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TRANSCRIPTOME STUDIES OF CELL-FATE AND AGING

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As means to an end,
where the end is the future

ABSTRACT

Microarrays enable the researcher to capture the expression levels from most genes in a single experiment. The methodology has opened up a new field with possibilities and challenges. This thesis focuses on both methodological and bioinformatics improvements in the application of microarrays; and in particular on the use of such approaches in cell-fate and aging research. We have introduced a method to fabricate microarrays on unmodified glass that enables a cost effective approach to produce microarrays; and refined the SAM (significance analysis of microarrays) approach, highlighting important technical aspects of this, the most widely used method to identify differentially expressed genes.

Cell-fate is a term to describe how a cell ends its normal life span. We have used microarrays to identify genes that are associated with cell-fate outcomes. Initially, we studied early changes of gene expression during induction of cellular senescence and separated them from changes that are associated with growth arrest. We thereby identified a set of genes that are induced during induction of cellular senescence but not during growth arrest. We also studied a model-system that recapitulates a characteristic of cancer cells, apoptosis resistance, mediated by overexpression of the translation initiation factor eIF4E. We identified a set of transcripts that are overtranslated when eIF4E is overexpressed and demonstrated that two of them act as mediators of apoptosis resistance. These studies have contributed to the understanding of how the transcriptome is regulated during induction cell-fates and how regulation of translation can influence cell-fate decisions.

Senescence has been hypothesized to cause aging. When analyzing microarray studies of cellular senescence and aging we found a common mammalian aging transcriptome that had at least two components and established that cellular senescence resembles aging in mice but not humans. These findings are important as it indicates that aging in mice and humans could be substantially different and that cellular senescence in tissues, probably does not contribute to aging in humans.

LIST OF PUBLICATIONS

I. **Larsson, O**., Scheele, C., Liang, Z., Moll, J., Karlsson, C., and Wahlestedt, C. Kinetics of Senescence-associated Changes of Gene Expression in an Epithelial, Temperature-sensitive SV40 Large T Antigen Model. Cancer Res, *64:* 482-489, 2004.

II. **Larsson, O**., Perlman, D. M., Fan, D., Reilly, C. S., Peterson, M., Dahlgren, C., Liang, Z., Li, S., Polunovsky, V. A., Wahlestedt, C., and Bitterman, P. B. Global Analysis of Apoptosis Resistance Downstream of eIF4E Identifies Translationally Activated Anti-Apoptotic Transcripts with Novel Structured RNA Elements. Submitted, 2004.

III. **Larsson, O**., Wahlestedt, C., and Timmons, J. A. Unpredictable instability in the application of the SAM (Significance analysis of microarrays) algorithm. Submitted 2004.

IV. Wennmalm, K., Wahlestedt, C., and **Larsson, O**. The mouse expression signature of *in vitro* senescence resembles mouse but not human aging. Submitted 2005.

V. Kumar, A., **Larsson, O**., Parodi, D., and Liang, Z. Silanized nucleic acids: a general platform for DNA immobilization. Nucleic Acids Res, *28:* E71, 2000.

VI. **Larsson, O**., Thormeyer, D., Asinger, A., Wihlen, B., Wahlestedt, C., and Liang, Z. Quantitative codon optimization of DNA libraries encoding sub-random peptides: design and characterization of a novel library encoding transmembrane domain peptides. Nucleic Acids Res, *30:* e133, 2002.

Contents

Abbreviations

CAGE	Cap analysis of gene expression
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
EF	Extraction factor
eIF4E	Translation elongation initiation factor 4E
FC	Fold change
G1	First gap phase
G2	Second gap phase
GO	Gene ontology
MEF	Mouse embryonic fibroblast
MM	Mis-match
mRNA	Messenger RNA
PCA	Principal components analysis
PCR	Polymerase chain reaction
PM	Perfect-match
RMA	Robust multiarray analysis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real-time PCR
S phase	DNA synthesis phase
SA-βGAL	Senescence associated beta galactosidase
SAGE	Serial analysis of gene expression
SAHF	Senescence associated heterochromatin foci
SAM	Significance Analysis of Microarrays
siRNA	Short interfering RNA
SV40	Simian virus 40
SVD	Singular value decomposition
TM	Trans-membrane
UTR	Untranslated region

1 INTRODUCTION

Microarrays are tools that capture the expression levels from most all genes, and thereby enable a characterization of the transcriptome. The methodology has driven a new way of thinking, where the global expression signature is studied to identify genes associated with, and thereby possibly explaining, the phenotype. This is different from traditional studies, where a single gene is studied at a time and assessed for its contribution to the phenotype.

Apoptosis and cellular senescence are two common cell-fates. While apoptosis leads to death and removal of the cell, senescent cells persist and survive for extended periods of time (unable to maintain their normal functions). Senescence and apoptosis are important for cancer prevention and genes that control senescence or apoptosis could be interesting from a cancer treatment perspective. Two of my studies aimed to identify genes that were associated with cell-fate decisions. In the first study (Paper I) we looked for genes that were associated with early induction of senescence rather than general growth arrest, and identified a set of genes that were unique to senescence. In the second study (Paper II) we used a model system that recapitulates apoptosis resistance. Apoptosis resistance, important for tumor progression, can occur through multiple mechanisms. We focused on a newly identified mechanism that involves the translation machinery and the translation initiation factor eIF4E. In this study we identified a set of transcripts that are overtranslated when eIF4E is overexpressed, and two of them appear to be mediators of apoptosis resistance in the model. Both these studies have generated new information about how the transcriptome is regulated during cell-fate and how translational control contributes to cell-fate decisions.

One of the most important improvments to enable large scale transcriptome studies, in addition to the technical achievements, is the development of bioinformatics tools. We have contributed to the understanding of a commonly used method to identify differentially expressed genes (Paper III), where we discovered some significant problems regarding preselection of data and use of some settings. One of the challenges in the microarray field has been to identify common and acceptable standards; our data suggests that even applications that appear simple to use can be unreliable. A major challenge within the data analysis field is how to combine data from different sources and ask new questions. We have performed a study across several species and plattforms and gained new information. Our study thereby provides an example of how such goals can be reached (Paper IV). We have also contributed to the technical development by introducing a new method to build microarrays on unmodified glass that enables a cost effective approach to make microarrays (Paper V).

Finally, senescence has traditionally been thought to contribute to aging. To test this hypothesis using transcriptome analysis, we used publicly available microarray data and compared the transcriptome of aging to that of cellular senescence (Paper IV). We were able to show a common aging transcriptome that had at least two components and established that the transcriptome of cellular senescence has similarities to that of aging in mice but not humans. These findings are important from an aging and cellular senescence perspective as it indicates that aging in mice and humans may be substantially different and that cellular senescence in tissues may not contribute to aging in humans.

2 AIMS OF THE THESIS

The overall aim of this thesis was to explore the transcriptome during induction of senescence; resistance to apoptosis; and during aging.

The specific aims were:

1. To identify genes which are activated during induction of senescence and describe their kinetic profiles.
2. To identify transcripts which are translationally activated by eIF4e and investigate if these also mediate the anti-apoptotic effects downstream of eIF4E.
3. To explore a commonly used method for identification of differentially expressed genes and test how it handles different restrictions in the initial data set.
4. To used a large set of transcriptome studies to assess if cellular senescence resembles aging.
5. To develop a chemistry that can be used to construct microarrays by attachment of modified cDNAs or oligo-nucleotides onto unmodified glass surfaces.
6. To develop and verify a method that can be used to construct sub-random peptide libraries encoded by oligo-nucleotides.

3 CELL FATE

3.1 INTRODUCTION

Cell fate is a term to describe the different end points of a normal cells life span. Cell fate can be divided into cell death (necrosis and apoptosis) and a form of "genetic" death called cellular senescence. While necrosis and apoptosis leads to removal of the cell in a tissue with or without an inflammatory response respectively, a senescent cell can persist, at least *in vitro*, incapable of performing its natural functions. The programs that control the different cell-fate end points differ in terms of their mechanisms and specificity. While necrosis is not controlled but occurs as a consequence of tissue damage, apoptosis is controlled by a well characterized genetic program. Cellular senescence is less well defined and partly relies on the same pathways that control apoptosis. The different cell-fates are important for several processes. Apoptosis is active during development and helps to prevent cancer progression; while senescence is believed to be important to prevent cancer progression and as a factor contributing to aging. In this thesis I will principally focus on senescence, but will discuss some aspects of apoptosis when appropriate.

3.2 SENESCENCE

3.2.1 History

In the end of the 1950: ies the common belief was that all cells could live forever *in vitro* if maintained under the proper conditions. This idea came from the Nobel Prize winner, Alexis Carrell, who reported a culture of rat reticulocytes that had been maintained for 34 years. However, his protocol included addition of an extract from chick embryo tissues that included some fresh living cells and hence the immortal culture [1]. When Hayflick found that his fibroblasts were unable to propagate in culture it contradicted the current theories of the day. However, instead of accepting the dogma he decided to challenge it. Two experiments convinced him that he was able to maintain the cells under proper conditions and that a normal cell has a finite life span. In the first experiment he mixed young female and old male cells in a culture while maintaining the original cultures as well (Fig 1) [2, 3]. When the old male culture had

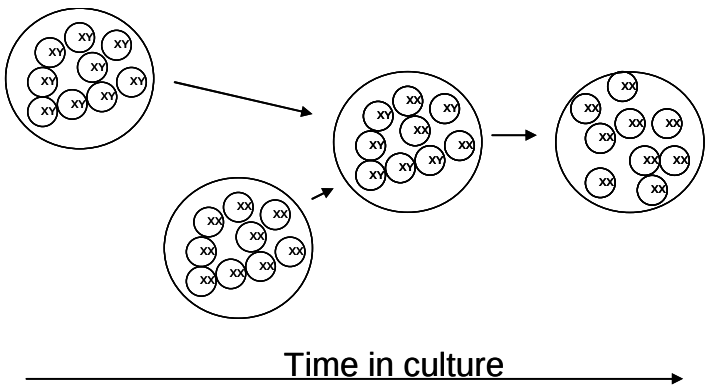


Figure 1: Hayflicks experiment to establish that senescence did not occur because of culture conditions. Female and male cells of different culture ages were mixed and surveyed later for gender.

stopped dividing he went back to the mixed culture and was only able to find female cells. He thereby concluded that there was no external factor that caused the growth arrest. In the second experiment, cells were freezed, stored for some time and thawed with the discovery that it is the time that the cells had been cultured that determines when they stop growing and not the cumulative time. He named the state when cells had stopped growing phase III and it was later called the Hayflick limit, cellular senescence or replicative senescence. His findings led to the understanding that in contrast to normal cells, cancer cells are essentially immortal.

3.2.2 Definitions

The senescence field still struggles to clearly define the processes involved in cellular senescence. There are three commonly used criteria that should be fulfilled, at the same time. The first is irreversible growth arrest. Although it seems like a criterion that is easy to define there are some uncertainties. In many studies one gene is overexpressed and shown to induce growth arrest. Is this irreversibility or should the overexpression be released to see if the growth arrest is dependent on continuous overexpression? Also there is data accumulating from traditional senescence models where the irreversible growth arrest can be reversed artificially [4, 5]. The second criterion is based on phenotypic changes including, morphology [6]; staining with a biochemical marker for senescence (senescence associated-β galactosidase (SA-βGAL)) [7]; and changes in gene expression that are inappropriate for that specific cell type. However, both the classical large flat morphology as well as staining of SA-βGAL can be reversed in human fibroblasts when the media is changed to low serum media [8]. Also, several pathways/genes have been discovered that mediate some of the morphological changes without contributing to the growth arrest. This indicates that the phenotypic changes may not be directly coupled to the growth arrest but result from the culture conditions [9-11]. The last criterion is apoptosis-resistance. Although this is commonly accepted, the mechanisms for the apoptosis resistance are poorly defined and the incidence of resistance is not well established. Senescent cells have been shown to be resistant to serum withdrawal [12] and p53 mediated apoptosis [13] but at least some cell types have increased sensitivity towards TNF-α [14]. Some cell types spontaneously die by apoptosis after prolonged maintenance *in vitro* although they are senescent by most definitions [15]. It therefore appears that the “anti-apoptosis” dogma requires further investigation.
In summary it is currently unclear what should be considered as senescence but the term is typically accepted if <5% of the cells proliferate and flat cellular morphology together with SA-βGAL staining, can be demonstrated.

3.2.3 Senescence pathways

Several pathways can trigger senescence in various cell types and under a variety of different conditions. The most common pathways described in relation to senescence are the p16/Rb and p53/p21 pathways.

3.2.3.1 The p16/Rb pathway

Rb mediates regulation of the cell cycle at the transition from first gap phase (G_1) to DNA synthesis phase (S phase). Rb is hypophosphorylated during G_1/G_0 and is bound to E2F whereby the activity of E2F is inhibited. When Rb is phosphorylated it releases E2F and this occurs before the G_1/S transition and through S-phase. E2F mediates transcription of a variety of genes necessary for G1 to S progression and replication including cyclin-E, cyclin-A and thymidine kinase [16]. Phosphorylation of Rb is mediated by cyclin dependent kinases (CDK) bound to cyclins (cyclin-D1/CDK4-6 and cyclin-E/CDK2). CDK4/cyclin-D is activated by mitogenic signaling through the RAS pathway by transcriptional induction of cyclin-D [16]. There are proteins called cyclin dependent kinase inhibitors that can inhibit the CDKs. One of them is p16 which inhibits phosphorylation of Rb and thereby G1 to S progression by inhibiting CDK4/cyclin-D [16]. p16 can in turn be regulated transcriptionally by several proteins of the polycomb group and seems to be a sensor for cellular stress (see under oncogene-induced senescence) (Fig 2).

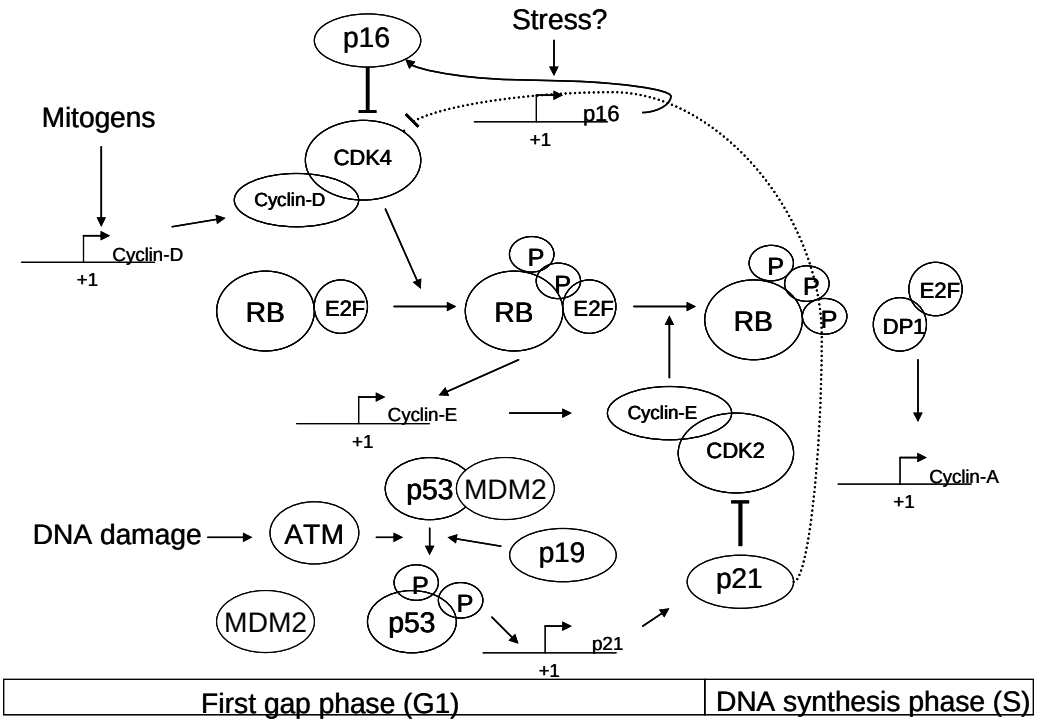


Figure 2: Regulation of the cell cycle. G1 to S transition.

There is extensive evidence for an important role for the p16/Rb pathway during the induction of senescence. Overexpression of p16 induces features of senescence including growth arrest [17] while knock-down of p16 using short interfering RNAs (siRNAs) inhibited RAS-induced senescence in epithelial cells [18]. Re-expression of Rb in a cancer cell line [19] or inhibition of E2F [20] also induces senescence, indicating that the p16/Rb pathway can induce senescence under several conditions.

3.2.3.2 The p53/p21 pathway

p53 has been named the “gate-keeper of the genome” and is mutated in 50% of all tumors. It acts as an integrator for various signals and can mediate cell cycle arrest, apoptosis and differentiation. There are several mechanisms that regulate the activity of p53. The DNA-damage-ATM/ATR-Chk1/Chk2 pathway activate p53 by phosphorylation [21] leading to displacement of the cellular protein MDM2, which relocates p53 from the nucleus to the cytoplasm and targets it for degradation [16]. MDM2 can also be regulated by $p19^{ARF}$, which inactivates MDM2 leading to an increased activity of p53 [22]. Many other proteins e.g. SUMO-1 [23] and Parc [24] can modulate p53 activity and the p53 activity can further be modulated by protein modifications (e.g. acetylation) [16, 21]. Once activated, p53 induces transcription of many genes involved with cell cycle arrest and apoptosis [25, 26]. One of the activated proteins that mediate the cell cycle arrest downstream of p53 is p21. p21 is a member of the “Cip/Kip” family of cyclin-dependent kinase inhibitors (CDKI) that inhibits CDK2/cyclin-E [16] and to a lesser extent CDK4/cyclin-D [25]. p21 is believed to be the main target for cell cycle arrest downstream of p53 (Fig 2).

The p53/p21 pathway has clear role during induction of senescence. Mouse embryonic fibroblasts (MEF) lacking either p53 [27] or p19 [28, 29] do not senesce. Human cells bypass senescence when both the p53 and the Rb pathway is inhibited by e.g. Simian virus 40 (SV40) large T antigen [30]. Furthermore, senescence induced by inactivation of SV40 large T antigen can be inhibited by introduction of a dominant negative p53 in some cell types [31]. Interestingly, inhibition of the p53 pathway in cells already senescent can reverse the phenotype as injection of a dominant negative p53 [4] or a SV40 large T antigen that only binds and inactivates p53 [5], can reinitiate DNA synthesis at least in some cell types. Also, overexpression of p53 can induce senescence in some tumor cell lines [32]. The downstream target of p53, p21 can induce senescence in tumor cell lines independent of p53 status [33-35], and human but not mouse fibroblasts lacking p21 bypass senescence [36-38]. This indicates an important role of p21 for induction of senescence in human cells and further indicates that induction of senescence differs between species. Reactive oxygen species (ROS) are possible mediators of the senescence response downstream of p53/p21 and a three-step process has been proposed after induction of p53 activity. The model includes transcription of ROS related genes, formation of ROS and alterations to the cell leading to either apoptosis or senescence [39]. In support for an involvement of ROS, both p53- and p21-induced senescence has been shown to be at least partly dependent on ROS [40, 41]. The p53/p21 pathway seems to be mainly activated during replicative senescence (see below).

3.2.4 Replicative senescence

3.2.4.1 The Telomere

The telomere is a structure located at the end of each chromosome. It consists of a repeated DNA sequence (TTAGGG.) and associates with several binding proteins. There are species variations in telomere biology, for example humans have longer telomeres compared to mice [42, 43]. Telomerase, a reverse transcriptase, can extend the telomere DNA by using a nucleus-encoded RNA as a template for its RNA-dependent DNA polymerization [44]. Each telomere ends with a 3´ single stranded sequence of about 200 nucleotides that folds back to the double stranded telomere sequence to form a loop structure called the t-loop [45, 46]. The t-loop together with the telomere binding proteins is believed to protect and hide the end of the telomere to avoid a DNA damage signal and/or regulate the length of the telomere. The first telomere binding protein, TRF1, was identified using the telomere structure as bait on magnetic beads in a protein extract [47]. TRF1 binds directly to the telomere repeat as a dimer and negatively regulates the length of the telomere [47, 48]. TRF1 also seems to bend the telomere DNA possibly to enhance the formation of the t-loop [49]. One of the main functions of TRF1 is to act as a scaffolding protein for other proteins, including TIN2 [50]. TIN2 links TRF1 to a second protein called POT1 [51, 52] through PTOP/PIP which binds both TIN2 and POT1 [53, 54]. POT1 is the only protein found so far that binds directly to the single stranded telomere DNA. The TRF1-TIN2-PTOP/PIP-POT1 interactions are thought to inhibit telomerase access to the repeat structure by maintaining the loop structure. TRF1 also interacts with PINX1 which is a direct negative regulator of telomerase [55]. TRF1 therefore negatively regulates telomere length by both maintaining a closed telomere structure as well as directly inhibiting telomerase.

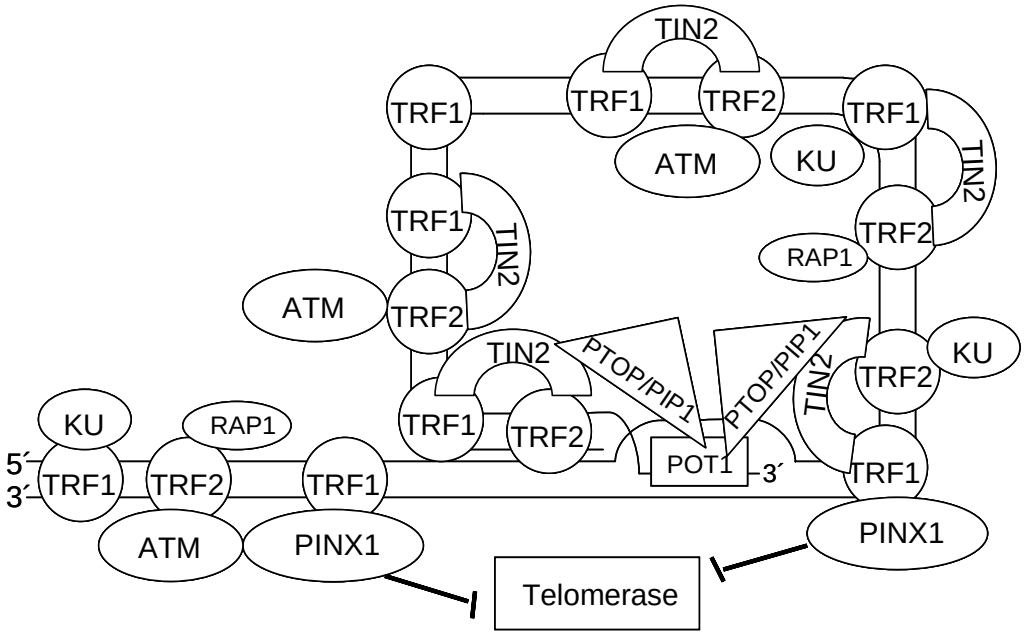


Figure 3: The telomere structure with associated proteins.

A third protein that binds directly to the telomere (after TRF1 and POT1) is called TRF2 [56]. Interestingly TRF1 binds to TRF2 through interactions with TIN2 [57] indicating that most of the proteins located at the telomere bind to each other through protein-protein interactions to ensure a tightly protected structure. TRF2 is essential for the formation of the t-loop and a dominant negative TRF2 protein can destroy the telomere structure to induce either apoptosis or senescence [58, 59]. TRF2 also binds to ATM which has an important role in the induction of telomere driven senescence (see below) [60]. Other proteins which binds to TRF2 includes RAP1 (which has no functional conservation to its yeast homologue that binds directly to the telomere) [61-64]. Some proteins of the DNA damage family have been found to be associated with the telomere including RAD50/MRE11/NBS1 which interact with TRF2 [65] and the KU proteins [66, 67] (Fig 3).

Together these data indicate that the telomere is regulated and kept in a closed structure and whenever it fails to maintain a protected structure it may rapidly induce a DNA damage response through the DNA damage proteins that are localized to the telomere.

3.2.4.2 Senescence downstream of the telomere

Telomere-induced senescence is thought to result from the "end-replication problem". The replication machinery can not start at the absolute end of the chromosome and therefore a piece of the telomere is lost following each round of replication [68]. Recently, t-loop sized deletions of the telomere have been detected in primary cells indicating that other mechanisms could contribute to telomere shortening [69]. The rate of telomere shortening is suggested to be related to the length of the single stranded telomere DNA. However, this may differ between cell lines, where telomere shortening can be proportional to the length of the single stranded DNA, and primary cells from different donors, where no correlation between lengths of the single stranded DNA and telomere shortening was found [70, 71].

The main experimental evidence that the telomere drives replicative senescence reflects experiments where overexpression of telomerase in several cell types allows those cells to bypass senescence [72-80]. However, in some cell types telomerase fails to overcome senescence as these cells senesce for other reasons then telomere shortening (see below) [81-83]. Initially it was uncertain what overexpression of telomerase really achieved as cells with telomerase (that escaped senescence) showed shorter telomeres than senescent cells [74, 84]. A reasonable explanation for this paradox was that it is not the average telomere length that determines when a cell enters senescence, rather senescence is triggered by the shortest telomere within each cell [85-87]. Interestingly, telomerase was initially reported to have no other role beyond maintaining the telomere [88, 89]. However, it is now clear that this is not true as telomerase can contribute to tumorigenesis by mechanisms

unrelated to telomere elongation [90], stimulate proliferation [91] and change the response to TGF-β [92]. It seems plausible that these other mechanisms could be important for the escape from senescence or the role telomerase has in tumor development (90% of all tumors overexpress telomerase, see Senescence and cancer). In addition, only a proportion of all cells are actually immortalized by telomerase expression when mass cultures (compared to clonal cell lines) are studied, indicating that other mechanisms and selection processes may contribute to escape from senescence by telomerase [93].

A critical question is what happens to the telomere when it becomes shorter and shorter. Currently there are two theories, and although they do not seem to rule each other out, it has led to a lot of controversy within the field. The first theory postulates that, upon shortening of the telomere, the proteins that usually cap the telomere are no longer able to protect and a DNA damage response is initiated. Inherited with the model is the assumption that the proteins that bind to the telomere suddenly are unable to do it, presumably because the telomere is too short. The second theory states that the single stranded telomere DNA is degraded or eroded; and that this makes that telomeric structure unstable and induces senescence. The telomere erosion theory does not imply that the telomere has to be particularly short, just that the single stranded overhang is lost. The erosion theory was initially supported by the finding that the single stranded DNA is lost during senescence [94]. However, recently this finding has been challenged, as no loss of the single stranded DNA was found in senescent cells [95]. This clearly questions the basis for the erosion theory. The difference between the two studies did not reflect the selected cell types, but may be related to the different methods used to measure single stranded telomere DNA lengths. The main evidence supporting that senescence is induced when the telomere proteins fail to protect the telomere structure, comes from experiments with TRF2. Overexpression of TRF2 leads to shortening of the telomere but a delayed senescence which indicates that excess TRF2 can maintain a proper telomere structure of short telomeres and that this is a key event that regulates onset of senescence [96]. Also, experiments with a dominant negative TRF2 lead to induction of senescence without loss of telomere DNA [56] but with cleavage of the single stranded telomere DNA dependent on the ERCC/XPF endonuclease [97].

In the last two years significant progress has been made through identification of the signaling pathways that are activated by the telomere and induce senescence. Several groups have identified a DNA damage “response” specifically originating from the telomere structure in senescent cells [98-100]. All studies detected a phosphorylated form of histone H2AX and several DNA damage related proteins. It therefore seems likely that the telomere structure is identified as a double strand break that signals through ATM, which can phosphorylate H2AX as well as CHK2 [101]. If telomere senescence is initiated through the ATM pathway, one might ask

what maintains the senescent state. Previously it has been shown that it is possible to reverse the “irreversible” growth arrest, at least in some cell types, by inhibiting p53 [4, 5], and it may therefore be plausible that the DNA damage signals from the telomere still persists in the fully senescent cell [98]. However in another cell type, the DNA damage signals disappeared once the cell became fully senescent [100]. The cells that maintained an active DNA damage response also demonstrate reversal of senescence by inhibition of p53 [5]. The cells that did not maintain the DNA damage signals have not yet been assessed for the irreversibility phenotype, but it seems possible that there might be cell type differences in this characteristic. Another group has challenged whether the DNA damage signals originate from the telomere and claim that they are randomly distributed upon induction of senescence [102]. However the data presented by d'Adda di Fagagna et al. included several methods to assess where the signals originate from and the data seems convincing [98].

Based on current data, a likely model for senescence downstream of the telomere could be as follows: (i) the telomere structure and/or function is compromised as a result of telomere shortening, (ii) the DNA ends of the chromosome become exposed and trigger a DNA damage response through the ATM pathway, (iii) depending on the cell type this DNA damage is un-repairable and the ATM signalling persists and maintains the cell in a non-dividing state or the DNA damage is repaired but other pathways have been activated, and the cell maintains an irreversible growth arrest.

What is downstream p53/p21 activation? Several microarray studies have looked at the transcriptome induced by p53/p21 or used bioinformatics approaches to identify p53 responsive genes [25, 26, 103-107]. We performed a detailed study of the gene expression kinetics [108] and identified a set of genes that are activated with varying kinetics, and were unique to senescence. New method-developments have enabled large functional screens for genes essential for senescence using siRNAs. Using a reversed genetics approach in the temperature sensitive Simian virus 40 large T antigen model, five genes were identified as being essential for senescence [109]. Although the identity of several of them was surprising, it indicates that senescence relies on large changes in basic cellular mechanisms. Another protein (Smurf 2) has been identified in a microarray study of replicative senescence, and seems to be specifically induced during telomere senescence compared to hydrogen peroxide induced senescence. Smurf2 can induce a senescent arrest through either the p53/p21 or the p16/Rb pathway but seems unlikely to be essential [110]. Smurf 2 is likely to be a target of telomere signalling, probably through p53, but why it is specifically induced during telomere senescence is unknown.

3.2.5 Oncogene-induced senescence

Activation of oncogenes renders the cell self sufficient in growth signaling and is important for cancer progression [111]. Interestingly, overexpression of several oncogenes also induce senescence *in vitro* and this may be an important strategy to avoid cancer progression. RAS [112] and its downstream targets RAF [113] and MEK [114], as well as ERBB2 [115], Akt [115], eIF4e [116] and E2F in some cellular contexts [117, 118] can all induce senescence. This indicates that senescence could be a general defence against activated oncogenes. RAS-induced senescence is best described in the literature but is species specific.

RAS-induced senescence MEFs is dependent on both p16/RB and p53 [112] but p53 activity alone is not sufficient to induce a full senescent phenotype in MEFs [119]. Further, in MEFs with functional p53, disruption of both Rb and the Rb-family member p107 was necessary to avoid senescence but not sufficient to induce transformation [120]. The activation of p53 and induction of senescence downstream of RAS in MEFs is dependent on Dmp1, which activates p19 through a Ets site in the INK4A promotor [121]. Therefore, it seems like both a functional p53 and Rb pathway is necessary to induce senescence in MEFs when RAS is overexpressed.

In human cells, RAS does not seem to be dependent on p53 to induce senescence as it was either unchanged [122, 123] or was upregulated but not essential for RAS-induced senescence [38, 112]. Some of the discrepancies of p53 induction during RAS-induced senescence may be related to the dual action of RAS on p53 status as RAS activates both MDM2 and p19 which inhibits and activates p53 respectively [124]. The outcome of RAS on p53 status may therefore be cell type and experimental condition specific. Also, RAS has been shown to have a direct effect on the p21 promoter indicating that it can bypass p53 for induction of p21 [125]. However, the significance of this is unclear as fibroblasts lacking p21 entered senescence following RAS overexpression similarly to p53 negative cells [38].

Unexpectedly the stress activated kinase p38 seems to have an important role in human RAS-induced senescence. p38 is activated as a consequence of RAS expression and inhibition of p38 by a small molecule inhibitor (for isoforms α and β) bypassed RAS-induced senescence [126]. There is data indicating that the activation of p38 could be related to an accumulation of reactive oxygen species as human fibroblasts overexpressing RAS did not senesce at low oxygen levels or in the presence of scavengers for hydrogen peroxide [127]. It is also possible that overexpression of RAS changes the metabolic balance in the cells which activates p38. The later suggestion comes from a study where overexpression of an enzyme needed for glycolysis bypassed RAS-induced senescence in MEFs [128]. The contribution of cell stress to RAS-induced senescence is further supported by a recent report where cells that showed lower stress levels (measured by p16 activity)

before addition of RAS, did not senesce after RAS overexpression [123]. Together these data favor a model in human cells where overexpression of RAS leads to an accumulation of ROS, increased p38 activity which could activate the p16 pathway, and induction of senescence. It is also possible that both p16 and p38 are activated by ROS in a parallel pathway and that the activity of both is necessary to induce senescence. In support of this model, fibroblasts from a melanoma prone family with p16 deficiency, but functional p19, did not senesce when RAS was overexpressed [129]; and fibroblasts from a patient with bialelic mutations of the INK4a/ARF locus showed resistance towards RAS-induced senescence [130].

What then is the link between RAS/stress and activation of p16? The polycomb group of proteins has been suggested to be mediators of RAS-induced senescence as they regulate transcription of p16, either positively or negatively. The first class represses transcription of p16 and would be expected to delay senescence when senescence is mediated by p16. Accordingly, the Id proteins have been shown to delay senescence in cell types where senescence is mainly p16 dependent [131-133]. Similar functions have been established for BMI-1 which inhibits both senescence and apoptosis induced by p19 [134, 135], TBX2 [136, 137] although it also has activities on the p21 promotor [138] and CBX7 [139]. Interestingly, only the stress induced senescence can be inhibited by all of these p16 repressors while hTERT expression is needed for full immortalization (see further below). The second class of polycomb proteins includes members that activate transcription of the p16 promotor, i.e. mainly the Ets proteins. The Ets proteins are direct targets of Ras-RAF-MEK signaling and can directly activate transcription of p16 [140]. They therefore provide interesting candidates for RAS-induced senescence but can not explain the dependence of ROS.

Several attempts have been made to identify genes that repress senescence downstream of RAS either when overexpressed or inhibited. PLM was identified as upregulated in a microarray study of RAS-induced senescence and was shown to be sufficient for induction of senescence in the absence of RAS in both human and mouse cells [141-144]. PML can modulate the activity of both the p53 and the RB pathway [141, 145]. Similarly to RAS-induced senescence, PML-induced senescence is dependent on Rb [146] and Rb mutants that are less efficient in binding to E2F can induce senescence through induction of PML-nuclear bodies (PML-NB). This indicates that PML recaptures some of the characteristics of RAS-induced senescence and that PML might be one additional target of Rb during senescence [147].

A genetic screen for genes that overcome RAS-induced senescence in MEFs, identified hDRIL, an E2F binding protein. Both the p53/p21 and the p16 pathways were activated during rescue from RAS-induced senescence mediated by hDRIL [148] and it was postulated that the anti RAS-induced senescence action of hDRIL

was through release of E2F from Rb. It was later established that the effect may also relate to PML as hDRIL disintegrate the PML-NBs which further indicates that RAS-induced senescence is somehow connected to the PML-NBs [143]. However overexpression of hDRIL in human cells induced senescence probably in an oncogene manner similar to E2F and inhibition of hDRIL lead to an accumulation of PML-NB and PML induced senescence [148]. hDRIL can therefore not be used in human cells to understand the impact of PML on RAS-induced senescence and seems unlikely to be a key mediator of RAS-induced senescence although it can modulate the pathways.

A reverse genetics approach to identify genes that mediate RAS-induced senescence in rat embryo fibroblasts identified Seladin-1 [149]. Originally described as a metabolic enzyme, Seladin-1 is activated by both RAS overexpression and hydrogen peroxide indicating that it could act as an oxidative stress sensor [149]. Downregulation of Seladin-1 was further shown to allow bypass of senescence both in mouse and human fibroblasts [149]. However, Seladin-1 activates the p53 pathway by releasing p53 from MDM2, which is surprising as the p53 pathway is not necessary for RAS-induced senescence in human cells [149]. It should be mentioned that the functional data of Seladin-1, in rescue from RAS-induced senescence, was established using one anti-sense sequence without any control. Therefore, it is in principle possible that the effect could be mediated by another protein although it seems less likely given the effect that Seladin-1 has on the p53 pathway. Future studies may demonstrate that Seladin-1 acts on the p16/Rb pathway and how it relates to the stress kinase p38. Regardless, these data further indicate that senescence downstream if RAS is stress dependent as it was activated by both RAS and hydrogen peroxide.

In summary there is some evidence that RAS-RAF-MEK-Ets plays a role during induction of senescence downstream of RAS but questions remain as inhibition of ROS, p38 or Seladin-1 also inhibits RAS-induced senescence. RAS-induced senescence seems likely to occur when p16 levels reach a critical level [123]. This level could be achieved when both the direct RAS-RAF-MEK-Ets pathway is activated as well as the stress pathway mediated by ROS/p38/Seladin-1/p16. The PML protein is likely to act as an amplifier, but may not be necessary if the stress level is high enough. The kinetics of RAS-induced senescence supports this theory. While RAS expression is induced immediately, p38 signaling is activated together with p16 after four days (the design of the study did not allow a separation of p38 and p16 activation) and the senescent phenotype appears after seven days [126]. Interestingly, overexpression of a constitutively active downstream target of p38, MKK6EE, induced senescence after 4 days [150]. These data indicate that protein synthesis is needed at each step and that there is time for accumulation of ROS damage or a shift in the metabolic balance to activate p38/p16 which then needs further time to manifest the full senescent phenotype.

Regardless of the mechanism for RAS-induced senescence, the critical question is whether oncogene-induced senescence is an *in vitro* artifact or if it occurs *in vivo*. Given the results described above where it seems to be dependent on an artificially high oxygen level (as RAS-induced senescence was inhibited at low oxygen levels) leading to an inappropriate stress response, it seems possible that it never happens *in vivo* but the studies to demonstrate this are still missing.

3.2.6 Stress-induced senescence

Several chemicals can induce senescence, e.g. low concentrations of hydrogen peroxide [151-154] and several chemotherapeutics at low concentrations [155-157]. As expected, senescence downstream of hydrogen peroxide, and presumably most chemicals, is independent of telomere shortening [158]. It is possible that all therapeutics that induce apoptosis can induce senescence at lower concentrations than they induce apoptosis [153, 159]. Other stimuli that can induce senescence include UVB which is dependent on TGF-β1 [160], tert-butylhydroperoxide [154, 161] and ethanol [161].

Hydrogen peroxide is the best studied chemically induced senescence possibly because it has been assumed to be more similar to *in vivo* stress. The G1 arrest associated with hydrogen peroxide mediated senescence can be inhibited by both papilloma virus E6 and/or E7 but the cells were unable to proliferate which indicate severe DNA damage leading to an irreversible growth arrest (cells stop in G2 instead of G1) [152]. Similarly to senescence downstream of RAS, p38 is activated [150, 162] but inhibition of p38 signaling by a small molecular drug was unable to prevent hydrogen peroxide induced senescence in U2OS cells [150]. Human fibroblasts release TGF-β1 after H2O2 treatment [163] and this release is necessary for a maintained p38 activation which in turn induces TGF-β1 creating a loop which can result in some phenotypic changes [162, 163].

In summary, senescence downstream of stress stimuli recapitulates features of both telomere-induced senescence and oncogene-induced senescence. Both the Rb and the p53 pathway seem to be active in restricting the proliferative capacity while TGF-β1 and p38 mediates some of the phenotypic changes.

3.2.7 Why did you senesce?

All primary human cells enter senescence after a certain number of cell divisions [2, 3]. However, senescence is defined by a set of shared characteristics, not by a common pathway. During the last couple of years it has become clear that species and cell types differ in how they induce senescence. From a simple perspective, each human cell can be described as entering senescence with p16/Rb or p53/p21 activation. This view of senescence comes from a few studies of senescence in single cells. The initial finding was that there was no gradual increase in p21

expression in human fibroblasts as reported previously [164], rather the increase was abrupt in the single cell [165]. The gradual increase reported is clearly a function of heterogeneous telomere lengths in a multi- cellular population, that results in slightly different replicative potential of different cells [166]. Similarly it was described that although senescing fibroblasts can show an increase in both p16 and p53/p21 activity, these pathways are not active in the same cell and telomeres induce senescence exclusively by activating the p53/p21 pathway [167]. However, all data do not agree with this view as cells with a mutant TRF2, that generate telomere dysfunction before any stress induced senescence should be active, entered senescence with elevated p16 and p53 activity and abrogation of senescence was only possible with both E6 and E7 expression [168]. However, it can not be excluded that the TRF mutant model simultaneously induced a stress response and hence the need for both Rb and p53 inactivation, or that the appearance of stress induced senescent cells occurs very early.

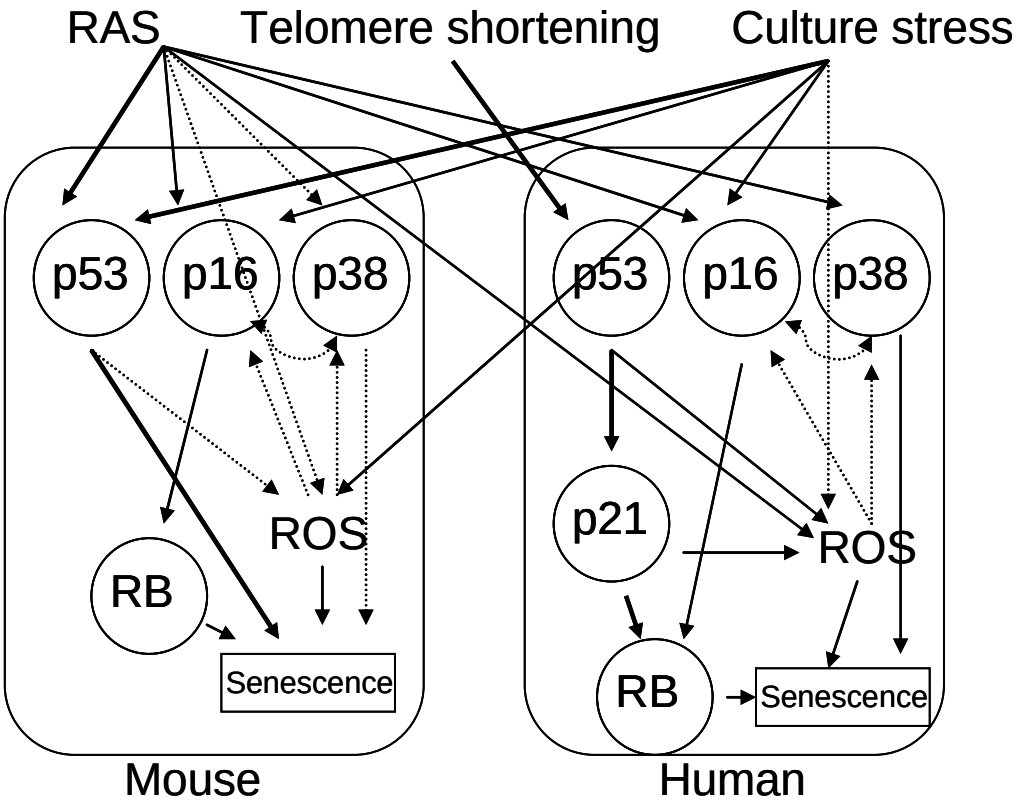


Figure 4: A summary of all senescence pathways described. Dotted lines are suggested/likely interactions/mechanisms and contious lines are experimentally validated. Bold lines show species differences.

The single cell studies indicate that senescence resulting from the telomere pathway and from culture stress can coexist in a population of cells. Therefore, one can define each cell type based on whether they are more likely to enter senescence as a result of culture stress or telomere erosion. The culture stress senescence appears to be similar to the senescence response driven by RAS and is characterized by p16

overexpression. Human epithelial cells [169-172] and keratinocytes [173] senesce with high levels of p16 but with long telomeres, and telomerase did not overcome senescence [81]. Human fibroblasts senesce mainly because of telomere erosion with p53/p21 induction. This probably reflects that human fibroblasts are more resistant to culture stress and therefore reach the telomere restriction point and activation of ATM-p53-p21 signaling [36, 38, 167, 174]. However, some fibroblast strains are also sensitive to culture stress and show a substantial stress induced senescence with p16 induction [18, 164, 175-177]. Further, human fibroblasts can be forced to senescence from stress [178] and can also enter a senescence state characterized by an increase in p16 expression if the telomere driven senescence is inhibited [179]. These data indicate that human fibroblasts can enter stress induced senescence similar to epithelial cells under some conditions, but normally senescence from telomere signaling.

Mouse cells do not senescence because of telomere erosion as they have substantially longer telomeres [42] and can grow indefinitely in low oxygen [180, 181] or low serum conditions [182]. In contrast to human cells that senesce from culture shock, both p53 and the p16/Rb pathway are necessary, but not p21 (discussed under senescence pathways and oncogene induced senescence). The TRF2 mutant senescence model further support that the basic mechanisms differ between mouse and human cells as mouse senescence induced by telomere damage was not dependent of p16 expression whereas human senescence was [168].

In summary, depending on cell type and species, the mechanisms for induction of senescence varies but importantly the end point is similar in terms of phenotypic characteristics and gene expression signatures [183] (Fig 4).

3.2.8 Why is senescence sometimes irreversible?

There seems to be a fundamental difference in the degree of irreversibility depending on which pathway that triggers senescence. Human fibroblasts that senesce with p16 activity do not reenter the cell cycle after microinjection of SV40 large T antigen whereas cells that senesced with p53/p21 activity do [5]. This effect could be a result of an establishment of senescence associated heterochromatin foci (SAHF) that was described in senescent cells with an active p16/Rb pathway [184]. SAHF leads to a stable repression of E2F target genes such as cyclin-A and cyclin-E [184]. The mechanism is likely to include BRG1, HDAC1, SUV39H1 and/or a transcriptionally repressive form of the histone protein H2A called macroH2A. All these proteins mediate changes on chromatin structure and have been linked to formation of SAHA or to the growth restrictive activities of Rb. BRG1 is a component of the SWI/SNF chromatin remodeling complex that can induce senescence in cells with functional Rb [185] and seems important for Rb mediated growth arrest [186] although some recent data indicate that the effects may not be directly through the physical interaction with Rb as BRG1 can induce p21 as well

[187]. Similarly, HDAC1 was found in complex with Rb and is also important for Rb mediated repression of cyclin-E but not necessary for Rb/SWI/SNF mediated repression of cyclin-A [188]. SUV39H1 associates with RB and corporate to repress cyclin-E probably through methylation of histone H3 followed by binding of HP1 to the chromatin and establishment of heterochromatin [189]. MacroH2A is enriched in SAHF and could affect the chromatin structure by removing the chromatin modifications [190]. Two chaperone proteins that can assemble macroH2A onto DNA (HIRA and Asf1a) are sufficient and necessary for establishment of SAHF and senescence at least in some cell types [190]. Interestingly there could be a role of the PML-NB as the proteins that localized to SAHF first associate with the PML-NB [190].

It is likely that some of these factors contribute to stably repress E2F target genes and thereby mediate the genetic death that is characteristic of some forms of senescence. An interesting question is why only the p16/Rb pathway leads to irreversibility and not the p53/p21 pathway. It could be related to the phosphorylation pattern of Rb which will differ if mainly CDK4/cyclin-D or CDK2/cyclin-E is inhibited by p16 or p21 respectively.

3.2.9 Senescence and cancer

Cancer cells proliferate beyond the normal point of replicative senescence and thus need to maintain telomere lengths to continue to divide. 90% of all tumors maintain stable telomeres by overexpression of telomerase while the remaining 10% use an alternative mechanism of telomere maintenance that involves recombination, called ALT [191]. Although this suggests that a mechanism that induces senescence *in vitro* is also needed for extensive proliferation *in vivo*; the detailed mechanisms *in vitro* and *in vivo* may not be the same. However, an interesting p53 mutant that is unable to induce apoptosis while still able to induce cell cycle arrest (at an intermediate level between wild type and p53-null), has provided some indications that senescence could operate *in vivo* and restrict cancer progression. Double mutants for this p53 allele did not develop early tumors compared to a p53 null mice and the tumors that eventually occurred were diploid showing that cell cycle arrest (and possibly senescence) is occurring *in vivo* [192]. This remains the strongest principal proof for senescence being a natural mechanism that counteracts tumor progression. There are indications that senescence is a response to ongoing chemotherapy treatment, as most cell lines are able to respond to doxorubicin by induction of senescence [155] and senescence can be an *in vivo* response to chemotherapy in mice [155, 193].

Could induction of senescence be a reasonable strategy for cancer treatment? There is data supporting this idea: Inhibition of telomerase by expression of a mutated telomerase RNA-component, inhibited proliferation of human cancer cells [194]. Induction of senescence was also achieved by adding single stranded oligo-

nucleotides (telomere repeat) to the medium of tumor cells [195], although a similar treatment has also been described to induce apoptosis in another cell type [196]. The mechanism involves disruption of the telomere structure, possibly by titration of some of the telomere binding proteins, and induction of a DNA damage response [197, 198]. Interestingly the effect is specific for the telomeric repeat sequence [195] and dependent on both the p53 and the Rb pathway, indicating that it probably mimics a normal replicative senescence response as well as a general stress response [197].

Another approach to induce senescence in human cells would be to inhibit telomerase. Several drug companies have tried to develop telomerase inhibitors but so far without success. However, a compound was recently reported to reduce the levels of telomerase indirectly, induce senescence *in vitro* and showed *in vivo* effects in a mouse model [199, 200]. However, the treated cells exhibited extensive genomic instability and it is therefore possible that inhibition of telomerase and possible induction of senescence/crisis could drive further tumor progression [199].

While limiting cancer cell growth by induction of senescence sounds like a good strategy, it is not entirely clear how beneficial this would be *in vivo*. Indeed it has been discovered that senescent cells can actually promote tumor cell growth, *in vitro* and *in vivo*, in a model with pre-malignant epithelial cells [201, 202]. Similar promotion of cancer progression, by one cell type influencing another, has been shown in a system where fibroblasts deficient in TGF-β signaling were able to promote cancer progression in adjacent epithelial cells [203]. The mechanisms were described to be mediated both by cell-cell interactions as well as paracrine stimulation [201, 203]. Also, senescent cells have previously been described to secrete growth factors; and media from senescent cells can be mitogenic and anti-apoptogenic [104]. In that sense the ability enter senescence may be beneficial for overall tumor survival. An increased knowledge of senescence may therefore lead to a reevaluation of the potential for senescence as a treatment strategy and possibly show that specific inhibition of senescence, with retained apoptosis induction, during treatment with standard chemotherapy is the way forward.

3.2.10 Senescence and aging

When Hayflick discovered that primary cells *in vitro* have a finite life span and enter senescence, one of the first theories that arose was that cells in a tissue would behave similarly and cause aging [2, 3]. According to the theory, these senescent cells have lost their original function and impair organ function. The aging phenotype was therefore the sum of all malfunctions in all organs that the senescent cells cause. While plausible and accepted among many researchers, the data is not convincing:

The theory dictates that the number of senescent cells increases with age in tissues and several attempts have been made to detect such an increase. Initial studies established cultures of primary cells from differently aged donors and measured their replicative life span. Some studies managed to find a decreased replicative lifespan from older subjects while others did not [204, 205]. However this approach may not be valid as there will be a clonal expansion of the cells with the longest telomeres and although there are senescent or close to senescent cells in the population, these could be difficult to detect. Another approach is to look for a decrease in telomere lengths as a function of life span and take this as an indication of a replicative decline in the tissue. When combining all such efforts the conclusion was that although the main differences in telomere lengths depend on the individual, there is a gradual decrease of the telomeres with age in some organs [206].

Neither of these approaches demonstrates that the senescent cells actually accumulate in a tissue. Therefore, several attempts have been made to identify an increase in senescent cells with age but the main setback of this approach has been the lack of markers for senescence, except the commonly used SA-βGAL. An increase in SA-βGAL staining cells with age has been detected in human skin [7] and in mouse kidney [207]. In the human study only cells close to the hair follicle were stained, while the whole kidney stained blue in the mouse study, which would indicate that almost all cells in the kidney were senescent. An alternative explanation in both these studies is a lack of specificity of SA-βGAL that has been described [208]. SA-βGAL staining cells have also been observed in mice after chemotherapy treatment [193], after liver hepactomy in third generation telomerase-activity deficient mice [209] and in a knock-out mouse for Bub1 that shows an accelerated aging phenotype [210].

Therefore, if one believes that SA-βGAL is a valid marker for senescence *in vivo* there seems to be evidence for senescent cells *in vivo* of the mouse but not humans as the increase of SA-βGAL cells in skin [7] could not be confirmed [208]. Interestingly both humans [211] and worms [212] with longer telomeres show extended life span. The human telomeres were measured from blood samples and the extended life span may at least partly reflect the immune system, as the increased mortality with short telomeres was attributed by the authors to death in infectious disease and increased heart disease. The extended life span in worms was somehow related to the main survival pathway in *C. elegans* controlled by DAF-16.

The best link between senescence and aging comes from a premature aging syndrome called Werner syndrome. Werner syndrome patients die at an median age of 47 with myocardial infarctions and cancer and show several signs of accelerated aging [213]. The Werner syndrome arises as a consequence of mutations of the Werner protein which is a RecQ DNA helicase [214]. Interestingly, primary

fibroblasts from Werner patients senesce early and show similar expression patterns as normal senescent cells indicating that the early senescence may drive an accelerated aging phenotype [215]. Several lines of evidence indicate that the early senescent phenotype is related to an inability to maintain the correct telomere structure/length as the Werner cells can be rescued from senescence by overexpression of telomerase [216]; a third generation telomerase activity deficient mouse with a Werner mutation shows an accelerated aging phenotype [217]; the Werner protein is associated with the telomere and can bind to TRF2 [218, 219]; and cells with the Werner mutation lose their lagging strand telomere at a high rate [220]. It has also been reported that the average telomere length in Werner cells is not different from normal cells [221] but that could be explained by the observation that some lagging strand telomeres are very short while others are normal [220]. Several other functions of the Werner protein has been reported including transcription [222] but it seems likely that it is the function related to the telomere structure that drives the early senescent phenotype and possibly also the early aging phenotype. There is no data suggesting that Werner patients have more senescent cells in their tissues but given the functions of the Werner protein the effects could be in stem cell compartments that would be depleted from replicatively competent cells.

In summary there is no substantial data showing that senescence drives aging or is accumulated as a function of age in humans. Senescent cells have been detected during aging as well as in an accelerated aging phenotype and some other conditions in mice, indicating that they could exist. Interestingly our data supports this species difference as the senescence transcriptome was found to be similar to that of mouse but not human aging [183].

3.3 APOPTOSIS

3.3.1 Characteristics

While senescence is characterized as a relatively undefined process, apoptosis on the other hand is well defined and conserved from *C. elegans* to mammals. Apoptosis was initially described as a series of morphological steps leading to the elimination of the cell without inflammation [223]. During apoptosis, the nuclear DNA is condensed and fragmented, the cell shrinks and is fragmented into several peaces that are phagocytosed *in vivo*.

3.3.2 The apoptosis pathways

There are two main pathways that induce apoptosis, the intrinsic and the extrinsic pathway. The extrinsic pathway is induced by ligand binding to the TNFR1 [224], FAS [225] or DR4/5 [226] receptors. The receptors, exemplified by the FAS receptor, undergo a trimerization upon ligand binding which enables assembly of DISC (death inducing signaling complex) [227]. An adaptor binds to the receptor

and recruits pro-caspase-8 which then becomes activated. Activated caspase-8 can both activate pro-caspase-3 and pro-caspase-7 and/or converge on the intrinsic pathway through cleavage of BID.

The intrinsic pathway is regulated by the BCL-2 family of proteins, which are either pro- or anti-apoptotic [228]. The BCL-2 family proteins affect the stability of the mitochondrial membrane and the balance of pro/anti-apoptotic proteins determines if cytochrome-c will be released from the mitochondrion or not. Released cytochrome-c can bind to apaf-1, which acts as an adaptor molecule for both cytochrome-c and pro-caspase-9. Activated caspase-9 activates caspase-3 and this represents a point of no return during the induction of apoptosis [229].

There are several members of the BCL-2 family of proteins. The anti-apoptotic BCL-2 and BCL-XL inhibits the pro-apoptotic BAK and BAX by binding to the BH3-only family of proteins and thereby inhibit oligomerization of BAK and BAX [230]. When active, BAK and BAX probably form a pore in the mitochondrial membrane and cytochrome-c can be released. The balance can be shifted by induction several proteins of the BH3-only family proteins which induce oligomerization of BAX/BAK and thereby release of cytochrome-c [228]. The BH3-only proteins can be induced or activated by different pathways. As mentioned above, the extrinsic pathway leads to a cleavage of BID into tBID. Similarly, p53 induces transcription of the BH3-only proteins NOXA [231] and PUMA [232] following DNA damage, while BIM is induced under several conditions [233].

3.3.3 Translational control of apoptosis

Inhibition of apoptosis is one of the hallmarks of cancer [111]. Apoptosis inhibition can be achieved by a variety of mechanisms e.g. overexpression of BCL-2. This occurs in follicular lymphoma [234], but essentially any mechanism that inhibit activation of apoptosis can promote cancer progression. A member of the translation initiation machinery, translation elongation initiation factor 4E (eIF4E), has been identified as a anti-apoptotic molecule [235]. eIF4E is believed to be the rate-limiting factor for initiation of translation [236] and the activity of eIF4E is tightly regulated through its binding proteins, eIF4E-BP1, -BP2 and -BP3. These binding proteins bind to eIF4E when they are hypophosphorylated, thereby inhibiting formation of the trimolecular complex eIF4F (which mediates translation initiation). eIF4F consists of eIF4E, which binds to the 5´ of the mRNA, eIF4G which acts as a scaffolding molecule and eIF4A which is an ATP dependent helicase that unwinds the mRNA. Both the AKT and the RAS pathway can activate eIF4E by phosphorylating the binding proteins and thereby release eIF4E and promote formation of the eIF4F complex [237].

There are several lines of evidence for an important role for eIF4E during cancer progression. Overexpression of eIF4E can transform NIH3T3 cells [238]. Further,

eIF4E activity is increased in all transformed cell lines tested [239], and so far, in tumors of breast [240, 241] and colon [242]. It is likely that an important activity of eIF4E in transformation is mediated by its anti-apoptotic activity as it can inhibit Myc mediated apoptosis [243] by inhibiting cytochrome-c release [244] and cooperate with Myc *in vivo* to promote tumor formation [116]. It is likely that eIF4E has a general function in inhibition of apoptosis as it also inhibits apoptosis induced by endoplasmic reticulum stress [245], serum starvation [235] and various chemotherapeutic drugs [243, 246]. Recently eIF4E was found to be an important downstream target of survival signaling from AKT in murine cells [116, 247] and essential for a malignant phenotype in human epithelial cells [248].

What then is the mechanism of eIF4E mediated apoptosis resistance? It is known that higher activity of eIF4E will lead to an dramatically increased translation of transcripts that show low affinity towards eIF4E, while the majority of transcripts are affected in a moderate manner [249]. Therefore, one possible anti-apoptotic mechanism is that eIF4E induces over-translation of some proteins with anti-apoptotic functions. In support of this, activation of AKT affects the transcription of only a few genes but induce overtranslation of several proteins probably through activation of eIF4E [250]. Similarly, we established that overexpression of eIF4E induces overtranslation of a set of transcripts and found that some of these mediate the anti-apoptotic functions of eIF4E [251]. However, the identity of the transcripts that mediate the anti apoptotic effects will be dictated by which mRNAs that are expressed in the cell. It is therefore likely that each tumor type will have a specific mechanism of rescue from apoptosis mediated by eIF4E.

3.3.4 Senescence vs. apoptosis

As described above, replicate senescence involves a DNA damage response that is also capable of promoting apoptosis; so why is senescence the outcome of telomere instability? The first thing to point out is that there is little evidence of senescence in human tissues, so far senescent cells have only been detected in skin of elderly people [7], a finding that could not be reproduced [208].

One obvious mechanism that could regulate the choice between senescence and apoptosis is if the apoptotic process was inhibited and senescence occurred instead as a default mechanism. The mitochondrial anti-apoptotic protein Bcl-2 has been proposed to represent such a mechanism. Unexpectedly, overexpression of Bcl-2 has been shown to induce senescence, judged by SA-βGAL staining, yet this may more resemble quiescence as p27 was overexpressed [252]. Bcl-2 can also accelerate RAS-induced senescence to some extent [253]. In support of the hypothesis, Bcl-2 has been described to shift the response from apoptosis to senescence when artificially overexpressed in rat cells [254].

In the report describing a shift from apoptosis to senescence upon Bcl-2 overexpression, p21 was found to be overexpressed. In fact, this could be the reason for the shift from apoptosis to senescence as p21 expression after DNA damage lead to senescence while absence of p21 induction after DNA damage lead to apoptosis [255]. Similarly, apoptosis was associated with low p21 levels whereas senescence was associated with high p21 levels in a cancer cells treated with interferon-γ [256]. If p21 decides if the response, downstream of p53induction, will be senescence or apoptosis, then an important question is why p53 sometimes induces p21 expression and sometimes not. Some of the regulation could be a result of the convergence of several pathways that directly regulate p21. For example both Miz-1 and CUGBP have been described to affect the transcription and translation of p21 respectively [255, 257]. Similarly, cisplatin can induce growth arrest at low concentration and this growth arrest is associated with translational activation of p21 by an unknown mechanism (George Thomas, personal communication). It is also possible that the decision, of whether or not to induce p21, occurs at the level of p53 activation. Interestingly, the phosphorylation patterns of p53 during induction of senescence and after a DNA damage treatment leading to apoptosis seems to differ [258, 259]. The question would then be what regulates the differential phosphorylation of p53 during senescence and apoptosis. Interestingly, there are some indications of how could be achieved. It appears that a large DNA damage response leads to apoptosis while a low but persistent activation of p53 induces senescence. For example, upon hydrogen peroxide treatment both senescence and apoptosis are possible outcomes; apoptosis was associated with higher levels of p53 and low levels of p21 while senescence was associated with lower levels of p53 and higher levels of p21 [153]. Similarly a TRF2 mutant that cause telomere dysfunction induced apoptosis or senescence, depending on the expression level and thereby the extent of telomere damage [59]; and substantial overexpression of p53 induced apoptosis while lower overexpression induced senescence [41].

In summary, it seems like p21 and the nature of the p53 response is the major determinant whether the p53 response will induce apoptosis or senescence. The differential regulation of p21 needs to be further clarified.

4 MICROARRAYS AS TOOLS TO STUDY CELL-FATE

4.1 INTRODUCTION TO MICROARRAYS

Going from studying the expression of one gene to all genes is an incredible improvement that has taken science to a new level. One can even say that the possibilities opened up by the methodology have driven a new approach to biological science. For example, it is not a hypothesis about a certain gene and function that drives the scientific process but rather, the methodology is used to generate new hypotheses by studying a phenotype and ask what genes are responsible for a given characteristic. However it has also created problems. The literature is contaminated by numerous data sets where the researcher has not understood the limitations of the approach. Part of the problem reflects the costs of performing a microarray study. Thus even though the results in terms of quality, would have been unacceptable to the researcher for a traditional experiment, the studies have still been published.

The microarray methodology is based on specific hybridisation of the four deoxyribonucleic acids described by Watson and Crick in 1953 [260]. In principle it is a scale up of southern blotting [261] or northern blotting [262] on a solid support [263-266]. Other methods have been developed to monitor large scale changes in expression including differential display [267], serial analysis of gene expression (SAGE) [268], suppressive subtractive cloning [269, 270] and cap analysis of gene expression (CAGE) [271]. In one sense the massive sequencing effort by the RIKEN consortium can be viewed as a qualitative expression study of the mouse [272, 273]. None of these other methods have the same potential as microarrays both in terms of cost and scale.

To run a successful microarray study it is vital to have a stable model system where the phenotype is as pure and defined as possible. Otherwise, secondary phenotypes can dominate the output. This is critical because typically over 10000 genes are studied at the same time, with limited prior knowledge of what to expect. To identify thousands of genes in a study as a result of an undefined phenotype is usually not ideal if one wants to pursue any of the genes in functional studies. It is possible to construct experiments that simulate likely confounding factors and then use these experiments as control experiments in the primary study. However, multiplying this approach is limited by the noise in each study and introduces an additional risk to lose important genes as a result of technical aspects.

The second undesirable scenario from a microarray study is that nothing is found as a result of a badly designed experiments or poor quality biological materials. One should also keep in mind that almost all data analysis approaches assume that most genes will be unchanged. If the study involves a phenotype that is not believed to apply to these criteria difficulties in normalisation could result. Another assumption

in the microarray method is that the phenotype needs to be defined by altered mRNA levels. Most phenotypes have an mRNA component but some aspects are probably only seen on the protein level. If the phenotype studied is more likely to be dominated by changes in protein modification or translational control, the standard microarray approach will not be useful.

In general, if a study is well designed, well informed and used to study a system where a reasonable amount of expression changes are expected, microarrays can help to generate a new layer of information previously not possible in biological sciences.

4.2 PLATFORMS - OVERVIEW

There are two main platforms for construction of microarrays: deposition of pre-synthesised materials and on-chip synthesis of DNA. Deposited materials can be in the form of cDNAs at a length of 200-800 nucleotides (nt) or oligos usually 50-70 nt long. Attachment of the DNA is achieved either by covalently binding of the oligos or by electrostatic interactions between the DNA backbone and the support material. On-chip synthesis was developed by Affymetrix and first described 1996 [263, 264]. Currently, this principle for making microarrays is used by several companies such as NimbleGen and Xeotron that circumvent the patents filed by Affymetrix in various ways [274].

4.3 DEPOSITION METHODOLOGIES

4.3.1 Background

The deposition methodology development was initiated in 1995 by Schena et al [265, 266]. At the time it represented an interesting alternative to the current dot-blot methodologies in terms of future large-scale possibilities. Running micro arrays during the following years was not easy as one had to build an arrayer to deposit the materials on to the slides, as well as to cope with the poor immobilisation chemistries. The most popular attachment surface - Poly-L-Lysine - was originally developed for tissue sections and “witch-craft” like skills were needed to produce a good Poly-L-Lysine coating. The slides that were commercially available from Sigma were of low and variable quality. Due to the nature of the attachment, a good blocking of the surface surrounding the deposited materials was also needed but also hard to produce uniformly and reproducibly. Therefore, various attempts were made to develop chemistries and surfaces that would make the process more reproducible and improve the quality in terms of signal to background levels. This led to the development of a few chemistries around 2000 including our approach [275] and other [276-278]. However, companies like Corning were also highly active and introduced their GAPS series of which there have been several versions. The GAPS slides rely on electrostatic interactions, but is much easier to block compared with Poly-L-Lysine slides. The current version is good and highly

popular as most of the previous issues have been solved. These improvements make today's research using deposition microarrays less difficult although some problems still remain.

4.3.2 Technical aspects

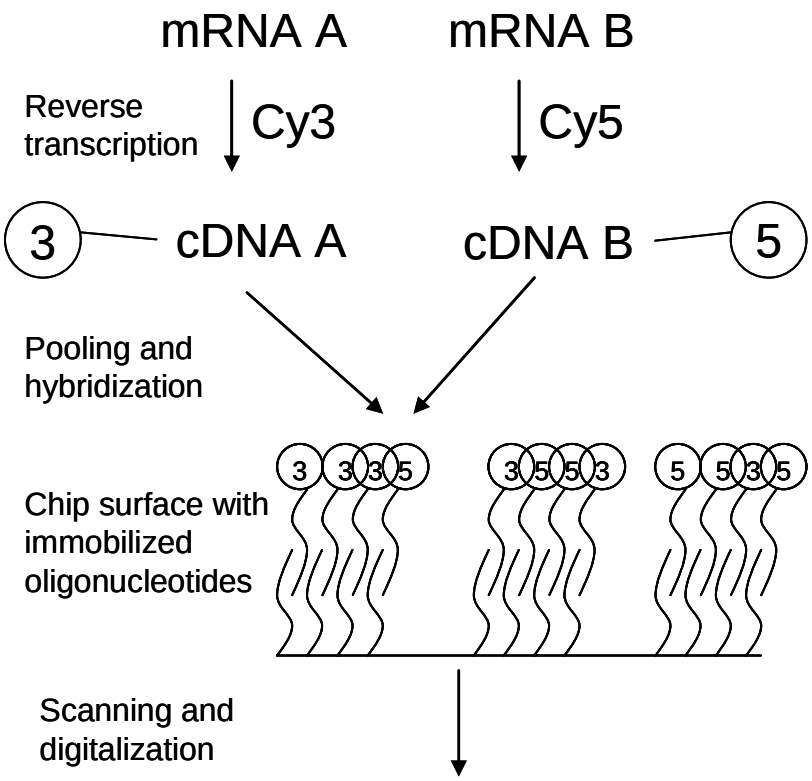
There are several environmental aspects that can severely limit the success of a microarray experiment. Both ozone and high humidity can quench the fluorophores, especially Cy5, used to label the cDNAs [279]. Currently, it is therefore advisable not to run cDNA chip studies that rely on Cy5 during the summer, unless the room environment is controlled. Alternatively, there are some ways to stabilise the Cy5 in high ozone, using "dye-savers" available from several companies, but this adds another possible source of variation. Another important factor is dust, which can severely increase the rate of false positive and negative results by appearing as spots on the slides causing problems during the image analysis stage.

Initially, only cDNAs were deposited on the microarray. These were either amplified from a random or sub-random library (like a subtractive library) or from a clone collection like the NIH15k collection. Today, oligos are popular as a lot of the tedious work needed for cDNA preparation is omitted (PCR, PCR-cleanup and dilution of the PCR-product at a standard concentration) and the risk to contaminate the stocks is no longer an issue. There are oligo collections sold as libraries that cover a high percentage of all known and predicted genes from several companies (e.g. Operon). The oligos can be deposited onto GAPS slides as well as used with amino-allyl coated slides and seem to be more sensitive then cDNA chips [280]. One obvious advantage of oligo based chips compared to cDNA chips is that it is possible to design oligos that are specific for the measured gene. A cDNA might not only target the gene it is believed to target, but potentially a whole family of genes.

The material is deposited from 96- or 384-well plates using a robot with typically 24 or 48 pins. Production of good quality slides is challenging and requires a lot of patience in terms of optimisation of e.g. washing steps, settings of the robot and combinations of similar pins. Extensive quality control is necessary throughout the process to ensure that the obtained slides are of high quality. Even so there is still a large risk that slides of low or variable quality will be obtained. There are indications that academic arrays are considerable less consistent compared to commercial arrays [280].

4.3.2.1 Running an experiment

Competitive hybridisation is usually performed when deposition chips are used. There is a need for competitive hybridisation as the amount of material that is deposited by the pins can vary by more then 100% from slide to slide. In the competitive approach, the two RNA (total or mRNA) are separately reverse-transcribed in the presence of nucleotides bound to fluorophores (usually Cy3 and



Gene	mRNA A	mRNA B
A	3	1
B	2	2
C	1	3

Figure 5: A competitive hybridization using on-chip synthesis microarrays.

Cy5) or modified so that that the fluorophores can be bound after the cDNA synthesis is performed. The two differently labelled cDNAs are then mixed and compete for binding to the cDNAs/oligos that have been deposited onto the surface. The hybridisation is performed under conditions that are optimised in terms of stringency so that the desired sequence binds with high specificity. After hybridisation for 12-18h the chips are washed and scanned using a confocal scanner at 10 or 5 μM resolution generating two different images in 16 bit TIFF format (Fig 5).

4.3.3 Study design

If the deposition methodology is selected for a study there are some important aspects related to the nature of the technology that needs to be considered. The competitive approach has two major set-backs. First, as the two labelled cDNAs are left to compete for binding, the sensitivity is reduced to half. Second, if mRNA species is present in one of the populations but essentially missing from the other, there is no competitive hybridization. This has drastic consequences, as the analysis

methods are based on the comparisons of the ratios between the two fluorophores. If a close to background signal is achieved from one of the cDNA population, the ratio is likely to be variable and the gene will therefore be lost whenever applying statistical tests. This is one of the most important reasons for using a reference design compared to the direct design (Fig 6). In the reference design, each sample competes for binding with a third population of RNA, which is used as a link between the two or more populations of interest. The reference should ideally bind to all probes on the chip and thereby ensure that stable ratios will be obtained. The reference can either be a mix of all the mRNA populations to be studied or a mix of different cell lines and tissues. The reference design becomes more and more useful, the larger the study as direct comparisons increase the number of chips needed dramatically. Also, if one uses the reference design it is always possible to add an extra sample to the study as long as it is possible to reproduce the reference RNA.

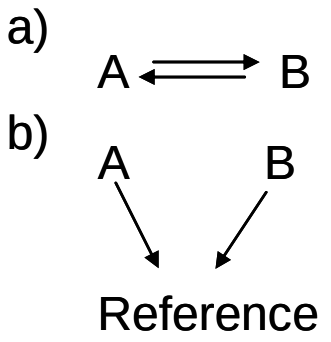


Figure 6: A direct (a) and an indirect (b) comparison of two mRNA populations.

A second important aspect of the study design is replication. It is difficult to assess how many replications that are needed in micorarray experiments. Ideally it would be good to have 6-8 independent measurements from each comparison [281-283]. However, due to the cost, this is usually not possible and 2-4 independent replicates are used. However, if most expression changes will occur among the low expression genes, it is essential to have more replicates.

4.3.4 Data extraction

After the competitive hybridisation and scanning, two TIFF images are generated and digitalized. Several software packages exist to analyse the images, including ArrayVision, Quantarray, Imagequant and Spot. Initially a grid is created using the theoretical measurements of distances between the spots and the number of spots in each grid. Then, algorithms attempt to either use a fixed or variable circle to allocate each of the spots. However, no spotting-robots are able to produce a perfect chip where every spot is located at its theoretical position. Therefore, an algorithm moves the circles from the theoretical location to look for local maxima. This creates a problem as the risk of identifying signals that are appearing from dust or other artefacts increase as the allowed distance for searching for local maxima increase. One way to address this problem is a manual correction. While this approach seems reasonable on a spot to spot basis, it introduces another layer of variation and a high workload. It is clear that it is not possible to manually perform a consistent correction across tens or hundreds of slides. The approach that should be used is to optimise the settings that are available in terms of distances that are allowed from the theoretical location as well as sizes that are accepted to define a

spot. The same settings should then be used for all the arrays used in the same batch/study. As a result it is advisable that all arrays in the same study should come from the same batch, otherwise different optimizations of the image analysis is needed, leading to an increased risk of variation.

It has been suggested that multiple scanning (three times) of the same chip to remove variation from the scanning process and image analysis is an advantage for downstream analysis, although it considerably increases the time needed for data generation and extraction [284]. Another important point about the scanning procedure is the need to obtain comparable images from the two channels. Although less recognised, my experience is that this can determine whether a study yields a publishable data set. This aspect is now incorporated into most modern scanner programs but can also be achieved by obtaining a few scans of different intensities. These images, from the Cy3 channel and the Cy5 channel, can be compared to match overall signal intensities. Our experience is that there should not be more then a 2-fold difference between the sums from the Cy3 and Cy5 channel [251]. The reason behind this is that a high scan in one channel, matched by a low scan in the other channel, will not generate any additional information, instead it will cause problems during the normalisation by contributing to the local mean (see below) [285].

Another aspect that can severely affect downstream analysis is background subtraction. Historically it is claimed that the signal observed has two components, one from the background and one from the hybridized cDNA. Most programs create an area surrounding either the individual spot or a part of the array to approximate the background. However, the surface in the spot is not identical to the surface surrounding the spot and it is therefore not certain that it provides a good approximation of the background. In fact, spots can show lower signals compared to the background, generating negative spots. This clearly indicates that the background surrounding the spot is a bad approximation of the real background. A second consideration about the background comes from downstream data analysis approaches. Low numbers or even negative numbers are difficult to handle as unreliable large ratios are created when a signal is compared to a signal that is approaching zero. If the background is left as a signal that can be used in a ratio, it may be possible to use data from a gene where a signal only appears in one channel. In addition, just the addition of another round of data collection for background subtractions adds another source of variation and is likely to increases the noise in the data set. It is therefore not advisable to use background subtracted data if the technical standard is high.

4.3.5 Normalization

Variation in microarray experiments can be separated into biological and technical variation. A biological variation can arise as no biological system is static and gene

expression fluctuates over time, for example as a consequence of forward and backward loops [286]. However, the effect of these variations should be limited on a cell-population basis but may apply when microarrays are used to analyse gene expression in single cells [287-289]. Factors outside the cell are more important and may give rise to systematic biological variation. The main factors for *in vitro* systems include growth conditions such as confluence, media and temperature. For *in vivo* studies there is almost an endless number of possible sources of variation relating to that different aspects e.g. that different individuals show different signatures [290]. Even if we assume that all persons are closely matched and almost identical in their gene expression signature, problems may still arise e.g. because of timing of sample donation in relation to sleep and meals [291]. Technical random and non-random variation can arise at all steps of the microarray procedure including RNA preparation, cDNA labelling, cDNA purification, hybridisation, scanning and data extraction.

Normalisation is an attempt to correct variation but is limited to variation that affects all genes measured in a similar manner or all genes within a defined intensity range in a similar manner. All other variation can only be controlled by replication. The first approach used for normalization applied the standard procedures in e.g. western blotting, and normalized to house keeping genes. The assumption in the house keeper approach is that the expression of the house keeping gene(s) is stable across all conditions. There is a fundamental difference between using a house keeper when studying one gene in one northern or western blot and studying thousands of genes on a microarray. While small true or random differences of the house keeper abundance are less likely to have effects during a northern blot it may severely change the output from a microarray study. As a result, another approach was proposed called global normalisation. During the global normalisation, the average ratio is set to 0 on the $\log_2$ scale. The major assumption is that the average true ratio is 0 ($\log_2$). During the period when global normalisation was initiated this was usually not valid as small arrays with less then 1000 genes were used. Therefore, the concept of using spike-in controls emerged. In the spike-in approach, a set of external RNA species are added to the labelling reaction and are used to set the channels at the same level. The problems are obvious as one assumes that exactly the same amount of RNA from both the samples and spikes are used in both channels. The bias with spike-in normalization is therefore probably similar compared the house keeper approach.

Since microarrays became larger, the global approach has become more valid and can still be used, if the experiment is performed well. The main problem with the global approach is the non-linear, non-random variation observed in some studies that seems to be dependent on the intensities of the two channels [285]. Therefore, an intensity dependent normalisation strategy was developed to normalise the ratios of the two channels using loess regression. In the loess approach, the normalisation

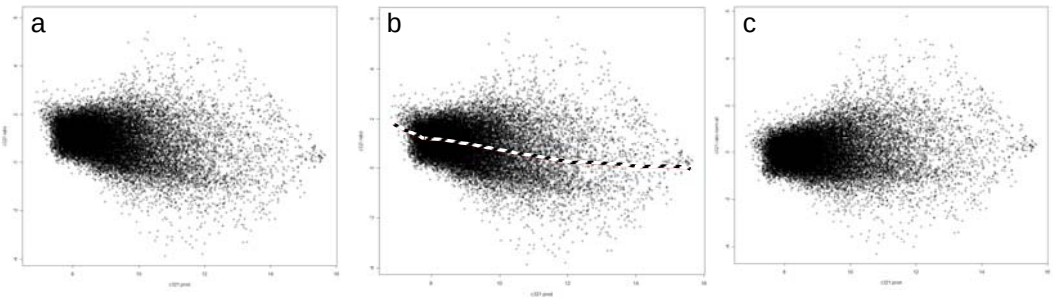


Figure 7: Normalisation for deposition methodology chips; x-axis: $\log_2 \sqrt{(G*R)}$; y-axis: $\log_2$ (R/G). **A** The un-normalized data. **B** The un-normalized data with the local regression line. **C** The normalized data

is based on a local regression of the log2 ratios of the two channels ($\log_2$ (R/G)) (R and G are used to describe the two different cDNA populations labelled with different fluorophores that has competitively hybridized on the same chip) as a function of the $\log_2$ square root of the products of the two channels ($\log_2 \sqrt{(G*R)}$) [285]. Loess normalisation can be viewed as a local global normalisation procedure although only a fraction of all data points within a delta $\log_2 \sqrt{(G*R)}$ are used (Fig 7).

Our experience is that the reason for the intensity dependent systematic behaviour is poor selection of intensity-matched images. As stated above, we suggest that the sum of the intensities should not differ more then two-fold. In fact, as the channel sums get more and more similar the loess normalization approaches a standard global normalization. It also seems like some information is lost if excessive loess normalization is needed. A possible reason for this is that the genes with a low $\log_2 \sqrt{(G*R)}$, as a result of small true R and G values, will be grouped with ratios that have a high G and low or close to background R. The normalization will try to fit all the ratios to an average ratio of 0 and some true ratios will be "dominated" by the ratios arising from bad image selection. Also, there is no intuitive reason to believe that $\log_2 \sqrt{(G*R)}$ should be optimal to separate the genes on the x-axis. One effect is that the images used for the normalization should have similar overall quality throughout the study. If the quality varies across the arrays in the study, the researched will lose information and the output will partly be dictated by the lowest quality chip (see below – Quality control).

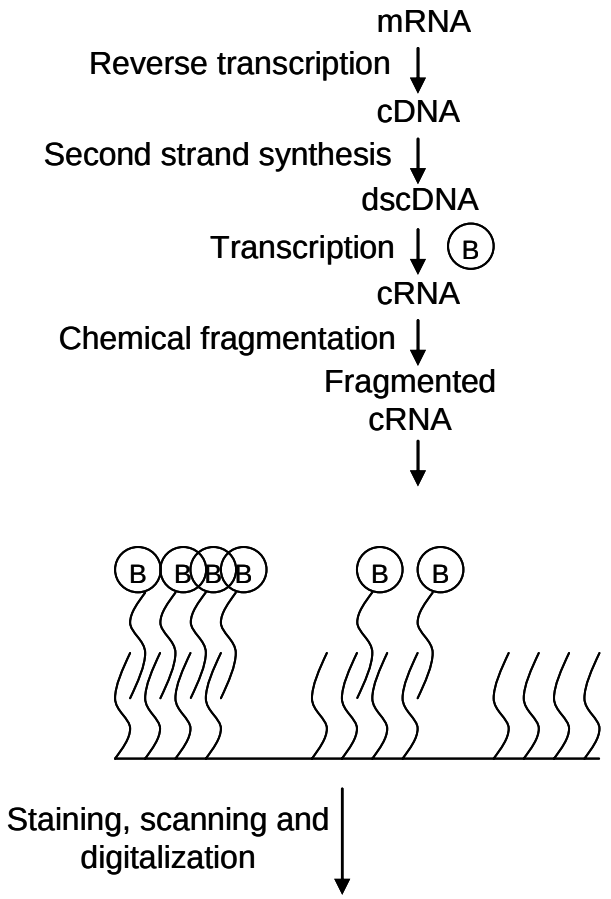
4.4 ON CHIP SYNTHESIS

4.4.1 Background

The on chip synthesis method was relatively mature when it was launched [263, 264]. This was a result of the industrial production needed to produce the arrays as well as patents that enabled the platform to emerge homogenously. Initially, it was essential to use chips within the same batch due to inconsistent synthesis. Other problems involved how to deal with the amount of information obtained from each gene (see below). For several years Affymetrix was the only company producing on-chip synthesised chips but today several companies use variations of the theme [274]. I will only describe the Affymetrix platform as it is the most commonly used.

4.4.2 Technical aspects

In the on-chip synthesis methodology the oligo is synthesized directly onto a solid support. The continuous elongation of the desired oligo depends on spatial restricted photolithographic chemistry. The nucleotides are protected by a photolithographic group which are de-protected upon light exposure. For each round of elongation, a photolithographic mask is used to direct the light to the desired feature and the protected nucleotides are allowed to couple with the de-protected oligo-nucleotide. Once the nucleotides have bound to the oligo, the surface is washed and the second round starts with a new mask and another nucleotide. Initial problems were related to the slow generation turnover as production of the mask was the most expensive process in the methodology and changing only one of the oligos meant that all/most masks had to be redesigned. On the other hand this was also an advantage as the same chip-version was used by several labs during a few years and thoroughly validated. The labelling procedure differs between the deposition methodologies and Affymetrix mainly as a result of the non-competitive approach that can be used on this platform. This is due to high reproducibility in terms of amount of oligo synthesized at each feature initially across chips in the same batch but now also across batches.



Feature	Signal
A (PM)	4
B (MM)	2
C (PM)	0

Figure 8: A hybridization using the Affymetrix on-chip synthesis platform

In the Affymetrix approach, the RNA is reversed transcribed using a bacterophage T7-oligo-dT primer and the second strand of the cDNA is synthesized. The cDNA is transcribed by the T7 RNA polymerase in the presence of biotinylated nucleotides to generate cRNA. The cRNA is chemically degraded to generate

fragments with a size of 35-200 nt to enhance the binding to the short oligos to the chip. Hybridisation is carried out overnight. The chip is washed and incubated with fluorescently labelled streptavidin, a biotinylated anti-body that binds to the biotin-streptavidin complex followed by another fluorescently labelled streptavidin to amplify the signal. The chip is scanned with a confocal scanner (Fig 8).

4.4.3 Study design

A study design for Affymetrix arrays has fewer issues compared to the deposition methodology as a measurement reflecting the absolute amount of a transcript is achieved, which is easier to use in downstream analysis (see below). All aspects relating to the need to obtain a stable ratio, and not comparing samples where all or nothing differences are expected directly to each other, are circumvented. Also, the correlation of technical replicates is almost perfect which lowers the number of chips needed. However, Affymetrix is not always a better choice. For example, if only two samples are compared to each other and the direct comparison could be used, the Affymetrix approach doubles the number of chips needed. Also, there is more flexibility in the on-chip synthesis approach as one only has to order a new oligo and include it in the next batch of chips if a particular gene appears to be interesting. Therefore, depending of the study design and the budget, it can sometimes be better to use a deposition chip compared to the Affymetrix platform.

4.4.4 Data extraction

The image analysis process for Affymetrix arrays is more standardized and automated compared to the on-chip deposition methods. There are a few fixed positions that the image analysis program uses to automatically allocate itself on the right position of the image, but still some experience is needed to make sure that the grid is positioned correctly. As the mask process is highly reproducible the problems in the deposition methodologies regarding imperfect location of spots is not an issue. Each of the spots (called features) consists of 100 or 50 pixels and the software uses the central pixels to quantify the signal intensity. Progress is being made towards reducing the size of the features further and thereby increasing the number of genes that can be fitted on one chip. The output from the process is a .CEL file, which is a file with all features and their respective signals.

4.4.5 Normalization

While the deposition approaches typically generate one signal for one gene, each gene on an Affymetrix array is represented by 11-20 oligo sets (25 mers) with a perfect match oligo to the target (PM) and a mis-match oligo at position 13 (MM) [292]. All features need to be summarized to obtain a measurement for a particular gene, but the question has been how to achieve this. The first algorithm was developed by Affymetrix (MAS 4). In MAS 4 the 11-20 differences between the PM-MM oligos in each pair are summarized to yield an average difference

measurement for each gene. The idea was that the MM sequences approximate the non-specific binding of the PM oligo and the difference between PM and MM represents true abundance. There were severe problems with the approach as one third of the MM oligos produced higher signals compared to the corresponding PM oligos [293]. This generated negative expression values and it has been questioned whether the approach was valid at all.

Therefore Affymetrix proposed a new algorithm (MAS5). MAS5 uses a One-Step Turkey's Biweight estimate of the average PM-MM in log scale. The One-Step Turkey's Biweight estimate reduces the effect from outliers and an additional step was introduced where negative PM-MMs were replaced by switching the true MM to an estimated MM. While MAS5 was good in terms of not producing negative expression values it was criticized for leaving out some of the best potential estimators of gene expression levels. Depending on secondary structures and base composition of both the target oligo and the probe, it is not surprising that some oligos produce higher intensities compared to others, even if they measure the same transcript. For low expression genes, only a few of the oligos are able detect the probe and these are potentially removed as outliers. This obviously led to an underperformance in assigning expression levels to low-expression genes.

The complexity of the output from the Affymetrix platform attracted a lot of attention and several algorithms have been proposed from academic sources. One such method is Robust Multiarray Analysis (RMA) [293-295]. After background subtraction (2% of lowest intensity features), RMA uses log expression values and ranks all features of each array. Thereafter, the average of each rank position, across all arrays, substitutes all values at that rank (Fig 9). This provides a normalization of all arrays in terms of their signal distribution from highest to lowest. Then, the features for each gene are summarized without using the MM oligos. The advantage of the methodology is the robustness especially at the low expression genes. However, the dynamic range in terms of relative expression levels is reduced. This occurs as RMA does not try to separate noise from the signal and therefore a proportion of the signal that is affected by a "real" change in expression could be dominated by noise. The result is that one of the layers of information from the microarray is corrupted. The problems is illustrated by relative expression changes not being compared easily across genes as different compositions of the signal in terms of percentage of true signal and noise will affect the observed difference. The gain is that a lot of genes that were impossible to detect as statistically differentially expressed in MAS5 or MAS4 can now be identified.

The creators of RMA recently presented a new version called GC-RMA [296]. In this version, some of the MM information is used to remove the noise from each PM probe and thereby improve the relative expression range. Each PM is adjusted based on the MM values with similar affinity, assessed by base composition. The

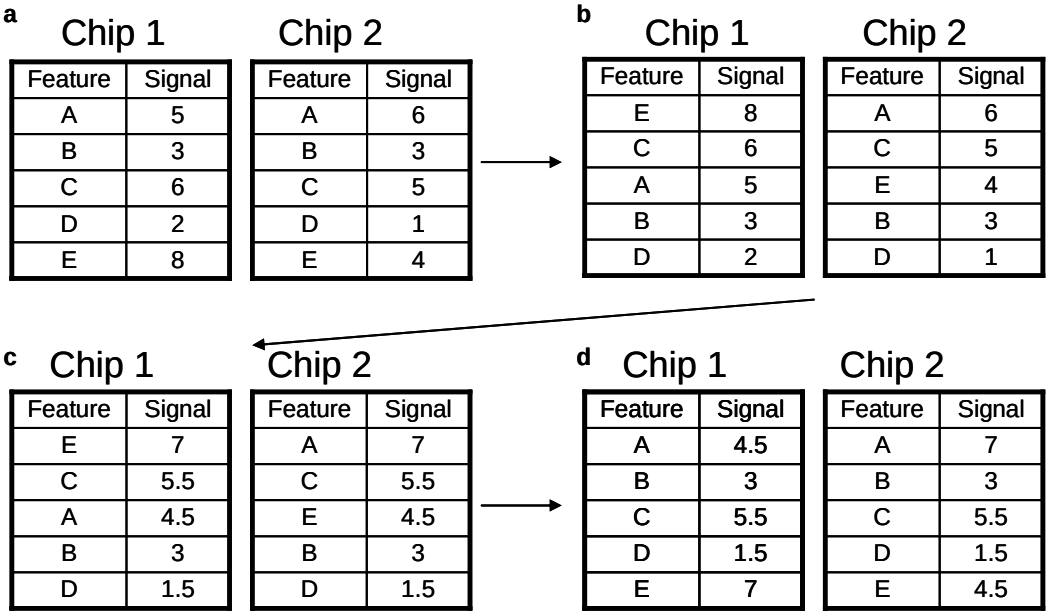


Figure 9: RMA normalization. The raw data **(a)** is ranked from highest to lowest signal **(b)** and the average at each rank substitute the original values **(c)**. After that the order of the data is restored **(d)**.

advantage is clear in terms of restoring the fold change scale without losing too much of the robustness compared to RMA. Interestingly this method only requires in the order of 1000 MM oligos to approximate the background. Currently there are over 100 000 MM features on a standard array [296] and an adaptation to GC-RMA would reduce of the space needed for MMs.

Another algorithm that use sequence information to achieve robust background corrected values is PerfectMatch. However, neither PerfectMatch nor another algorithm called D-chip [297, 298] out-perform GC-RMA [299]. Given the big differences in approaches it may not be surprising that these algorithms will yield different results. In one of our studies we used both RMA and MAS5 and RMA yielded a higher number of differentially expressed genes (about 5 fold) compared to MAS5. However, some of the genes were only identified as differentially expressed using MAS5. We were unable to see any difference in terms of truly differentially expressed genes in validation studies that included both QRT-PCR and literature comparisons [300]. Our somewhat preliminary results indicate that MAS5 and RMA are not equivalent and that RMA out-performs MAS5 on low expression genes, although MAS5 is still able to add expression measures to some genes in a robust manner. The failure of RMA in our comparison could be related to a reduced relative expression range as our analysis strategy included a fold change selection as well as a statistical test.

4.5 DATA ANALYSIS

4.5.1 Quality control

Due to the enormous amount of data generated in a microarray study and the difficulties to assess whether a given change is expected from a biological perspective or not, it is essential to monitor the overall quality of the experiment. As an example, in a cancer study, almost any selection of genes will appear as reasonable if one uses prior biological knowledge from e.g. PUBMED. One way of dealing with the problem is to, before that study starts, identify a set of genes that are expected to be differentially expressed and one set that is not, and then look for them in subsequent data analyses steps. This assumes that there are such sets of genes and that they are efficiently measured on the chip. However, there are other ways to assure that the quality of the experiment is good.

On the Affymetrix platform there are controls to assess the RNA quality, as oligos have been designed to target the 3´, middle and the ´5 end of the mRNA for a few "housekeeping" genes. Also, when several Affymetrix chips are to be paired together, the scaling factor, which is the factor needed to reach a given target signal in the MAS5 global normalization approach can be used to assess overall quality. If the scaling factors differ substantially it indicates that the sensitivities are different that they therefore are difficult to compare. It is also important to look at the images to see that no strange artefacts have arisen.

For deposition chips there are usually no 3´, middle and 5´ oligos/cDNAs but with the emerging oligo approach there will probably be some shortly. Similarly, there are no standardised ways to compare the chips that are brought together in a set but as I mentioned in 4.3.4 there is a need to make sure that the images used in the data-set resemble each other in terms of quality and sensitivity. There are software to look for artefacts on the images from deposition methodologies [301].

In an ideal situation, all samples should be prepared at the same time. Similarly, all mRNA labelling reactions and all hybridisations should be done on the same day. This is usually impossible to achieve and whenever it is not achieved, one could expect differences between experiments that are unrelated to the sample identities. Some methods have been proposed to remove such problems [302] but the most important aspect is to control the data set for such events. This can be done using correlation measures, clustering or dimension reduction approaches (see below). If a big effect is seen depending on something else then sample identity, a part of the study can and should be left out. Even if there are only three replicates and one is substantially different, the sample that is different could be excluded, with an increase in information as a consequence [183]. We have seen that the interpretation of a data set can substantially change by using these simple means to increase the homogeneity of the data set [183]. The exclusion should not be related to any obvious aspect of the samples like fluorophore.

4.5.2 Identification of differentially expressed genes

In most microarray experiments the aim is to identify a set of differentially expressed genes. There are two layers of information in a microarray output, both qualitative and quantitative information. The range of possible fold changes is limited because of several reasons. First, as indicated above for the RMA procedure, some normalization approaches reduces the fold change range. Second, the roof of the dynamic range is often reached. This leads to that a lot of genes can not show high fold difference. Third, outliers might affect the fold change measure a lot. Fourth, a biological meaningful fold change is hard to define and will depend on the specific gene that is studied. Therefore it is important to not base the gene selection on the fold change parameter alone. However, from a biological point of view, it is good if a reasonable fold change is observed if a gene is to be selected for down stream validation and functional assays. Therefore it is common that a statistical test is used in combination with a low threshold fold change.

There is one major problem with the use of classical statistical test in microarray studies, as a lot of measurements are performed with only a few replicates. Parametric tests with a p-value of e.g. 0.05 will by definition falsely identify 5% of genes as being differentially expressed. Usually more than 10 000 tests are performed and 5% therefore becomes a large number of false positives. There are several algorithms that can be used to correct for multiple testing like Bonferroni; Benjamini and Hochberg false discovery rate; and Westfall and Young permutations. However the usual microarray study is not replicated enough to enable multiple correction and the result is usually that no genes pass the multiple correction [283]. This has led to strategies that reduce the number of hypothesizes that are tested. For example, I could decide that I am only interested in genes that show a more then two fold change between the conditions and then run the statistical test. As the number of tests is reduced I would therefore identify a lower number of false positives. The paradox arises as this is statistically different compared to when the statistical test is applied first, followed by a selection of genes that show a two fold difference. The two approaches will generate different number of “true” positives depending on which order the filters are applied when the analysis is combined with a multiple testing correction. This is hard to rationalize and indicates that the approaches to control for multiple testing are not well adapted to the microarray scenario. A test without multiple corrections provides a ranking of the genes that can be used to identify those that are most likely to be differentially expressed. Several measures have been developed to rank genes in addition to standard t-test and ANOVA [282] e.g. “signal-to-noise-ratio” [303-305].

Some nonparametric tests have been developed specifically for analysis of microarrays. One such method is Significance Analysis of Microarrays (SAM)

which approximates the false discovery rate by assessing the real variation within the data set using a similar ranking measure as “signal-to-noise” [306]. However, the SAM algorithm is very sensitive to any pre-filtering of the data. In addition we have found some settings in SAM that can severely change the biological interpretation [307]. Others have tried rank products to assess FDR, which seems to work well [308].

Regardless of which method is used, it is important to remember that some of the information will be lost due to experimental variation. Therefore there is no true list of all differentially expressed genes in an experiment. It is a matter of selecting the significance levels that appear valid for a given data set. Also, a negative result, that a gene is not differentially expressed, is more difficult to interpret. If the negative findings are considered important they should be extensively validated. Currently, identification of differentially expressed genes in microarray studies is sometimes more like an art-form then a rigorous scientific process as a combination of different methods, platforms, cutoffs, pre-filtering and multiple testing approaches can be used.

4.5.3 Clustering and dimension reduction methods

Clustering was proposed as a method to identify genes that share the same expression pattern. The assumption is that genes that show a similar expression profile are also more likely to share similar functions. The approach has been overused as it produces “nice” pictures. An experienced microarray analyser can produce a clustering picture that matches a desired pattern by doing a series of more or less valid modifications of the data. However, the most important aspect of clustering is that there is no statistical assessment of whether two genes really cluster together based on chance or if the similarities are true. A clustering is therefore not an objective way to analyze the data. It is advisable to try a range of settings and methods if one wants to identify genes that show similar expression patterns. It is also important to filter for genes that show differential expression and are truly expressed. Otherwise, genes with very small non-significant differences will be included and influence the overall pattern of differentially expressed genes.

Hierarchical clustering was introduced in 1998 [309] and generates a dendrogram for genes and experiments. The experiment dendrogram is useful as a control method to look for systematic problems within data sets (see above). There is a range of linkage methods (e.g. average linkage) as well as a range of distance methods (e.g. Euclidian distances) and the output usually changes with different combinations of linkage and distance measures. Other clustering methods include SOM [310] and K-means [311] which both requires a pre specified number of clusters to be defined. These methods are therefore more difficult to handle as it is difficult to assess what is the true number of cluster in the data set. Several

improvements of these methods are available but clustering remains difficult to use and should never be used to identify differentially expressed genes.

Dimension reduction methods are used to reduce the complexity of the data set to a manageable number of dimensions that can be visualised. Exactly what the difference is between the samples in the different dimensions is not defined. Dimension reduction methods are useful when the quality of a data set is assessed as biases are usually captured in one of the components. The most popular methods include principal component analysis (PCA) [312] and singular value decomposition (SVD) [313]

4.5.4 Analysis of biological context

Hundreds of genes are typically identified as differentially expressed in the standard microarray study. The challenge of how to integrate all this information into biologically meaningful conclusions is apparent. A lot of the current methods to look for biological meaning are based on gene ontologies. The gene ontology (GO) consortium has created a hierarchical classification of genes from several species [314-316]. The annotation has several layers of significance and one of the high quality annotations is e.g. called “inferred by curator” while the lower quality annotations are e.g. called “non-traceable author statement” or “inferred from expression pattern”.

The goal in a gene ontology analysis is to look for overrepresentation of functions (like biological processes and features), typically in terms of gene ontology terms, in a group of genes compared to a background (usually defined as all genes present on the chip). The analysis produces a table of categories that are overrepresented and should be but in context with the study. There are several programs that can perform the analysis of two different gene lists, one to be tested and the other as background, like Gominer [317] and DAVID/EASE [318, 319]. Similarly to the differential expression analysis, there is a multiple testing issue in a gene ontology analysis as several hundreds or thousands ontologies will be tested for overrepresentation. Therefore, e.g. global false discovery rate are usually implemented. Other methods have been developed that aim to detect small changes in gene ontology categories that may not be identified by standard statistical methods based on differential expression. In these methods small shifts of significance measures (e.g. t-test or signal-to-noise) in the GO group are compared to the global shift of all genes [320-322]. The validity of such methods is questioned as they seem to be unstable and their use is currently under debate [323].

Pubgene is another method to link genes together into groups based on their occurrence in the same title/abstract in PubMed [324]. Pubgene can be combined with gene ontology to generate maps of genes and ontology groups (http://www.pubgene.org/).

4.5.5 Meta-analysis

As the microarray technologies have improved and data has accumulated, there is a large source of public data sets. Similar data sets can be combined to ask questions about how general gene expression patterns are, if different processes are related or if two apparently similar phenotypes really are similar in terms of expression profiles. Also, it is becoming more and more important to compare even a small study to what others have done as part of a manuscript aimed for publication. As described, there are lots of different methods and approaches during all steps of the microarray procedure. Therefore the combination of several data sets from several platforms is a challenge.

Early attempts to compare expression data was based on comparisons of gene lists obtained from different studies. This is a naive approach as different labs use different standards and data analysis approaches. To be able to do a non-biased comparison it is essential to obtain raw data that can be processed in a similar manner. The two main data-repositories, Gene Expression Omnibus (http://www.ncbi.nih.gov/geo/) and ArrayExpress [325-327] are good initiatives in the sense that they have tried to create databases that collect microarray data. To our surprise, data sets that are no longer accessible seems to be a major concern in the field as several researchers claim that their published data sets can not be located [183]. In addition, the data formats that are deposited in ArrayExpress and Gene Expression Omnibus are usually less useful because of data formats. For Affymetrix studies it is essential to have the .CEL files or the .DAT (image file) as these are the only files that have not been processed. Any file with normalized values will only reflect the current normalization approaches. In that sense the Stanford Microarray Database, the largest source of cDNA chip studies (but now extended to Affymetrix studies), is also a disappointment as it has only MAS5 and D-chip normalized data [328]. These issues may seem trivial but are actually essential to enable genuine comparisons between different microarray studies.

There are some examples of within-species meta-analysis in cancer [329-331] as well as cross species comparisons of aging [332] and an attempt to identify genes that are co-regulated across many species [333]. However, to-date there is no hypothesis driven meta-analysis published. Our comparisons of *in vitro* senescence and *in vivo* aging is therefore an important new meta-analysis example [183].

There are two major approaches to a microarray meta-analysis. Either each individual study is analysed in a similar manner to identify a set of differentially expressed genes followed by identification of overrepresentation of differentially expressed genes. The obtained result is compared to a simulation of how often a gene would be found in several studies by chance [330]. The main problem is to decide a threshold, over which genes are called significantly differentially

expressed. An iterative approach has been developed to search for the “right” threshold across all studies that are included [330] but it may be difficult to establish a threshold that is optimal for several studies. Some studies could therefore report an “optimal” number of differentially expressed genes while other studies, due to a higher or lower variation and replication, reports a sub-optimal number of genes.

The second approach is to look for similarities between data sets. This can be done by comparing expression on the same platform [334] but is probably only possible on the Affymetrix platform as deposition chips will vary too much. Large similarities are normally found across all studies due to e.g. expression of housekeepers and it is therefore necessary to filter for those genes that show a difference. Usually one would like to combine data from different deposition platforms, different Affymetrix chips and different species. In this case it is necessary to look at changes in gene expression in each individual study and then study the correlation of the orthologs that are represented [183, 332]. The change in gene expression could either be expressed as a statistical measure or as a fold change measure. It seems likely that although fold changes have several problems, statistical measures could vary even more and are hard to interpret. Importantly, the method is sensitive as most genes will not change and will therefore not contribute to the correlation. This enables the genes that do correlate to dominate the correlation measure. A setback of the approach is the risk to have two principal gene populations, one that shows a good correlation between the conditions and another that anti-correlates and thereby cancels the “true” correlation. It is therefore essential to have conditions that are as similar as possible in all characteristics except the phenotypes that are compared [183]. Both the significant gene approach and the correlation approach should be strengthened by various gene ontology studies to show a non-random similarity [183, 332].

5 RESULTS AND DISCUSSIONS

5.1 PAPER I

Identification of genes downstream of tumor suppressors such as p53 or p16 that are active as inducers of senescence could be important from a cancer treatment perspective. The possibility to induce senescence *in vivo* has recently been reported and highlights the potential of the strategy [193]. In this study, we used a highly controllable senescence model to isolate candidate genes involved in the induction of senescence. In the SV40 T antigen model, a release of p53 and RB from SV40 large T antigen contributes to the induction of senescence and one expects to identify genes that act downstream of p53 and/or RB but also other genes whose transcription is affected by the SV40 large T antigen. In contrast to most other comparable studies we used epithelial cells. This is important from a cancer perspective because the majority of human cancers are of epithelial origin and induction of senescence is known to be cell type specific. The model was established in mouse cells because mouse cells with over-expressed SV40 large T antigen do not enter crisis. Using a mouse cell model to study senescence compared to a model established in human cells could affect the interpretation of the results. However, although the initiation signals for *in vitro* senescence caused by serial passaging might differ between human and mouse cells, execution of mouse senescence is likely to be similar to human senescence [183].

We had two primary goals in our expression study of the temperature sensitive SV40 large T antigen model. The first goal was to study early events that lead to senescence rather than the stationary senescent phenotype and the second goal was to separate primary genes related to the induction of the senescence phenotype from secondary genes related to the growth arrest that is a consequence of senescence. To accomplish these goals we looked at early and late time-points of senescence induction and also made a comparison to cells, growth arrested by high confluence. After removal of genes that are similarly regulated in wt- and temperature sensitive SV40 large T antigen expressing cells, 60 % of the remaining genes were shared between cells arrested by inactivation of SV40 T antigen and by confluence. We identified 125 upregulated and 39 downregulated candidate genes/ESTs that are regulated upon SV40 T antigen inactivation and not during heat shock or confluence and classified these based on their kinetic profiles.

Senescence has been studied using microarray approaches in several other models e.g. a model where senescence is induced by adding doxorubicin to HCT116 cells [335] or a model where a temperature sensitive papilloma virus E2 is used to induce senescence in HeLa cells [106]. Both of these models use human cells but the induced senescence differs largely in terms of cell cycle profile in the senescent population that they generate. Senescence induced by introduction of doxorubicin leads to an arrest in G2 whereas induction of E2 activity leads to G1 arrest. As our

model involves mainly G1 arrest and, like the E2 model, does not involve any external stimuli except for the temperature change (in the E2 model the temperature is reduced) one would expect a larger overlap between the E2 model and our SV40 large T antigen model. To investigate if this was the case, and to study the similarities between the three studies in detail, we compared the genes identified as differentially expressed in these two studies to the genes identified as differentially expressed in our SV40 large T antigen model. Out of 703 differentially expressed genes/ESTs from the E2 study, we were able to link and find an overlap compared to our identified genes for 47 genes. Thirty out of 47 genes/ESTs were down-regulated and as expected, this group included mainly genes related to DNA replication or G2/M phase progression. 17 genes were upregulated in both the E2 model and the SV40 large T antigen model. 13 of these were senescence specific (not induced in confluent cells in our study). When we linked the genes from the doxorubicin study with our identified genes we found an overlap for 19 genes (~5% of our genes). Interestingly all of these overlapping genes were down-regulated in both studies which indicate that there might be a difference between senescence induced by the physiological stimulus used in both the E2 and the SV40 large T antigen model compared to doxorubicin. The down-regulated pool that is shared between the SV40 large T antigen model and the doxorubicin model is characterized by genes that are associated with replication and cell cycle progression like Mcmd, Top2a, and Rrm1 but also to DNA damage responses like Rad51. Only 8 genes/ESTs were differentially expressed (downregulated) in all three studies (the known are Stk5, Mad2l1, Cdc2a, Bub1, Ts (Thymidylate synthase) and Mcmd2) and they probably reflect that the cells are no longer dividing in any of the models as only Stk5 and Cdc2a were repressed more during senescence compared to confluence in our study. The overlap between the SV40 large T antigen model and the E2 model or the doxorubicin model can be summarized by two major points. First, the overlap seems to be greater compared to the E2 model than to the doxorubicin model (11% compared to 5%). This probably reflects the different methods used to induce senescence but the percentage of shared genes could also be affected by the data analysis approaches. Secondly, the genes that are shared with the doxorubicin model are all downregulated hence no similarities in the possible activating genes were found, which is in contrast to the E2 model where several of the shared upregulated genes were signaling genes and potentially represents senescence inducing genes. The overlap of induced senescence specific genes that are shared between the E2 model and the SV40 large T antigen model indicates co-regulation of senescence in mouse and human cells.

5.2 PAPER II

Translational control is recognized to have a central role in normal and malignant cell growth and differentiation. Recent studies have elucidated critical apical steps linking normal and pathological extracellular growth and survival signals through their cognate receptors to signaling intermediates which converge on eIF4E, the

mRNA cap-binding component of the protein synthesis initiation machinery. While a number of investigations have deduced the identity of individual transcripts which are targets for translational activation and mediate aspects of the anti-apoptotic and neoplastic function of eIF4E – no systematic, functionally verified (where the identified genes are shown to affect the phenotype) genome-wide examination of the repertoire of translationally activated transcripts has been reported. We sought to identify those transcripts functioning in the downstream mechanism of eIF4E-mediated apoptosis resistance by combining estimates of mRNA translational efficiency and abundance to inform functional studies using RNA interference, and to identify shared nucleotide sequences in the identified transcripts that might mediate the co-regulation. The proposed approach stratifies the mRNA population of a cell, based on translational activity using polyribosome preparations, a well defined procedure for separating transcripts based on the number of ribosomes each transcript has bound [336], and probes the stratified transcripts with microarrays. Our global analysis of gene expression represents an advance over prior studies of translational control in which RNA from polyribosome preparations has been used to study recruitment of ribosomes to individual transcripts of interest using Northern blotting or real-time PCR (RT-PCR) [336, 337]. In addition, while some prior studies have addressed the downstream effects of activating eIF4E using polyribosome preparations in combination with microarrays; they have used rapamycin as a tool to inactivate eIF4E - an approach that also alters other key cellular functions including ribosomal biogenesis [338, 339] and have not systematically addressed the mechanism of eIF4E–mediated apoptotic rescue.

Using this global profiling of translational efficiency, we identified a set of 244 transcripts whose translation is selectively activated in the context of eIF4E rescue from apoptosis. The translationally activated mRNAs we identified encode a diverse group of regulatory and structural proteins. In principle, our gene list should include those with known - or to be discovered - functions in mediating the potent and pleotropic ability of an activated translation initiation apparatus to regulate cell fate. Based on recently published studies in murine hematopoetic malignancy models and human breast cancer it is now understood that the translation initiation apparatus functions as a master integrator of trophic extracellular signals – both physiological and pathological – mediating their morphogenic, survival and oncogenic function. To orchestrate the output of proteins with such profound and coordinated impact on cell function, we expect that the set of translationally activated mRNA would be heavily biased towards those associated with regulatory functions. In this regard, we found a large number of transcripts for transcription factors and signaling intermediates. In accord with the trophic function of the initiation machinery, we also identified translational activation of mRNA encoding mitochondrial proteins, proteins functioning in intermediary metabolism and components of the translational machinery itself.

Functional studies using RNA interference demonstrate that at least two of these transcripts, those encoding c-Ski and osteopontin, participate in the anti-apoptotic function of eIF4E in our model. These results validate our experimental approach to profile global translational; identify important candidate transcripts as mediators of the anti-apoptotic function of eIF4E; and illustrate the validity of our model to identify transcripts that are associated with apoptosis resistance in other non-transformed and transformed cell systems. These results also lend further support to the pleotropic nature of survival signaling emanating from eIF4E. *c-Ski* is a proto-oncogene involved in TGF-beta signaling and implicated in tumor development. Osteopontin is a phosphorylated acidic glycoprotein involved in mediation of the inflammatory response and may have a role in tumor progression as well. Of note, knock-down of each diminished rescue of cells from apoptosis by eIF4E – but did not ablate it. Thus, our data begin to shed light on specific transcripts subject to translational control that may serve critical functions in mediating the physiological and pathological functions of the translation initiation apparatus. It also has the potential to provide insight into the properties of those messages most influenced by the translational activity state of the cell.

Regulatory elements residing in the 3´ UTR and 5´ UTR of mRNAs have important roles in translational control. We show that no known mRNA elements (as collected in UTRsite) were associated with the efficiency of ribosome loading onto transcripts when cells were rescued from apoptosis by enforced activation of the translation initiation apparatus. However, two novel elements, a 5´ 55-mer and a 3´ 26-mer, were found to be conserved in these translationally activated transcripts; and the 5’ 55-mer was predicted to fold into a secondary structure. This indicates that although we do not know the full nature of the differences in affinity of eIF4E towards different transcripts, at least some of the specificity during apoptosis rescue lies in the elements we have discovered as well as in other elements to be discovered.

While powerful, our experimental approach to global translational profiling is not without its limitations. Polyribosome analysis as a tool to assess translational activity may over- or under- represent transcripts translated in different sub-cellular niches, loci or organelles; may overestimate translational activity as a function of increasing transcript size; and may fail to accurately identify transcripts activated due to increased nuclear-cytoplasmic transport. Despite these limitations, our findings now enable biological and biomedical scientists to systematically study translational control in a wide variety of biological systems and human diseases in an unbiased and reproducible manner and provide the potential of identifying novel mRNA regulatory elements that are involved in translational control regulation.

5.3 PAPER III

Analysis of microarray data is an area of research where several different competences need to be combined. To understand the meaning of any microarray data set a deep knowledge of the particular biological area is needed. In addition, the microarray researcher needs to have reasonable understanding of the various methods used for data extraction, normalization, statistical and post hoc analysis. It is usually difficult to combine all of these skills and it is possible that one competence may dominate the analysis strategy, so that the average researcher is highly dependent on the use of 'standard procedures' for their analysis.

One such common procedure is SAM, yet surprisingly no thorough examination of the impact of a seemingly arbitrary filtering option in the Microsoft Excel Addin (we have called this the FC hurdle to distinguish from FC criteria – which is the *final* inclusion criteria) or data qualification based on data exclusion (e.g. present or absent call thresholds) has been made. This is despite the observation that SAM is one of the most widely used methods (currently 701 citations) and the basis of the method implicates that it could be affected by data set restrictions. We have examined the effect of discrete data selection criteria (qualification criteria for inclusion) and response thresholds (out-put filtering) on the number of significant genes reported by SAM. Our findings demonstrate that commonly utilized arbitrary thresholds can alter, in an unpredictable fashion, the number of reported "significant" genes by more than 100%. For each final FC criteria there is an optimal FC hurdle that can be used within Microsoft Excel to *maximize* the number of reported genes. The reason for this is that the FC hurdle changes the composition of the relative difference values in the control data set, and a different significance level (q-value) is obtained for any given gene.

A clear problem arises when SAM is utilized on different software platforms. In the R package *Siggenes* no hurdle criteria for FC can be made unless an additional function is implemented by the individual researcher. This is in contrast to the more widely used Microsoft Excel *SAM Addin* where the researcher can introduce a FC hurdle prior to the q-value calculations but maintain the SAM parameters that were used in the initial analysis of the full data set. No guidance or discussion is given to the impact of the FC hurdle and we can only presume that it has not been previously appreciated how critical this step can be. As we have shown, the number of genes reported as significant with a certain fold change will depend on the FC hurdle setting used when running SAM. This might be expected as data is selected that show a FC when the correct sample categories. This part of the data would be expected to show a lower tendency towards producing high d(i) when the sample categories are permuted to generate the control data set. Typically the FC hurdle used during SAM to yield the largest significant data set is lower than the final FC criteria being used to define the significant gene list. However, as we have demonstrated, this is not always the case. Thus by applying, what the researcher

believes to be more ‘strict’ data inclusion criteria, the final size of the significant gene list can either be increased or decreased, in a less than predictable fashion.

One might question the entire basis of the SAM methodology if it is heavily dependant on both pre SAM data selection and within SAM (Excel) data filtering. However, one of the appreciated strengths of SAM is that the real data set is used to estimate variation and these effects are thereby hard to avoid. One could also question whether it is valid to reduce the data set prior to using SAM. It would seem obvious that much of the data being removed using a FC hurdle during SAM operation is below an acceptable response level to be considered as being biologically relevant. However, the filter will also remove data that may be essential for an accurate estimation of the noise within the gene set that passes the final hurdle criteria. The challenge would then be to remove noise from non-expressing genes without removing genes that are genuinely expressed and necessary to approximate the data set variation. The FC strategy may not be the most efficient way to achieve this. We have also demonstrated that the same principle has been applied unwittingly during the original analysis of the impact of aging on the human brain [340]. In this case, the reduced data set used by the authors (using a Present/Absent threshold scheme) identifies more “significant” genes for a given final statistical threshold, indicating that reducing data ‘noise’ by alternative means also affects the output list.

Any microarray study underreports the number of truly differentially expressed genes. It appears that failure to act on our observation, when using SAM, may further exacerbate the under-reporting limitation of microarray technologies. We would therefore argue that the SAM algorithm could be modified to use the FC setting as means to select the transcript expression data set that displays the lowest noise, thus enhancing the statistical power of the experiment. We would like to designate the FC hurdle within SAM as an ‘extraction factor’ (EF) that can be modulated to maximize the number of significant genes reported for a given fixed and final statistical threshold. This should be an acceptable strategy, if one accepts the principle that minimizing noise prior to calculating the q-value is valid, which as we have shown is already done by most investigators. In theory, our approach to optimizing each data set is dynamic and responds to the individual data set characteristics. It is clear, however, that investigators must be made aware that the impact of ‘qualification criteria for inclusion’ and ‘out-put filtering’ is less than predictable, when using SAM.

5.4 PAPER IV

If a coordinated cellular program for aging exists in mammals, the transcriptome should be conserved across species. Similarly, if cellular senescence was a pronounced phenotype of an aging tissue it should a component of the aging transcriptome. Given the expansion of the microarray field during the last years, we

concluded that sufficient data was present to enable a comprehensive analysis of gene expression signatures in aged mammals and cellular senescence. To make our study possible, we had to control for a possible bias across platforms and species. To achieve this, we calculated a log-transformed ratio of change in gene expression between old and young tissues or senescent and control cells, for each individual gene in each study. These relative gene expression measures could then be compared to the corresponding genes, or orthologous genes, across datasets. The extent of similarity in a comparison can be calculated by correlation measures (e.g. by using Pearson, Spearman or Kendal correlation measurements). However, it is also necessary to establish what should be considered a significant correlation and what should not. To do this, we used a randomization approach. The simulation approximates the likelihood of finding correlation in a comparison if there is no underlying similarity, and can be used to calculate a p-value for the observed correlation.

Our study is the first to use microarrays to perform a meta-analysis of the components of a complex biological phenotype. Calculation of correlations between changes in gene expression that occur during aging and senescence, respectively, was the fundamental tool used in the present study. The validity of these calculations and the accuracy of the significance estimations are therefore important. The method applied requires that the conditions analyzed should have as similar controls as possible in terms of general biological characteristics; otherwise such variations could mask any similarity. Distributions of random correlations were produced for each study-to-study comparison and used to assess the significance of any observed correlation. It might be argued that such simulations may underestimate the variance of the true distribution as it assumes that all probes (or genes) will display similar changes in signal intensity. However, in a study [332] where genes were randomly paired within quantiles of overall hybridization intensity, the resulting distributions of correlations did not show a significantly increased variance. We claim that a valid result can be obtained if (i) the unmodified and robust Monte Carlo simulation is used to approximate significance, (ii) if the similarities obtained are present in several comparisons and (iii) components of discrete biological processes are identified.

Using these methods, we observed an expression signature for mammalian aging. Furthermore, we found that aging is more different across species than across organs of the same species. This finding is not unexpected, given the differences between species in terms of life span. From this perspective, it might even seem surprising that any of the cross species (but within tissue comparisons) give rise to significant correlations. Our findings highlight the complexity of the aging program: one component is species specific, manifested in all organs, while another component is tissue specific, appearing only in some tissues.

Importantly, the present study establishes a senescence-like expression pattern during aging in mice. It may appear contradictive that mice, with a shorter life span and longer telomeres than humans, would display an *in vivo* cellular senescence phenotype, as senescence is believed to reflect extended cell division, and telomere erosion. However, mouse cells cultured *in vitro* do not develop a senescent phenotype if they are maintained under low oxygen levels. Thus, the overlap between aging and senescence that we observed could reflect stress-induced senescence *in vivo*. Importantly, the signaling pathways downstream of telomere induced senescence and stress induced senescence are believed to overlap. This is supported by our study where the transcriptional changes with senescence, induced by telomere shortening and stress correlate highly. This indicates that a senescence response in mice independent of telomere erosion could not be distinguished from senescence induced by eroded telomeres. Further, if stress-induced senescence occurs *in vivo* in mice, then one might expect cellular p16 to accumulate with age. This is supported by a recent study where p16 was identified as a biomarker of aging in mice in a range of organs [207]. Taken together this indicates that aging mouse cells could be stressed *in vivo* and thus *in vivo* senescence could occur as a consequence of cellular stress.

Intriguingly, we were unable to establish any similarities between cellular senescence and human tissue aging. One might argue that the low availability of human senescence studies where senescent cells could be compared to quiescent cells limits our conclusion. However, this is unlikely, as human cellular senescence actually showed similarities to mouse aging. This clearly indicates that the similarities between mouse aging and senescence are robust and less dependent on the nature of the senescence model. Given the robustness of the senescence to mouse aging comparisons, and the overlap between mouse aging and human senescence, it seems likely that if senescence was a pronounced component of human aging, it should have been observed.

A possible explanation for the difference between the correlation between senescence and aging in mouse and humans, respectively, is the difference in sensitivity to reactive oxygen species. Mice have less protection mechanisms against ROS**.** Hence, ROS may elicit more pronounced damage in mice than in humans. This idea is supported by the fact that human cells can proliferate indefinitely if telomerase is expressed while mouse cells with over expressed telomerase still enter senescence. Accumulation of ROS could therefore induce expression of p16 and senescence, which in turn might be part of the process of aging in mice. In humans the oxidative stress effect could be expected to be less pronounced and may not induce senescence. The contribution of cellular senescence in human aging may therefore be restricted to the telomere driven replicative senescence.

One could speculate that senescence indeed occurs *in vivo* in humans, but to a much lesser extent compared to mice or that the inbred mice are more homogenous in their aging process compared to humans. The effect of the senescent cells on the overall function of the organ and thereby their contribution to aging, would nevertheless be expected to be less drastic. Other possibilities are that apoptosis is the end point of telomere erosion *in vivo*, and that senescence down-stream of telomere erosion is an *in vitro* phenomenon. Indeed, senescence may only affect stem cells when these fail to escape stress induced senescence, or are unable to maintain their telomeres. Regardless of the explanation, it seems likely that the senescent phenotype does not dominate the transcription profile of human aging tissues.

5.5 PAPER V

Initially a lot of focus within the deposition methodology research was focused on development of chemistries that would allow immobilization of cDNAs or oligo-nucleotides onto solid surfaces. The cheapest surface is a plain glass surface but at the time there were no efficient methods to attach DNA to it. As glass is hydrophobic it was also interesting from a non-specific background perspective as it would not be expected to bind to DNA.

We established that an unmodified glass surface was superior in terms of background signals and continued to develop a method for simultaneous deposition and covalent cross-linking of oligonucleotides or PCR products on unmodified glass surfaces. This was achieved by covalently conjugate an active silyl moiety onto oligonucleotides or cDNAs in solutions followed by manual or automatic deposition. The immobilization was efficient and stable as up to three washings by boiling followed by rehybridization could be preformed without extensive signal loss.

Our method provided a simple and rapid, yet very efficient solution to the immobilization of prefabricated oligonucleotides and cDNA for chip production. Also, the use of unmodified glass surfaces was cheap. However, several competitors also developed surfaces and chemistries for deposition chip production at a higher speed and the method was therefore not pursued further.

5.6 PAPER VI

Peptide ligands are important resources for functional proteomics and drug development. Different partial randomized schemes have been employed for constructing peptide libraries through chemical synthesis. By exploiting that amino acids share some bases in their triplets and have similar properties form clusters in the codon table, it is possible to design encoded peptide libraries with different properties using codon biased library design. This is done by defining each base of the triplet as a mixture of all bases: ($T_{A\%}C_{B\%}A_{C\%}G_{D\%}$) where A+B+C+D = 100%.

The different proportions of the bases are mixed and used during the conventional synthesis of the oligo so that a biased randomness is created, which also leads to a greater coverage of the theoretical complexity.

Design of random peptides that span the membrane has several possible applications including molecular targeting and membrane penetration.The amino acid distribution in trans-membrane (TM) domain peptides shows an amino acid bias which could be compatible with codon biased library design. The hydrophobic amino acids, which are highly abundant in natural TM domains, appear to form a group that has T as the second base in their triplet. Based on such observations we designed an oligonucleotide encoded TM library.

If all peptides that are either truncated or show a non-TM amino acid distribution are regarded as non-functional, our best TM library theoretically contained 86% functional peptides that can enter the membrane, in contrast to 0.5% for a totally randomized library. To verify that the chemically synthesized DNA library can be constructed according to the theoretical design, 50 independent clones from TM library 3 were sequenced. 82% of the sequenced clones from library 3 were predicted to encode TM peptides. To study where the TM peptides localize within the cell, 13 peptides were fused to GFP and expressed in Cos 7 or 293 cells. All 13 peptides were highly expressed and non-toxic. 10 out of 13 peptides were able to target the GFP to the plasma membrane. In summary, we drastically increased the occurrence of functional TM peptides in our TM library (10/13) compared to a fully randomized library (0/20).

By using codon biased library design it is possible to formulate a systematic strategy to generate different peptide libraries with small subsets of all amino acids or a strong bias towards some amino acids with shared properties. Compared to the chemical synthesis of peptide libraries, the generation and re-generation of codon-biased libraries is much easier and very flexible. The construction of the library only involves standard molecular biology techniques, and the library can be easily integrated into phage-display, bacterial display or ribosome-display protocols for high throughput screening.The main limitation of the codon directed approach is that the flexibility in terms of amino acid combinations is limited by the geographic distribution of codons on the codon table. This is reflected by the unwanted incorporation of stop codons and other amino acids when they are not desired or a partial loss of some amino acids (especially Trp due to the geographic linkage with the stop codons). Even with severe bias against Trp in the TM libraries, the abundance of functional TM peptides containing this residue is still higher in our libraries compared to a fully randomized library. This is mainly due to the fact that our library has an elevated abundance of TM peptides by about 160 fold. Similar mathematics could apply to many of the codon biased libraries.

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