Absence of premature senescence in Werner's syndrome keratinocytes.

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Abstract

Werner's syndrome (WS) is an autosomal recessive genetic disorder caused by loss of function mutation in *wrn* and is a useful model of premature *in vivo* ageing. Cellular senescence is a plausible causal mechanism of mammalian ageing and, at the cellular level, WS fibroblasts show premature senescence resulting from a combination of telomeric attrition and replication fork stalling. Over 90% of WS fibroblast cultures achieve less than 20 population doublings (PD) *in vitro* compared to wild type human fibroblast cultures.

It has been proposed that some cell types, capable of proliferation, will fail to show a premature senescence phenotype in response to *wrn* mutations. To test this hypothesis, human dermal keratinocytes (derived from both WS and wild type patients) were cultured long term. WS Keratinocytes showed a replicative lifespan in excess of 100 population doublings but maintained functional growth arrest mechanisms based on p16 and p53. The karyotype of the cells was superficially normal and the cultures retained markers characteristic of keratinocyte holoclones (stem cells) including p63 expression and telomerase activity. Accordingly we conclude that, in contrast to WS fibroblasts, WS keratinocytes do not demonstrate slow growth rates or features of premature senescence. These findings suggest that the epidermis is amongst the tissue types that do not display symptoms of premature ageing caused by loss of function of *wrn.* This is in support that Werner's syndrome is a segmental progeroid syndrome.

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1 Primarily this authors work.

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1.0 Introduction

The ageing phenotype is a complex one, which has the potential to be moulded by a wide combination of genetic and environmental effects. Population-based studies, which seek to identify candidate genes involved in successful ageing through the study of long-lived populations, are potentially both feasible and informative but their interpretation is not always straightforward (Kirkwood 1996; Leckie 1992).

Alternative, and possibly simpler, approaches rely on either (i) the identification in a simple model organism of single gene mutations which significantly extend healthy lifespan (Kimura et al. 1997; Clancy et al. 2001) or (ii) the study of heritable human genetic diseases, or "progeroid syndromes", which mimic some, but not all, features of the ageing process in order to gain insights into how the ageing process functions in normal individuals (Martin 1982; Kipling & Faragher 1997).

Werner syndrome (WS, MCK277700) is a well-known progeroid syndrome caused by loss of function mutation in the *wrn* gene. The enzyme is involved in DNA recombination, repair and the re-initiation of stalled DNA replication forks (Yu et al. 1996; Cox & Faragher 2007) and the disease itself is the highest ranked candidate for a disorder which accelerates multiple features of normal human ageing (Martin 1985; Cox & Faragher 2007)

The challenge with using model systems to understand ageing is to separate out disease or species specific observations from those that are of general applicability to the ageing population. This requires that data, generated using models, must be evaluated within the context of theories designed to explain how normal ageing operates. In this regard, Werner syndrome is particularly utile because some tissue and organ systems (e.g. the cardiovascular system) are severely affected whilst in others the "ageing" pathology appears to be limited or absent (e.g. the immune system). Clear mechanistic hypotheses thus allow researchers to design experiments which can separate correlative relationships from those with the potential to be causal.

Two theories, which also have explanatory power for the normal ageing process, have been used to explain the phenotypic presentation of WS patients and *wrn* knockout animals. The first places an emphasis on oxidative damage, with concomitant mutation accumulation, as the primary driver of pathology (Labbé et al. 2010; 2012). The second identifies the accumulation of senescent cells as the major causal mechanism (Ostler et al. 2002; Cox & Faragher 2007). These theories are not mutually exclusive (Kudlow et al. 2007) but the differences in emphasis between them are large enough to give rise to different predictions in the context of Werner's syndrome. In particular, any theory used to explain the phenotype of WS must be able, at least in

principle, to explain why some tissues are severely affected and others much less when the disease is caused by the loss of function of a ubiquitously expressed gene.

Senescent cells are the viable, but permanently growth arrested form of cells from mitotic populations (Burton & Faragher 2015; Norsgarrd 1996). The senescent state often results from extended cell division but can also occur through cell stress or oncogene activation. A variety of molecular pathways, including critical telomere shortening, drive entry into senescence resulting in a cell with a profoundly altered phenotype. This phenotype typically involves the elevated secretion of pro-inflammatory cytokines and matrix metalloproteinases (termed the senescence associated secretory phenotype or SASP) but can also involve pro-calcificatory changes or the loss of the ability to contribute to effect immune responses (Burton & Faragher 2015).

The cell senescence hypothesis invokes the known differences in the controls on the replicative lifespans of different cell types to explain the lack of disease phenotypes in some WS tissues. It considers senescent cells to be the primary drivers of the disease pathology and thus predicts that cell populations from affected WS tissues will show premature senescence *in vivo* whilst those derived from unaffected tissues will have a normal replicative lifespan. One of the cardinal *in vitro* features of WS is the exceptionally short replicative lifespan of dermal fibroblasts derived from patients. This is typically less than 20 population doublings and is the result of greatly increased rates of cell cycle exit (Faragher et al. 1993). This exit is primarily driven by intra-S phase arrest. However, abbreviated replicative lifespans are not universal among WS cell populations. This has been demonstrated in cultures of T cells derived from WS patients, which did not show a difference in replicative lifespans compared to normal controls (James et al. 2000).

The normal proliferative capacity of WS T cells may be attributed to the natural expression of telomerase in this cell type (Bodnar et al. 1996). Several studies have shown that telomerase expression can have a protective effect that prevents premature senescence in WS. For instance, ectopic expression of telomerase results in lifespan extension of WS fibroblasts (Wyllie et al. 2000). Reprogramming WS cells to specific lineages that naturally express telomerase also result in lifespan extension as was demonstrated in induced pluripotent and neuronal progenitor cells (Shimamoto et al. 2014; Cheung et al. 2014).

In keratinocytes, senescence does not take place directly, the cells must undergo clonal evolution from stem cell to transient amplifying cells prior to growth arrest. Keratinocytes in the basal layer naturally express telomerase and are known to grow in stem cell colonies (holoclones). Keratinocyte stem cells then stochastically divide into stem cells or transient amplifying cells. The transient amplifying cell population can undergo a series of divisions before they either senesce or commit to terminal differentiation (Watt 1998; Barrandon & Green 1987; Dellambra et al. 2000).

In Werner's syndrome, dermal fibroblasts enter premature senescence and features of dermal skin disorders mainly scleroderma-like skin, are evident. In contrast to the dermis, the epidermal layer of the skin appears to be less effected in WS patients (Epstein et al. 1966; Goto 1997). Accordingly the cell senescence hypothesis was tested by culturing and characterising the growth and phenotype of keratinocytes derived from the epidermal basal layer of WS patients. WS keratinocytes were grown in parallel to wild type keratinocytes under optimum conditions (co-cultured with feeder cells) to promote proliferation and maintenance in the stem cell compartment (Rheinwald & Green 1977). WS cells were then examined for normal keratinocyte characteristics including differentiation, senescence and stem cell properties of the basal layer. This study is aimed to improve our understanding of the state of keratinocytes in WS and its involvement in the premature age pathology of WS.

2.0 Materials & Methods

2.1 Initiation and culture of primary human keratinocytes

Primary keratinocytes were isolated mainly from the basal layer of the epidermis by Drs M. Illsley and S.E. James at the Blonde McIndoe Centre, East Grinstead, UK (James et al. 2010). The strains were obtained from 26 year old male patient (SK206AK) and 26 year old female Werner's syndrome patient (WSK368).
Keratinocytes were routinely seeded at densities between 1.2 x 10^4 and 2 x 10^4 cells/cm^2 co-cultured with a layer of γ-irradiated (9000 rads) 3T3 cells (mouse embryonic fibroblasts, National Institute for Biological Standards and Control, Hertfordshire, UK) seeded at 2.4x10^4-4x10^4 cells / cm^2. The cells were cultured in Rheinwald and Green culture medium (60% v/v Dulbecco's modified Eagle's medium (DMEM: Gibco®, Life Technologies, Paisley, UK), 20% (v/v) Ham's F12 (Gibco®, Life Technologies) and 20% (v/v) Foetal Bovine Serum (Gibco®, Life Technologies) supplemented with 10 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich Company Ltd., Poole, UK), 400 ng/ml hydrocortisone (Sigma-Aldrich Company Ltd.), and 10^{-10} M cholera toxin (Sigma-Aldrich Company Ltd.)) (Green et al. 1977; Rheinwald & Green 1977). The medium was replaced every 2 to 3 days and the keratinocytes were passaged when they had reached 80% confluency. Keratinocytes were co cultured with feeder cells in all experiments, except when transferred to defined keratinocyte serum free media (K-SFM) (Gibco®, Life Technologies) to test the reactions of cells to change in culture conditions. Cells were maintained at 37°C in a humidified atmosphere containing 10% CO_2 and 90% air. The number of population doublings (PD) per passage were calculated and plotted against the total time in culture ($PD = \log(N_t / N_o)/\log 2$, where N_t is number of cells counted and N_o is number of cells seeded).
For growth arrest studies, keratinocytes were treated with 1µM Adriamycin (doxorubicin) ($C_{27}H_{29}NO_{11}$) (Sigma-Aldrich Company Ltd.) for two hours followed by replacement with drug free media.

2.2 Senescence-associated β-galactosidase assay (SA-β-Gal)

Keratinocytes were grown in a 12 well plate (Iwaki®, Sterilin Ltd, Cambridge, UK) for 48 hours and fixed with 3% formalin (Sigma-Aldrich Company Ltd.). Senescence-associated β-galactosidase staining was carried out as described by Dimri and co-workers (1995).

2.3 Indirect immunofluorescence

Keratinocytes were seeded on 13 mm coverslips (VWR International, Radnor, PA, USA) and maintained to approximately 60-80% confluence. The samples were fixed with a 1:1 methanol:acetone solution, except for WRN detection, where the cells were fixed with 4% paraformaldehyde followed by permeablization in 0.1% Triton X-100. The cells were then incubated in primary antibody solutions overnight at 4°C and in secondary antibody solutions for 45 minutes at room temperature. For Ki67 labelling polyclonal rabbit anti-human Ki67 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA); 1:30 followed by FITC conjugated swine anti-rabbit IgG (Dako Agilent Technologies, Glostrup, Denmark); 1:30 were used. For cytokeratin staining monoclonal mouse anti-human cytokeratin (Dako Agilent Technologies); 1:30 followed by FITC conjugated rabbit anti-mouse IgG (Dako Agilent Technologies); 1:30 were used. For involucrin labelling monoclonal mouse anti-human involucrin (Abcam® plc., Cambridge, UK); 1:100 followed by Rhodamine red conjugated Donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA); 1:200 were used. For WRN detection monoclonal mouse anti-human WRN helicase [4H12] (Abcam® plc.); 1:200 followed by FITC conjugated Rabbit anti-mouse IgG (Dako Agilent Technologies); 1:30 were used. All antibodies were diluted in 0.1 % (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS, Oxoid Ltd, UK). The cells were washed in PBS after incubation with each antibody. Finally, the cells were mounted in Vectashield mountant, containing 4'6-diamidino-2-phenylindole 4′6-diamidino-2-phenylindole (DAPI; Vector Laboratories Ltd., Peterborough, UK) as nuclear counter stain. The cells were viewed using an inverted fluorescence microscope (Axiovert 25, Carl Zeiss Ltd., Oberkochen, Germany).

2.4 Western blotting

Total protein was extracted by treating the cells with protease inhibitor cocktail (Sigma-Aldrich Company Ltd.) and lysing in Totex buffer (20 mM Hepes pH7.8, 350 mM $NaCl_2$, 20% Glycerol, 1% (w/v) Igepal, 1mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA). Separation was carried by electrophoresis on acrylamide/bis-acrylamide gel (29:1) with 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.05% (w/v) ammonium persulphate and 0.05% (v/v) Temed and the samples transferred onto nitrocellulose membranes. The membranes were probed with either: mouse monoclonal anti human WRN [4H12] (Abcam® plc.); 1:500, mouse monoclonal anti human p63 (Abcam® plc.); 1:1000 or rabbit polyclonal anti human p16 (Santa Cruz Biotechnology, Inc.); 1:200. Secondary antibodies used were: goat anti mouse IgG horse radish peroxidase conjugate (Dako Agilent Technologies); 1:2000 or goat anti rabbit IgG horse radish peroxidase conjugate (Dako Agilent Technologies); 1:500 respectively. All antibodies were

prepared in a diluent buffer (23.1 mM Tris-HCl, 137 mM NaCl, pH 7.6, 0.1% (v/v) Tween 20, 5% (w/v) milk powder). Bands were visualized using an ECL solution and exposed to ECL Hyperfilm (Amersham, GE Healthcare, Little Chalfont, UK).

2.5 Detection of telomerase activity

Detection of telomerase activity, using a variant of the telomere repeat amplification protocol (TRAP) assay (Kim *et al.* 1994), with the Telo TTAGGG telomerase kit (Roche Diagnostics Ltd., Burgess Hill, UK). Briefly, 2 x 10^5 cells were harvested and pelleted by centrifuging at 3000g. The cells were lysed and prepared for amplification using the reagents provided in the kit. The reaction was initiated with primer elongation (25°C for 30 minutes) and telomerase inactivation (94°C for 5 minutes). This was followed by 30 cycles of denaturation (94° C for 30 seconds), annealing (50°C for 30 seconds), and polymerization (72°C for 90 seconds). A final extension step was carried out at 72°C for 10 minutes.
The PCR products were separated by electrophoresis on acrylamide/ bis-acrylamide gel (19:1) with 0.5 x TBE, 0.1 % (w/v) ammonium persulphate and 0.1 % (v/v) Temed) at 400V. The gel was stained with Sybr Gold (Life Technologies Ltd, Paisley, UK) and viewed under ultraviolet light. Extracts from EK1Br.hTERT and EK1Br cells were used as positive and negative controls respectively. Further controls were provided by heat inactivation of telomerase for each sample at 92° C for 5 minutes.

2.6 Statistical analysis

The data were confirmed for normal distribution using Shapiro Wilks and Kolmogorov-Smirnov tests of normality. Two-tailed T test was performed to examine differences between the two keratinocyte strains for pKi67 and involucrin expression. Univariate analysis of variance was performed to examine differences in the growth rates between SK206AK and WSK368. P values <0.05 were considered statistically significant. All statistical analysis were carried out using the SPSS software (version 22, IBM).

3.0 Results

3.1 Culture and growth of primary human keratinocytes

Cell cultures were initiated from a dermal biopsy of a Werner's syndrome patient under conditions that would promote the selective growth of keratinocytes rather than dermal fibroblasts. The resulting cell culture (designated WSK368) demonstrated classical keratinocyte morphology (Figure 1) and grew at an initial rate of 0.4±0.13 population doublings per day. WSK368 cells stained positive for cytokeratin's by indirect immuno-fluorescence (Figure 2) and were negative for the presence of WRN protein as assayed both by indirect immuno-fluorescence and Western blotting (Figure 3). In tandem, a fibroblast culture (WSF368) was initiated from part of the same biopsy material. Although viable cells emerged from the biopsy this failed to achieve the

degree of growth needed to be passaged despite being maintained in culture for more than 40 days (data not shown).

A strain of wild type human dermal keratinocytes initiated under the same conditions as WSK368 (SK206AK) reached 61 Cumulative Population Doublings (CPD) in 259 days at an average growth rate of ~0.27 ± 0.1 PD day^{-1}. WSK368 achieved 66 CPD in 263 days with a mean growth rate of ~0.26 ± 0.2 PD day^{-1}. Univariate analysis of variance demonstrated no significant differences between the growth rate of these wild type and progeroid keratinocyte cultures (p=0.966).

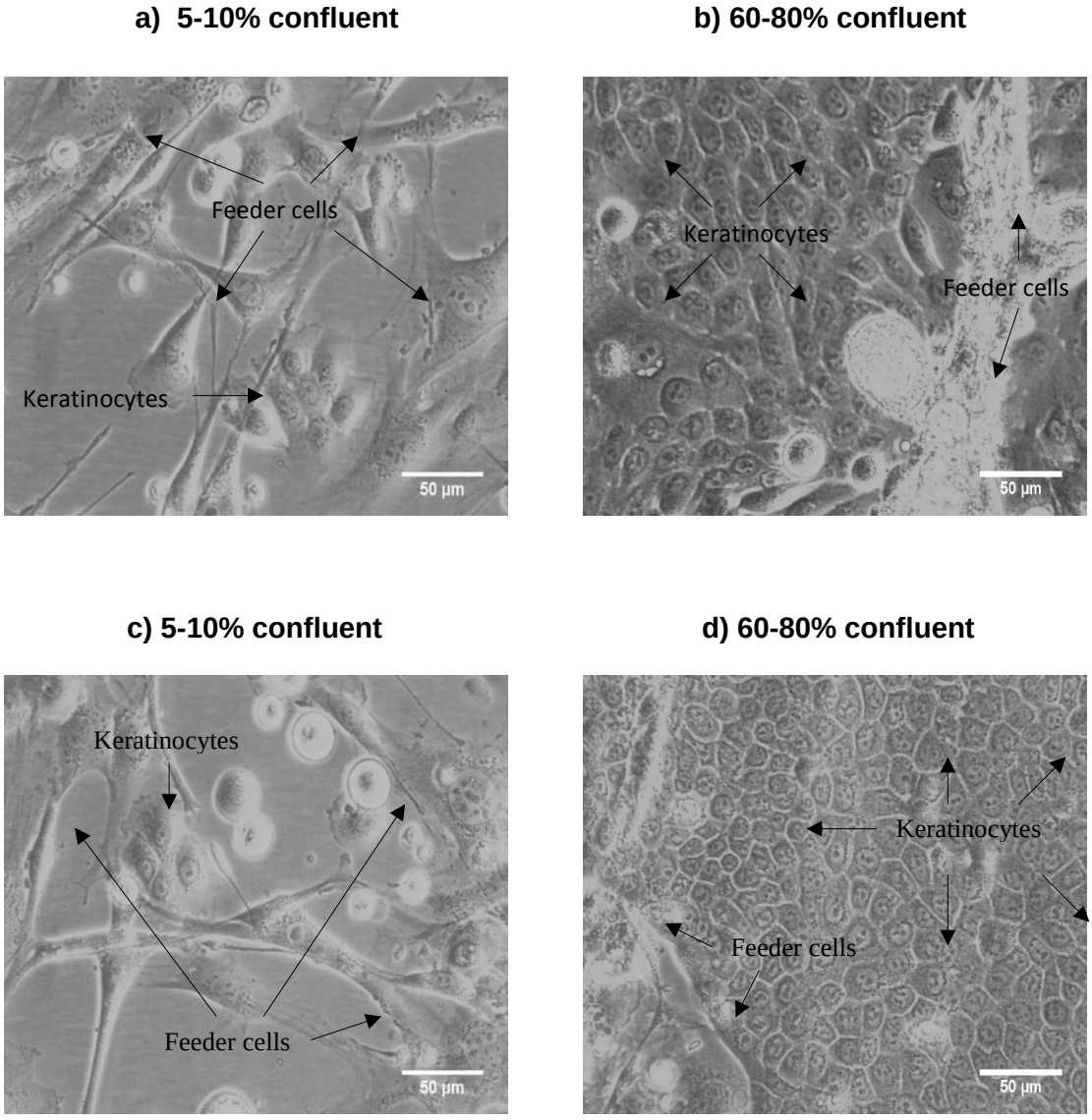
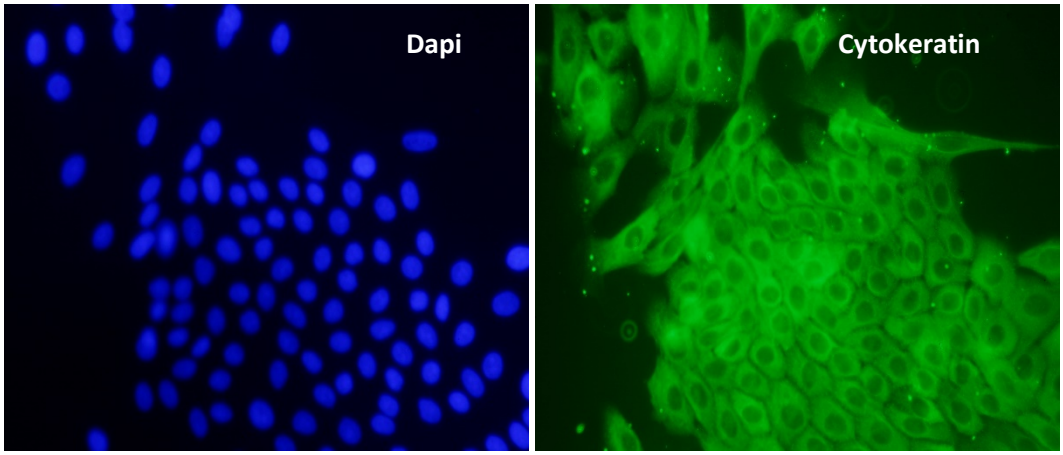


Figure 1. Photographs of cultured keratinocytes. Wild type keratinocytes SK206AK (passage 24, 60.7 CPD) at a) early stage (< 10% confluent) and b) late stage (60-80% confluent). Werner's syndrome keratinocytes WSK368 (passage 38, 96.28 CPD) at c) early stage (<10% confluent) and d) late stage (60-80% confluent).

a)



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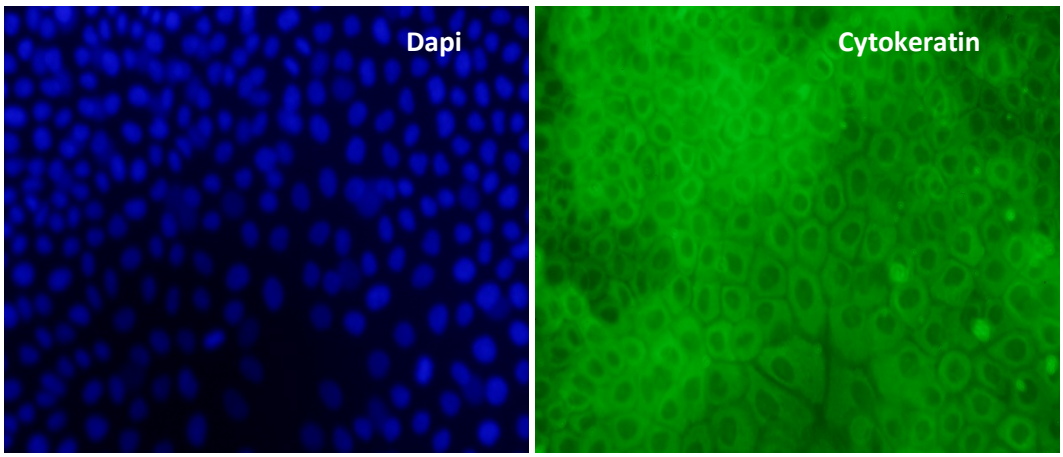

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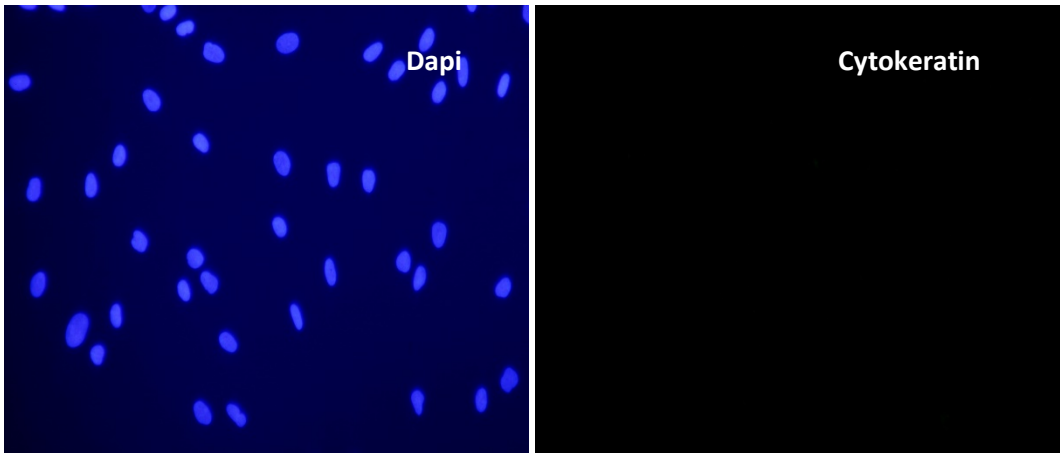
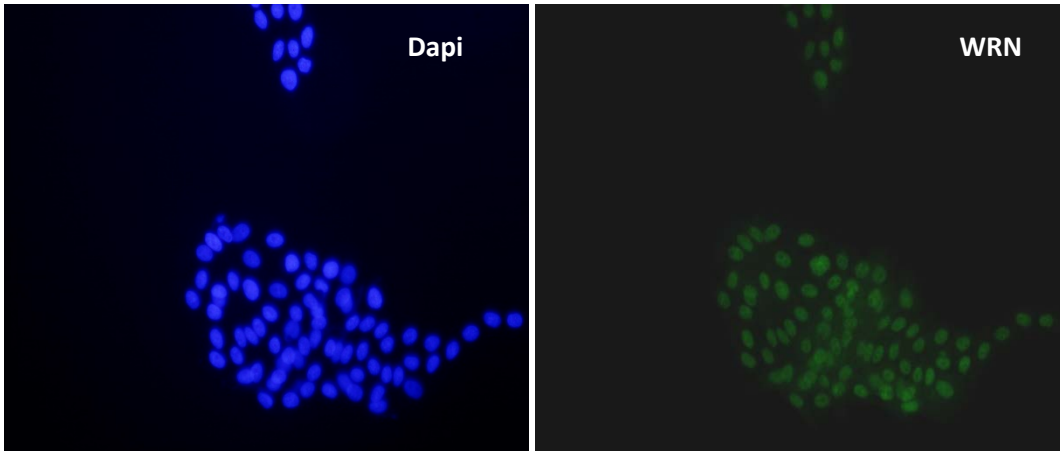


Figure 2. Immunocytochemical detection of cytokeratin in wild type and WS keratinocytes. DAPI (nuclear stain, blue) and anti-cytokeratin (cytokeratin 5, 6, 8, 17 and probably 19) FITC (cytoplasmic stain, green) staining of a) wild type keratinocytes (SK206AK, 46.79 CPD), b) WS keratinocytes (WSK368, 108.81 CPD) and c) wild type dermal fibroblasts (SKF276) used as negative control.

a)



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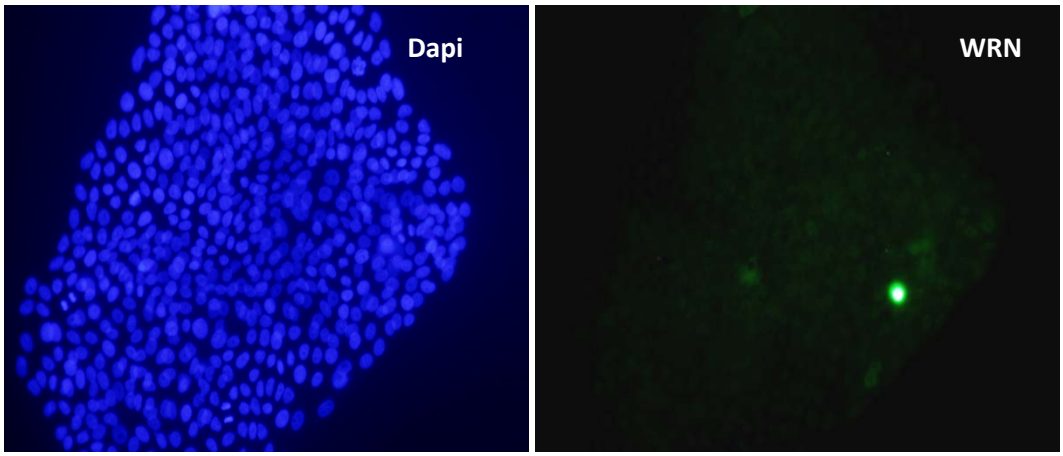

c)

SK206AK **WSK368**

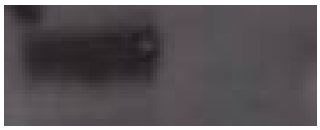

Figure 3. Immunocytochemical detection of WRN. Imunofluorescence staining with DAPI (blue, nuclear) and for WRN [4H12] (FITC (green), nuclear) in a) SK206AK (passage 19, 46.79 CPD) and b) WSK368 (passage 41,108.81 CPD). c) Western blot demonstrates the presence of the ~ 160 kDa band of WRN in wild type keratinocytes (SK206AK) but is absent in Werner's syndrome keratinocytes (WSK368).

WSK368 were maintained in log phase culture for 400 days reaching 130 CPD before continuous culture was terminated (Figure 4). The growth rate of this culture at termination appeared higher (0.331 ± 0.17 PD day^{-1}) than that seen in the initial phase.

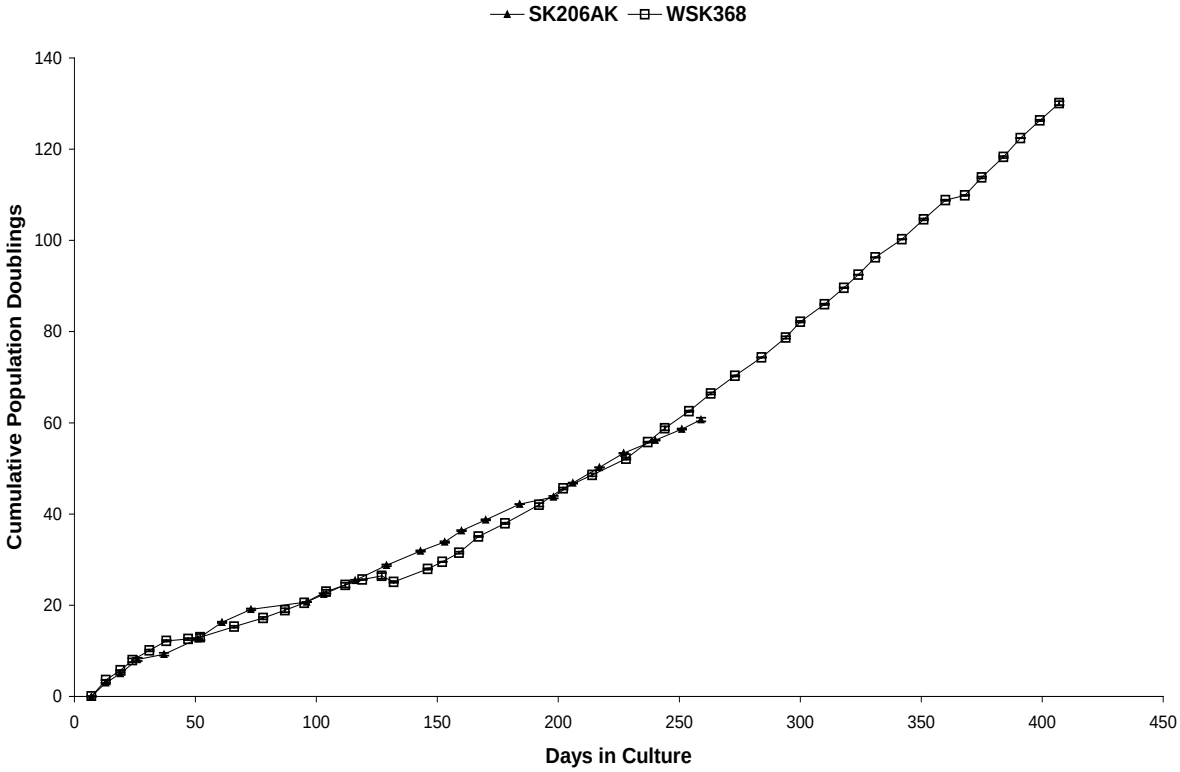

Figure 4. Comparison of wild type and Werner's syndrome keratinocyte growth. Growth curves of wild type (SK206AK) and WS (WSK368) keratinocytes were grown for 259 and 400 days respectively before termination of the model at those time points. Both strains showed extended growth and did not express a reduction in growth rate, signs of replicative senescence or immortality during the model growth period. Error bars= ± standard deviation.

3.2 Population dynamics of WSK368

The growth fraction of WSK368 was captured at intervals throughout serial passage by quantified indirect immuno-fluorescence using antibodies directed against the well characterised proliferation marker pKi67. There was no detectable loss of growth fraction during serial passage of WSK368 and the mean labelling indices of WSK368 and SK206AK was statistically indistinguishable (Figure 5, p=0.177 two tailed T-test).

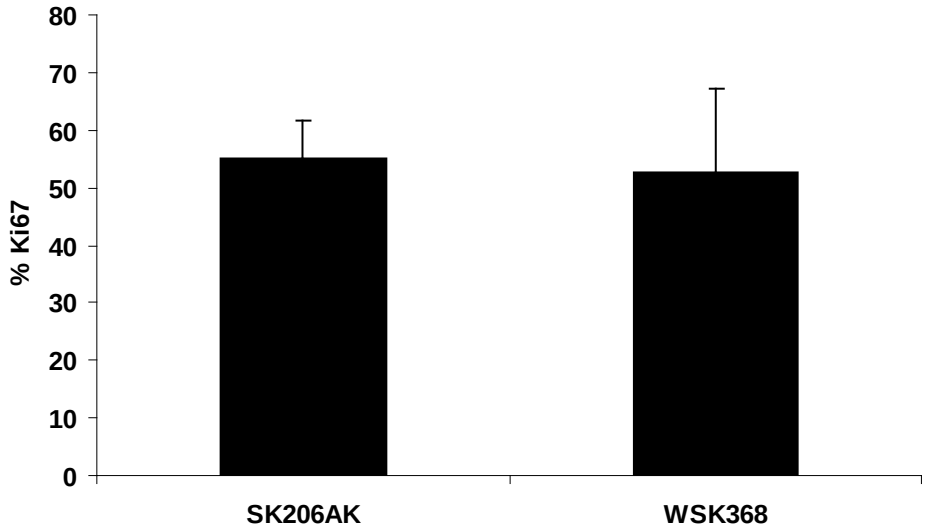

Figure 5. Ki67 labelling index in wild type and Werner's syndrome keratinocytes. Mean Ki67 labelling percentages in wild type (SK206AK) (n=12) and Werner's syndrome keratinocytes (WSK368) (n=38). Error bars= standard deviation.

As a proxy measure of the fraction of differentiating cells within the keratinocyte cultures, the fraction of involucrin positive cells were determined at regular intervals across the culture from 5-120 population doublings. The mean percentages of involucrin positive cells are shown in Figure 6 and did not differ significantly between SK206AK and WSK368 (two tailed T-test, p=0.709). In addition, no senescence associated beta galactosidase positive cells were observed in cultures of either SK206AK (assayed at n=7 separate time points from 6-48 CPD) or WSK368 (assayed at n=18 separate time points from 4-104 CPD) despite the presence of detectable control beta galactosidase activity in both strains of keratinocytes at pH4.0 and in late passage human dermal fibroblasts at pH6.0 (data not shown).

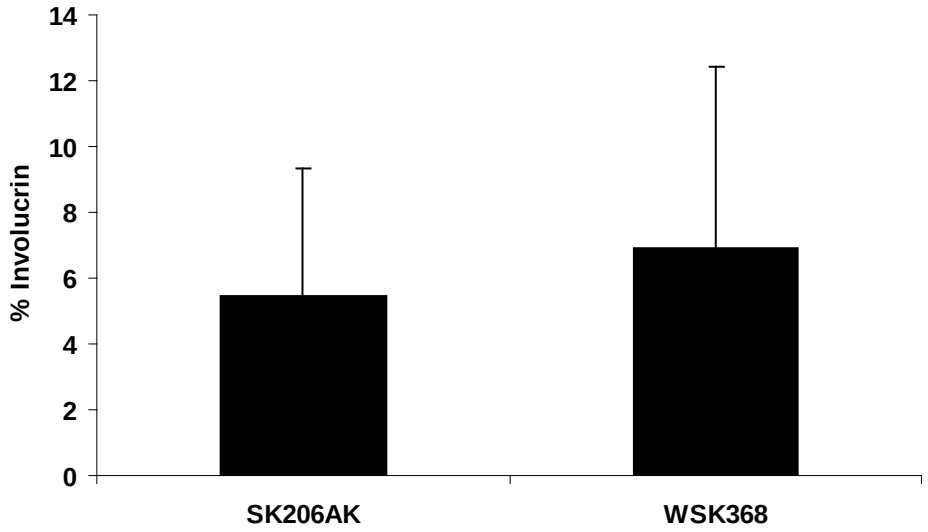

Figure 6. Involucrin labelling index in wild type and Werner's syndrome keratinocytes. Mean involucrin labelling percentages in wild type (SK206AK) (n=3) and Werner's syndrome keratinocytes (WSK368) (n=4). Error bars= standard deviation.

3.3 WSK368 keratinocytes show detectable telomerase activity and are positive for the stem cell marker p63

Given that the extended growth of human cell populations requires stabilisation of telomere length, the presence of telomerase activity was determined using a commercial TRAP assay (Figure 7). A ladder of bands consistent with the presence of active telomerase was observed in samples taken from both SK206AK and WSK368 (together with appropriate positive controls). No laddering was observed in human corneal fibroblasts (strain Ek1.Br) which are known to be telomerase negative (Wallis et al. 2004).

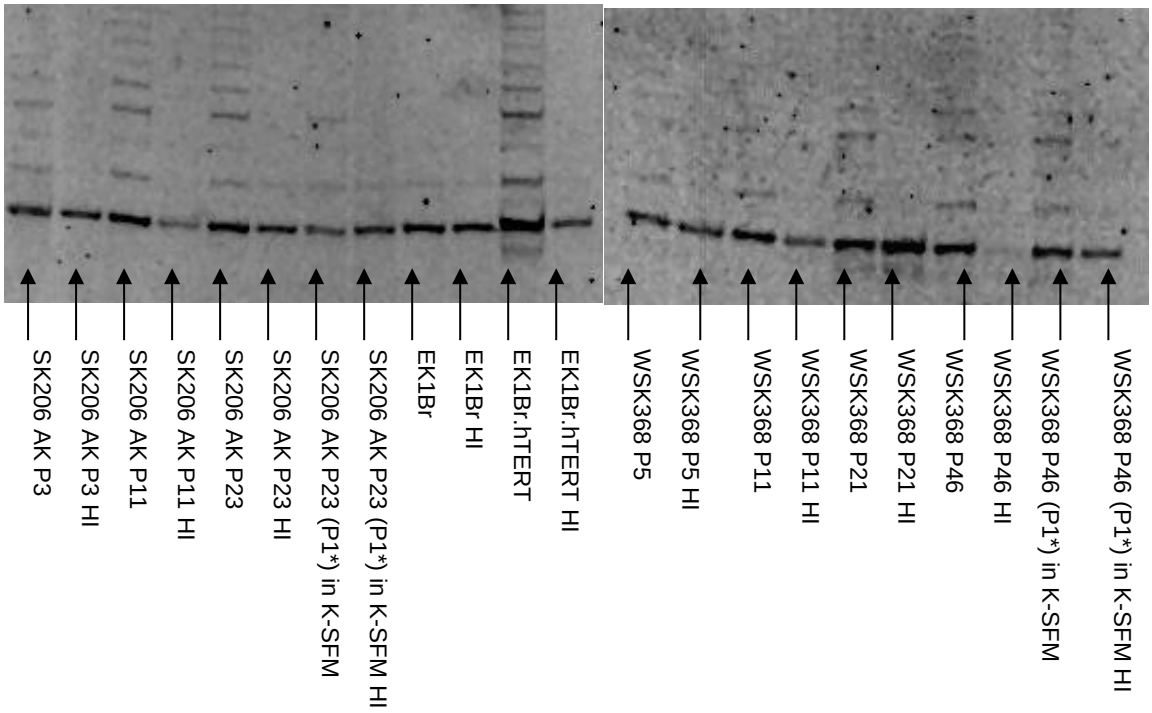

Figure 7. TRAP assay of wild type and Werner's syndrome keratinocytes. TRAP assay of SK206AK (P3 (5 CPD), P11 (25 CPD) and P23 (59 CPD) in the feeder system and passage 1* in K-SFM) and WSK368 (P5 (10 CPD), P11 (19 CPD), P21 (35 CPD) and P46 (126 CPD) in the feeder system and p1* in K-SFM) at different passages. Primary fibroblast cell strain EK1Br and immortalised EK1Br.hTERT were used as negative and positive controls respectively. Each sample was paralleled by a heat inactivated (HI) counterpart as negative control.

In tandem with this analysis the presence of the keratinocyte stem cell marker protein p63 in both SK206AK and WSK368 cultures at a range of CPDs was determined by Western blotting. As can be seen in Figure 8 both wild type and WS keratinocytes were positive for this protein.

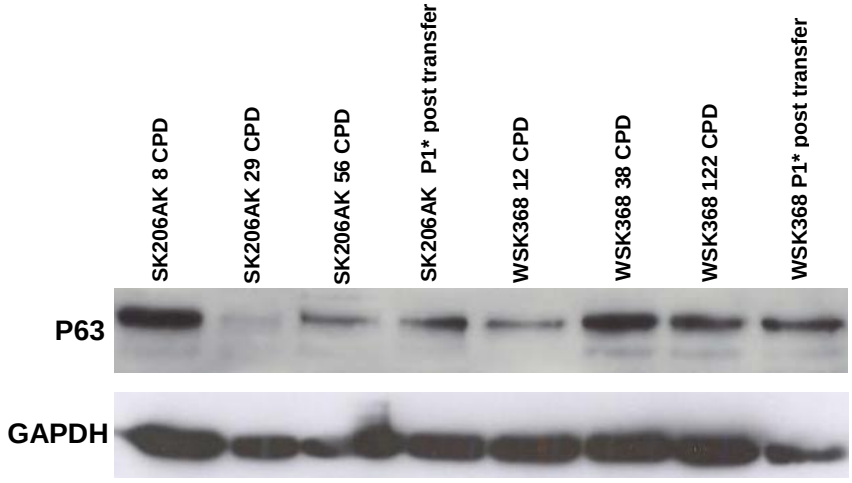

Figure 8. Western blot detection of p63 in wild type and Werner's syndrome keratinocytes.

Western blots of P63 (upper panel) in wild type (SK206AK) and Werner's syndrome (WSK368) at early, mid and late cumulative population doublings (CPD). Once cultures were transferred to plastic they were

designated passage 0* (P0*). Cells were harvested at P1* post transfer to plastic in K-SFM media. Using densitometry the bands were normalised against their corresponding GAPDH (lower panel).

3.4 WSK368 keratinocytes show functional growth arrest checkpoints and anchorage dependence.

To determine if WSK368 cells retained the capacity to a growth arrested state after prolonged *in vitro* culture, populations of WSK368 (at 118 CPD) were transferred into keratinocyte serum-free medium (K-SFM) without feeder cells (alongside parallel populations of SK206AK, at 56 CPD, were also transferred). Figure 9, shows the shift into K-SFM triggered a rapid decline in the growth of both cultures. Neither cell population proliferated for more than two passages under these conditions.

a) **b)**

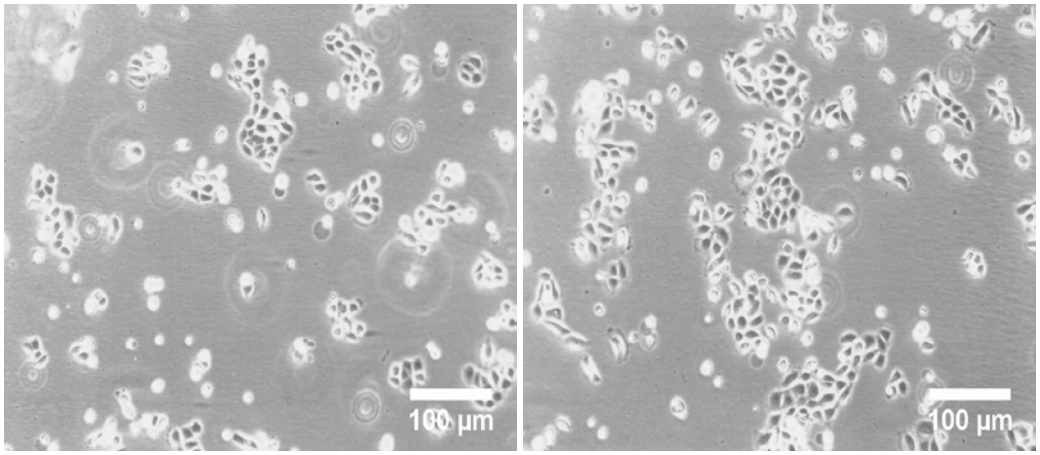

Figure 9. Photographs of wild type and Werner's syndrome keratinocytes after transfer to K-SFM. Keratinocytes from a) Wild type (SK206AK) (56.17 CPD) and b) Werner's syndrome (WSK368) (118.28 CPD) strains transferred to K-SFM. (Size bar=100µm).

This cessation of growth was associated with increased levels of the cyclin-dependent kinase inhibitor $p16^{ink4a}$, as measured by Western blotting analysis (Figure 10). Both cultures, post transfer, were seen to show an up-regulation of $p16^{ink4a}$ as indicated in figure 10 (P1* post transfer) for both wild type and WS cultures compared to the late CPD samples.

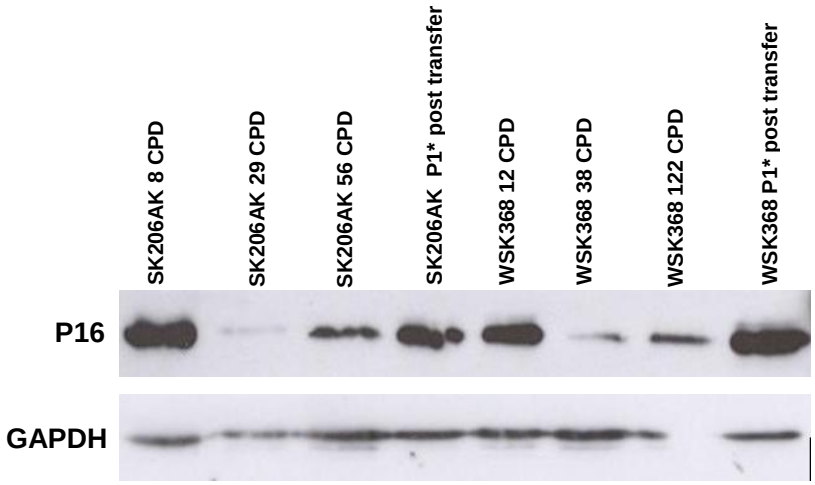

Figure 10. Western blot detection of p16 in wild type (WT) and Werner's syndrome (WS) keratinocytes. Western blots of p16 (upper panel) in wild type (SK206AK) and WS (WSK368) keratinocytes at early, mid and late cumulative population doublings (CPD). Once cultures were transferred to plastic they were designated Passage 0* (P0*). Cells were harvested at P1* post transfer to plastic in K-SFM media. Using densitometry the bands were normalised against their corresponding GAPDH (lower panel).

Similarly, transfer of cultures of both wild type and Werner's syndrome keratinocytes onto methylcellulose led to a cessation of proliferation (no growth for 18 days in the case of SK206AK and a growth rate of less than 0.003 CPD day^{-1} over the same period for WSK368).

Treatment of populations of WSK368 and SK206AK grown under standard culture conditions in the presence of feeder cells for two hours with $1\mu M$ adriamycin resulted in a significant change in cell size and the presence of detectable senescence associated β galactosidase activity (data not shown).

4.0 Discussion

Fibroblasts from patients with WS are known to have a significantly reduced replicative lifespan *in vitro* when compared with wild type fibroblasts. Salk *et al.,* (1981) studied the growth, *in vitro,* of 20 different Werner's syndrome fibroblast strains and concluded that there was no overlap in growth potential between WS and wild type fibroblast cultures. The mean cumulative population doubling level reached by the progeriod strains was only ~27% of that obtained using wild type controls (n=10 cultures, obtained from donors whose age ranged from newborn to 34 years). Several studies confirm these findings and show that 90% of Werner's syndrome cultures have an *in vitro* lifespan of less than 20 population doublings (Tollefsbol & Cohen 1984). The short lifespan is due to a three to five fold elevation in the fraction of fibroblasts exiting the cell cycle and becoming senescent (Faragher et al. 1993). Premature cell senescence is one of the most obvious and striking manifestations of the disorder *in*

vitro, which can lead to the presumption that poor proliferative capacity is a universal feature of WS cell populations.

Here we have initiated cultures of WS keratinocytes *de novo* from a patient whose dermal fibroblasts had entered premature senescence and found no evidence for premature replicative senescence compared to wild type controls. If WS keratinocytes grew as poorly as WS fibroblasts, then a deficit in proliferation should have been clearly observable. Instead, WSK368 keratinocytes were able to proliferate for more than 100 population doublings and similar levels of proliferation were found in SK206AK wild type controls. Cross contamination of WSK368 with SK206AK was excluded because western blot showed WRN protein in SK206AK and its absence in WSK368. Wild type and WS keratinocytes expressed both cytokeratin and involucrin, consistent with a keratinocyte origin and the maintenance of differentiation capacity.

A problem when any cell type shows particularly good growth is the difficulty of distinguishing between long term growth in the 'normal' state and the acquisition of genetic changes which have caused the cell population to become immortal or enter an extended lifespan phase. The replicative lifespan of keratinocytes grown on feeder layers is known to be very long compared to growth in keratinocyte serum free medium (typically at least 60-80 population doublings with up to 150 population doublings being reported (Rheinwald *et al.* 1977; Parkinson *et al.* 1992) compared to 20-40 population doublings in serum free medium (Kiyono *et al.* 1998; Opitz *et al.* 2001)). Furthermore, wild type keratinocytes that are cultured in the feeder system are maintained in the stem cell compartment as holoclones and can potentially be immortal (Dellambra et al. 2000). WSK368 is thus long lived but not abnormally so.

Maintenance of involucrin expression is not in itself strong evidence against immortalisation because keratinocyte cell lines can, under some circumstances, retain differentiation capacity *in vitro* (Boukamp et al. 1988). Since keratinocyte lifespan can be extended due to inactivation of p53, $p16^{ink4a}$ or both (Opitz et al. 2001) we set out to determine if these important cell cycle checkpoints remained intact. If functional $p16^{ink4a}$ is present within keratinocytes then growing the cells on plastic alone in keratinocyte serum free medium is known to induce growth arrest (Ramirez et al. 2001). Both WSK368 and SK206AK responded to such a change in culture conditions by ceasing proliferation and by upregulating p16 at the protein level. These data are therefore consistent with a functional p16 pathway and argue against immortality as a result of loss or substantial reduction in p16 expression. Similarly both WSK368 and SK206AK responded to a brief treatment with the DNA damaging agent adriamycin by entering senescence. Since adriamycin requires a functional p53 pathway to exert this effect (Elmore et al. 2002), these data are not consistent with extended lifespan as a consequence of p53 mutation. Rather, the presence at the protein level of the putative keratinocyte stem cell marker p63 suggests that the high proliferative capacity we have observed is the result of maintenance of a stem cell pool within cultures of both WSK368 and SK206AK (Pellegrini et al. 2001). This is further supported by the observation of telomerase expression in both cultures, which is also characteristic of

keratinocyte stem cells (Härle-Bachor & Boukamp 1996; Barrandon & Green 1987; Dellambra et al. 2000). It cannot be formally ruled out that the cells may be transformed, in either population, as spontaneously transformed cell lines have been previously reported (Boukamp et al. 1988). However, the lack of anchorage independent growth in methylcellulose argues against acquisition of the transformed phenotype (Allen-Hoffmann et al. 2000).

We therefore conclude that both WSK368 and SK206AK are essentially normal and that premature replicative senescence is not a feature of WS keratinocytes. Similar observations have been made in WS T lymphocytes, which demonstrated normal replicative lifespans when compared to normal controls (James et al. 2000). Concurrently, the immune system of WS patients appears to be clinically normal (Goto et al. 1985). Since wild type and WS T lymphocytes naturally express telomerase, it appears that telomere maintenance plays a pivotal role in the prevention of WS pathology (James et al. 2000). Keratinocytes also naturally express telomerase and maintain a stem cell growth when cultured under the feeder system. WS keratinocytes are therefore protected from premature senescence by telomere maintenance. These findings suggest that the underlying cause of the skin pathology seen in WS does not involve keratinocyte premature senescence. However, WS keratinocytes lack functional WRN protein which leaves open the possibility that exposure to severe DNA damaging agents (e.g. UV light) might produce a more stringent response in a WS keratinocyte population than a wild type one and thus a subtle epidermal pathology in patients. This coincides with the statement that WS is a segmental progeroid syndrome and some of the symptoms of the disease may not correlate with premature ageing. These findings may help improve our understanding of the role of senescence in ageing by distinguishing premature ageing from non-ageing pathologies of WS.

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**********ENDS**********

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