

Cell Senescence *In Vitro*

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Introductory article

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Cell senescence *in vitro* refers to the multitude of physiological, structural, biochemical and molecular changes that occur progressively during serial subcultivation of normal diploid cells, culminating in the permanent cessation of cell division. The whole duration of serial passaging is considered as the process of cellular ageing, and the end-stage irreversible growth arrest is termed as replicative senescence. The limited proliferative capacity of normal, diploid and differentiated cells is also known as the Hayflick limit, and the overall phenomenon is called the Hayflick phenomenon. A progressive loss of global deoxyribonucleic acid (DNA) methylation and of telomeres is considered to be the critical determinants of the Hayflick limit. However, *in vivo*, it is not the absolute number of senescent cells which is responsible for age-related impairments, but it is the drift in cell functions occurring during repeated cell division that is relevant to ageing and age-related diseases. Cell senescence in the body is also considered to be a regulatory mechanism against cancer.

Introduction

In a wide variety of multicellular organisms, including human beings, several cell types retain the capacity to divide during embryonic, postnatal and adult life. These proliferative cell populations are required to divide frequently (e.g. in the skin and the gut) or infrequently (e.g. during the immune response and in the endothelium) in carrying out various functions of the body. These functions include the immune response, blood formation, bone formation, epidermal turnover, gut renewal, and repair and regeneration of tissues. Endothelial cells, epithelial cells, epidermal basal cells, fibroblasts, glial cells, lymphocytes, myoblasts and osteoblasts constitute major differentiated

and proliferating cell types of an organism, and are distinct from the pluripotent stem cells. Not only are their differentiated and specialised functions critical for the organism, but also their capacity to divide is an integral part of their role in organismic growth, development, maintenance and survival. The study of age-related changes in the physiology, biochemistry and molecular biology of isolated cell populations has greatly expanded our understanding of some of the fundamental aspects of ageing. **See also:** [Ageing; Cell Cycle](#)

In modern biogerontology, the terms ‘cellular ageing’, ‘cell senescence’ or ‘replicative senescence’ imply the study of normal diploid cells in culture, which – upon serial subcultivation – progressively undergo a multitude of changes culminating in the cessation of cell division. This process of intrinsic and progressive cellular ageing *in vitro* is generally known as the Hayflick phenomenon, and the limited division potential of normal cells is called the Hayflick limit, in recognition of the observations first reported by Leonard Hayflick in 1961 (Hayflick and Moorhead, 1961; Hayflick, 1965, 2000). In more recent times, the term ‘replicative senescence’ has been used more frequently, and it underlines the ultimate senescent phenotype of serially subcultivated cells as irreversibly growth-arrested cells (Cristofalo *et al.*, 2004; Campisi and d’Adda di Fagagna, 2007).

Serial Subcultivation *In Vitro*

Although normal diploid fibroblasts of mesodermal origin have been the most frequently used cells for studies on cellular ageing *in vitro*, a variety of other cell types including epithelial cells, endothelial cells, keratinocytes, melanocytes, glial cells, lymphocytes, osteoblasts, bone marrow cells, chondrocytes, articular cartilage cells and muscle satellite cells have also been used. Once the primary culture of normal cells is established in culture from the normal tissue by way of any of the standard methods, such as the explant growth and enzymic dissociation of cells, the primary culture can then be subcultivated as a cell strain repeatedly at 1:2, 1:4 or 1:8 ratio, generally after reaching confluence as a monolayer. Although the exact culturing conditions (such as the type of the culture medium, the source of growth factors, the use of antibiotics and the incubation temperature, humidity and gaseous

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composition) may vary for different cell types, serial subcultivation (also known as serial passaging) of normal diploid cells can be performed only a limited number of times. This is in contrast to the high proliferative capacity of transformed, cancerous and immortalised cells, whose cultures can be subcultivated and maintained indefinitely.

See also: [Primary Cell Cultures and Immortal Cell Lines](#)

The cumulative number of cell proliferations, represented as population doublings (PD), is estimated by determining the number of times a population of cells undergoes doubling between each subculturing. The cumulative PD level (CPDL), which can be achieved by a specific cell type during serial passaging *in vitro*, depends upon several biological factors. These factors include the maximum lifespan of the species, developmental and adult age of the donor of the tissue biopsy, the site of the biopsy and the health status of the donor (Cristofalo *et al.*, 2004; Macieira-Coelho, 2011). For example, for human fibroblasts the range of CPDL for

the cell strains originating from embryonic tissues is between 50 and 70, whereas for those originating from adult biopsies it is generally less than 50 CPDL. Additionally, gaseous composition, especially oxygen levels, and the quality of the nutritional serum added to the culture medium, can significantly affect the proliferative lifespan of cells *in vitro*. It is generally believed that culturing of cells *in vitro* in the air with approximately 20% oxygen levels significantly reduces their lifespan, which could be otherwise achieved at low level (2%) concentration akin to *in vivo* conditions (Wright and Shay, 2002).

The Senescent Phenotype

Serial subcultivation of normal cells is accompanied by a progressive accumulation of a wide variety of changes before the final cessation of cell replication occurs. The

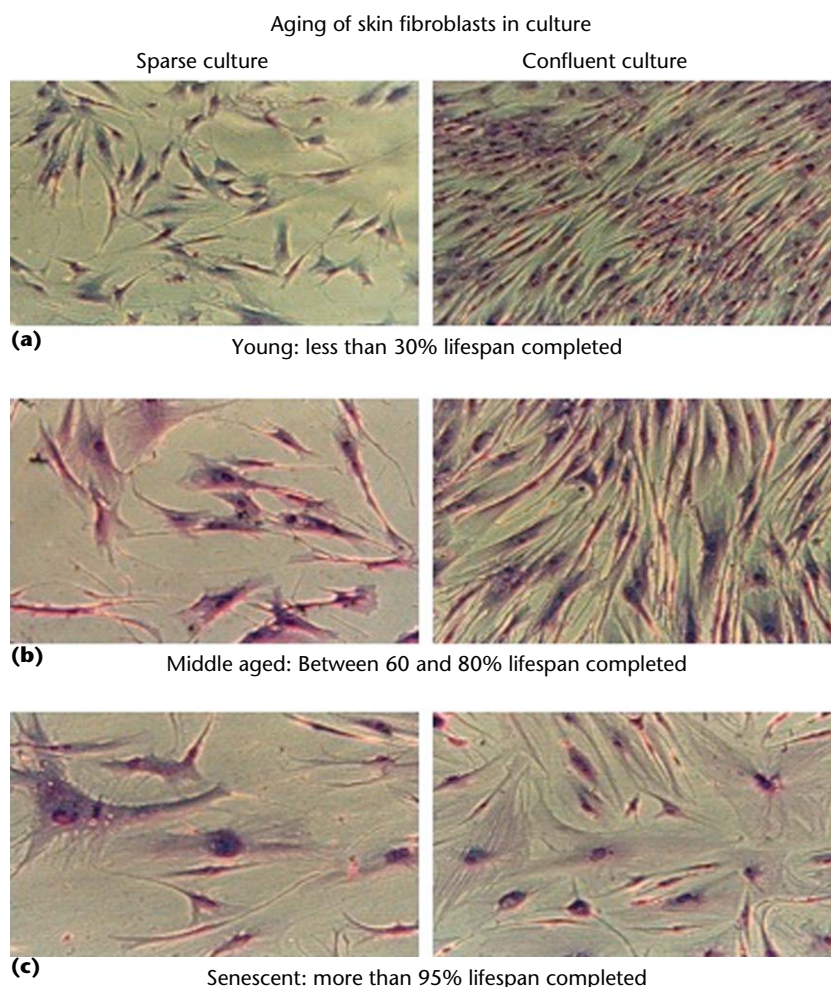


Figure 1 Cellular ageing *in vitro*. Giemsa-stained light microscopic phase-contrast pictures of serially passaged human skin fibroblasts at various points in their *in vitro* lifespan. Sparse and confluent cultures at three stages during replicative lifespan are compared: (a) early passage young adult skin fibroblasts with less than 30% lifespan completed; (b) middle-aged cells with 60–80% replicative lifespan completed; and (c) late passage senescent cells with more than 95% lifespan completed. Reproduced with Rattou SIS (2010). Aging of skin cells in culture, In: M.A. Farage, K.W. Miller, H.I. Maibach (eds), *Textbook of Aging Skin*, DOI: 10.1007/978-3-540-89656-2_50. Springer Verlag: Berlin Heidelberg 2010, with kind permission from Springer Science & Business Media.

progressively emerging senescent phenotype of serially passaged normal diploid cells can be categorised into the structural, physiological and biochemical and molecular phenotypes, which can be used as biomarkers of cellular ageing *in vitro*.

- **Structural phenotype** – characterised by increase in cell size; change of shape from thin, long and spindle-like to flattened and irregular; loss of whorl-like spiral arrangement in parallel arrays on the cell culture substrate; increased number of vacuoles and dense lysosomal autophagous bodies containing ultraviolet (UV)-fluorescent age-pigments such as lipofuscin; polymerised actin filaments and disorganised microtubules in the cytoskeleton; mitochondrial distortions, and increased level of chromosomal aberrations, chromatin condensation, nucleolar fragmentation and multinucleation (Holliday, 1995; Macieira-Coelho, 1998) (see, **Figure 1**). **See also:** [Cytoskeleton](#)
- **Physiological phenotype** – characterised by reduced response to growth factors and other mitogens; increased sensitivity to toxins, drugs, irradiation and other stresses; altered calcium flux, pH, viscosity and membrane potential; reduced activity of ionic pumps; reduced mobility; reduced respiration and energy production and increased duration of G₁ phase of the cell cycle. **See also:** [Checkpoints in the Cell Cycle](#)
- **Biochemical and molecular phenotype** – characterised by altered activity, specificity and fidelity of numerous enzymes; accumulation of stochastically damaged macromolecules deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and protein–lipid conjugates leading to increased molecular heterogeneity; increased levels of post-translationally modified and inactivated proteins; reduced rates of protein synthesis and degradation; reduced levels of methylated cytosines; reduced length of telomeres and altered (increased or decreased) expression of thousands of genes (Macieira-Coelho, 2011; Rattan, 2006, 2008a). **See also:** [DNA Methylation in Development](#); [Telomere](#)

In addition to the above age-related changes that occur progressively during serial subcultivation of normal cells, the ultimate senescent phenotype is the permanent growth arrest in late G₁ phase of the cell cycle near the S phase boundary. This phenotype is accompanied and maintained by an increased expression of several so-called senescence-specific genes for ageing, which are considered to be critical for cell cycle regulation and tumour suppression (Cristofalo *et al.*, 2004; Campisi and d'Adda di Fagagna, 2007; Kaul *et al.*, 2007; Passos *et al.*, 2010). **See also:** [Cell Cycle: Regulation by Cyclins](#)

Modulators of Cellular Senescence *In Vitro*

The Hayflick system of cellular ageing *in vitro* is primarily a model for the study of slow and progressive accumulation

of damage resulting in the arrest of cells in a non-proliferative state followed by cellular degradation and death. Several physical, chemical and biological modulators have been tested for understanding various aspects of this phenomenon of cellular ageing *in vitro* and their implications in the origin of diseases, such as cancer (Campisi and d'Adda di Fagagna, 2007; Cheung *et al.*, 2010). For example, irradiation, severe oxidative stress and transfection with various genes have been used to cause a sudden and rapid increase in molecular damage, resulting in premature induction of the ultimate senescent phenotype (Toussaint *et al.*, 2000; Sejersen and Rattan, 2009). However, insertion of catalytically active component of the telomerase gene can completely bypass the Hayflick limit in many cell types, and such cells can proliferate indefinitely with or without becoming transformed (Simonsen *et al.*, 2002). Similarly, normal diploid cells can be transformed and immortalised by chemical carcinogens, irradiation and viral genes. Such approaches are helpful for unravelling the molecular details of cell cycle regulation in normal cells and its dysregulation in cancer cells (Kaul *et al.*, 2007; Rondo, 2006; Collado *et al.*, 2007). **See also:** [Telomeres in Cell Function: Cancer and Ageing](#)

The Hayflick system of cellular ageing *in vitro* has also been very useful for testing various natural and synthetic molecules and treatments that slow down the accumulation of damage and delay the onset of various age-related changes. Several growth factors including cytokinins kinetin and zeatin, a dipeptide carnosine and other extracts from various medicinal plants and herbs have been tested and discovered to have antiageing effects (Barciszewski *et al.*, 2007; Rattan and Clark, 1994; McFarland and Holliday, 1994; Rattan and Sodagam, 2005). Several of these tests have resulted in the development, production and marketing of various products with pharmaceutical, cosmeceutical and nutritional applications. The model system of cellular ageing *in vitro* has also been used extensively to test the applicability of hormesis (the phenomenon of attaining beneficial biological effects by repeated exposure to low level stress, such as heat shock, irradiation and pro-oxidants) in ageing research and interventions. For example, human skin fibroblasts and keratinocytes exposed to repeated mild heat stress (41°C, 1 h, twice a week) have more active protein degradation pathways, higher levels of chaperones, increased resistance to other stresses and slightly increased proliferative lifespan *in vitro* (Rattan, 2008b; Rattan *et al.*, 2009).

From Cellular Ageing *In Vitro* to Understanding Ageing *In Vivo*

A loss of proliferative capacity of any of the cell types has a deteriorative impact on the functioning and survival of the entire organism. A loss or slowing-down of proliferation of osteoblasts, glial cells, myoblasts, epithelial cells, lymphocytes and fibroblasts can lead to the onset of many

age-related diseases and impairments including osteoporosis, arthritis, immune deficiency, altered drug clearance, delayed wound healing and altered functioning of the brain. However, the presence of a significant number of senescent cells in various tissues and organs in the body during normal ageing is yet to be fully documented, and the commonly used senescent markers, such as the senescence-specific beta-galactosidase and DNA damage foci, have serious limitations and artifacts (Macieira-Coelho, 2011, 2010; Yang and Hu, 2005). Furthermore, in the body, it is not the absolute number of senescent cells that is responsible for the age-related impairments; rather it is the drift in cell functions occurring during repeated cell division that is relevant to ageing and age-related diseases (Macieira-Coelho, 2011). Increased cellular heterogeneity in terms of the occurrence of fully senescent or near-senescent cells among actively proliferating cells *in vivo* can promote dysfunctioning of the other tissues by producing harmful signals, and can also promote and stimulate the growth of other precancerous and cancerous cells.

The Hayflick system of cellular ageing *in vitro* has proved to be very useful in developing the cellular and molecular understanding of the overall process of ageing, which is characterised by a progressive accumulation of macromolecular damage. The resulting increase in molecular heterogeneity leads to interrupted molecular networks and illegitimate molecular interactions, which are the ultimate cause of ageing and age-associated diseases.

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