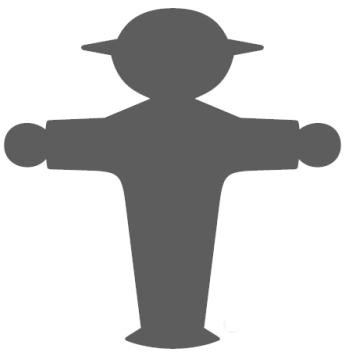
Chapter 8

General discussion



General discussion

This thesis aimed to investigate the cellular processes responsible for differences in human longevity. We studied *in vitro* stress responses of dermal fibroblast strains derived from offspring of nonagenarian siblings with the propensity for longevity and compared them with the responses of fibroblast strains derived from the partners of the offspring, representing the general population. As a proof-of-principle we also compared fibroblast strains from chronologically young and old subjects, hypothesizing that differences between offspring and partners should be similar, at least in terms of direction, to differences between fibroblast strains from chronologically young and old subjects.

Main findings

In **chapter 2** we describe the optimization of a flow cytometric method described earlier (1) to better differentiate between populations of fibroblasts in degrees of SA-β-gal activity. SA-β-gal activity is a widely used marker for cellular senenescence and is routinely detected cytochemically, manually discriminating negative from positive cells (2;3). This method is time-consuming, subjective and therefore prone to operator-error. Skin fibroblasts were isolated from young and very old participants of the Leiden 85-plus Study (4;5). To induce stress-induced senescence, fibroblasts were exposed to rotenone and senescence was assessed measuring SA-β-gal activity by cytochemistry and by flow cytometry. Under non-stressed conditions, fibroblasts from old subjects showed higher SA-β-gal activity than fibroblasts from young subjects and this difference was found for both the flow cytometric and cytochemical methods. However, under stress-induced conditions the flow cytometric method but not the cytochemical method revealed significantly higher SA-β-gal activity in fibroblasts from very old compared with young subjects. We concluded that the modified flow cytometric method for measuring SA-β-gal activity is superior in discriminating between degrees of senescence in different populations of fibroblasts.

Chapter 3 describes the cellular responses to stress in skin fibroblasts that were isolated from young and old participants of the Leiden 85-plus Study (4;5). These responses were compared with the responses of isolated fibroblast strains from participants of the Leiden Longevity Study, offspring of nonagenarian siblings, and their partners, representatives of the general population (6). Under non-stressed conditions SA-β-gal activity was lower and levels of apoptosis/cell death were higher in fibroblasts from young subjects when compared with fibroblasts from old subjects, as were stress-induced increases. Numbers and total size of colonies under non-stressed conditions were higher for fibroblasts from young subjects. Under non-stressed conditions there were no differences in levels of SA-β-gal activity and apoptosis/cell death between fibroblasts from offspring and partners. Stress-induced increases of SA-β-gal activity were smaller and levels of apoptosis/cell death higher in fibroblast strains of offspring when compared with strains of partners. Numbers and total size of colonies under non-stressed conditions were higher for fibroblasts from offspring whereas rotenone-induced decreases were lower. These results suggest that under stressed conditions, fibroblast strains from offspring resemble fibroblast strains from chronologically young subjects and provide support for the hypothesis that *in vitro* cellular responses to stress reflect the propensity for human longevity.

Cellular senescence, an important factor in aging phenotypes, can be induced by replicative exhaustion or by stress. **Chapter 4** reports on the relation between maximum replicative capacity, telomere length, stress-induced cellular senescence and apoptosis/cell death in human primary fibroblast strains obtained from nonagenarians of the Leiden 85-plus Study. Fibroblast strains cultured until replicative senescence (5) were stressed with rotenone at low passage. Fibroblast strains with a higher replicative capacity had longer telomeres. In non-stressed conditions replicative capacity was not associated with SA-β-gal activity and negatively associated with cell death. In rotenone-stressed conditions replicative capacity was negatively associated with senescence and positively associated with cell death. These data indicate that fibroblast strains with a higher maximum replicative capacity have longer telomeres, are less prone to go into stress-induced cellular senescence and more prone to die after stress.

The free radical theory of aging states that function of the respiratory chain becomes less efficient with age (7). The concomitant increased levels of ROS damage proteins and DNA. To model this process *in vitro*, we chronically exposed fibroblasts to a low dose of the mitochondrial complex I inhibitor rotenone. In **chapter 5** we describe the different responses between fibroblast strains from young and old subjects, and between offspring of nonagenarian siblings and their partners. Under non-stressed conditions, fibroblast strains from young subjects showed lower ROS levels and a higher growth speed when compared with old subjects, but telomere length and shortening rate were not different. Significantly increased ROS levels were observed after chronic exposure of fibroblasts to rotenone, whereas growth speed was inhibited and telomeres had shortened. Under stressed conditions, lower ROS levels and a larger decrease in growth speed were found for fibroblast strains from young subjects when compared with strains from old subjects. However, no differences in telomere shortening rate were observed. The stress-induced decrease in growth speed was larger in strains from offspring of nonagenarian siblings when compared with their age matched partners from the general population. Summarizing, fibroblast strains from young and old people are differentially affected by chronic inhibition of the respiratory chain and responses in fibroblast from offspring of nonagenarian siblings resemble the responses of strains from young subjects.

Not much is known about the complex interplay between upstream and downstream pathways leading to senescence. In **chapter** 6 we investigated the signaling pathways responsible for the different *in vitro* phenotypes between fibroblast strains from young and old subjects. We determined whole genome gene expression of non-stressed and rotenone-stressed human fibroblast strains from young and old subjects and identified gene sets involved in cell cycle, mitochondria and transcription. The gene whose expression differed most between fibroblast strains from young and old subjects was the cell cycle inhibitor p16, being higher in expression in fibroblast strains from old subjects. p16 is regarded as a robust marker for cellular aging (8) and increasing numbers of p16-positive cells can be found in mitotic aging of aging primates (9;10). Furthermore, p16 plays an important role in senescence (11). Unexpectedly p16 mRNA decreased after rotenone. To verify these results, we performed independent experiments and p16 was measured at both the mRNA level and

the protein level. Rotenone again resulted in decreased p16 mRNA expression, but this time p16 mRNA expression was lower in fibroblast strains from old subjects. This discrepancy might be explained by differences in seeding density and/or differences in incident diseases in the two different samples used for the microarray and replication experiments. No rotenone-induced changes or young-old differences in protein expression could be measured by Western blotting, whereas by immunocytochemistry fibroblast strains from old subjects showed higher percentages of p16-positive cells in both non-stressed and rotenone-stressed conditions.

When compared with the general population, nonagenarian siblings and their offspring showed a lower risk of diabetes mellitus (12), better insulin sensitivity and glucose handling (13;14) and a different lipid metabolism (15), indicating differences in metabolism in general. In **chapter 7** we investigated if fibroblast strains from offspring and partners also show differences in cellular metabolism *in vitro*. Ala-gln and glucose consumption were higher for fibroblast strains derived from offspring, when compared with fibroblast strains from their partners. Production of glutamine, alanine, lactate and pyroglutamic acid was found to be higher for fibroblast strains derived from offspring, when compared with fibroblast strains from their partners. In conclusion, the metabolic profiles of human fibroblasts *in vitro* reflect differences between offspring from families with the propensity for longevity when compared with fibroblasts from their partners.

Methodological issues

All the results described in this thesis were derived from *in vitro* experiments. As already alluded to in the introduction, translation of *in vitro* results to the *in vivo* situation requires much cautiousness (16). An important strength of our study is the fact that we can directly link the *in vitro* data to the *in vivo* data of the subjects the fibroblast strains were derived from. However, cells *in vitro* have been taken out of their natural context and are thus likely to display a very different cellular phenotype, making it difficult to interpret the relevance of *in vitro* results for the *in vivo* situation. Depending on the proliferative state of cells, they will be more or less responsive to stimuli and especially dermal fibroblasts are not as proliferative *in vivo* as they are *in vitro*.

Results can also be different depending on culture conditions. All cell types in the body experience oxygen tensions much lower than the oxygen tension of ambient air (17) under which cells are usually cultured. It is likely that ambient oxygen tension actually stresses cells and this background stress might make it difficult to measure stress responses induced by an experimental stressor (rotenone in our case). Cells can be handled and cultured under low oxygen tension conditions but the equipment necessary to do this is very costly and this is not feasible for many laboratories. Other culture conditions that will affect the results are the presence or absence of factors in the medium (e.g. growth factors, cytokines etc.), mostly derived from the foetal calf serum added to the medium but also often added separately. Different batches of foetal calf serum can yield different results so it is of paramount importance that when experiments are replicated, the same batch of serum is used and one batch of foetal calf serum will only allow for a limited number of experiments. We used the same batches of serum for our experiments, but the serum that was used for the Leiden 85-plus Study fibroblast strains was different than the batch used for the Leiden Longevity Study fibroblast strains, making one-on-one comparisons impossible. When comparing the results of the fibroblast strains of these studies, we could only look at the direction of differences. Furthermore, the sizes of the biopsies the fibroblasts were isolated from were different for the Leiden 85-plus Study (3 mm) and the Leiden Longevity Study (4 mm).

Another factor which should be taken into account when interpreting *in vitro* data is the cell type. The dermal fibroblast has been a popular *in vitro* model since this cell type is easily accessible and grows readily *in vitro*, providing ample (yet finite) experimental material. However, results generated with this model are not necessarily valid for other cell types. In the experiments described in this thesis we aimed to oxidatively stress the fibroblasts. One of the most commonly used oxidative stressors is hydrogen peroxide, but due to its reactive properties it is very unstable and it will readily react with other components in the culture medium, making it difficult to determine what final concentration is applied. Since one of the theories of aging states that a decreasing efficiency in oxidative phosphorylation results in mitochondria-derived increased ROS levels, we decided to use rotenone which inhibits complex I in the oxidative phosphorylation chain in the mitochondria. Although rotenone is extensively used in mitochondrial research, it is not used much in the field of aging, senescence and apoptosis, complicating comparison of our results with other workers.

Implications and future research

We have now shown that fibroblasts *in vitro* do reflect the population of subjects from which they were derived. More specifically, we have shown differences in *in vitro* stress responses dependent on chronological and biological age *in vivo*. Fibroblast strains of offspring with the propensity for longevity showed stress-induced responses that tended to be more like the responses from chronologically young subjects. Fibroblast strains from offspring of nonagenarian siblings showed less senescence and more apoptosis/cell death. The latter could be interpreted as being a tumor suppressive mechanism, removing damaged cells from the proliferative pool of cells before they become tumorigenic. Removed cells should then be replaced with healthy cells from a pool of replenishing stem cells. This could be a topic for further research.

Although there was much interindividual variation, *in vitro* stress responses might be regarded as a marker for biological age. Having found these *in vitro* differences, we performed initial experiments using a transcriptomics approach on the comparison of fibroblast strains of chronological young versus old subjects, to gain more insight in the pathway driving these differences. This approach should now be extended to the comparison of fibroblast strains of biologically young (offspring) with old (partners) subjects to determine if the pathways driving the differences in chronological age are similar to those driving biological age and to identify (combinations) of candidate genes as possible markers for biological age. Once pathways and key regulators in these pathways are defined, genetic variation in the responsible genes should be studied to see to what extent this variation can explain differences in responses to stress that ultimately lead to increased longevity. Ideally this approach should also be applied to cell types and stressors other than those described in this thesis. Once common pathways and genes responsible for the differences in chronological and biological age are identified, these pathways might be modulated by compounds resulting in biologically old cells becoming biologically younger, i.e. resemble chronologically younger cells.

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