Differential Protein Expression in Senescent Human Skin Fibroblasts and Stress Induced Premature Senescence (SIPS) Fibroblasts

(Perbezaan Pengekspresan Protein dalam Fibroblas Kulit Manusia Senesen dan Fibroblas Senesen Pramatang Teraruh Tekanan)

Goon Jo Aan*, Haryati Ahmad Hairi*, Suzana Makpol, Mariati Abdul Rahman,
Saiful Anuar Karsani & Wan Zurinah Wan Ngah

ABSTRACT

Replicative senescence of human diploid fibroblasts (HDFs) occurs when cells lose their capacity to proliferate and enter a phase of irreversible growth arrest. Stress-induced premature senescence (SIPS) on the other hand is caused by subcytotoxic concentrations of various oxidants which trigger accelerated cellular senescence. In this study, a SIPS model was established by exposing human diploid fibroblasts (HDFs) to 20 µM H_2O_2 for 2 weeks. A proteomic comparison between young, senescent and SIPS cells was done using two dimensional gel electrophoresis (2DGE) to elucidate the changes in protein expression associated with cellular aging. Our analysis showed that 28 protein spots were differentially expressed in senescent cells whereas 10 protein spots were differentially expressed in SIPS as compared to young cells. Three similar protein spots were differentially expressed in both senescent and SIPS cells when compared to the young cells. These results indicate that a difference in protein expression exists between senescent cells and SIPS cells compared to young cells.

Keywords: Fibroblast; senescent cells; SIPS; two dimensional gel electrophoresis (2DGE)

ABSTRAK

Senesen replikatif fibroblas diploid manusia berlaku apabila sel hilang kapasiti untuk berproliferasi dan memasuki fasa pertumbuhan terencat secara tidak berbalik. Senesen pramatang teraruh tekanan (SIPS) pula berpunca daripada kepekatan subsitotoksik pelbagai oksidan yang boleh mengaruh senesen sel. Dalam kajian ini, model SIPS telah dihasilkan dengan mendedahkan fibroblas diploid manusia kepada 20 µM H_2O_2 selama 2 minggu. Suatu perbandingan proteomik antara sel muda, sel tua dan model SIPS telah dilakukan dengan menggunakan elektroforesis gel dua dimensi (2DGE) untuk menunjukkan perubahan dalam pengekspresan protein berkaitan dengan penuaan selular. Keputusan menunjukkan 28 titik protein yang diekspreskan secara berbeza dalam sel senesen manakala SIPS menunjukkan 10 titik protein yang diekspreskan secara berbeza berbanding dengan sel muda. Tiga titik protein yang sama diekspreskan secara berbeza dalam kedua-dua sel senesen dan SIPS berbanding sel muda. Keputusan ini menunjukkan bahawa terdapat perbezaan dalam ekspresi protein antara sel senesen dan sel SIPS berbanding dengan sel muda.

Kata kunci: Elektroforesis gel dua dimensi; fibroblas; sel senesen; SIPS

INTRODUCTION

The Hayflick limit is a theory that covers the definition of *in vitro* replicative senescence. It states that proliferating cells reach the limit of replicative potential after a series of sub-cultivations (de Magalhaes 2004; Hayflick & Moorhead 1961; Toussaint et al. 2000). This phenomenon is characterized by a decreasing rate of proliferation and cell growth even when cells are alive and metabolically reactive (Duan et al. 2005; Toussaint et al. 2000). This theory has been used in the development of various premature senescence models to study the process of *in vitro* aging (Brack et al. 2000; Dierick et al. 2002; Duan et al. 2005).

It has been proposed that oxygen free radicals or reactive oxygen species are the major agents responsible for cellular aging (Deaton & Martin 2003; Harman 1956).

This theory together with the Hayflick limit is used for the development of stress induced premature senescence (SIPS) (Brack et al. 2000). In this model, human diploid fibroblasts (HDFs) at early cumulative population doublings (CPDs) are exposed to subcytotoxic concentrations of numerous types of oxidants such as ethanol, *tert*-butylhydroperoxide, hydrogen peroxide, hyperoxia and ultraviolet radiation (UV) (Boraldi et al. 2007; Borlon et al. 2007; Dierick et al. 2002; Duan et al. 2005). This led to an increase in the level of reactive oxygen species (ROS) within the cells.

HDFs in SIPS share similar characteristics with cells undergoing replicative senescence. These similarities include typical senescence morphology, senescence-associated β-galactosidase activity and growth arrest in the G1 phase of the cell cycle (de Magalhaes et al. 2004; Dierick et al. 2002; Dimri et al. 1995; Duan et al. 2005).

There is also significant increase of gene expressions such as *p21* and *gadd45*, increased *p53* binding activity as well as decreased DNA repair capability, accelerated telomere shortening and altered regulation of growth arrest (Cyclin-dependent kinase inhibitor overexpression, retinoblastoma protein underphosphorylation) (Duan et al. 2005; Frippiat et al. 2003) in both types of cells. However, whether HDFs in SIPS share similar expression of functional proteins with replicative senescence remains to be clarified.

Among the detrimental consequences of oxidative stress on cellular function is protein damage (Grune et al. 2003). ROS can cause modifications to the amino acid side chain of proteins. These modifications can lead to functional changes that disrupt cellular metabolism which may in turn lead to pathophysiological disorders such as neurodegenerative diseases, diabetes and atherosclerosis (Bagnato et al. 2008; Purecelli et al. 2006; Zhang et al. 2008). Thus, protein profiling has become an important tool to understand the pathophysiology of various diseases and disorders.

The objective of this study was to compare the expression of proteins between young cells, SIPS model and senescent cells. Because proteomics enables the analysis of the total protein complement of a biological system at a given moment, it is the recommended technique to determine changes in protein expression (Dierick et al. 2002a). As a fundamental step in establishing a proteomic study, a protocol for profiling the cellular proteome was developed using two-dimensional gel electrophoresis (2DGE). Analysis of the resulting 2DGE gel images using the Image Master Platinum 6 software allowed us to perform a quantitative analysis of these gel images and identify proteins that changed in expression with various treatments. The SIPS model was established with prolonged low dose treatment with H_2O_2 for 2 weeks and markers for cellular senescence such as morphology and senescence associated β-galactosidase (SA- β-gal) expression were examined.

MATERIALS AND METHODS

CELL CULTURE

Foreskins were obtained with informed consent from three healthy subjects aged between 8 and 12 years following circumcision. After extraction, foreskins were washed with sterilised 70% ethanol and PBS with penicillin/ streptomycin (Hyclone, US). They were then separated into dermis and epidermis. Dermis was incubated overnight in Type I Collagenase (Sigma, Aldrich) at 4°C. This was followed by shaking at 37°C at 200 rpm for 5 h. The resulting pellet was resuspended and cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, USA), 100 µg/mL penicillin and 100 µg/mL streptomycin (Hyclone, USA) at 37°C in a 5% CO_2 incubator. After 5-6 days, the growing-out dermal fibroblasts were harvested

and sub-cultured into culture flasks (Falcon BD, USA). The early passage cells with a population doubling (PD) of 26±2 were used as young cells and treated with 20 µM H_2O_2 for 2 weeks for the development of SIPS. Cells with PD 56±4 were virtually growth arrested and used as senescent cells (normally cultures of PD >50 with no detectable doubling in cell number for two weeks were considered senescent) (Hayflick & Moorhead 1961). For the SIPS model, cells at early passage (PD 26±2) were treated with 20 µM H_2O_2 for 2 weeks in 75 cm² flasks (Falcon BD, USA). Prolonged H_2O_2 exposure was performed by adding 20 µM H_2O_2 with medium change every three days followed by three days of recovery. Treatment was done for two weeks before any further experiments were carried out. Following treatment with H_2O_2, cells were examined and compared to parallel cultured control cells.

CELL MORPHOLOGY AND SENESCENCE ASSOCIATED β GALACTOSIDASE (SA-β-GAL) STAINING

Cell morphology is known to change with H_2O_2 treatment. In this study, cells were treated with H_2O_2 for two weeks followed by three days of recovery parallel to young and senescent cells. They were then examined using an inverted microscope at 200× magnification. Determination of SA-β-gal activity was performed using a Senescent Cells Staining Kit (Sigma, Aldrich) as described by Dimri et al. (1995) based on a histochemical stain for β-galactosidase activity at pH6. Dimri et al. (1995) reported that β-galactosidase activity was detectable in senescent cells and SIPS. Briefly, the cells were washed twice with PBS and fixed in wells with fixation buffer. The plate was then incubated for 7 min at room temperature. This was followed by rinsing the cells three times with PBS before the addition of the staining mixture (Staining solution, Reagent B and C, X-gal solution). The cells were left to stain at 37°C for 4 h. The proportion of SA-β-gal positive cells was determined by counting 100 cells per well under an inverted microscope. The results of cells with positive staining were shown as percentages of the total number of cells counted. SA-β-gal histochemical staining was performed with subconfluent cells to avoid nonspecific staining which may arise from cell confluency.

SAMPLE PREPARATION FOR 2DGE

At the end of recovery following two weeks of H_2O_2 treatment SIPS fibroblast and parallel cultured control cells were trypsinized and centrifuged for 10 min. They were counted using a haemocytometer at a density of 2 × 10⁶ cells. After washing with cold PBS, the resulting pellet was resuspended in 200 µL of lysis buffer (8M urea, 2% dithiothreitol (DTT), 4% 3-[(3-cholamidopropyl) dimethylamonio]-1-propanesulphonate (CHAPS), 2% IPG buffer containing protease inhibitor mix. The cells were then incubated on ice for 30 min and centrifuged at 15 × 10³ g for 30 min at 4°C. The supernatant was collected and transferred to fresh microcentrifuge tubes. Protein

concentration was then determined by Bradford assay (1976).

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2DGE)

Isoelectric focusing (IEF) was initially carried out on immobiline drystrips with a linear pH range of 3–10. Subsequently the IEF runs were repeated using a linear pH range of 4-7. Twenty four centimeter strips were used to obtain the best resolution in 2DGE and to allow larger amounts of sample to be loaded. The strips were rehydrated in 450 μL rehydration buffer (8M urea, 2% 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulphonate (CHAPS), 1% pH 4-7 IPG buffer, and trace amounts of bromophenol blue) overnight. Samples containing an equivalent of 60 μg (analytical gels) of protein were loaded onto sample cups. Isoelectric focusing (IEF) was performed according to the conditions shown in Table 1 (Gork 2004). Following IEF, equilibration was carried out in two steps using 10 mL equilibration buffer (6M urea, 75 mM Tris-HCl, pH 8.8, 29.3% Glycerol, 2% SDS, 0.002% bromophenol blue) with 0.1 g DTT for 15 min (reducing step) and followed by 10 mL equilibration buffer with 0.25 g iodoacetamide for an additional 15 min (alkylation step). Second dimensional gel electrophoresis was then performed on an ETTAN DALT II electrophoresis system (GE Healthcare) on 12.5% SDS slab gels with the IPG strips sealed on the top of the gels using 0.5% agarose. All samples were run in triplicate.

GEL STAINING AND IMAGE ANALYSIS

Analytical gels were stained with silver nitrate as described in the Plus One Silver Staining Kit (GE Healthcare) with modifications. Silver stained gels were scanned using a UMAX scanner (model UTA-2100 XL) and images were stored as TIFF files. Gel images were then analyzed using the 2D Image Master Platinum software Version 6.0 (GE Healthcare). Following background subtraction, protein spots were automatically defined and the volume of each spot in a gel was normalized as a percentage of the total volume of all spots detected on the gel. Within the same experimental conditions, only spots that were consistently different in expression between the different samples (more than 2-fold) were selected as spots of interest in this study.

STATISTICAL ANALYSIS

Statistical analysis of the SA-β-gal assay data between each group (young, SIPS and senescent cells) were performed using one way ANOVA. For 2DGE image analysis, the normalized volume of individual protein spots was used as a basis to compare protein expression between groups (senescent-young, and SIPS-young). Statistical analysis was performed by two-tailed unpaired t-test. A value of $P<0.05$ was considered as statistically significant.

RESULTS

CELL MORPHOLOGY AND SA-B-GAL STAINING

In cellular senescence, normal human diploid fibroblasts (HDFs) at PD 56±4 showed changes in their morphology from a spindle shape to an enlarged, flattened and irregular shape (Figure 1). Human diploid fibroblast cells (HDFs) at PD 26±2 that were exposed to 20 μM H_2O_2 for 2 weeks displayed similar morphological phenotype as senescent cells. The number of blue stained cells (positive for β-galactosidase) at pH 6 was shown to increase significantly in senescence and SIPS. Cells that had undergone senescence at PD 56±2 were found to have a 61.8% increase in β-gal staining while in SIPS cells, an increase of 51.2% was observed.

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2DGE)

2DGE of proteins extracted from young cells, senescent and SIPS produced well resolved, high resolution protein maps with very little inter- and intra-sample variation. Image analysis using the Image Master software showed that a total of ~1000 spots were resolved in silver stained pH 3-10 gels whereas a total of ~1500 were resolved on pH 4-7 gels under similar conditions. Comparison of 2DGE images was carried out – senescent vs young cells and SIPS vs young cells. Representative gels are shown in Figures 2 and 3. In a comparison between young and senescent cells, a total of 28 protein spots were found to be differentially expressed. Twenty-seven protein spots were up-regulated in senescent cells, while a single protein spot was down-regulated. In a comparison between young and SIPS cells, ten protein spots were found to be differentially

TABLE 1. The protocols for running 24cm Immobiline Drystrip gels on Ettan IPGphor Isoelectric Focusing Unit (Gork 2004)

Step	Step voltage mode	Voltage (V)	kVh
1	Step and Hold	500	0.5
2	Gradient	1000	0.8
3	Gradient	8000	13.5
4	Step and Hold	8000	pH 3-10: 30 pH 4-7: 45

expressed in SIPS cells. Three protein spots shared a similar expression profile in both senescent and SIPS cells when compared to young cells (Figure 4).

DISCUSSION

Morphological changes reflect alterations in cellular processes. These processes may include migration, differentiation, apoptosis, necrosis and senescence. Altered
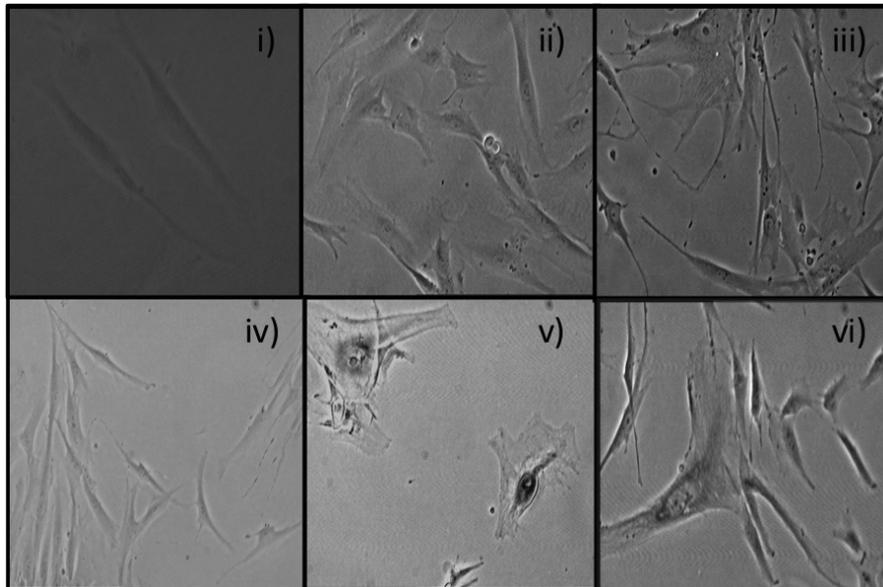
FIGURE 1. Morphological changes from (i & iv) young to (ii & v) SIPS and (iii & vi) senescent cells. Senescent cells and SIPS change their morphology from a spindle shape to an enlarged, flattened and irregular shape. Blue positive β-galactosidase stained cells were observed in both SIPS (v) and senescent cells (vi)
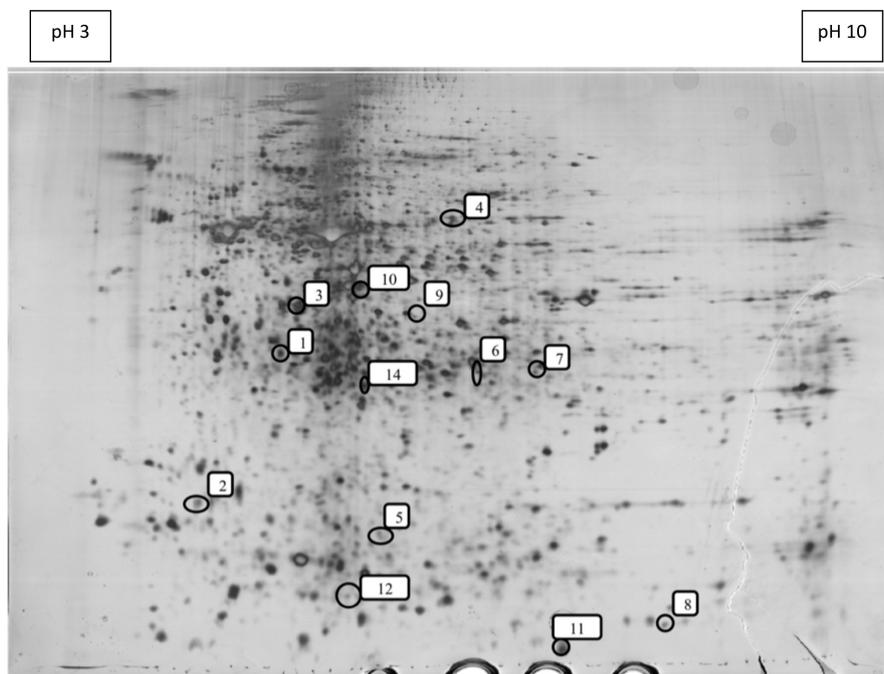
1-7 → protein upregulated in senescent cells as compared to young cells
8-10 → protein upregulated in senescent cells and SIPS as compared to young cells
11-14 → protein upregulated in SIPS as compared to young cells

FIGURE 2. Representative 2-DGE maps of senescent skin fibroblast cells using pH3-10. The boxed labels show the spot number of the proteins of interest in this study for both groups as compared to the young cells
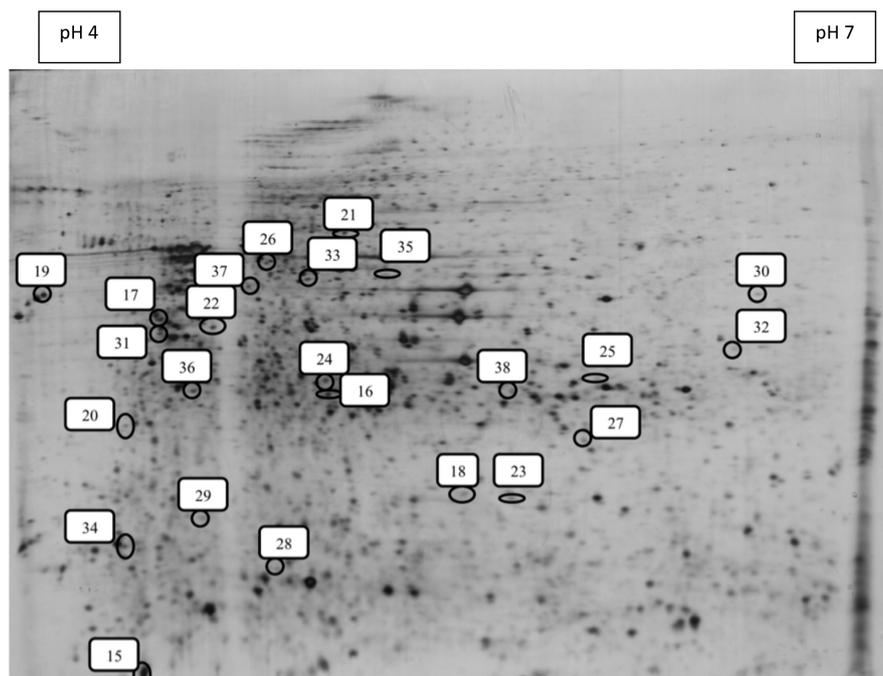
15-31 → protein upregulated in senescent cells as compared to young cells
32 → protein downregulated in senescent cells as compared to young cells
33-38 → protein upregulated in SIPS as compared to young cells

FIGURE 3. Representative 2-DGE maps of senescent skin fibroblast cells using pH4-7.
The boxed labels show the spot number of the proteins of interest in this study
for both groups as compared to the young cellsa

.
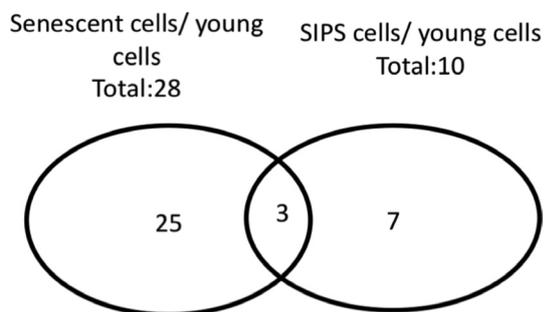
FIGURE 4. Summary of the results of the proteomic analysis in
senescent cells and SIPS cells as compared to young cells

morphology is an important criterion for apoptosis or necrosis (Chen et al. 2000). During cellular senescence, senescent cells and SIPS undergo morphological change from a spindle shape to an enlarged, flattened and irregular shape (Dimri et al. 1995). Toussaint et al. (2000) reported that cells in SIPS display a stable senescent like morphology. This is supported by β-galactosidase expression which is the most extensively utilized biomarker to identify senescent proliferating cells and induced senescence *in vivo* and *in vitro* (Dimri et al. 1995). β-galactosidase activity is detected by X-gal which forms a local blue precipitate upon cleavage, independent of DNA measurements (Dimri et al. 1995). Our results showed that β-Galactosidase

positive cells were significantly increased in senescent and SIPS cells. These results suggest that low doses of H_2O_2 induced SIPS which resemble replicative senescence at the cellular level.

An understanding of alterations in protein expression within senescent cells and SIPS is essential to understand and elucidate underlying mechanisms that correlate with ageing. Senescent cells and SIPS cells were shown to have a number of up-regulated proteins when compared to that of control cells. The theory of stability in equilibrium states that any modifications occurring within biological systems that affect cellular ageing were dependant on a balance between the level of damage and the kinetics of generation of damage and the potential of the cells themselves to respond to the stress (Touissaint et al. 1991; Touissaint et al. 2002). In other words, when stressed, cells will respond by attempting to remove the cause of stress and attempt to repair the damage generated. A change in protein expression may be an indication of the occurrence of this process.

In this study our primary objective was to compare the changes in the expression of proteins in replicative senescence and stress-induced premature senescence (SIPS) fibroblasts by 2DGE analysis. We accomplished protein separation and proceeded to analyze the resulting gel images and compare the resulting protein expression profiles. Of particular interest to us was how similar or different these profiles were under the different stress

conditions. Only a small proportion of the total visible proteome (38 protein spots or ~3%) changed in expression. Image analysis showed that from a total of 38 differentially expressed proteins, 25 were observed only in senescent cells whereas 10 were observed only in SIPS cells. This suggested that levels of protein expression were different between senescent and SIPS cells. These differentially expressed proteins that are unique to the different cells may be considered as molecular scars or proteins that are specific either to senescence or the effects of long term stress (Brack et al. 2000; Dierick et al. 2002. Toussaint et al. 2002). A similar observation has been reported by Dierick and coworkers (2002) who showed 14-3-3 tau to be present in SIPS induced by ethanol or *tert*-butylhydroperoxide but not in replicative senescence whereas creatine kinase BC and HSP27 were present in senescent cells but not in SIPS.

Interestingly, three protein spots were found to be differentially expressed in both senescent and SIPS cells suggesting that the change of expression of certain proteins was common in both senescent and SIPS cells. These differentially expressed proteins will be identified by mass spectrometry to further understand the roles of changes in the expression of proteins in replicative senescence and SIPS.

CONCLUSIONS

We demonstrated that senescent cells and SIPS cells are different at the level of protein expression even though they share similar characteristics such as morphology and β- galactosidase expression. It is interesting to note that these cells showed morphological changes as compared to young cells with only a small change in protein expression (~3%). Identification of these proteins in our future work will shed light onto the complex mechanisms of ageing, thus providing potential targets for intervention.

REFERENCES

Bagnato, C., Thumar, J., Mayya, V., Hwang, S., Zebroski, H., Claffey, K.P., Haudenschild, C., Eng, J.K., Lundgren, D.H. & Han, D.K. 2007. Proteomics analysis of human coronary atherosclerotic plague. A feasibility study of direct tissue proteomics by liquid chromatography and random mass spectrometry. *Molecular and Cellular Proteomics* 6.6. 1088-1102.

Bradford, M.M. 1976. A dye binding assay for protein. *Analytical Biochemistry* 72: 248-254.

Boraldi, F., Bini, L., Liberatori, S., Armini, A., Pallini, V., Tiozzo, R., Pasqualironchetti, I. & Quaglino, D. 2003. Proteome analysis of dermal fibroblasts cultured in vitro from human healthy subjects of different ages. *Proteomics* 3: 917-929.

Borlon, C., Debacq-Chainiaux, F., Hinrichs C., Scharffetter-Kochanek, K., Toussaint, O. & Wlaschek, M. 2007. The gene expression profile of psoralen plus UVA-induced premature senescence in skin fibroblasts resembles a combined DNA-damage and stress-induced cellular senescence response phenotype. *Experimental Gerontology* 42: 911-923.

Brack, C, Lithgow G.J., Osiewacs, H.D. & Toissaint, O. 2000. Molecular & Cellular Gerontology. *EMBO Journal* 19: 1929-1934.

Chen, Q.M., Tu, V.C. & Liu, J. 2000. Measurements of hydrogen peroxide induced premature senescence: senescence-associated β galactosidase and DNA synthesis index in human diploid fibroblasts with down-regulated p53 or Rb. *Biogerontology* 1: 335-339.

Deaton, C.M. & Marlin, D.J. 2003. Exercise-associated oxidative stress. *Clinical Techniquesin Equine Practice* 2(3): 278-291.

de Magalhaes, J.P., Chainiaux, F., de Longueville, F., Mainfroid, V., Migeot, V., Marcq, L., Remacle, J., Salmon, M. & Toussaint, O. 2004. Gene expression and regulation in H_2O_2-induced premature senescence of human foreskin fibroblasts expressing or not telomerase. *Experimental Gerontology* 39: 1379-1389.

Dierick, J.F., Eliaters, F., Remacle, J., Raes, M., Fey, S.J., Larsen, P.M. & Toussaint, O. 2002a. Stress-induced premature senescence and replicative senescence are different phenotypes, proteomic evidence. *Biochemical Pharmacology* 64: 1011-1017.

Dierick, J.F., Kalume, D.E., Wenders, F., Salmon, M., Dieu, M., Ress, M., Roepstorff, P. & Toissaint, O. 2002b. Identification of 30 protein species involved in replicative senescence and stress-induced premature senescence. *FEBS Letters* 531: 499-504.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M. & Campisi, J. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences USA* 92: 9363-9367.

Duan, J., Duan, J., Zhang, Z. & Tong, T. 2005. Irreversible cellular senescence induced by prolonged exposure to hydrogen peroxide involves DNA-damage-and-repair genes and telomere shortening. *The International Journal of Biochemistry & Cell Biology* 37: 1407-1420.

Frippiat, C., Remacle, J. & Toussaint, O. 2003. Down-regulation and decreased activity of cyclin-dependent kinase 2 in H_2O_2–induced premature senescence. *The International Journal of Biochemistry & Cell Biology* 35: 246-254.

Gork, A. 2004. *2-D Electrophoresis. Principles and Methods.* Handbooks from GE Healthcare. Sweden

Grune, T., Reinheckel, T. & Davies, K.J. 2003. Degradation of oxidized proteins in mammalian cells. *The Federation of American Societies for Experimental Biology Journal* 11: 526-534.

Hayflick, L. & Moorhead, P.S. 1961. The serial cultivation of human diploid cell strains. *Experimental Cell Research* 25: 585-621

Harman, D. 1956. Aging: A theory based on free radical and radiation chemistry. *The Journal of Gerontology* 11: 298-300.

Puricelli, L., Iori, E., Milioni, R., Arrigoni, G., James, P., Vedoyato, M. & Tessari, P. 2006. Proteome analysis of cultured fibroblasts from type 1 diabetic patients and

normal subjects. *The Journal of Clinical Endocrinology & Metabolism* 91(9): 3507-3514.

Toussaint, O., Medrano, E.E. & von Zglinicki, T. 2000. Stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Experimental Gerontology* 35: 927-945.

Toussaint, O., Raed, M. & Remackle, J. 1999. Ageing as a multi-step process characterized by a lowering of entropy production leading the cell to a sequence of defined stages. *Mechanisms of Ageing and Development* 61: 45-54.

Toussaint, O., Remacle, J., Dierick, J.F., Pascal, T., Frippiat, C., Royer, V. & Chainiaux, F. 2002. Approach of evolutionary theories of ageing, stress, senescence-like phenotypes, caloric restriction and hormesis from the view point of far-equilibrium thermodynamics. *Mechanisms of Ageing and Development* 123: 937-946.

Zhang, J., Keene, C.D., Pan, C., Montine, S.K. & Montine, T.J. 2008. Proteomics of human neurodegenerative diseases. *Journal of Neuropathology & Experimental Neurology* 67(10): 923-932.

Goon Jo Aan*, Haryati Ahmad Hairi, Suzana Makpol & Wan Zurinah Wan Ngah
Jabatan Biokimia
Fakulti Perubatan
Universiti Kebangsaan Malaysia
50300 Kuala Lumpur, Malaysia

Mariati Abdul Rahman
Jabatan Biologi Mulut Klinikal
Fakulti Pergigian
Universiti Kebangsaan Malaysia
50300 Kuala Lumpur, Malaysia

Saiful Anuar Karsani
Institut Sains Biologi
Fakulti Sains
50603 Universiti Malaya
Kuala Lumpur, Malaysia

*Corresponding author; email: joaan@medic.ukm.my