Alterations of gene expression during *in vitro* ageing: The mammalian fibroblast model

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Ageing is a complex biological phenomenon of immense importance. As all ageing changes have a cellular basis, the phenomenon is best studied at cellular level under defined and controlled environmental conditions. Molecular biology techniques have been applied to cultured mammalian fibroblasts to evaluate the leading hypotheses. The study of the difference in signalling pathways between young and senescent cells has given evidence of the involvement of several known genes, including oncogenes, oncosuppressor genes, cell cycle control genes and their inhibitors, during the *in vitro* ageing process. Furthermore, applications of different cloning approaches have resulted in the identification of several additional genes that are associated with cellular ageing such as fibronectin, osteonectin, aI-procollagen, Apo J, SM22, prohibitin, SAG, vimentin, and *mot-1* genes. The results of these *in vitro* studies may advance our understanding of *in vivo* ageing and focus future research efforts. The ultimate target is to provide the scientific foundation to enhance the quality of life for people suffering from deteriorating ageing process.

Cellular senescence

Cellular senescence is a term that describes the physiological processes that a cell culture undergoes when it reaches the end of its proliferative life span. The ageing of a whole organism is by far a more complex and sophisticated process that involves biological changes in many organ systems that lead to the eventual decline of the organism. Since the study of ageing of an organism is prohibitively complex, models of *in vitro* ageing systems have been established as tools to investigate the genetic and physiological changes that occur at the cellular levels. *In vitro* models of cellular senescence have been developed for a variety of cell types, including fibroblasts[1], endothelial cells[2], keratinocytes[3] and lymphocytes[4].

Normal diploid fibroblasts during serial subculturing undergo a period of rapid proliferation followed by a progressive loss of replicative capacity, manifested as slower population growth rates. The cell population in culture finally reaches a state of growth arrest where all cells fail to divide. During this period, cells undergo a multitude of age-related changes culminating in the complete inhibition of cellular proliferation[5]. Senescent cells may be maintained in culture for a prolonged period of time, they become bigger, more flattened and irregular in shape, their cytoskeletal network is organized differently and the constitution of their cell membrane is changed[6–9].

Difference in expression of genes in signalling pathways between proliferating and senescent cells

One approach used to study senescence is to understand the differences in expression of genes involved in different signalling pathways between proliferating and senescent cells. The expression of genes, such as histone[10] and insulin-like growth factor-binding protein-3 (ref. 11) is higher in senescent cells than in their proliferating cell counterparts. In addition, in some cellular systems (human fibroblasts, rat brain), the genes encoding for ornithine decarboxylase[10], *c-myc*, *H-ras* and thymidine kinase[12] are still mitogen-responsive in stimulated senescent cells, while *c-fos* gene is downregulated[13]. The loss of *c-fos* gene expression in these senescent cells in turn leads to loss of *fos-jun* heterodimers, a complex of the AP-1 transcription factor family. In contrast, in senescent rat embryonic fibroblasts serum stimulation leads to *c-fos* expression but not to *c-myc*[14]. These data demonstrate that the putative role of these genes in cellular senescence is likely to be species and perhaps tissue specific.

In addition to the mentioned changes of gene expression, detailed studies of the mechanisms of cell cycle progression have produced additional avenues of investigation for potential mediators of cellular senescence (Table 1). The retinoblastoma (RB) protein is a tumour supressor gene product, that is lost in a variety of human tumours[15]. The activity of RB proteins is regulated by phosphorylation which varies through the cell cycle. RB is active in its underphosphorylated form during the

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Table 1. Regulation of cell cycle components in senescent and proliferating cells

Cell cycle regulatory components	Proliferating cells	Senescent cells
c-fos mRNA[13,14,38]	Increases Go/G1 transition	varies in different species/tissues
c-myc mRNA[12,14]	Increases early G1	varies in different species/tissues
cdk2 mRNA[39]	Expressed	Not expressed
Cyclin A mRNA[40,41]	Cell cycle regulated	Not expressed
Cyclin C, D, E mRNA[40]	Cell cycle regulated	Expressed
E2F-1 mRNA[42]	Increases through G1	Present at low level
p21 mRNA[14,24]	Expressed	Elevated several fold
RB protein[44]	Phosphorylated at G1/S	Underphosphorylated
Cyclin A, B proteins[39]	Cell cycle regulated	Down-regulated
MAP kinase[40]	Expressed	Expressed
AP-1 (ref. 45)	Expressed	Expressed
p53 (ref. 40)	Expressed	Expressed

early and mid-G1 phase of the cell cycle, where it blocks cellular proliferation[16]. Phosphorylation of RB by agents such as cyclins of the D class (D1, D2, and D3) or cyclins E results in its functional inactivation. Other agents such as TGF-β, or cAMP, in turn, block RB phosphorylation by modulating the activities of the *cdks* that are responsible for RB phosphorylation, thereby preventing the cell's advance into late G1 (ref. 17). Interestingly, senescent cells fail to phosphorylate Rb in response to mitogens[18]. The underphosphorylated form of RB associates with members of the E2F transcription factor family (E2F-1, E2F-2 and E2F-3) as a complex with cyclins and *cdks*. E2F binding sites are found in the promoter elements of several cell growth regulated genes such as *c-myc*, *N-myc*, *c-myb*, cdc2, thymidine kinase, DHFR, EGF receptor and E2F-1 gene itself[19]. It is likely that RB regulates the transcription of cell growth regulated genes by sequestering E2F. Therefore, it is believed that RB function in the G1 phase of the cell cycle is to negatively regulate growth, and that RB phosphorylation inactivates these negative functions[20].

An additional class of cell cycle regulatory molecules have been discovered that are known as *cdk*-inhibitors. Harper and co-workers[21] while screening for proteins that bind to *cdk*-cyclin complexes found a small 21 kDa protein that inhibits the kinase activity of these complexes. The p21 gene is a universal inhibitor of cyclin-dependent kinases[22] and a PCNA inhibitor[23]. Interestingly, the p21 gene has also been found overexpressed in senescent fibroblasts[24], while its expression correlates with terminal differentiation in several lineages[25]. p21 is regulated by p53 (ref. 26), but can also be induced by other factors such as TGF-β (ref. 27).

Molecular markers of *in vitro* ageing

Many different cloning approaches have been applied to identify the genes involved in the regulation of cellular ageing. Several genes have been cloned that include: prohibitin, that has been shown to block DNA synthesis in proliferating normal fibroblasts[28], vimentin that has been found overexpressed in senescent human diploid fibroblasts[29], SAG a marker of senescence in human diploid fibroblasts[30] and *mot*-1, which is capable of inducing cellular senescence in NIH 3T3 cells[31]. In addition, Goldstein and his colleagues isolated 18 different cDNA clones encoding predominantly extracellular matrix proteins and repetitive sequences, which were found over-expressed in Werner's Syndrome fibroblasts, as well as in senescent human diploid fibroblasts[32,33], while Linskens and his colleagues identified several additional senescence-specific genes in human diploid fibroblasts[34]. Finally, to isolate the genes that regulate cellular ageing, we have employed a clonal system of conditional SV40 T antigen rat embryo fibroblast cell lines which undergo senescence upon T antigen inactivation[14]. Construction of cDNA libraries from two conditional cell lines and application of differential screening and subtractive hybridization techniques have resulted in the cloning of eight genes (SGP-2/Apo J, fibronectin, α-procollagen, SM22, osteonectin, GTP-α, cytochrome C oxidase and a novel gene) which were found over-expressed in the growth arrested conditional cells, as well as in rat and human fibroblasts undergoing ageing *in vitro*[35]. However, recent experiments in human fibroblasts from several donors of different ages in our laboratory indicate that the expression of some but not all of the above senescence-induced genes is associated with ageing *in vivo* (Petropoulou & Gonos, unpublished data). Therefore, the relationship between *in vitro* data and *in vivo* ageing itself remains to be demonstrated, as well as whether the observed changes in the expression of genes cause cellular ageing or, whether the change in expression is a consequence of *in vitro* ageing.

Conclusions

Maximum life span varies between and within species and it is likely that there are particular genes which are involved in the ageing process. An insight into the nature of these genes is provided by evolutionary studies such as the antagonistic pleiotropy model[36], which postulated that the ageing-facilitating genes have advantageous effects in the organism during the early phase of its life span (particularly during reproduction) and deleterious effects during later phases of the life span. The disposable soma theory[37] has formalized and developed this concept. Ageing is perceived as a by-product of the optimization by natural selection for the repro-

ductive success of the organism and this is under genetic control. Although this model appears to satisfy the evolutionary paradox of how genes can evolve that cause deleterious effect on an organism, the precise biochemical pathways which control the underlying partitioning of resources is unclear. Further research in this field will help not only to understand the mechanism of cellular ageing, but also to produce the future target therapeutics molecules for age-related diseases.

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