 ± 7.8^b	
		Effect of FA, $p < 0.0001$			Interaction, $p = 0.0009$
	Day 0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
	Day 14	17.0 ± 7.1^a	4.5 ± 3.5^b	1.0 ± 0.0^b	Effect of time
Frequency of BN with	Day 28	5.0 ± 1.4	0.5 ± 0.7	0.0 ± 0.0	$p = 0.0597$
1 or more NBud	Day 35	9.0 ± 11.3	0.5 ± 0.7	1.0 ± 1.4	
	Day 42	5.0 ± 4.2	0.5 ± 0.7	7.0 ± 5.7	
		Effect of FA, $p = 0.0085$			Interaction, $p = 0.1655$
	Day 0	41.5 ± 3.5	41.5 ± 3.5	41.5 ± 3.5	
Frequency of BN with	Day 14	184.5 ± 16.3^a	101.5 ± 12.0^b	36.5 ± 0.7^c	Effect of time
1 or more DNA damage	Day 28	165.0 ± 5.7^a	74.0 ± 14.1^b	28.5 ± 2.1^b	$p < 0.0001$
biomarker	Day 35	236.0 ± 50.9^a	64.0 ± 14.1^b	37.0 ± 0.0^b	
	Day 42	217.5 ± 13.4^a	116.5 ± 2.1^b	97.0 ± 5.7^b	
		Effect of FA, $p < 0.0001$			Interaction, $p < 0.0001$

4.3.3.4 Total number of DNA damage biomarkers per 1000 BN cells (42 day study)

The results presented in the previous section (4.3.3.3) show the frequency of BN cells with one or more of the biomarkers of DNA damage examined in the CBMN Cyt assay (MNi, NPB, NBuds), per 1000 BN cells. Tthe actual number of DNA damage biomarkers present per 1000 BN is presented in this section, and in Figure 4.11. The data presented here shows the total number of each biomarker (MNi, NPBs or NBuds, individually, and collectively) recorded per 1000 BN cells, at each time point for each FA treatment over 42 days.

The number of MNi increased in cells grown in low FA medium (30nM or 300nM) with ANOVA analysis indicating that FA deficiency was responsible for 52.1% of variance (p < 0.0001), 28.1% was due to time (p < 0.0001), and 17.7% of variance was due to interaction of FA and time (p < 0.0001) (Figure 4.11A).

The total number of NPB present also increased, with 57.9% of the overall variance due to FA concentration (p < 0.0001), 18.8% being attributable to time (p < 0.0001), and 17.9% to the interaction of both factors (p = 0.0012) (Figure 4.11B). The number of NBuds increased in the lowest FA treatment, with FA concentration being responsible for 24.7% of the variance (p = 0.009) (Figure 4.11C). Two-way analysis of all DNA damage events combined (MNi plus NPB plus NBuds) per 1000 BN cells showed that 56% of variance was attributable to FA concentration (p < 0.0001), 24.8% to time (p < 0.0001) and 17.6% to the interaction of the two (p < 0.0001) (Figure 4.11D).

A strong positive correlation was observed between the areas under the curves for telomere length with time (AUC TL, from Figure 4.5), and AUC total DNA damage biomarkers (from Figure 4.11D) (r = 0.84, p = 0.037) (Figure 4.12 & Table 4.6). A strong negative relationship was observed between FA concentration in medium and AUC of total number of damage biomarkers with time (r = -0.98, p = 0.0007) (Figure 4.13 & Table 4.7).

Overall, these results confirmed, and extended those of the 21-day study indicating that MNi, NPB and NBuds increased in cells cultured in conditions of FA deficiency, and are paralleled by increases in TL.
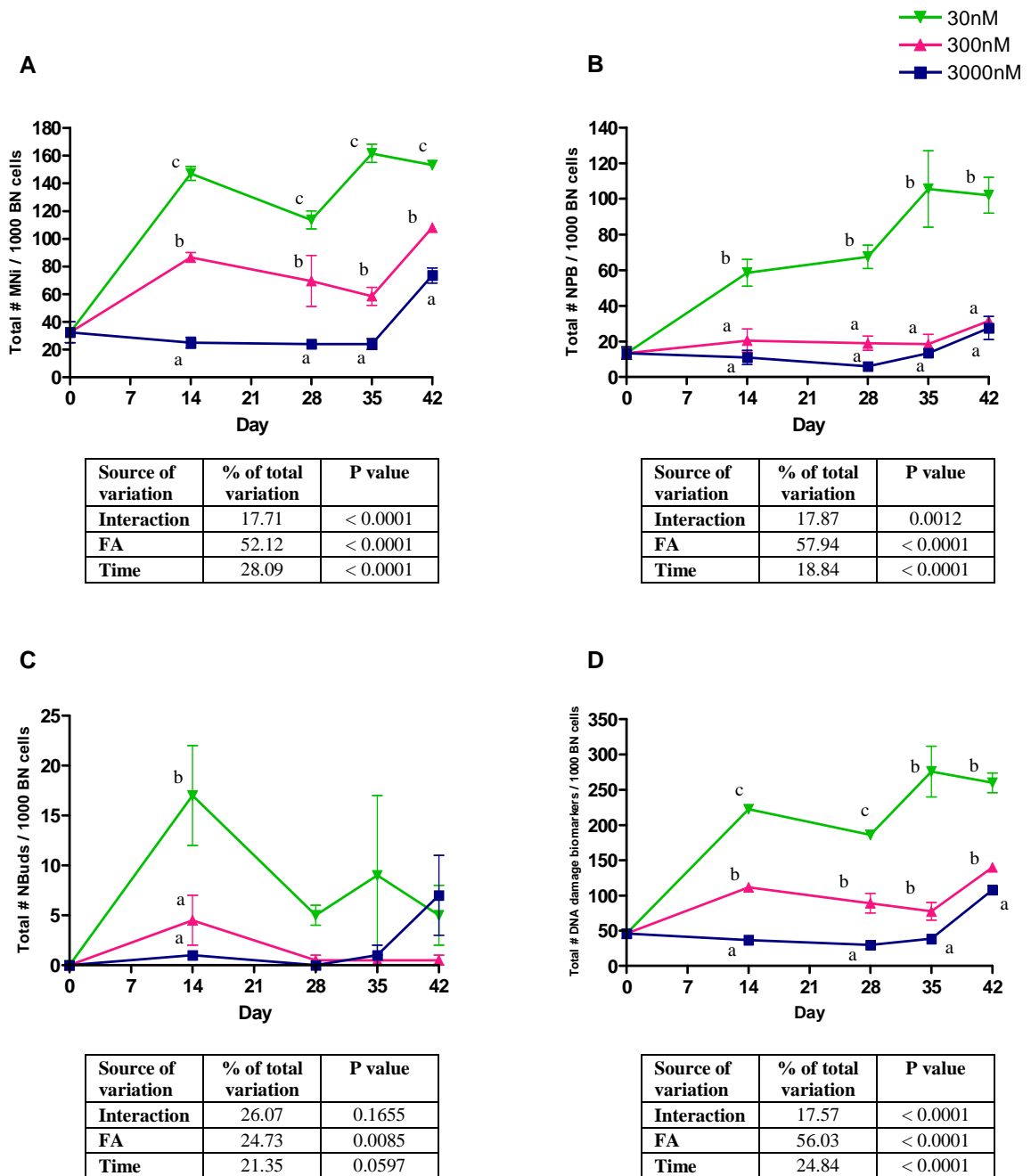
A

Source of variation	% of total variation	P value
Interaction	17.71	< 0.0001
FA	52.12	< 0.0001
Time	28.09	< 0.0001

B

Source of variation	% of total variation	P value
Interaction	17.87	0.0012
FA	57.94	< 0.0001
Time	18.84	< 0.0001

C

Source of variation	% of total variation	P value
Interaction	26.07	0.1655
FA	24.73	0.0085
Time	21.35	0.0597

D

Source of variation	% of total variation	P value
Interaction	17.57	< 0.0001
FA	56.03	< 0.0001
Time	24.84	< 0.0001

Figure 4.11: **Total number of DNA damage biomarkers in WIL2-NS grown in medium containing either 30, 300 or 3000nM folic acid (FA) for 42 days, measured by the CBMN Cytome assay**. Data represent the total number of DNA damage biomarker events present per 1000 BN cells. (A) Total micronuclei (MN); (B) Total nucleoplasmic bridges (NPB); (C) Total nuclear buds (NBud); and (D) Total number of damage events in BN cells (MN, NPB and NBuds combined). (N = 2. Groups not sharing the same letter at each time point differ significantly from each other, as measured by Bonferroni post-test. Error bars indicate SD).
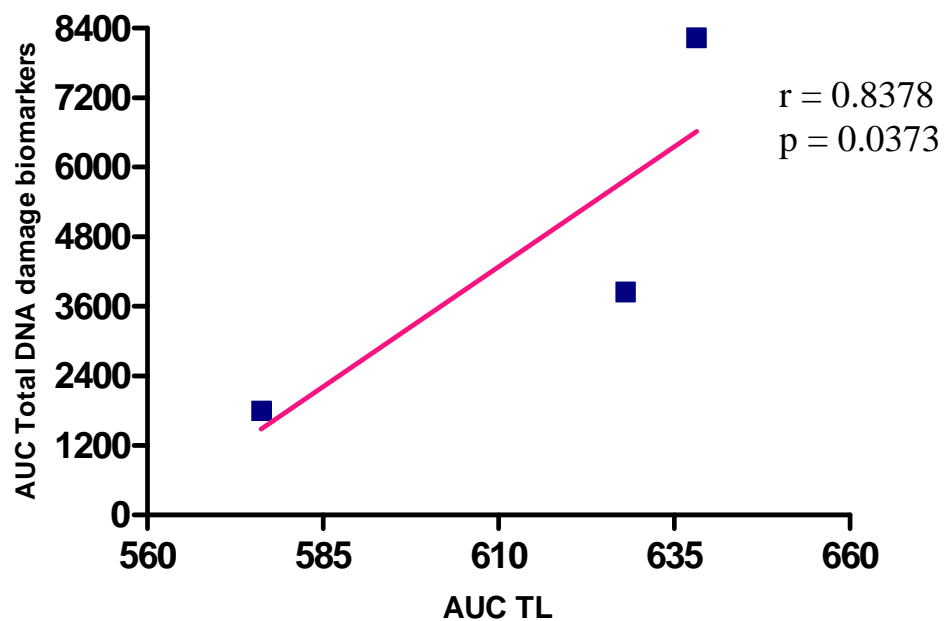
$r = 0.8378$
$p = 0.0373$

Figure 4.12 Correlation between area under the curve (AUC) of telomere length with time (AUC TL, obtained from Figure 4.5) and AUC total DNA damage biomarkers (obtained from Figure 4.11D) in WIL2-NS cells following 42 day culture in 30, 300 or 3000nM FA.

Table 4.6 Correlations between AUC TL and AUC MNi, NPB and NBuds (individually and combined) in WIL2-NS cells following 42 day culture in 30, 300 or 3000nM FA.

	Pearson's r	P value
Total MNi	0.9035	0.0135
Total NPB	0.7240	0.1037
Total NBuds	0.6845	0.1336
Total DNA damage biomarkers	0.8378	0.0373
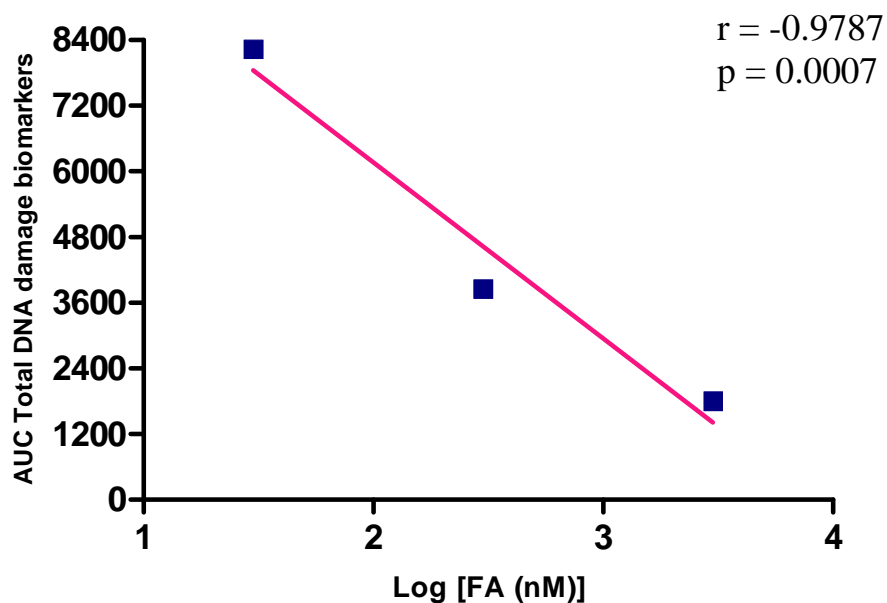
Figure 4.13 Correlation between FA concentration in medium (nM) and areas under the curves (AUC) for total DNA damage biomarkers with time (obtained from Figure 4.11D) in WIL2-NS cells following 42 day culture in 30, 300 or 3000nM FA.

Table 4.7 Correlations between AUC TL and AUC MNi, NPB and NBuds (individually and combined) in WIL2-NS cells following 42 day culture in 30, 300 or 3000nM FA.

	Pearson's r	P value
Total MNi	-0.9974	< 0.0001
Total NPB	-0.9248	0.0083
Total NBuds	-0.9022	0.0139
Total DNA damage biomarkers	-0.9787	0.0007

4.3.4   Homocysteine in spent medium

Homocysteine (Hcy) measurements were performed on fresh medium at day 0, and in spent medium collected at the day 42 time point of the long term study. Viable cell counts were performed on the contents of each flask, so that Hcy concentrations (µmol/L) could be calculated per million cells. To ensure accurate comparison between treatments, the Hcy concentration was then converted, using the formula weight for Hcy and the volume of each flask, to µg/ Hcy produced per million viable cells.

After 42 days in culture, cells grown in 30nM FA produced a significantly greater amount of Hcy per 1×10^6 viable cells (4.5µg), compared with cells cultured in 300nM (1.8µg) and 3000nM FA (1.7µg) (Figure 4.14). One way ANOVA analysis of day 42 data showed a significant difference in the amounts of Hcy produced between 30nM compared with 300 and 3000nM, but not between the 300 and 3000nM conditions ($p < 0.0001$).
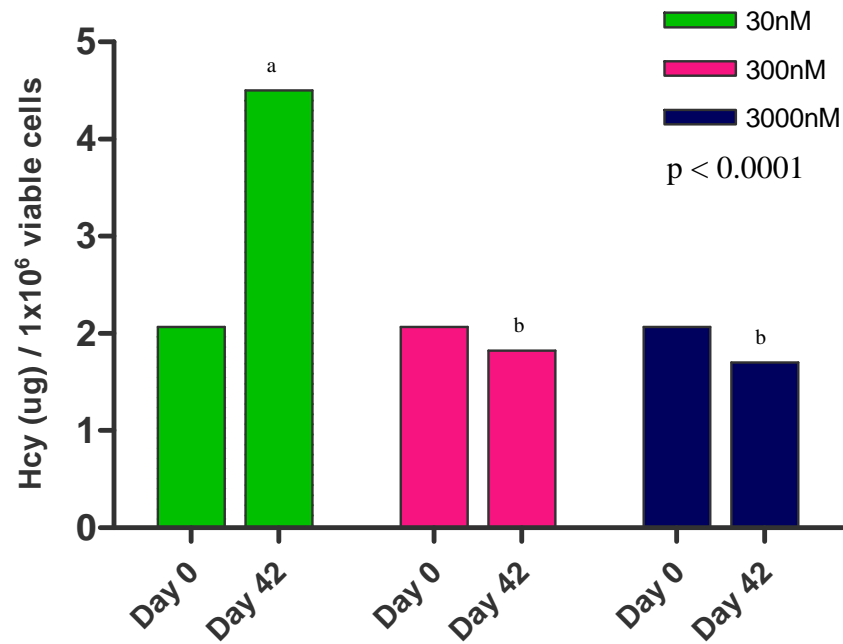
Figure 4.14 Homocysteine (Hcy) produced by WIL2-NS cells cultured in 30, 300 or 3000nM FA for 42 days. Data represents the amount of Hcy (µg) produced by 1×10^6 viable WIL2-NS cells grown in 30, 300 or 3000nM FA.   For purposes of comparison, the concentration of Hcy in fresh medium at day 0 and spent medium at day 42 of culture was converted to µg per million viable cells (n = 1. P value represents one-way ANOVA analysis at day 42. Columns not sharing the same letter at day 42 of each treatment differ significantly from each other, as measured by Tukey's post-test).

4.3.5 Effects of FA on levels of expression of telomerase in WIL2-NS cells

The increase in telomere content observed in WIL2-NS cells when grown in FA-deficient conditions raised the question as to whether this cell line expresses telomerase. The findings of a preliminary *in vitro* study, conducted earlier by the author, were relevant in answering this question, and these are provided below in summary form.

4.3.5.1 Telomerase Inhibitor study

This preliminary study involved growing WIL2-NS cells over 42 days in complete medium containing one of six telomerase inhibitor (TI) treatments (Table 4.7 below). Treatment medium contained either 0 or 3mM 3-aminobenzamide (3AB), combined with 0, 1 or 10μM MST-312. MST-312 is a synthetic analogue of epigallocatechin gallate (EGCG), a component of green tea, which had been found to inhibit telomerase activity. MST-312 has ten fold higher potency as an inhibitor of telomerase as compared with EGCG, with telomere shortening observed in human monoblastoid leukemia cells (U937) after culture for 30 days[320]. 3AB is a poly-ADP ribose polymerase (PARP)-inhibitor that has been used *in vitro* to inhibit tankyrase activity, thus indirectly inhibiting the effect of telomerase in the cell[321]. Tankyrase is a PARP molecule that, by poly-ADP ribosylation of telomere-bound TRF1 molecules, causes their release from the telosome, thus allowing telomerase to bind and extend telomeric DNA[321,322]. Individually, these two compounds had shown effective inhibition of telomerase-mediated telomere elongation[321]. Combination of the two compounds had, however, shown a synergistic effect, resulting in enhancement of telomerase inhibition[321]. The design of the preliminary experiment is shown in Table 4.7.

Table 4.7 Experimental design for testing the effects of telomerase inhibition on telomere length in WIL2-NS cells.

		3AB concentration (mM)	
		0	3
MST-312 concentration (μM)	0	Treatment #1 Solvent (DMSO) only	Treatment #4 3AB only
	1	Treatment #2 1μM MST only	Treatment #5 1μM MST & 3mM 3AB
	10	Treatment #3 10μM MST only	Treatment #6 10μM MST & 3mM 3AB

WIL2-NS cells were cultured for 42 days in the presence of the chemical combinations shown, and cells were sampled every 14 days for TL measurement by flow cytometry. Cells cultured in 10μM MST-312 (Treatment #3 and #6) died early in the study, while cells in the three remaining treatments and in the control culture (DMSO only) grew successfully for 42 days.

Telomere length decreased significantly over the 42 day observation period. However, as a similar decline in TL was observed in the control culture, this effect was due to the length of time in culture rather than any detectable effects of the treatments with telomerase inhibitors. Two-way ANOVA analysis indicated that only 0.41% of observed variance was attributable to treatment (p = 0.06), whereas 96.1% was attributable to time (p < 0.0001). 2.8% of observed variance resulted from the interaction of treatment and time (p = 0.0005) (Figure 4.15).

This study was initially designed for the purpose of producing WIL2-NS cells with shortened TL for use as a negative control in the TL studies. As the WIL2-NS cells cultured in medium containing DMSO only were to be the positive control, no further positive controls had been incorporated into the study design. Given the lack of a control to demonstrate the functionality of the inhibitors, the possibility that the compounds were inactive, or that the response in WIL2-NS differed, somehow, from cell lines used in previous studies, can not be excluded. However, as TI treatment had no discernible effect on TL of WIL2-NS cells, it may also be possible to conclude from these preliminary data that telomerase may either not be present in WIL2-NS, or may only be present at very low levels.
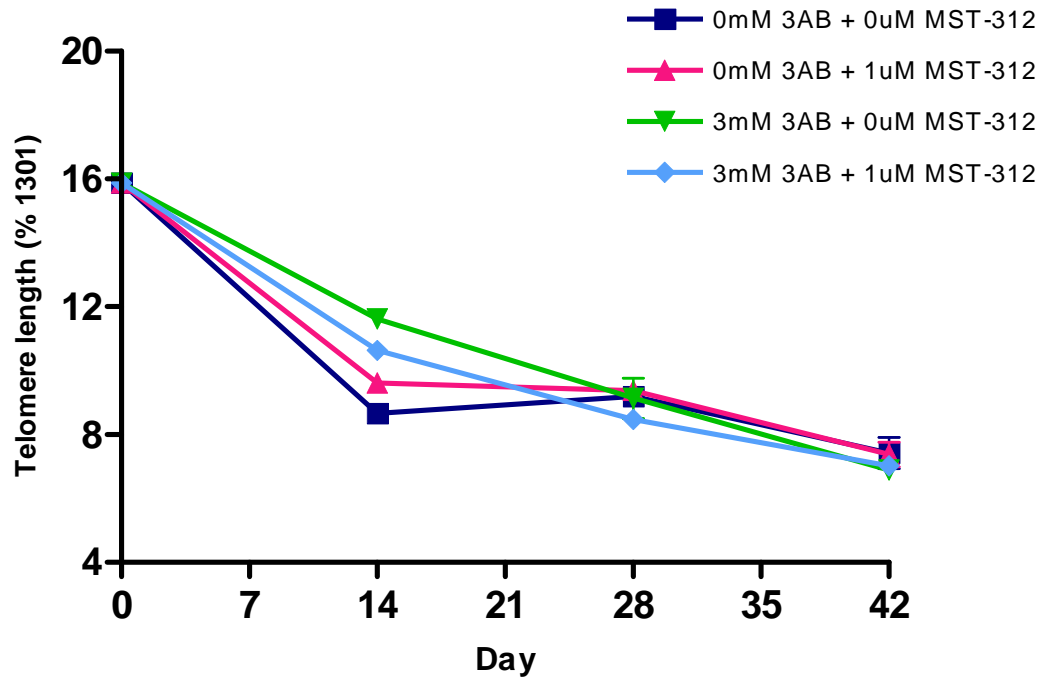
Source of variation	% of total variation	P value
Interaction	2.76	0.0005
TI Treatment	0.41	0.0604
Time	96.11	< 0.0001

Figure 4.15 **Telomere length (TL) of WIL2-NS cells cultured in complete medium containing telomerase inhibitors for 42 days, measured by flow cytometry**. (N = 10 at day 0, n = 4 at all points thereafter. TL of sample is calculated relative to the TL of 1301 cell line standard. Data table represents result of analysis by two-way ANOVA. Error bars indicate SD).

4.3.5.2 hTERT Expression analysis

Preliminary findings of the telomerase inhibitor (TI) study suggested that WIL2-NS cells may not have significant levels of telomerase activity. It is possible, however, that FA-deficient culture conditions might impact on expression of *hTERT*, the catalytic component of the telomerase enzyme. Ideally the Telomeric Repeat Amplification Protocol (TRAP assay) would have been used to determine telomerase activity, however, this assay was not established in this laboratory. Instead, expression of *hTERT* was quantified in samples obtained during the long term (42 day) FA-deficiency study to provide a representative measure of telomerase expression. This was achieved by estimation of *hTERT* RNA transcripts by quantitative RT-PCR (qPCR), normalisation based on levels of transcripts encoding the house-keeping gene GAPDH, and expressing the results relative to the level of *hTERT* transcripts at day 0 (WIL2-NS). Two-way ANOVA analysis of these data showed no significant differences between treatments, with 5.2% of variance attributable to FA concentration in medium ($p = 0.77$), and 18.2% of variance due to time ($p = 0.75$) (Figure 4.16).
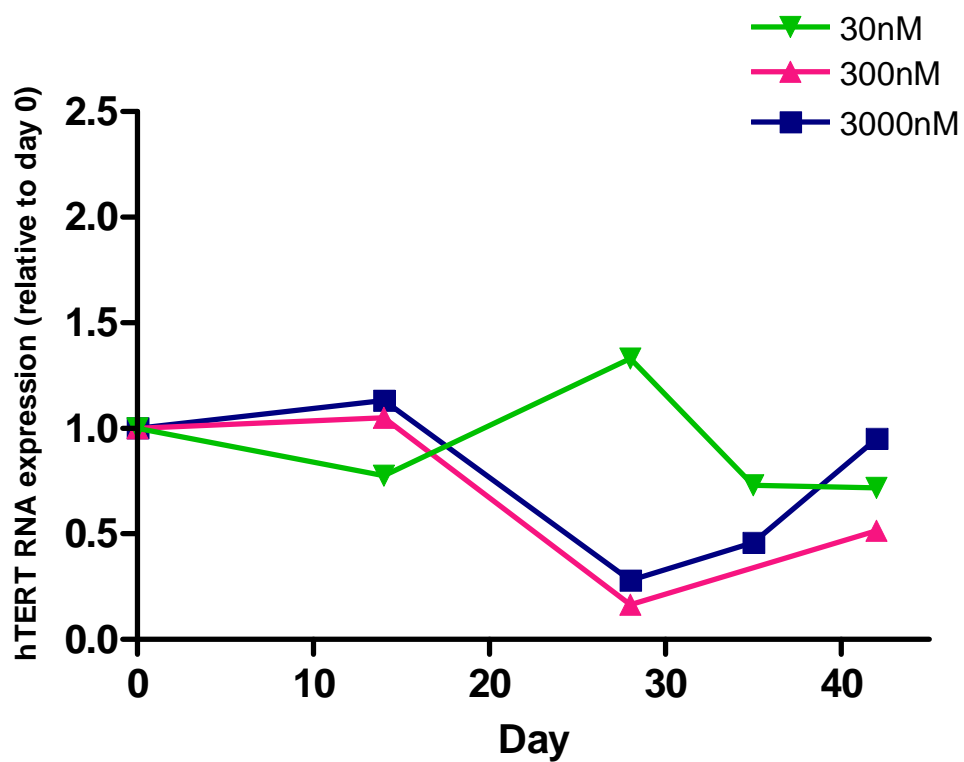
Figure 4.16 Levels of *hTERT* transcripts in WIL2-NS cells grown in medium containing 30, 300 or 3000nM FA*, measured by qPCR. Results are displayed as fold change from hTERT expression of wildtype WIL2-NS (n = 2).

4.4 DISCUSSION

4.4.1 Impact of FA depletion on telomere length

Estimation of telomere length (TL) was a vital component of this study. Preliminary attempts were made to measure absolute TL in DNA isolated from cells in the course of the FA studies, using a novel qPCR method developed in this laboratory[308]. This method has been validated against Southern blot methodology (telomere restriction fragments (TRF)) using mononuclear cells isolated from fresh blood samples. However, the preliminary data suggested that the method was not suitable for samples that contained high proportions of damaged and necrotic cells (data not shown). The qPCR method amplifies telomeric DNA, irrespective of cell viability or stage in the cell cycle, thus making comparison of TL between folate deplete and replete cells difficult. Additionally, polymerase activity in the PCR assay may be slowed where DNA damage (*eg.* abasic sites) is encountered. This is an additional confounder in studies of folate deficiency, where uracil incorporation promotes formation of abasic sites and there is likely to be severe cell damage. In contrast, flow cytometry allows selection of only in viable $G_{0/}1$ cells that have a full 2N nuclear complement of DNA. In this context, flow cytometry provides a more reliable comparison of TL, regardless of folate status. For these reasons, and because the preliminary data obtained by qPCR were not satisfactory, flow cytometry was selected as the most appropriate method for the current studies.

Previous studies have shown that during FA deficiency, uracil is incorporated into DNA as a result of the scarcity of methyl groups available for the synthesis of thymidine[91,93,323]. Where FA is limiting, imbalance in the intracellular thymidine:uracil ratio leads to increased incorporation of uracil and as a consequence, an increased frequency of abasic sites[91,93,323]. The abasic sites arise by excision of the aberrant nucleotide by uracil glycosylase. Where abasic sites exist in close proximity on opposing strands, there is a ten-fold increase in the occurrence of double stranded breaks (DSBs)[75]. For this reason it was hypothesised that telomere sequences would be vulnerable to DSBs in FA-deficient conditions due to their high thymidine content and that this would result in shortening of telomeres.

An additional risk to telomeres arises from the possibility that damage in telomeric DNA may not be repaired as effectively as in other regions of the chromosome[271]. It has been proposed that the telomere, being "expendable" by design, may in some way be a lower priority for

repair. The specific mechanism responsible for this difference in repair efficiency has not been identified. Work conducted by Ricchetti *et al* (2003) in yeast showed that DSB repair efficiency is reduced the closer the damage is to the telomere, possibly due to the non-histone chromatin structure in this region[271]. A more recent study, also in yeast, provided further evidence for this effect, showing that telomeric repeats inhibit DNA repair mechanisms, regardless of the location of telomere sequence in the chromosome[272]. Michelson *et al* (2005) found that internal tracts of telomeric repeats inhibited DNA damage checkpoint signaling from adjacent DSBs. Another study, by Richter *et al*, explored the mechanism underlying inhibited repair of single strand breaks (SSB) within the telomere[177]. TRF2 over-expression was used to examine whether binding of TRF2 inhibited SSB repair, thus leaving the telomere vulnerable to accelerated shortening. While the study by Richter *et al* was based on SSBs arising from damage induced by reactive oxygen species (ROS), it is plausible that a similar effect could occur following lesions induced by FA insufficiency. From their findings, both Richter *et al* and Michelson *et al* concluded that the three-dimensional structure of proteins bound to the telomere could sterically hinder repair processes[177,272].

When the effects of folate deficiency were examined in WIL2-NS cells, there was an unexpected increase in TL following culture for14 days in medium containing 30nM FA. The increase was statistically significant and it occurred in a dose-dependent manner. In the cultures containing 30nM or 300nM FA TL declined during the final 14-21day period, while it remained relatively constant throughout the 21 days in cells grown in FA-replete medium (3000nM).

This late decline in TL prompted a longer-term study, with a common data point at 14 days, followed by culture for an extended time frame in FA-deficient media, to 42 days. The flow cytometric estimates of TL confirmed the trends observed in the initial study. Again, TL increased sharply during the first 14 days of culture in 30nM FA, and this increase was dependent on FA concentration. Furthermore, the downward trend in TL observed at day 14 after commencement of culture in 30nM FA was also confirmed, and this decline continued in a linear fashion through to day 42. There was a similar trend in cells cultured in 300nM FA, although it developed less rapidly and was less marked, while TL remained relatively constant throughout the culture period in folate-sufficient medium. By 42 days, TL was similar in all culture conditions. Thus, although TL increased in folate-deficient conditions, this change was not sustained. While this decline was consistent with the initial hypothesis, there was no net decrease in TL over the observation period as a whole. Until the mechanism responsible for

the initial increase in TL is known, it is difficult to assess whether the later shortening is a confirmation of hypothesis one.

The unexpected increase in TL (208% increase in the 21-day, and 142% in the 42-day study) during the first 14 days of culture in 30nM FA clearly did not fit with the hypothesis that uracil incorporation under conditions of folate deficiency will lead to shortening of telomeres. There were numerous possible explanations for these findings, several of which could act in concert. An initial consideration was that the phenomenon may have been due to an altered balance in metabolic enzyme activity. The ability of cells to utilise folate is dependent on the action of enzymes in the folate/methionine cycle (Figure1.4)[91,93,323]. One key enzyme that can affect the folate/homocysteine balance is methylene tetrahydrofolate reductase (MTHFR). When folate is limiting, common polymorphisms (MTHFR C677T) have been shown to affect activity of this enzyme, altering the rate of thymidine synthesis[91,93,323]. There is also evidence that individuals who carry the variant (T) allele have different rates of chromosomal instability compared with non-carriers and that methylation status of DNA could also be affected[91,93,323]. However, when the WIL2-NS cell line was genotyped for the $MTHFR_{C677T}$ polymorphism (data not shown), allelic discrimination analysis showed that the WIL2-NS cell line is homozygous for the more frequent C allele. This suggested that neither the incorporation of uracil, nor DNA methylation, were likely to be affected in this pathway. Accordingly, it was concluded that genetically imposed reduction in MTHFR activity was an unlikely cause of increased TL during folate deficiency.

A second possibility is that telomerase itself could be directly responsible for the observed increase in TL. An earlier pilot study using telomerase inhibitors had suggested that WIL2-NS cells may not have detectable levels of telomerase activity, however, this may have been affected by FA deficiency. Accordingly, in the absence of TRAP assay data, *hTERT* expression levels were measured in the current study in cells grown in FA-deficient medium. Previous evidence has indicated that regulation of telomerase activity is dependent upon presence of *hTERT,* the active component of the enzyme, and that regulation of *hTERT* most likely occurs at the level of transcription[324]. In general, epigenetic methylation has an important role in the control of gene expression at two key points; post-translational methylation of histones is necessary to maintain the closed conformation of heterochromatin, resulting in gene silencing[310]; and secondly, at the level of the DNA sequence, methylation of CpG islands within a promoter region directly controls the binding of transcriptional machinery to the promoter[310]. In the majority of cases, promoter methylation results in gene silencing. However, in the case of *hTERT* the situation is reversed and it appears that

hypermethylation of the *hTERT* promoter is associated with increased gene expression and increased telomerase activity[184,325]. While FA insufficiency could result in hypomethylation of CpG in the *hTERT* promoter[323], it is unclear whether this would alter induction in *hTERT* expression. Therefore, the possibility that *hTERT* expression might have been affected by FA deficiency was investigated using qPCR to estimate levels of *hTERT* transcripts. Results from the long-term (42 day) experiment showed that expression of *hTERT* were not significantly altered under FA-deficient conditions and that an increase in *hTERT* expression (and thus in telomerase activity) was not a likely explanation for the early increase in TL during folate deprivation.

A third possible explanation for telomere elongation under folate deficiency is a change in the three dimensional structure of the telosome that facilitates access by telomerase, thus allowing extension of the telomeres. van Steensel and de Lange (1997) have previously shown that TRF1 acts as a negative regulator of TL[168]. Overexpression of TRF1 in a telomerase-positive tumour cell line resulted in progressive telomere shortening, while the introduction of a dominant negative TRF1 mutant, which inhibited binding of endogenous TRF1, led to telomere elongation. The authors concluded that telomere-bound TRF1 physically inhibits the binding of telomerase to the ends of telomeres[168]. A later study provided more detail regarding the structure and function of the telosome. It was found that TRF1 and TRF2 were linked in yeast via TIN2 and TINT1, the latter functioning as a negative regulator of telomerase-mediated telomere elongation[151]. These studies confirmed that disruption to binding of TRF1 increased the access by telomerase, and that this was followed by telomere elongation until a plateau was reached. The authors proposed a degree of functional redundancy for TRF1 and TRF2, whereby the latter is able to overcome a TRF1 deficiency, initially stabilising TL and then restoring TL homeostasis[151].

Work conducted in the Bohr laboratory by Opresko *et al* (2005) provided specific evidence that base lesions in the telomere sequence directly inhibited the binding of capping proteins TRF1 and TRF2[139]. The addition of a single 8-oxo-guanine (8oxoG) within the binding sites of these proteins resulted in approximately 50% reduction in bound substrate. Substitution of a single guanine within each telomere repeat with an 8-oxo-G led to a reduction in the amount of bound TRF1 or TRF2 to barely detectable levels[139]. Interestingly, these workers also explored the effects of base excision repair (BER) intermediate states, such as single strand nicks (which had no effect) or single nucleotide gaps (abasic sites). The abasic site within the telomere sequence resulted in a three-fold reduction in binding of TRF1 and TRF2. Given that no gross distortions of the DNA structure occurred as a result of these alterations, the authors concluded

that the reduction in TRF1 and TRF2 binding was due to the disruption of the specific contacts required for recognition of the telomeric sequence[139].

Taken together these findings suggest that the binding kinetics of telomere capping proteins are highly sensitive to the presence of base lesions and/or abasic sites. Similar changes may have occurred due to uracil incorporation during FA insufficiency and accordingly, this may have inhibited the binding of capping proteins. A consequence of this might have been greater access to telomerase, thus providing an explanation for the elongated telomeres observed in these studies. Unfortunately, time did not allow this hypothesis to be pursued. It should be noted, however, that the hypothesis does not provide an explanation for the telomere shortening that was observed after 14 days of exposure to low FA. It is possible that folate insufficiency accelerates a separate process of telomere shortening and that there is a transition phase when this comes to exceed the rate of telomere lengthening.

Since the commencement of this project, evidence has emerged that hypomethylation of the subtelomere may cause telomere elongation[148]. Garcia-Cao in the Blasco laboratory had shown that Knockout mice that are null for histone methyltransferases (HMTases) Suv39h1 and Suv39h2 have reduced histone methylation, and this appears to lead to longer telomeres[326]. These mice were shown to be mono-methylated at lysine 9 of histone 3 (H3-Lys9), which is normally a site of di- or tri-methylation. The same animals were found to have abnormally long telomeres, compared to wild type[326]. The findings indicated that epigenetic factors are involved in homeostasis of TL. Further work in the same laboratory has demonstrated that demethylation at the histone level in mouse embryonic fibroblasts (MEFs) also results in increased frequencies of telomeric recombination[327]. Extending from this work, and more directly relevant to the FA deficiency studies described herein, are the results of studies on the role of DNA methyltransferases (DNMTs) in TL homeostasis[328]. These studies showed that genetic deficiency in DNMT1, DNMT3a or DNMT3b resulted in a reduction in methylation of CpG sites in the (normally heavily methylated) subtelomere, while histone methylation remained unchanged[328]. Importantly, this reduction in subtelomeric methylation was found to cause dramatically elongated telomeres, increased telomeric recombination (sister-chromatid exchanges) and the presence of ALT-associated promyelocytic leukaemia (PML) bodies[328]. Re-introduction of DNMT3a and 3b into these cells facilitated a rescue of the wild type phenotype, with re-methylation of the subtelomeric domains and decreased homologous recombination[328]. Accordingly, it has been proposed that DNA methylation may regulate the ALT mechanism for telomere elongation via recombination[148].

These epigenetic findings have subsequently been tested in a range of human cancer cells, where it was shown that the subtelomeres in neoplastic cells expressing telomerase are heavily (almost completely) methylated[184]. In contrast, in cells that utilise the ALT pathway, these epigenetic markers were less dense, and the pattern of subtelomeric CpG methylation was more heterogeneous[184]. A further novel observation from this study was the difference in epigenetic markers between telomerase-positive cancer cells and cells which were positive for the ALT phenotype, and the presence of telomeric repeat-containing-RNA (TelRNA) which inhibit telomerase activity (possibly by blocking the enzyme's RNA template site). Since the coding region for TelRNA is located in the subtelomere, the authors propose that the heavy cytosine methylation in this region in telomerase-positive cells may act as a positive regulator of telomerase activity, by silencing TelRNA transcription[184]. Although interesting, these findings are not consistent with the hypothesis that the demethylation caused by FA deficiency is responsible for the dramatic short term increase in TL that was observed in WIL2-NS cells. If FA deficiency led to hypomethylation of TelRNA, it would be expected that expression of telomerase would be diminished. Further experimental work was performed as part of this thesis to explore whether epigenetic changes were associated with the telomere elongation observed with FA deficiency, and these results are presented in the following chapters (Chapters 5 and 6).

A final mechanism that was considered as a plausible explanation for telomere elongation was the amplification of telomeric sequences by the breakage-fusion-bridge (BFB) cycle. Numerous studies have reported increased incidence of anaphase (nucleoplasmic) bridges (NPBs) where telomere length and/or integrity has been compromised, resulting in BFB cycles[19,32,172,276-279,284,329-331]. BFB cycles are generated when end fusions occur, generating a dicentric chromosome (see Figure 1.2). When breakage of the dicentric chromosome occurs at anaphase, the breakage site is randomly located and results in an uneven allocation of genes to the daughter cells. The newly formed dysfunctional, and potentially uncapped, chromosomes rejoin, forming new chromosome structures which may be copied again in the subsequent division, thus perpetuating the cycle of breakage and gene amplification[19,30]. BFB cycles resulting from telomere end fusion could, theoretically, also result in amplification of telomere sequences.

There is a widely held view that critically short telomeres are key instigators of BFB cycles. Additionally, there is evidence that altered epigenetic markers at telomere ends[329], compromised TRF2 binding[172], and recombination due to ALT, are additional instigators of

chromosome end fusions[197]. All of these factors could potentially contribute to the TL lengthening described herein.

Studies using cells expressing a dominant negative form of TRF2 have shown the presence of end fusions that contain substantial lengths of telomere in the nucleoplasmic bridges. These observations are consistent with merging of intact telomeres, as opposed to a fusion between critically short ends[172]. These workers also found evidence of fusions between telomeres that have lost the G-tail. This suggests that TRF2 is required for G-tail integrity, which in turn is involved in preventing end fusions[172]. Slijepcevic *et al* tested the fusigenic potential of shortened telomeres in Chinese hamster and mouse cell lines. Their findings were particularly interesting in the context of the results described herein. Not only was no clear link found between short telomeres and chromosome fusigenic potential, but the frequencies of telomeric associations were actually higher in cell lines with longer telomeres[332]. Accordingly, the authors proposed that fusigenic potential may be determined by chromatin structure and integrity[332].

Taken together with previous evidence that the frequency of NPBs is increased under conditions of FA deficiency[28], it is plausible that BFB cycles contributed to the increased TL observed in WIL2-NS cells during the first 14 days of culture in low concentrations of FA. Accordingly, this mechanism was explored further using the CBMN Cytome assay, and the results obtained using this approach are discussed in 4.4.3. Additionally, the frequency of binucleated cells containing multiple NPBs was analysed and the results are discussed further in Chapter 6 of this thesis.

4.4.2   FA depletion and chromosome instability

The CBMN Cytome assay was used to analyse cells from both the short- and long-term studies on the effects of FA insufficiency. The results from this assay were in general agreement with findings from previous studies on primary lymphocytes in which FA concentration was found to correlate significantly and negatively with markers of chromosome damage (MNi, NPBs and NBuds)[28,333]. A strong cross-correlation had previously observed between all three markers, prompting the authors to propose the existence of a common mechanism that is initiated by FA deficiency-induced DNA breaks[28]. The authors proposed that the underlying cause of the damage might be chromosomal instability generated by repeated BFB cycles[28]. Accordingly, the incidence of NPB was of particular interest in the current experiments.

The results of the current studies showed that the frequency of binucleated (BN) cells displaying one or more NPB, as well as the total number of NPB recorded, was significantly higher in cells grown in 30nM FA than in cells grown in either 300 or 3000nM FA. As mentioned earlier, the efficiency of DSB repair in yeast is reduced with increasing proximity to telomeres[271]. Poor repair suggests that lesions in the telomere may accumulate, thus increasing the likelihood of breakage and loss of telomeric DNA[27]. Taken together these findings suggest a model in which inefficient repair of DSBs that arise from uracil incorporation in the telomere sequence predisposes to end fusions involving the telomeres, which in turn leads to enhanced instigation of BFB cycles. Alternatively, reduced TRF1 and TRF2 binding to telomeres containing uracil:thymine substitutions, or uracil-induced abasic site lesions, may increase telomere fusion events.

The incidence of MNi and NBuds also showed significant inverse correlation with FA concentration in the growth medium. MNi represent chromosome breakage or loss, while NBuds are understood to have a function in expelling amplified genetic material from the genome[14,15]. Amplified sequences are believed to be eliminated when there is recombination between homologous regions and formation of mini-circles of acentric and atelomeric DNA (double minutes) that contain the amplified regions[14]. Accordingly, it is plausible that the increased incidence of NBuds observed may be due to gene amplification and homologous recombination, possibly arising from hypomethylation of the subtelomere. A study by Lindberg *et al* (2007) recently characterised the contents of MNi and NBuds in 9-day cultures of normal and folate (FA and 5-methyltetrahydrofolate)-deprived human lymphocytes. These workers found that these markers of genomic damage arise by different mechanisms[15]. In normal lymphocytes, 43% of NBuds contained interstitial DNA and did not contain either

centromere or telomere sequences, while only 13% of MNi contained neither. In contrast, 62% of MNi were labeled by a telomere-specific probe and 22% were labeled when probes specific to telomere and centromere sequences were used in combination. These findings indicate that MNi derive from lagging chromosomes and terminal acentric fragments during mitosis[15]. Characterisation of the contents of 894 NBuds and 1392 MNi using FISH in normal and folate-deprived 9-day cultures of human lymphocytes demonstrated that, while the frequency of BN cells containing NBuds and MNi increased with folate depletion, the proportion of MNi containing telomeric DNA remained unchanged (87%) and NBuds with telomeric DNA increased from 56% in 120nM to 63% in 12nM FA[15]. The results of the Lindberg study provide support for the model in which telomere loss is enhanced under FA deficient conditions as a result of reduced efficiency of DSB repair. At the same time, given that NBuds are an indication of gene amplification, the study also supports the possibility that there is amplification of telomeres.

In both the short- and the long-term FA deprivation studies, the cells cultured in the lowest FA concentration (30nM) exhibited both the longest TL (at Day 14) and the greatest levels of DNA damage. In the case of the long-term study, these two events occurred at different time points. The longest telomeres occurred at day 14, prior to a decrease in TL from days 14-42, while chromosomal damage markers tended to increase gradually over time in culture. Analysis of the relationship between DNA damage biomarkers and TL using area under the curve provided an unexpected result with a significant positive correlation being observed between these two parameters. This appears to be the first time that this correlation has been observed, challenging the paradigm that TL and chromosomal instability have an inverse relationship.

4.5 CONCLUSIONS

- The results of the experiments presented in this chapter indicate that FA-deficiency impacts on TL and chromosomal stability in WIL2-NS cells, in a dose dependent manner. These *in vitro* findings indicate that FA insufficiency causes a rapid, short term increase in TL (days zero to fourteen), followed by a rapid reduction back to commencing levels. The elongation of telomeres observed in the low FA cultures does not support the hypothesis that FA depletion will result in telomere shortening, at least in the short term. It is possible that beyond day 14 other mechanisms, such as deletions in telomeric DNA, may come into play and cause TL shortening, superseding or overwhelming the mechanisms causing telomere elongation.

- The findings presented in this chapter refute the hypothesis that longer telomeres constitute a positive biomarker for chromosome stability, by demonstrating that cells maintained in the lowest concentrations of FA exhibited not only the longest telomeres, but also the highest levels of chromosomal damage biomarkers. The significant, positive relationship between chromosomal damage markers and TL, using area under the curve, was an unexpected result. This appears to be the first time that this correlation has been observed, challenging the paradigm that TL and chromosomal instability have an inverse relationship.

- Further research is needed to explore potential mechanisms for these findings, several of which are discussed in subsequent chapters of this thesis.