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Telomere lengths in human oocytes, cleavage stage embryos and blastocysts

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Abstract

Telomeres are repeated sequences that protect the ends of chromosomes and harbour DNA-repair proteins. Telomeres shorten during each cell division in the absence of telomerase. When telomere length becomes critically short, cell senescence occurs. Telomere length therefore reflects both cellular ageing and capacity for division. We have measured telomere length in human germinal vesicle (GV) oocytes and pre-implantation embryos, by quantitative fluorescence *in-situ* hybridisation (Q-FISH), providing baseline data towards our hypothesis that telomere length is a marker of embryo quality. The numbers of fluorescent foci suggest that extensive clustering of telomeres occurs in mature GV stage oocytes, and in pre-implantation embryos. When calculating average telomere length by assuming that each signal presents one telomere, the calculated telomere length decreased from the oocyte to the cleavage stages, and increased between the cleavage stages and the blastocyst (11.12 vs 8.43 vs 12.22kb respectively, $p<0.001$). Other methods of calculation, based upon expected maximum and minimum numbers of telomeres, confirm that telomere length in blastocysts is significantly longer than cleavage stages. Individual blastomeres within an embryo showed substantial variation in calculated average telomere length. This study implies that telomere length changes according to the stage of pre-implantation embryo development.

Key words: Embryo/oocyte/telomere length/human/chromosome

Introduction

Telomeres are the ends of chromosomes, comprising hexamer repeats (TTAGGG) associated with proteins that protect the DNA from degradation by exonucleases (Blackburn, 2005). With each cell division, a small amount of the telomere is lost due to the 'end replication problem' (Bailey and Cornforth, 2007). The precise amount of shortening is affected by other variables and may be accelerated by oxidative stress (Valdes et al, 2005). Sufficient telomere length to accommodate such shortening is therefore essential for cells that replicate. Ultimately, after many cell divisions, critically short telomere length results in cell senescence associated with chromosome instability and end-to-end joining, unless other mechanisms actively restore telomere length. One such mechanism involves the reverse transcriptase enzyme telomerase which adds hexamer repeats to telomeres (Blackburn, 2005). Telomerase is present in stem cells, most cancers and immortalised cell lines, but not in normal somatic cells (Holt and Shay, 1999).

In addition to the generalised, age-related shortening of telomeres due to cell division, sporadic loss or truncation of one or more telomeres may occur in a particular cell due to exogenous DNA damage, problems with DNA repair, or spontaneously, irrespective of whether telomerase is present (Baird, 2008). Substantially shortened telomeres may either be rectified by telomere 'healing', or the cellular response may lead to severe consequences associated with chromosome instability (Murnane, 2006).

Telomerase effects gradual lengthening of telomeres, but 'quantum' lengthening may also occur on rare occasions. One means is by non-reciprocal translocations whereby a damaged chromosome captures a telomere from another chromosome, thus perpetuating chromosomal instability (Murnane, 2006). A further mechanism of telomere lengthening, known as Alternative Lengthening of Chromosomes (ALT), involves recombination and results in heterogeneous telomere lengths (Reddel et al, 1997).

During meiosis, telomeres control chromosome movements, particularly bouquet formation at the leptotene stage, homologous pairing and interaction with microtubules, and spindles (de la Roche Saint-Andre, 2008; Morelli et al, 2008). In telomerase null mice, short telomeres are associated with infertility, abnormal spindles and misalignment of metaphase chromosomes in oocytes (Liu et al, 2004). However, mouse telomeres are much longer than those of humans, and telomere erosion in mice takes several generations to reach a critical point. In humans, telomere length in human unfertilised oocytes correlated with the morphological appearance of embryo quality in sibling fertilised oocytes as well as the likelihood of pregnancy (Keefe et al, 2005; 2007), however, no measurements of telomere length in human embryos were made directly.

Telomere lengths in human sperm vary across a wide range within individuals (Baird et al, 2006). Generalised telomere length in sperm increases with male age (Alsopp et al, 1992) and counter-intuitively, is inversely proportional to telomerase during the maturation of germline cells in rats (Achi et al, 2000). This indicates that a mechanism of telomere extension other than telomerase is also active. Baird et al (2006) estimated that only 19% of human sperm have normal length telomeres at all chromosomes. Critically short telomeres are associated with sperm DNA fragmentation in mice

(Rodriguez et al, 2005). In humans, fragmented sperm DNA is associated with abnormal embryonic development (Evenson et al, 2002).

The oocyte is well known for its capacity to modify and repair damaged DNA (Derijck et al, 2008), and this capacity is also found in the pre-implantation embryo although the mechanisms differ after fertilisation, at least in mice (Fiorenza et al, 2001). At fertilisation, the oocyte and sperm telomeres coexist in the same cell and the mechanisms of telomere extension and shortening mentioned above may become active. Liu et al (2002a) showed that failures of fertilisation and pre-implantation embryonic development in telomerase null mice were caused approximately equally by the sperm and the oocyte.

Liu et al (2007) found that oocyte telomere length was surprisingly short in mice, but significant lengthening occurred between the zygote and 2-cell stage. In contrast, Meerdo et al (2005) observed significant shortening of the telomere-to-centromere ratio in cattle between the oocyte and cleavage stages. Liu et al (2007) showed that both telomerase and ALT sister chromatid exchange are active in mice. Wright et al (2001) found that telomerase is present in human cleavage stage embryos at lower levels than in oocytes or blastocysts. The blastocyst stage is believed to be the main point at which telomere length increase becomes established for the new generation (Schaetzlein et al, 2004; cattle), however, significantly shorter telomeres were observed at this stage in cattle by Meerdo et al (2005).

In view of the importance of telomere length for cell replication and mitosis, meiosis and early embryonic development demonstrated in mice, and the intriguing and partial information arising from human studies to date, our objective has been to measure telomere lengths directly in human oocytes and pre-implantation embryos.

MATERIALS AND METHODS

Human material

The project was approved in advance by Coventry Research Ethics Committee (04/Q2802/26) and licensed by the Human Fertilisation and Embryology Authority (R0155). Patients undergoing in vitro fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI) treatment gave informed consent to donating oocytes or embryos to this project. Only material that could not be used for the patients' treatment was accessed for research.

Material for analysis was collected as follows:

(1) Immature oocytes containing a single germinal vesicle at oocyte collection for ICSI treatment. Four oocytes collected from 4 different women were used.

(2) Embryos not selected for transfer or cryopreservation during IVF or ICSI treatment, as a result of relatively poor morphological appearance.

(3) Embryos stored frozen, for couples undergoing IVF or ICSI treatment, and subsequently donated to research after the couples had completed their family or desired no further treatment. The frozen-thawed embryos had been stored in liquid nitrogen at the pronuclear (PN) or early cleavage stages using propanediol as cryoprotectant (Lassalle et al, 1985). Four of the six couples donating frozen embryos had undergone treatment that resulted in a live birth, and embryos had

been positively selected for cryopreservation according to their morphological features, therefore these embryos were considered relatively good quality.

Some embryos were fixed on slides immediately while others were cultured for up to 4 days with a view to obtaining a range of pre-implantation embryonic stages for analysis. A total of 17 frozen and 6 fresh embryos from 9 couples were successfully analysed for this study.

Embryo culture

Where necessary, embryos were thawed and cultured according to standard clinical protocols using commercially available reagents (Medicult, Redhill, UK). Individual or small groups of embryos were cultured at 37°C in drops of 20µl medium under mineral oil, pre-equilibrated with a gas mixture of 5% CO_2 in air. For culture, embryos thawed on day 1 or 2 were placed into Medicult embryo assist medium. If still in culture on day 3, they were transferred into blastassist medium. The embryos were transferred to fresh drops of equilibrated culture medium every 2 days.

Preparation of slides

Oocytes and embryos were spread according to the method of Dozortsev and McGinnis (2001). Briefly, the oocyte or embryo was placed into a 5µl drop of acid Tyrodes solution on a precleaned glass slide at room temperature until the zona pellucida had dissolved. It was then moved into a 5µl drop of Ca^{2+}/Mg^{2+}-free solution (Biopsy medium, Medicult) on the same slide for 2 min to weaken bonds between blastomeres. Individual oocytes or morulae/blastocysts that consisted of multiple adherent cells were also passed through this solution which served to remove the acid Tyrodes. Individual blastomeres or whole oocytes/embryos were then rinsed for 5-10s in a 5µl drop of hypotonic solution (1% sodium citrate and 0.02mg/ml human serum albumin) to induce swelling for clearer

visualisation of the nucleus. The cell or embryo was then transferred to a 1µl drop containing Tween 20 (1% in 0.01M HCl) in a pre-marked circle on a glass slide. The drop was allowed to evaporate completely and then 2 drops of fresh 3:1 methanol/acetic acid were added to the site to fix the nuclei. Slides were air dried at room temperature and stored at 4°C. Oocytes and embryos were mounted individually. Blastomeres from a single embryo were individually mounted close together on one slide for analysis.

Quantitative fluorescence in situ hybridisation (Q-FISH)

Q-FISH was performed according to the protocol provided by the manufacturer of the pan-telomeric PNA probe (DAKO, Denmark; Telomere PNA FISH kit/FITC, K5325) with modifications. To provide a quantitative control enabling comparison between slides prepared on different occasions, a suspension of L-5178Y-S cells, which have known telomere length, was added to the slides where the oocytes or embryos were fixed. All cells on the slide were processed together for Q-FISH.

For Q-FISH, slides were placed on a heating block at 55°C overnight. The following day, the slides were washed with Tris-balanced salt solution (TBS) and fixed with 3.7% formaldehyde solution for 5 minutes. After washing with TBS, the slides were treated with pepsin (1mg/ml) at 37°C for 10 minutes, washed with TBS and fixed again with formaldehyde solution. After washing off the formaldehyde solution with TBS, the slides were dehydrated through 70%, 80% and 90% ethanol, and then air-dried. Fifteen µl of telomere probe (DAKO) was added to each slide and denatured by placing on the heating block at 80°C. Slides were then placed into a dark box for two hours to allow hybridisation, and then washed with 70% formamide solution twice and TBS three times before dehydration through the ethanol series and air-drying. The slides were counterstained and mounted with Vectashield containing DAPI.

Microscopy

The cells were viewed under an Olympus IX81 microscope with a Xenon 150W arc burner for fluorescence viewing. Images were viewed at x96 magnification. Chromatin was viewed with a DAPI filter (excitation wavelength 359nm, emission wavelength 461 nm) while telomeres utilised a FITC filter (excitation wavelength 490nm, emission wavelength 525nm). The images were viewed digitally, stored and analysed with the Cell^{M} system (Olympus, Watford, UK) coupled with a digital CCD camera C4742-80-12AG (Hamamatsu). Images of the telomeres were captured with a fixed exposure time of 1000 milliseconds. The maximum and minimum thresholds were set at automatic and recorded.

Image analysis

Telomere images were exported as 8 bit tagged image file format (tiff) files and analysed with TFL-telo program (freely available at www.flintbox.com), developed by Peter Lansdorp and SS Poon (Zijlmans et al 1997). Individual dot fluorescent densities were measured. The average density of each dot was calculated. The average dot density of 20 L5178Y-S nuclei was used as the quantitative standard for telomere total length in the foci of human oocytes and blastomeres. Nuclear areas, and the number and size of signals were also recorded for each nucleus analysed.

In order to standardise the Q-FISH procedure, in every experiment, the fluorescence intensity of cells with known telomere length L-51784-S (7kb, McIlrath et al, 2001) was used as a reference for staining intensity. The average telomere lengths of the oocyte and embryo nuclei were calculated by the total fluorescence intensity of all the telomere signals divided by the number of telomere signals visible in each nucleus, and quantified according to the relative intensity of L-51784-S cells (Wong and Slijepcevic, 2004).. An additional analysis divided the total fluorescence by the number of telomeres predicted

to be present in GV oocytes (n=184) and the maximum and minimum numbers predicted to be present in mitosing blastomeres (184 or 92). This analysis was undertaken because the clustering of telomeres and cell cycle variation in embryos renders the exact number of telomeres in any given nucleus uncertain.

For embryos where more than one blastomere was analysed, the individual blastomere results were averaged to give the average calculated telomere length for that embryo. For blastocysts, average telomere lengths were calculated only for those nuclei which could be analysed. Control analyses were undertaken in order to test whether the smaller nuclear area analysed during successive embryonic cleavages might influence the fluorescence intensity.

Interpretation of data

The number of signals varies at different stages of the cell cycle according to the amount of DNA and telomere replication and with clustering of telomeres. Our analysis cannot distinguish telomere clustering from single bright telomeres. Also, telomere signals that were very small and could not be distinguished from background were omitted. Therefore, the interpretation of data is subject to potential errors because these factors cannot be quantified in human material. For analysis, GV stage oocytes were used as a standard, because such oocytes are arrested in the diplotene stage of meiotic prophase I, their DNA having already replicated, and further replication being inhibited at this stage (Fulka et al, 1997; Whitmire et al, 2002). In view of the expected variation in the stage of the mitotic cycle in blastomeres, all blastomeres would be predicted to contain between half and the same number of telomere signals as for GV oocytes (which we confirm in our results), assuming a similar extent of telomere clustering. Any signal intensity results that exceed those for GV oocytes would therefore indicate an increase in telomere length in chromosomally normal cells.

Data analysis was performed using Microsoft Excel. Telomere lengths are presented as box plots in order to show the distribution characteristics. Comparisons of groups were performed by Kruskal Wallis test. A probability <0.05 was considered significant.

RESULTS

Oocyte and embryo analysis

The 23 embryos were classified into groups as follows: 2-4 cells (13), 5-8 cells (6), morula (1) and blastocyst (3). Overall, 68 individual nuclei from 19 cleavage stage embryos, 8 nuclei from a morula, and 11, 9 and 46 nuclei from three blastocysts, were analysed by Q-FISH. The numbers of nuclei and blastomeres were not always coincident, as expected for human embryos. Additionally, some nuclei were lost during preparation and fixation. Telomeres were sometimes analysable in nuclei from blastomeres that had lysed during cryopreservation. Some example images are shown in Figure 1.

Characteristics of telomere signals

The four oocytes examined had similar numbers of telomere signals identified per GV nucleus examined, on average 53 (56, 52, 58, 46). The mean spot size in GV oocytes was also consistent, averaging approximately 152 pixels in our system (142, 154, 163, 149). The number of telomeres in a meiotic cell with fully replicated DNA is 184, with sister chromatid association potentially reducing this to 92 signals, so these results indicate that telomere association is occurring to a consistent degree in GV oocytes.

The numbers of signals observed per nucleus during the stages of embryo development are presented in Figure 2. The number of signals varied, but as expected was fewer than that in GV oocytes for most embryos. Two 3-cell embryos from one couple (who were noted to have multinucleated blastomeres in other embryos that were not

analysed) had exceptionally high signal numbers (117, 149, 142) in one or two nuclei, and so were omitted from further analysis. One further 3-cell embryo was omitted because the number of signals observed was very low in both of the analysed nuclei.

No significant relationship between nuclear area and fluorescence was found, indicating that nuclear size differences at different stages of development were not confounding the data (Figure 3).

Calculations of telomere length

The number of telomeres present in a nucleus depends upon the stage of the cell cycle. We have therefore documented the numbers of signals identified in our studied nuclei and used this as the denominator to determine the average fluorescence per signal, which is related to the amount of telomere sequence present. The absolute level of signal is then calibrated against a standard cell type with known telomere length. In the event that telomeres are associating, then the denominator would be reduced, resulting in a higher calculated telomere length. We have demonstrated that telomere association is occurring in GV oocytes, and we consider it likely to be occurring in embryos, in view of the numbers of signals that we have documented (Figure 2). Therefore, our results may overestimate telomere length for a given fluorescence measurement. Calculated telomere length was unrelated to the number of signals detected but was linearly associated with the total fluorescence per nucleus, as expected (Figure 4b).

Telomere length in relation to developmental stages

Figure 5 presents the calculated average telomere lengths, on a per embryo basis, as a function of stages of development from the 2-cell to the blastocyst. The data were calculated assuming that each signal represented a single telomere. Using this method, the telomere lengths of cleavage stage embryos were significantly shorter than those of oocytes or blastocysts ($p<0.001$). If these results are overestimates in mitosing cells, as

explained above, then the correct results would be more significantly different. The general trend in calculated telomere length through the pre-implantation period was U-shaped, ie reducing from the oocyte to the cleavage stages, then increasing again to the blastocyst stage. This, together with the numbers of signals observed at different stages of development (Figure 2), implies that clustering of telomeres is unlikely to give rise to the observed increased fluorescence intensity in the blastocyst. However, when the results were calculated by dividing the GV oocyte fluorescence by 184, the number of telomeres known to be present in a GV nucleus, the calculated telomere length was considerably shorter, approximately 4kb, as a result of telomere clustering. For GV oocytes, this method is likely to be more accurate, since the number of telomeres is a constant.

Figure 6 shows the same results, on a per nucleus basis. In panel a, total nuclear fluorescence was divided by the number of telomere signals observed (assuming each to represent a single telomere). However, in panels b and c, total nuclear fluorescence was divided by the predicted number of telomeres per nucleus as follows: GV fluorescence was divided by 184 (the number of telomeres present in a normal GV nucleus), while the results for blastomeres were divided by 92 (panel b) or 184 (panel c). These numbers are the predicted minimum and maximum number of telomeres present in blastomeres, so provide lower and upper estimates of telomere length. The significant difference between cleavage stage and blastocyst nuclei ($p<0.001$) is therefore confirmed by both methods of calculation. However, oocyte telomere length appears similar to that of cleavage stage embryos at maximum, while it remains significantly higher than cleavage stage embryo telomere length at the minimum calculated length.

There was no significant difference in calculated telomere lengths between embryos at different stages of cleavage or between fresh and frozen-thawed embryos.

Telomere length variation among cohorts of embryos from different couples

The average calculated telomere lengths of embryos varied among cohorts from a particular couple. Figure 7 shows the average telomere lengths of the 19 cleavage stage embryos and the morula according to the couple from whom they originated. There was no obvious similarity in the telomere lengths of embryos originating from the same cohort, indicating that inter-embryo variation is greater than inter-couple variation.

Telomere length variation among individual nuclei of embryos

The calculated average telomere lengths in the individual blastomeres of each embryo are presented in Table 1, calculated according to the number of telomere signals observed. Calculated telomere lengths varied greatly among the blastomeres comprising an embryo. There was no correlation between the extent of variation among cells and the developmental stage of the embryo.

Discussion

This study is the first to measure telomere length in human pre-implantation embryos. Importantly, we have found that that telomeres lengthen at blastocyst formation and that average telomere length in cleavage stage embryos may be significantly shorter than that of germinal vesicle oocytes, depending upon the calculation method used.. Our methods of calculation of telomere length utilize the GV oocytes as a cell cycle standard, to which other nuclei can be compared. We have presented a number of control assessments that validate the methodology for assessment of relative telomere length, however, it is important to note that the absolute values of telomere length may be inaccurate because of the technical challenges of analyzing cells that are cycling, and the likelihood of telomere clustering. We also present novel findings on numbers of telomere signals in human oocytes and embryos. Telomere clustering at the nucleolar periphery is expected in the early stages of oocyte meiotic maturation, as shown in mice

(Longo et al, 2003), and telomere association at this stage is confirmed in our results, however, no data have previously been available on telomere association in early embryos. Our results present varying numbers of telomere signals identified at different stages of pre-implantation embryonic development, which could indicate stage-specific association and deserves further investigation.

We determined the average telomere length of all the cleavage stage embryos analysed to be 8.43 kb which was significantly shorter than the telomere length of blastocysts (12.22 kb) or GV oocytes (11.12 kb, $p<0.001$), based upon the assumption that each fluorescent focus represents a single telomere. When the results are calculated according to the estimated minimum and maximum numbers of telomeres present at different stages of the cell cycle, the results confirm the increase in telomere length at the blastocyst stage. However, the difference between oocytes and cleavage stage embryos depends upon whether the maximum or minimum estimates of blastomere telomere length are used for comparison. Since there are no other studies of telomere length in human pre-implantation embryos, this is the first time that such data have been published. The data reported on telomere lengths in animal embryos so far have been conflicting with no clear profile of pre-implantation telomere dynamics evident. In mice, telomere lengths in blastocysts and 2-cell embryos were found to be similar by Liu et al, (2007), although Schaetzlein et al (2004) had previously found a significant increase in telomere length between the 8-cell/morula and blastocyst stages. In cattle, Meerdo et al (2005) observed that telomere length increased between the cleavage and morula stages but decreased again at the blastocyst, while Schaetzlein et al (2004) observed significant increases in telomere length between the morula and blastocyst stages. Telomere lengthening around the morula/blastocyst transition was reported to be telomerase-dependent and confined to this stage of development (Schaetzlein and Rudolph, 2005). In addition, significantly longer telomere lengths were identified in

female compared to male bovine blastocysts, which probably reflects epigenetic differences between the sexes exerted on the sub-telomeric regions (Gonzalo et al, 2006; Bermejo-Alvarez et al, 2008). However, despite the variability in animal data, and the caveats associated with analysis of telomere lengths in mitosing cells, our results do appear to concur with the profile of telomerase activity in human pre-implantation embryos reported by Wright et al (2001). Their data show an approximately 5-fold increase in telomerase activity from the 8 cell stage to the blastocyst. In bovine embryos, telomerase activity also increased over the same period but authors differ over the extent of the increase: Xu and Yang (2001) reported an approximate doubling, while Betts and King (1999) found a forty-fold increase.

Keefe et al (2007) found that the telomere length of human unfertilised oocytes averaged 6-7kb, with a standard deviation of ~4kb. Our data on GV oocytes suggest telomere lengths >10kb when the signal number is used as the denominator, or approximately 4kb, when the predicted absolute telomere number is used for the calculation. These results are potentially compatible, however, the oocytes were examined at different stages and further variation could also result from differences in the measurement techniques applied. Keefe et al (2007) used Q-FISH, however full methodological details were not provided. Many researchers use relative units of measurement in order to represent differences in telomere length, but both Keefe et al and ourselves have attempted to produce measurements in kb, so some margin of difference may have resulted from the different controls and validation techniques involved in converting fluorescence intensity signals into kb.

Depending upon the method of calculation, we also identified a significant difference in telomere length between oocytes and cleavage stage embryos, which is worthy of further investigation. Our results are more similar to those observed in cattle, whose telomeres shortened between the oocyte and the cleavage stages (Meerdo et al, 2005),

than to those in mice, where lengthening of telomeres between the oocyte and the 2-cell stage was reported (Liu et al, 2007).

It is interesting that calculated telomere lengths differ greatly among the blastomeres of an embryo (Table 1), showing that blastomere division within an embryo does not produce daughter cells that are identical in terms of average telomere length. This variation may arise from a number of mechanisms, for example, telomerase activity differs substantially among human blastomeres (Wright et al, 2001). Also, the ALT pathway has not yet been investigated in human embryos but is active in mice (Liu et al, 2007). ALT could potentially result in highly variable telomere lengthening by a recombination-like mechanism in early embryos (Reddel et al, 1997). ALT is suppressed by telomerase, but there is ample evidence of the two mechanisms coexisting (Cerone et al, 2001). It is possible that ALT is most active while telomerase is relatively low in the cleavage stages of human pre-implantation development (Wright et al, 2001), thereby contributing to the inter-blastomere variability in average telomere length that we observed, although cell cycle stage may also have an effect in this regard. Variability among the cells of human embryos in terms of polarity and capacity to switch on the embryonic genome (Tesarik, 1989; Edwards, 2005), as well as chromosomal mosaicism (Daphnis et al, 2008) may also contribute. The telomere lengths of individual chromosomes differ in approximate proportion to the chromosome length and therefore selective loss or gain of individual chromosomes might affect the cell average telomere length, but should not affect the embryonic average telomere length.

Our data show that inter-embryonic variability in average telomere length is at least as large as variability among patients, within the small cohort that we have studied. This is not surprising as it is well known that human embryos are highly variable, even within a single stimulation cycle. The embryos used in this study were surplus to clinical requirements and are therefore not optimal research material. This is unavoidable,

however, we took care to include embryos from patients who had achieved a pregnancy, such embryos being the best quality available for research. Prolonged culture may affect embryos at a molecular level, however, these same conditions result in pregnancies from blastocyst culture.

Here, we present data showing that human GV oocytes have significantly longer telomeres than cleavage stage embryos, under the assumption that each spot represents one telomere. When the calculation was made with reference to the expected number of telomeres present, the difference remained significant when blastomeres were analysed at their minimum telomere length. Others have presented data that sperm also have relatively long telomeres, although many of them may not have a normal complement of telomeres (Baird et al, 2005). The question of what happens at fertilisation is intriguing. Older fathers have longer average telomere lengths in sperm, which is reflected in their offspring (Kimura et al, 2008), however, a range of environmental factors, such as smoking and obesity, that may operate through increased oxidative stress, are associated with shortened telomeres (Valdes et al, 2005). A relationship between oxidation and telomere length of oocytes in relation to ageing may be hypothesised (Keefe et al, 2007). Oxidative stress may also be experienced by oocytes during insemination, due to oxidative free radicals derived from spermatozoa (Aitken and De Iuliis, 2007). Significantly shorter telomeres were reported in bovine morulae produced *in vitro* compared to *in vivo* (Schaetzlein et al, 2004), providing some support for the idea that culture conditions may result in shorter telomere lengths. The possible impact of oxidative stress during gamete processing for human IVF may therefore be relevant in the current study (Betts and Madan, 2008), as may lifestyle factors, such as cigarette smoking in oocyte and embryo donors (Huang et al, 2010).

It remains uncertain how the oocyte is equipped to deal with the severely truncated telomeres or the long average telomere lengths identified in many sperm (Baird et al,

2006). Interestingly, children born after intracytoplasmic sperm injection (ICSI) appear to have a wider range of telomere lengths than controls (Robinson et al, 2005). Human embryos display a wide variety of genetic abnormalities, however, end-to-end joining has not been reported as a common occurrence in humans (Zhivkova et al, 2007). This finding might be taken to imply that the human oocyte effectively avoids such an eventuality under normal circumstances, although chromosome fusion can be induced in mouse embryos by exposure to toxic mitochondrial uncouplers (Liu et al, 2002b) or cigarette smoke condensate (Huang et al, 2010). In view of this, a mechanism in early embryos to distribute the available telomere material, arising from the sperm and the oocyte, into an appropriately balanced embryonic complement may be postulated. Since the oocyte is the major contributor to the early embryo's cellular components, it is likely that inherited maternal molecules play a major role in determining the telomere length of the cleavage stage embryo, at least until generalised embryonic genome activation. In addition to telomerase, the oocyte's ability to repair DNA, including that of the incoming male gamete, may have important consequences for the telomere length of the embryo. We therefore hypothesise that the oocyte modifies the sperm telomeres to result in the embryonic complement, by methods that include recombination and telomerase.

Data on the telomere lengths of human gametes and pronuclear embryos are necessary to complete our study of average telomere lengths during pre-implantation development and to provide data to test our hypothesis of how telomere lengths are modified at fertilisation. Our methods provide some estimates of relative length of telomeres in individual oocytes and blastomeres, that may provide a valuable resource for comparison with other methodologies, stages and species, providing insights into the early events of telomere maintenance by human embryos. The prospect of diagnosing potential for development based upon telomere length remains a possibility but requires

the factors controlling telomere length maintenance, clustering and cell cycle effects to be better understood.

In conclusion, this study presents quantitative data upon telomere length in human GV oocytes and pre-implantation embryos, demonstrating stage-specific variations in length, and significant differences between oocytes, cleavage stage embryos and blastocysts. Our findings bear some similarities to prior work in animal embryos, but also highlight interesting differences. The implication that there may be stage-specific clustering of telomeres needs to be further confirmed. Our future work will address telomere length in human gametes and pronuclear embryos, as well as the mechanisms responsible for control of telomere length in reproductive cells.

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Contributor Roles

ST Collected and analysed all oocytes and embryos, compiled and analysed data, telomere length calculations

H-PW Set up telomere measurements on embryos, established methods, advised ST, wrote first draft

JR Managed frozen embryo bank and gamete/embryo donations for research

GH HFEA license holder, led research programme, gained approvals, supervised researchers, led analysis and interpretation, completed writing of paper.

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Figure legends

Figure 1. Telomeres in human embryos.

Telomeres were stained using a FITC-labelled pan-telomeric probe (green). All nuclei were counterstained with DAPI (blue). (A) 6-cell embryo resulting in 5 nuclei with two polar bodies, E and E' (scale bar=44um) . Two of the nuclei (C and D, scale bar=8.5um) are also shown under higher magnification (scale bar=4um). (B) The telomeres in a fixed blastocyst (scale bar=55um).

Figure 2. Numbers of telomere signals identified in germinal vesicle oocytes (GV) and embryonic nuclei at different stages of development (2 cell – 7 cell, morula, blastocyst). Box plots represent the median and inter-quartile range, with whiskers representing the 5^{th} and 95^{th} percentiles.

Figure 3. Total telomere-associated fluorescence per nucleus in relation to nuclear area. There was no significant relationship between fluorescence and nuclear area.

Figure 4. Calculated telomere lengths and total fluorescence per nucleus in relation to the number of signals per nucleus. a) not significant, b) $p<0.01$.

Figure 5. Calculated telomere lengths of human oocyte germinal vesicle and embryonic nuclei at different stages of pre-implantation development, presented per embryo. Cleavage stage embryos have significantly shorter calculated telomere lengths than germinal vesicle or blastocyst nuclei ($p<0.001$).

Figure 6

Telomere lengths calculated per nucleus for human oocytes and embryos at different stages of pre-implantation development, using three different methods of calculation.

a. Telomere length calculated from total fluorescence of the nucleus, divided by the number of telomere signals observed in it.

b. Telomere length calculated in GV oocytes by division of the nuclear fluorescence by 184 and in blastomeres by division by 92. 184 is the number of telomeres known to be present in a normal GV nucleus. 92 is the minimum number of telomeres expected in a normal blastomere nucleus.

c. Telomere length in GV oocytes, calculated as in b, and in blastomeres by division by 184. 184 is the maximum number of telomeres expected in a normal blastomere nucleus.

b and c therefore present the upper and lower telomere length estimates of blastomeres according to predicted numbers of telomeres.

a and c, Telomere length shorter in cleavage stage blastomeres than blastocyst or GV nuclei. $p<0.001$. b, Telomere length longer ($p<0.001$) in blastocyst nuclei than cleavage stage or GV nuclei. No significant difference between GV nuclei and cleavage stage blastomeres.

Figure 7. Average calculated telomere lengths of individual human embryos originating from different patients.

The embryos are arranged into eight groups according to the couple from whom they originated. Couple 8 donated the morula, while the other embryos are from 2-cell to 8-cell stage.

Table 1. Summary of telomere length measurements in blastomeres of 23 human embryos at stages from 2-cell to blastocyst, calculated according to the number of signals detected. The left hand column indicates the cell stage of the embryo at fixation. Those indicated as, for example, 3/4 indicate a 4-cell embryo that had 3 surviving cells after cryopreservation. The numbers of nuclei for which telomere length could be analysed does not necessarily coincide with the number of cells in the embryo due to technical losses during processing and the expected biological variability of the embryonic material. Note: * indicates average telomere length for the embryo as a whole.

Table 1

Stage	Nuclei analysed	Individual blastomere average telomere lengths					Mean telomere length (kb)
1/2c	1	11.96					11.96
2c	2	4.69	5.69				5.19
2c	3	8.57	9.85	9.55			9.32
3c	3	6.96	16.03	12.17			11.72
3c	2	8.75	4.83				6.79
3c	3	12.62	5.02	3.34			6.99
3c	2	8.14	7.10				7.62
1/4c	3	9.03	8.25	8.23			8.50
2/4c	4	7.95	8.01	5.66	3.72		6.34
3/4c	2	14.37	13.32				13.85
4c	2	7.48	7.45				7.47
4c	5	2.54	4.73	4.73	9.54	10.34	6.38
4c	4	10.04	12.75	5.12	2.32		7.56
4/6c	2	8.60	8.95				8.77
5/6c	5	11.92	5.52	5.40	3.53	3.60	6.00

5/6c	6	2.53	3.46	8.46	7.12	6.72	5.91						5.70
6c	6	6.71	4.02	9.32	7.29	9.51	12.70						8.26
6/7c	7	9.49	10.00	13.07	11.17	7.27	12.77	9.10					10.41
7c	6	5.22	8.60	9.04	11.93	9.78	9.61						9.03
Morula	8	11.17	13.48	8.39	5.75	5.29	5.54	8.65	7.30				8.20
Blastocyst	9	10.00	14.81	11.41	11.65	5.90	6.57	9.83	10.58	13.58			10.48
Blastocyst	10	19.84	15.53	11.05	13.30	13.88	14.16	11.21	19.09	14.18	11.78	20.26	14.93
Blastocyst	46	*11.27											11.27

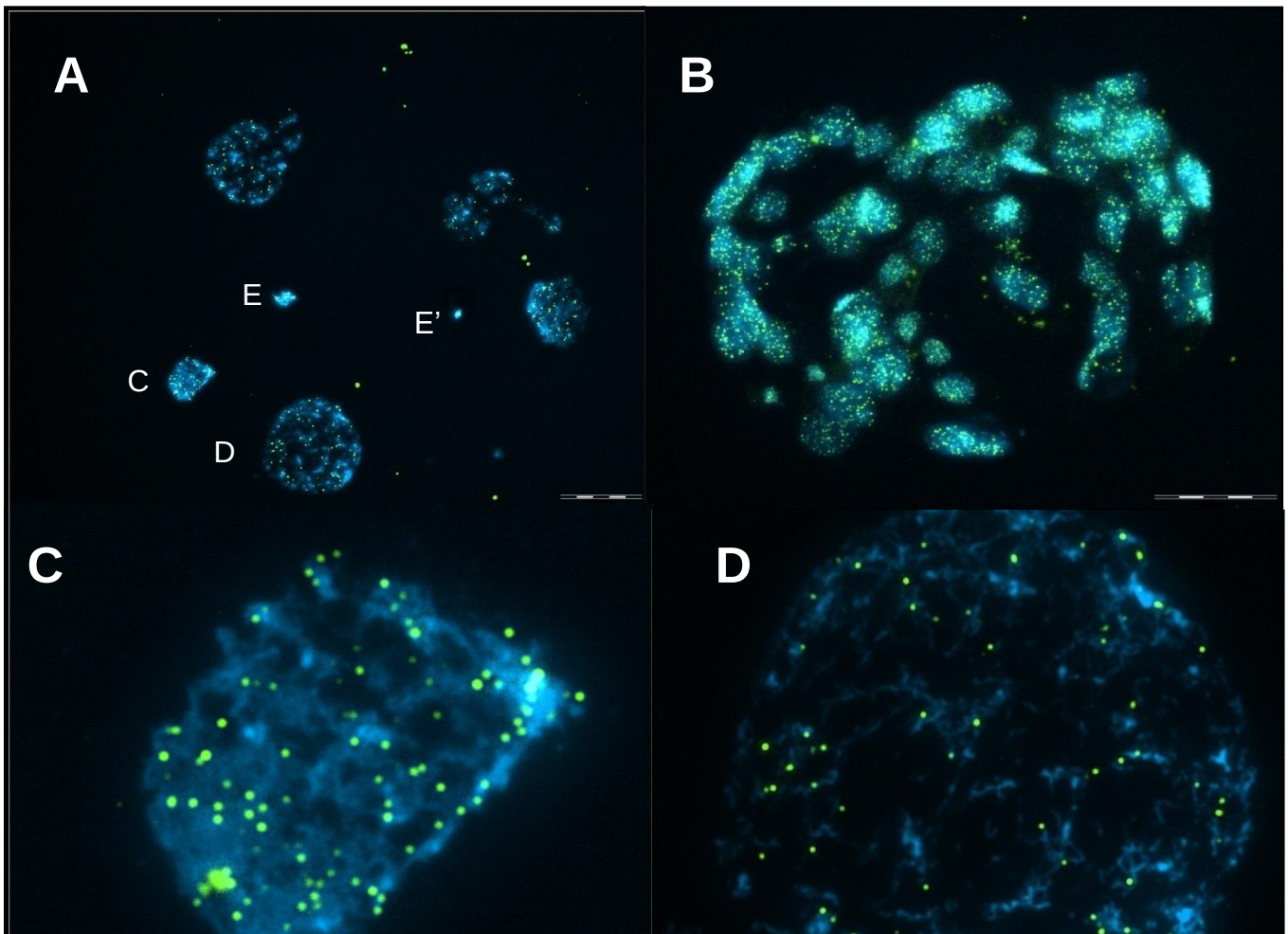


Figure 1

Figure 2

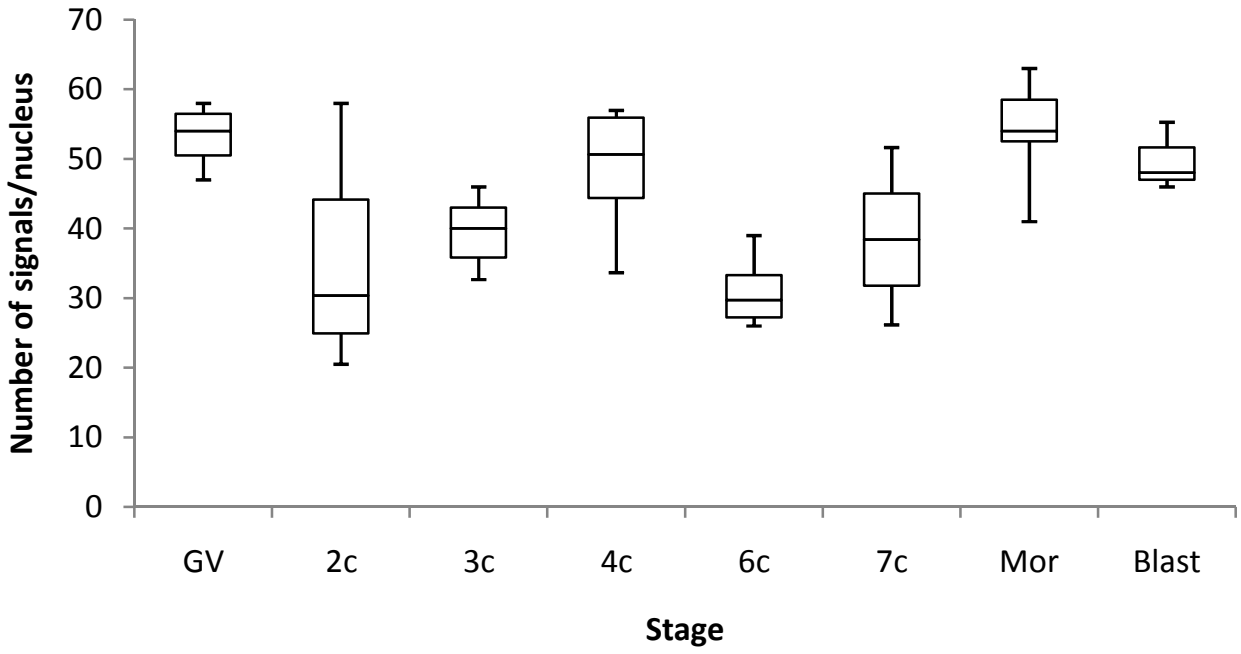
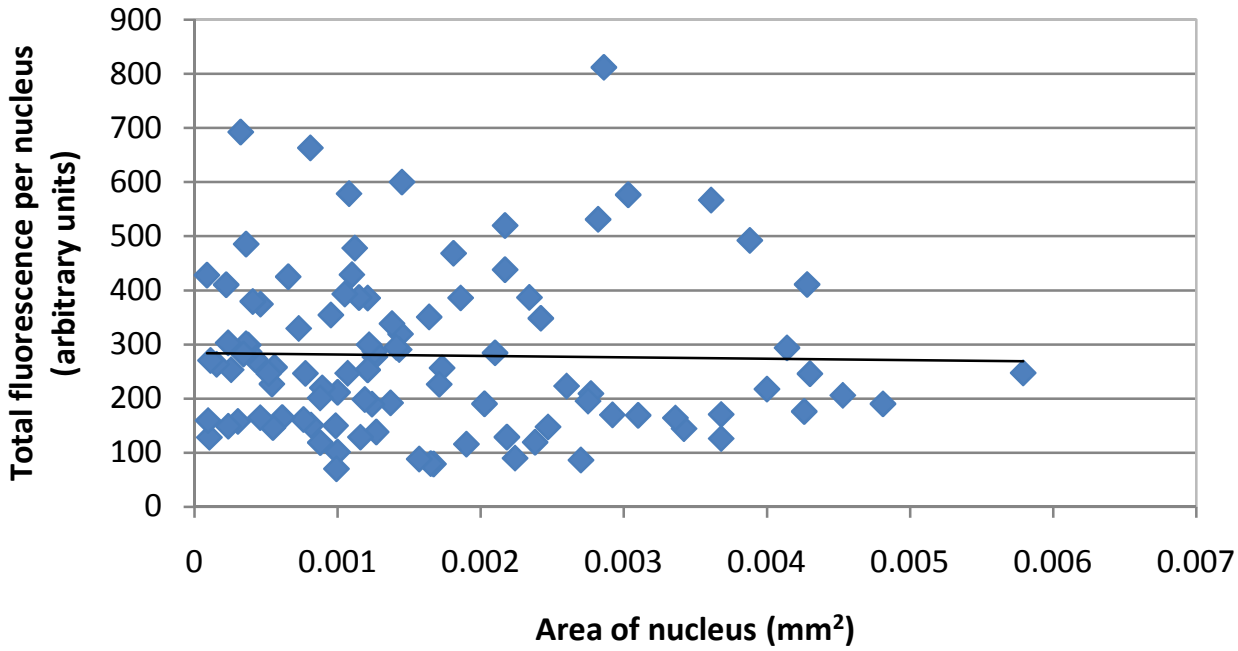
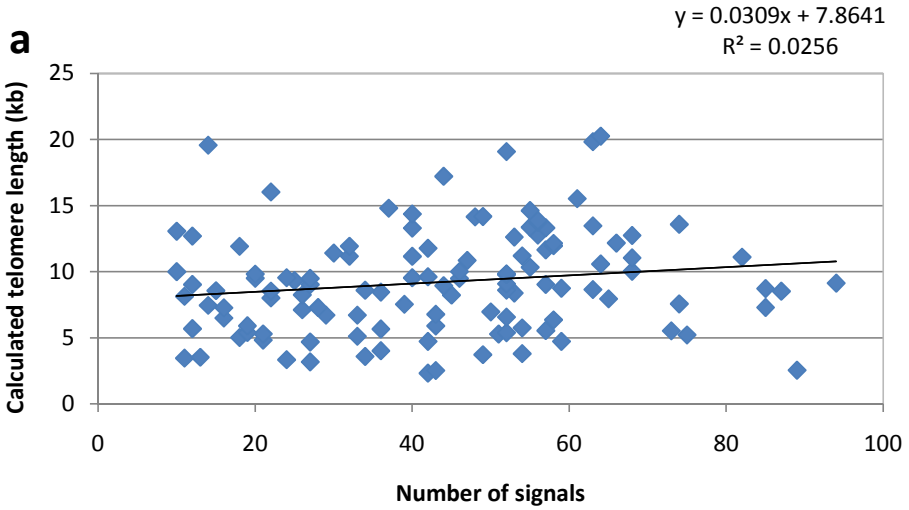

70
60
50
40
30
20
10
0
Number of signals/nucleus
GV
2c
3c
4c
6c
7c
Mor
Blast
Stage

Figure 3


Total fluorescence per nucleus (arbitrary units)
900
800
700
600
500
400
300
200
100
0
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
Area of nucleus (mm²)


a
y = 0.0309x + 7.8641
R² = 0.0256
Calculated telomere length (kb)
25
20
15
10
5
0
0
20
40
60
80
100
Number of signals

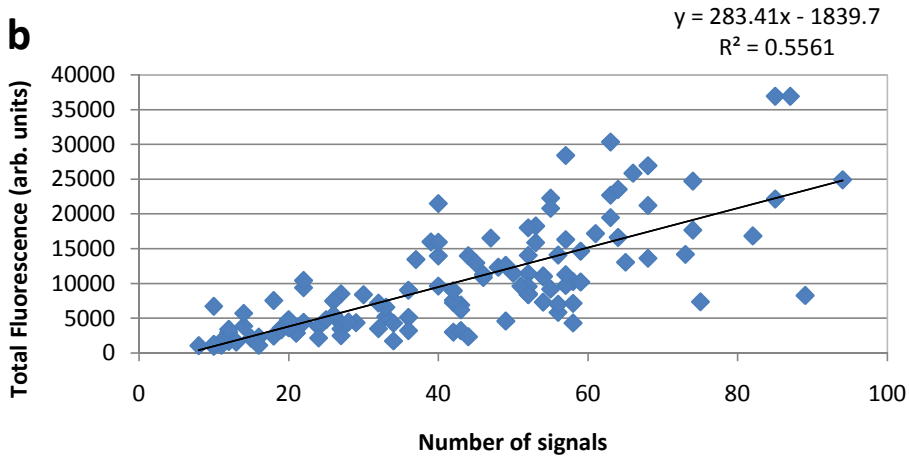

b
y = 283.41x - 1839.7
R² = 0.5561
Total Fluorescence (arb. units)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
20
40
60
80
100
Number of signals

Figure 4

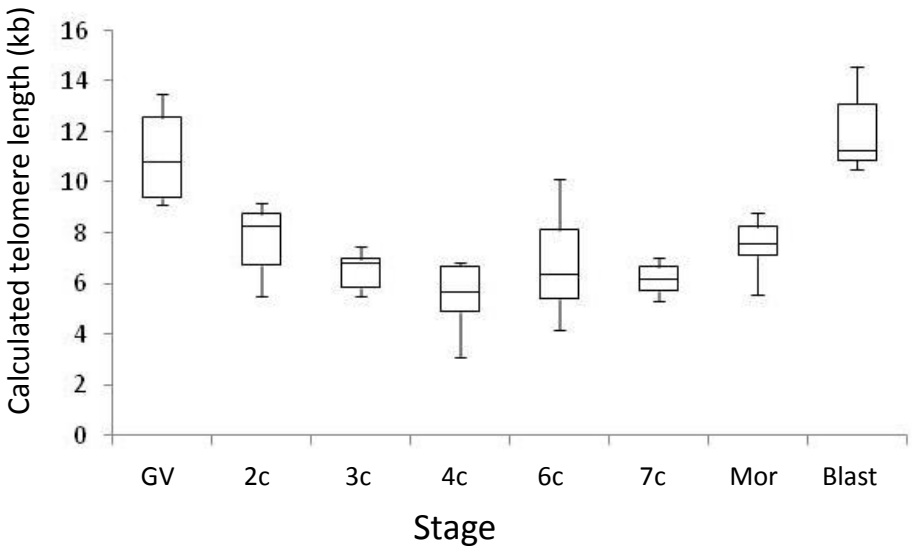

Calculated telomere length (kb)
16
14
12
10
8
6
4
2
0
GV
2c
3c
4c
6c
7c
Mor
Blast
Stage

Figure 5

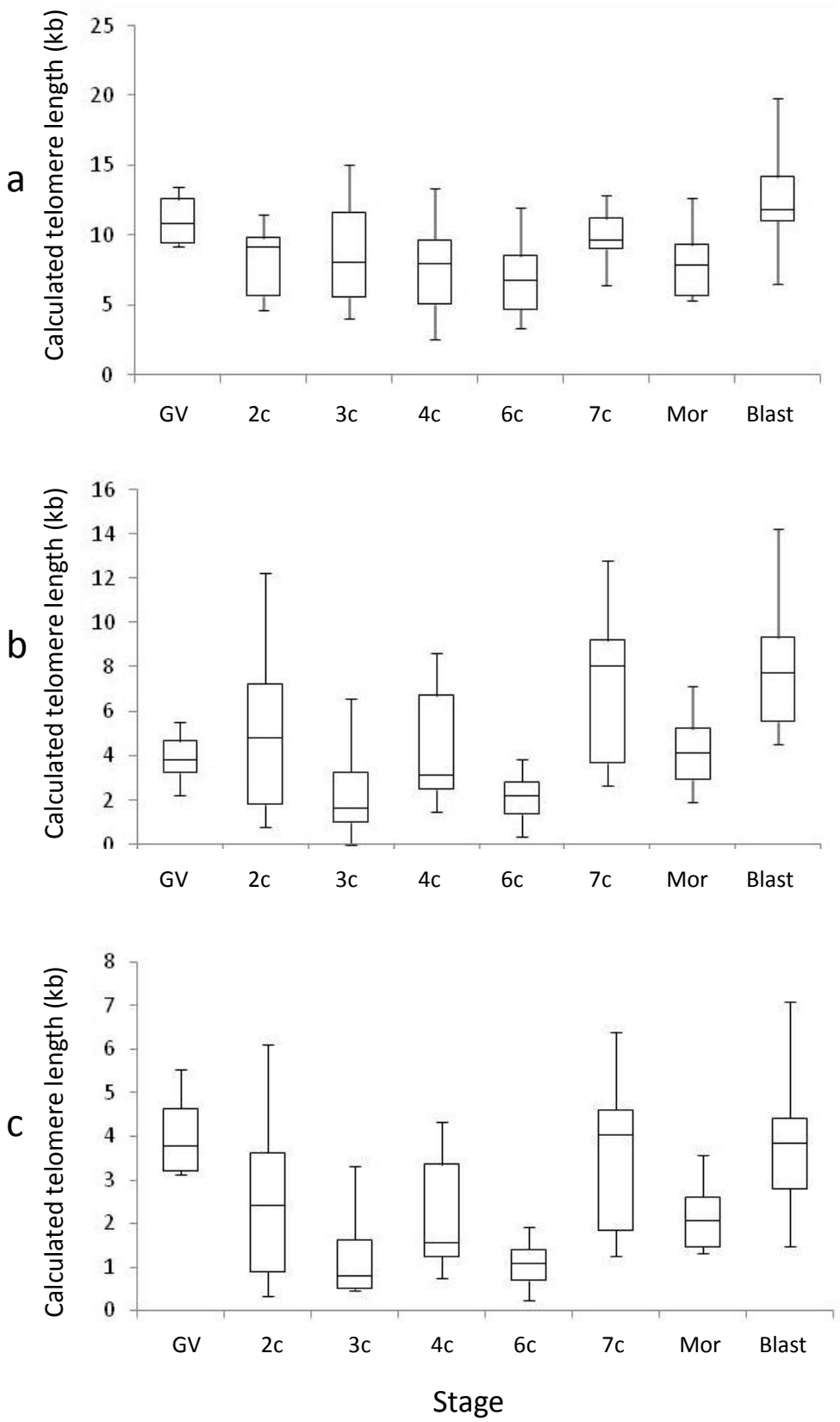
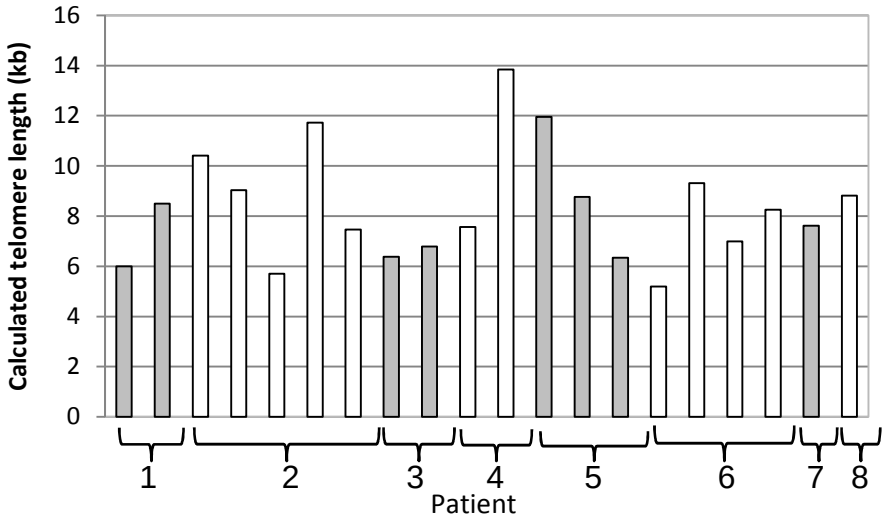

a
Calculated telomere length (kb)
25
20
15
10
5
0
GV
2c
3c
4c
6c
7c
Mor
Blast
b
Calculated telomere length (kb)
16
14
12
10
8
6
4
2
0
GV
2c
3c
4c
6c
7c
Mor
Blast
c
Calculated telomere length (kb)
8
7
6
5
4
3
2
1
0
GV
2c
3c
4c
6c
7c
Mor
Blast
Stage

Figure 6

Figure 7


Calculated telomere length (kb)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
Patient