DEPARTMENT OF ONCOLOGY-PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

Analytical strategies for identifying relevant phenotypes in microarray data

Kristian Wennmalm

Karolinska Institutet

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ABSTRACT

With microarray technology, the transcription thousands of genes can be determined simultaneously. The large number of genes, often assessed in a relatively small number of samples, presents a challenge. The risk of making false positive discoveries is substantial, and making biological sense of hundreds of identified genes is difficult. In response to this, a variety of methods for computerized analysis have been developed yet implementation of these is still fraught with challenges. This thesis focuses on the application of such methods in three areas of biomedical science, where the underlying biology needs more detailed characterization; cellular senescence, cell differentiation, and breast cancer.

Cellular senescence describes a state of growth arrest *in vitro* (cell cultures) believed to be of relevance for aging in mammals. In a comparison of seven microarray data sets addressing aging in human mouse and rat, and four data sets addressing cellular senescence in human and mouse, we discovered similarities between gene expression changes in the aging and senescence experiments, respectively. Resemblance between aging and cellular senescence could only be demonstrated between senescent cells and aging mice, not human. This finding indicates that aging in mice and humans can be substantially different, and that the cellular senescence process may not be a prominent feature of aging human tissues *in vivo*.

Adipogenesis requires exquisite control of cell-cycle proteins in two diverse types of adipocytes, brown and white. Brown adipose tissue, in contrast to white, can consume energy to generate heat. In a microarray experiment contrasting brown and white preadipocyte differentiation, we identified a novel transcriptional program in brown cells involving early expression of myogenic transcription factors previously thought to be unique to differentiation of muscle. We applied a novel array analysis strategy to understand which genes may be responsible for the brown adipocyte maturation and final unique cell phenotype. Our findings add a new dimension to current ideas on the developmental origin of brown adipose tissue.

In the last 40 years, survival in breast cancer patients has improved through the combined effects of earlier detection through mammography screening and adjuvant therapies. To achieve further progress, developing new prognostic markers, treatment predictive markers, and tailored therapy is important.

In two population based cohorts with 402 expression profiled primary breast cancers, we found that five proposed molecular subtypes of breast cancer could be collapsed to form two groups on the basis of gene expression in the long arm of chromosome 16, in agreement with histological grade.

We also explored the possibility to predict the sites of distant recurrences and found that lung and liver metastasis could be predicted. Prediction was characterized by poor sensitivity, numerous false positives, and strong dependence on biology underpinning histopathological grade and HER-2/neu status.

These findings indicate an important role for biology related to histopathological grade in breast cancer, and further investigation may provide means for better prognostication and treatment prediction.

LIST OF PUBLICATIONS

I. **Wennmalm K**, Wahlestedt C, Larsson O. The expression signature of in vitro senescence resembles mouse but not human aging.
Genome Biol. 2005;6(13):R109

II. Timmons JA*, **Wennmalm K***, Larsson O*, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, Nedergaard J, Cannon B. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages.
Proc Natl Acad Sci U S A. 2007 Mar 13;104(11):4401-6

III. **Wennmalm K**, Calza S, Ploner A, Hall P, Bjöhle J, Klaar S, Smeds J, Pawitan Y, Bergh J. Gene expression in 16q is associated with survival and differs between Sørlie breast cancer subtypes.
Genes Chromosomes Cancer. 2007 Jan;46(1):87-97.

IV. **Wennmalm K***, Bjöhle J*, Smeds J, Klaar S, Ploner A, Bergh J. Prediction of distant metastasis site in primary breast cancers - results from two population derived cohorts.
Manuscript.

* Authors contributed equally to the manuscript.

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LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BAT	Brown adipose tissue
C/EBPα, β	CCAAT/enhancer binding protein (C/EBP), alpha and beta
cDNA	Complementary DNA
Chk1/Chk2	CHK1 / CHK2 checkpoint homologs (S. pombe)
cRNA	Complementary RNA
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
E2F	E2F family of transcription factors
EASE	Expression Analysis Systematic Explorer
ER, ERα, β	Estrogen receptor, estrogen receptors alpha and beta
ERBB2	Alias for HER-2/neu (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2)
FDR	False discovery rate
GO	Gene ontology
HER-2/neu	Human Epidermal Growth Factor Receptor 2
hTERT	Telomerase reverse transcriptase
IM	Ideal match
IRS	Insulin receptor substrate
MAS 4, 5	Microarray Analysis Suite Versions 4, 5
MM	Mismatch
mRNA	Messenger RNA
MyoD	Myogenic differentiation 1
PCA	Principal component analysis
PGC1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PM	Perfect match
PPARγ	Peroxisome proliferator activated receptor gamma
PR -A / -B	Progesterone receptor isoforms A and B
RB	Retinoblastoma 1
RFS	Recurrence-free survival
RMA	Robust multichip average
RNA	Ribonucleic acid
RT-qPCR	Reverse transcriptase - quantitative polymerase chain reaction
SA ß-gal.	Senescence associated ß – galactosidase
SAM	Significance Analysis of Microarrays
SIRT1, 3	Sirtuin (silent mating type information regulation 2 homolog) 1 and 3
UCP1	Uncoupling protein 1
WAT	White adipose tissue

1 GENERAL INTRODUCTION

Expression microarray technology is well suited for exploring areas of biomedical research where the underlying processes are unclear, or suspected to be imprecisely represented by current terms and biological markers. The transcription levels of thousands of specific genes can be estimated in parallel, allowing the researcher to investigate how studied cells transcribe an identical set of genes to achieve a distinct phenotype. The large number of variables, assessed in a relatively small number of samples does however present a challenge to the interpretation of microarray data. A significant risk that irrelevant genes will appear correlated with an endpoint of interest will have to be acknowledged given the amount of measurement error associated with simultaneous detection of thousands of transcripts. Also, the potentially large number of findings is a concern with regards to interpretation: many genes will be unknown to the investigator, and reviewing published literature for hundreds of genes is time consuming. A vast array of analysis software has been designed to automate these tasks, and a major weakness has become evident: genes are annotated according to discovered functions, and accordingly, biological processes implicated in a microarray experiment seem to confirm rather than represent discoveries. Direct comparisons between microarray experiments are likely to yield more novel understanding, and will be enabled by public repositories if deposition of published experiments becomes widespread. This thesis focuses on analytical strategies for identifying phenotypes – as assessed with microarray technology – relevant to three biological fields where it is unclear how well current terms and notions correspond to the underlying biology: cellular senescence, since 40 years a putative cause for aging that still resists extension to the in-vivo setting owing to a definition not useful outside culture dishes; brown fat differentiation, whose molecular underpinnings needs to be separated from that of white fat in order to become a possible way to combat obesity; and breast cancer, where considerable heterogeneity has been acknowledged for decades and presents an obstacle to research into new drugs and prognostic markers.

2 SENESCENCE

2.1 INTRODUCTION

The function of multi-cellular tissues is maintained through a balance between cell renewal and cell death. Stem cells with unlimited capacity to enter and exit the cell division cycle – which consists of distinct phases (G1, S, G2, and M) leading to duplication of DNA and subsequent division – give rise to new cells that differentiate to attain the phenotype necessary for tissue function. In contrast, cell death can either be as a consequence of irreparable damage (necrosis), or apoptosis (programmed cell death). The latter process can be viewed as a mechanism for controlled induction of death, when this is beneficial to the organism. For example, apoptosis occurs during development, to remove redundant cells, and as a consequence of DNA damage, presumably to prevent cancer progression. Senescence represents an alternative cell fate, and renders cells non-functional and incapable of dividing, but alive. Senescence is the focus of this chapter, and the potential importance of this mechanism will be discussed.

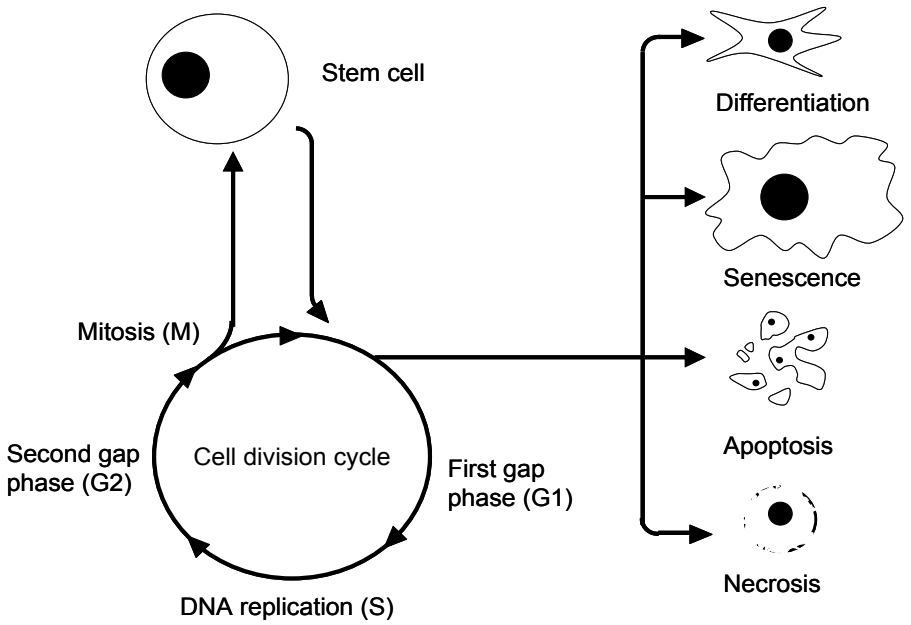

Figure 1. The cell division cycle and cell fate.

2.2 SIGNIFICANCE

In the cell culture setting, the term senescence is used to denote cells that have lost their ability to divide further, typically as a consequence of extended in-vitro growth. It was described by Leonard Hayflick in 1965, who noticed that there was a limit to the number of times fibroblasts could divide in culture[3]. An obvious interpretation of this finding was made early on; this limit may be the microscopic appearance of aging, and by extrapolation, time dependent deterioration of many

organisms may at least in part be caused by accumulation of non-dividing senescent cells. It has subsequently become clear that this possibility differs between cell types and species. Stem cells and cancer cells represent an extreme – they can go through a large or possibly infinite number of cell divisions without entering senescence. Hayflick's finding thus highlights a key characteristic of cancer cells: immortality. In contrast to normal differentiated cells, cancer cells seem to circumvent senescence, which in turn suggests that senescence may exist to prevent malignant development. If no cell could divide more that 50 times, it is not easy to see how the inefficient multi-step process of tumor evolution, spread, and distant growth could ever be completed. The prospect of understanding the reason for aging as well as a seemingly crucial barrier against tumor progression has, not surprisingly, attracted a great deal of interest in biomedical research.

2.3 PATHWAYS OF SENESCENCE INDUCTION

Senescence can be induced in several ways, and several terms have been coined to account for this. Here, the terms telomere-dependent and telomere-independent senescence will be used to discriminate between replicative senescence on one hand, and the terms premature, oncogene-induced, and stress-induced senescence on the other.

2.3.1 Telomere-dependent senescence

Hayflick established that it is the time a cell has been cultured that determines when it stops dividing, not chronological time. For instance, if cells are frozen, the time spent in the freezer does not affect the timing of senescence. He therefore proposed the existence of an internal counting function, a 'replicometer'. A potential mechanism was suggested by Olovnikov in the early 1970s. He described a problem related to the DNA polymerases responsible for duplicating chromosomes during cell division: the requirement of RNA primers for polymerase function should shorten the 5' end of a linear chromosome progeny with each round of cell division[4]. Subsequent investigations have demonstrated that chromosome ends in fibroblasts and various other human cells become shorter with accumulating mitoses[5-8]. The suggestion that chromosome ends –or telomeres - may be involved in limiting the replicative lifespan of eukaryotic cells led to intense studying of these specialized structures during the 1980s. Human telomeres were shown to consist of repeats of the TTAGGG sequence [9], ranging in size from about 5 to 15 kb depending on cell type. Furthermore, they are associated with several binding proteins and a 3' single stranded overhang of a few hundred nucleotides. The overhang seems to bend back to the double-strand portion of the telomere and form a loop. The associated proteins play a part in determining telomere length, formation of the loop, and protecting the structure[10, 11]. Notably, several proteins known to be a part of the DNA damage family (RAD50, MRE11, NBS1) have been found to associate to telomeres[12].

Compelling evidence in favor for an important role for telomeres in replicative senescence comes from experiments where hTERT (human telomerase reverse transcriptase) has been over-expressed. This ribonucleoprotein uses a nuclear

encoded RNA template to extend telomere ends, thereby counteracting telomere shortening. It is not expressed in many cells known to undergo replicative senescence, whereas stem cells and ~90% of cancer cells express it[13]. When it is aberrantly expressed in fibroblasts and retinal epithelial cells, telomeres become significantly longer and their lifespan is extended far beyond the 40-60 population doublings achievable with corresponding wild-type cells[14]. D'Adda di Fagagna and co-workers have performed another experiment that strongly implicate telomeres in replicative senescence. They speculated that a DNA damage response is activated by critically short telomeres, and were able to show that senescent fibroblasts display foci staining for phosphorylated histone H2AX and other proteins associated with double-strand DNA breaks. By chromatin immunoprecipitation and microarray experiments, they further demonstrated that these proteins primarily associated with telomere DNA, thereby showing that this is where the response is elicited[15]. DNA damage is known to induce several responses in cells that ultimately lead to repair or programmed cell death (apoptosis)[16]. A system of sensors – ATM, ATR, and other proteins – sense different types of DNA damage. Chk1 and Chk2 act as signal transducers by phosphorylating CDC25A, B and C, as well as p53, which can result in initiation and maintenance of cell cycle arrest through transcriptional induction of p21 and inhibition of cyclin-dependent kinases (discussed further below), presumably to allow for repair of compromised DNA. The connection between this DNA damage response and senescence highlights an additional possible outcome of DNA insults: terminal growth arrest.

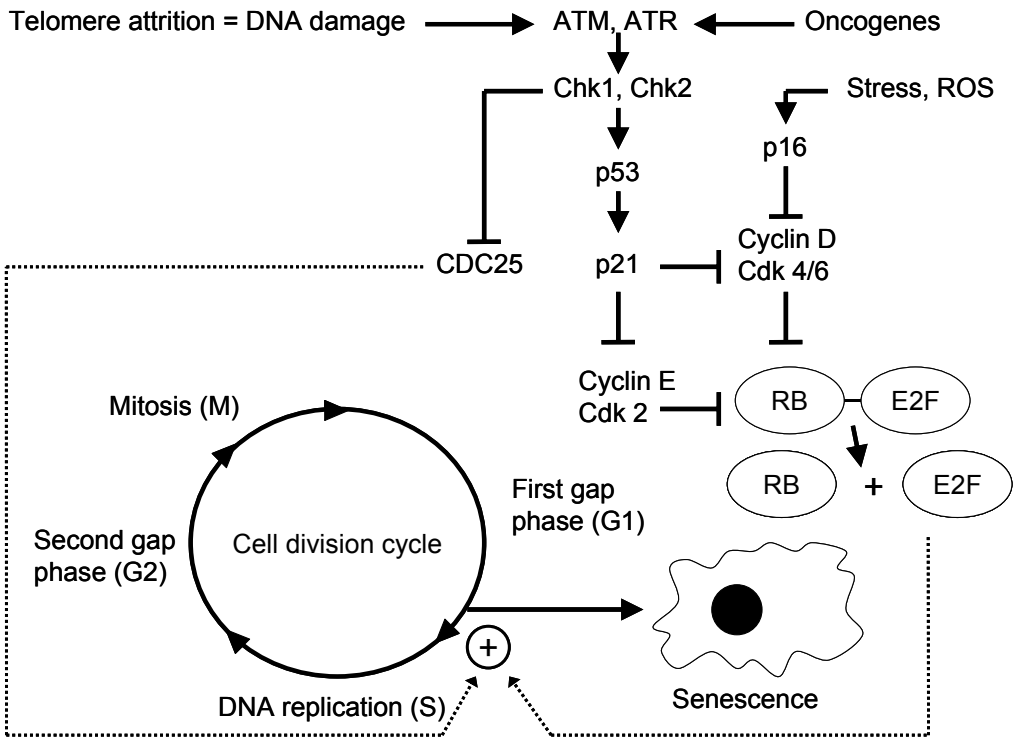

Figure 2. The p53 and RB pathways in cellular senescence.
In response to telomere attrition (a DNA damage response) or cellular stress, the p53 and RB pathways induce cell cycle arrest via suppression of cyclins / cyclin dependent kinases, resulting in hypo-phosphorylated RB, and suppression of E2F-responsive genes.

In summary, it seems clear that telomeres do shorten as a consequence of mitosis, and that the elongation of telomeres is necessary to achieve cell immortality. The activation of the ATM/ATR – Chk1/Chk2 – p53/p21 pathway seems important for detecting short or altered telomeres[15, 17]. How telomeres shorten is still unclear, however. Shortening has been suggested to be related to the size of the 3' overhang, but this conclusion has been challenged[18, 19]. In addition to Olovnikovs incomplete replication mechanism, others have been suggested: recombination events and deletions[20, 21]. When senescence is triggered is also unclear. Average telomere length has been shown to continue to decrease even after introduction of telomerase activity[22, 23], although this may be accounted for by a mechanism that selectively targets short telomeres for telomerase elongation. This would avoid below-threshold telomere lengths, but allow for average length to shorten without senescence induction[21].

2.3.2 Telomere-independent senescence

Forced expression of hTERT is not always sufficient to achieve unlimited proliferation. For example, human mammary epithelial cells (HMECs) and keratinocytes were not immortalized by hTERT expression alone in a study by Kiyono and co-workers: impairment of the p16/Rb pathway was also necessary[24]. Several other human epithelial cells have been shown to enter a premature senescence-like state[25] with increased p16 expression[26, 27]. Interestingly, over-expression of p16 induces a senescence-like growth arrest in fibroblasts [28], and re-expression of Rb induced senescence in a cancer cell line[29]. Furthermore, this pathway is so frequently targeted in cancer, that its inactivation has been suggested to be essential for tumor formation[30]. The p16/Rb pathway regulates transition from gap phase (G1) to the DNA synthesis phase (S), and can respond to stress, such as non-physiologic culturing conditions. Repressive cell-cycle control is exerted by hypophosphorylated RB (and RB family members p107 and p130) through inhibition of the E2F family of transcriptional regulators, which in turn promotes transcription of genes necessary for DNA replication. The phosphorylation status of RB is controlled by D-type cyclins and associated cyclin-dependent kinases. p16 is one of four INK4 proteins that inhibit D-type cyclins, and responds to environmental stress, such as non-physiologic culturing conditions. That this might form a seemingly telomere-independent growth barrier is supported by a report of HMECs and keratinocytes that were immortalized by hTERT expression alone in a setting with appropriate growth conditions[31]. This stress-imposed barrier has been suggested to explain the low number of divisions achievable in rodent cells in culture compared to human cells in culture[32].

Oncogene signaling can also trigger telomere-independent senescence. This premature form of senescence has primarily been linked to p16 and E2F rather than DNA damage and p53 activation[33, 34]. In a seminal paper by Bartkova and colleagues, involvement of the double-strand break checkpoint and the p53 pathway is demonstrated in oncogene-induced senescence. Several oncogenes were over-expressed in fibroblasts, and markers of DNA damage were induced

(phosphorylated H2AX, Chk2) together with p53, p21, p16 and Senescence Associated β-galactosidase (SA β-gal) staining. Furthermore, foci of DNA damage co-localized with sites of DNA replication, and signs of prematurely terminated replication forks were found, suggesting that DNA replication stress in response to high levels of oncogene expression is causative[35]. A related study by Di Miccio and co-workers had similar results[36]. Somewhat surprisingly, the ATM/ATR – Chk1/Chk2 – p53/p21 pathway previously implicated in telomere dependent senescence seems important for telomere-independent oncogene-induced senescence as well. Further complexity is added by results regarding other oncogenes. Over-expression of oncogenic RAF, a direct downstream target of RAS, can induce senescence independent of p53 in fibroblasts and independent of both p53 and p16 in mammary epithelial cells[37, 38]. This underscores the importance of the specific biological context in which experiments have been conducted. Several chemicals such as hydrogen peroxide and chemotherapeutics can induce senescence, and at least in the case of hydrogen peroxide, this is telomere-independent[39]. Some investigators describe a p53 dependent senescence response to γ-irradiation and chemotherapeutic agents [40, 41], while others report that neither p53 nor p21 is necessary, at least in the case of chemotherapy-induced senescence[42]. A final interesting finding is that loss of tumor suppressor function also seems capable of inducing telomere-independent senescence[43]. Thus, it seems that several noxious or potentially dangerous stimuli can trigger telomere-independent senescence, and in this context the p16/Rb and p53/p21 pathways are important.

2.4 IN VIVO STUDIES

Although cells have been declared senescent in numerous reports of in-vitro experiments, the term is vague and a precise definition is lacking. A major complication is that the central feature of *senescence* - suggesting that it might be important for cancer progression and aging - is not unique to senescence. Cessation of cell division, which seems incompatible with cancer and may accompany aging, is widespread in multi-cellular organisms; growth arrest accompanying terminal differentiation is essential for organ structure and functioning. It can also be achieved by crowding cells in a culture dish (referred to as quiescence or confluence). Which are then the remaining common characteristics of senescent cells that could be used as unambiguous markers? Irreversible cell cycle arrest has been proposed characteristic of senescent cells, although irreversibility does not always seem to be the case[44]. Apart from ceased cell division, senescent cells display an enlarged and flattened morphology with nuclear and other aberrations, *in vitro*[32]. Gene expression changes do take place, as well as epigenetic events[33], but no markers are accepted as entirely specific. The widely used SA β-gal staining has been questioned[45]. As we have seen, the molecular mechanisms most intensely examined in relation to senescence have not demonstrated one canonical pathway of senescence induction.

It seems fair to suggest that these limitations to the concept of senescence have made it difficult to establish a role for senescence outside the laboratory bench.

That 90% of human malignancies express telomerase, and that the remainder manages to maintain their telomeres through alternative mechanisms is obviously a strong argument in favor of a role for telomere–dependent senescence in preventing cancer. Recently, a role in suppression of pre-cancerous lesions has been implicated by findings in melanocytic nevi. Several benign tumors of the skin, including nevi, stop growing after reaching a certain size. Also, they show a puzzling lack of mitoses[46]. Melanocytic nevi frequently harbor oncogenic mutations, such as BRAF(V600E), but rarely progress to malignancy. Michaloglou and co-workers demonstrated that sustained BRAF(V600E) expression results in cell cycle arrest and induction of p16 and SA β-gal, and this was also verified in real nevi[47]. Other recent investigations have provided support for the notion that oncogene-induced senescence is an in vivo mechanism that contributes to protection against cancer development[48, 49]. A role in aging seems more uncertain. Results regarding the effect of donor age on the replicative lifespan of cells in vitro are conflicting[50, 51]. Increased SA β-gal staining in aged humans has been reported[52], but the significance of this finding depends on the true specificity of SA β-gal. Longer telomeres in blood cells have been associated with longer lifespan in humans, and the decreased mortality was attributed to infections and heart disease, perhaps reflecting decline in immune system function[53]. The premature aging syndrome of Werner implicates telomeres, but the relevance to normal aging is uncertain [54].The lack of specific in-vivo characteristics of senescence has presented an obvious obstacle in extending the term to this setting.

In spite of the uncertainties regarding senescence, most researchers seem to consider it a valid biological entity, and that it represents a specific cell phenotype. This implies that there should be a collection of RNAs, transcribed at certain levels, that identifies senescent cells. If such an expression signature can be identified across many of the specific cell types and experimental conditions were senescence has been considered present, clarifying the contribution of this phenotype to organism aging should be possible.

3 ADIPOCYTE DIFFERENTIATION

3.1 SIGNIFICANCE

There are two major variations on adipose tissue phenotype. Brown fat can be found in a number of mammals, such as rodents, cats, dogs, cattle and humans. Its darker appearance, compared to white fat, is due to a higher degree of vascularization as well as cellular differences: the cells of brown fat (herein referred to as brown adipose tissue - BAT) contain many more mitochondria, and several small fat droplets, whereas white fat cells (white adipocytes or WAT) contain fewer mitochondria and typically one large droplet of fat. Also, the brown adipose tissue in highly innervated by the sympathetic nervous system [55] and this controls BAT phenotype. One distinctive molecular characteristic of brown adipocytes is expression of uncoupling protein 1 (UCP1). This protein, localizing to the inner mitochondrial membrane, has the ability to uncouple oxidation of fuel substrates from the production of ATP, thereby generating heat. In rodents (which are well studied in this context) this can occur as a response to food intake and low ambient temperature, through sympathetic signaling, norepinephrine release, and subsequent activation of adrenergic receptors on the brown adipocytes[56]. Thus, brown adipocytes essentially do the inverse of white adipocytes: they consume energy rather than store it, thereby providing heat in response to a cold environment [57].

Early indications that BAT function might affect energy balance in rodents came from experiments where BAT was surgically removed or denervated. In some reports, this caused obesity [58]. Genetically altered mice, where UCP1 or related genes have been either disrupted or over-expressed generally support the view that UCP1 confers resistance to cold and obesity, whereas its absence produces the opposite effect [57, 59, 60]. The traditional view that BAT is largely replaced by WAT short after birth in humans may seem to question the relevance for human obesity. Unexpected findings has however provided evidence for BAT in adults [61]. Today, much research effort is being directed towards the prospect of trans-differentiation of adipose tissue, or at least to make WAT attain the major phenotypical characteristics of BAT, so conferring resistance to obesity. In the laboratory setting, experiments have demonstrated that this might be possible: over-expression of the peroxisome-proliferator receptor γ (PPARγ) co-activator 1α (PGC1α) induces UCP1, respiratory chain proteins, and fatty acid oxidation enzymes in human white adipocytes [62]. Effect on BAT of the oral hypoglycemic drug ciglitazone was reported already in the 1980s. Drugs of this class (thiazolidinediones) seemed to induce the capacity for thermogenesis in rodent BAT [63]. It has subsequently become clear that these drugs can induce UCP1 expression in brown adipocytes [64], and that they are PPARγ agonists [65]. Their effect on blood glucose is considered due to increased insulin sensitivity, perhaps through mitochondrial remodeling in adipose tissue [66].

3.2 MOLECULAR DETERMINANTS OF FAT CELL DIFFERENTIATION

A number of factors have been implicated in brown and white fat cell differentiation. The following description is not only a reflection of those factors which might be considered well studied or important, but also of those that were assessed in our microarray experiment, or which have been investigated in other recent microarray experiments.

3.2.1 Regulators of adipogenesis

PPARγ has been proposed a master regulator of adipogenesis, being necessary for both BAT and WAT differentiation and survival [55]. It was identified as one of two components (the other being the retinoid X receptor – RXR) of a transcriptional unit targeting an important enhancer element of the adipocyte specific gene aP2 [67]. In the nucleus it forms a dimer with RXR, and mediates the effect of fatty and retinoic acids.

C/EBPα and C/EBPβ were the first identified transcriptional regulators of the UCP1 gene [68]. In C/EBPα knockout mice, lipid accumulation in BAT is absent and PPARγ, PGC1α, and UCP1 expression is decreased or delayed [69]. This is not likely to reflect effects of the knockout in brown adipose tissue *per se*, since mice that express C/EBPα in liver only have largely normal BAT but significantly reduced WAT[70]. Thus C/EBPβ and C/EBPδ seem to play an important role during development of both BAT and WAT, but minor roles in mature adipose tissue [71, 72]. Clearly these factors are not especially likely candidates for controlling BAT versus WAT phenotype from precursor stem cells.

Insulin and IGF-1 signal through cell surface receptors that, in turn, trigger phosphorlyation of insulin receptor substrates (IRS). Insulin/IGF-1 signaling promotes adipocyte differentiation [73], and mice lacking IRS-1 and IRS-3 have reduced amounts of WAT [74]. Although a subset of IRS proteins are required for adipose conversion in immortalized brown preadipocyte cell lines, BAT in IRS-1 and IRS-2 knockout mice seems unaffected [75]. A comparison of microarray data derived from preadipocytes, lacking individual IRS proteins, implicated a panel of genes including necdin as covariates of inability to differentiate into brown preadipocytes [76].

3.2.2 Factors implicated in brown adipogenesis

The ability to induce UCP1 expression with thiazolidinediones has proven dependent on PGC1α [77]. This protein is more highly expressed in BAT than in WAT, and is also cold inducible[55], making it a strong candidate for determining adipocyte phenotype, at least *in vitro*. It interacts with numerous nuclear receptors and seems to induce a more “oxidative” phenotype in several tissues, including the promotion of mitochondrial biogenesis, and in the case of brown adipose tissue, UCP1 (when combined with adrenergic activation) [55, 78]. The ability of

thiazolidinediones to induce mitochondrial biogenesis in white adipocytes (with inherently lower PGC1α expression) may be due to this drug's ability to also induce expression of PGC1α [79].

Norepinephrine-induced activation of β-adrenergic receptors is important for both activation of existing BAT and recruitment of new brown adipocytes [78]. Also, most physiologically induced events of recruitment, such as a cold environment or over-eating, can be understood as a consequence of chronic sympathetic stimulation of the tissue [78]. Mice lacking all three types of β-adrenergic receptors are highly sensitive to diet-induced obesity and cold, and their BAT lacks some morphologic characteristics and does not induce UCP1 expression in response to cold [80, 81]. Interestingly, in-vitro and in-vivo findings have not demonstrated a clear-cut effect of β-adrenergic stimulation on PPARγ, and although PGC1α expression is enhanced by norepinephrine, this does not seem to mediate the effect of norepinephrine on UCP1 expression [78]. SIRT1 and SIRT3 are members of a family of NAD dependent deacytelators/ADP-ribosylators. Both SIRT1 and SIRT3 seem to interact with PGC1α[82, 83], and be of potential importance for mitochondrial biogenesis. The SIRT1 ortholog Sir2 has also been shown to retard in-vitro muscle differentiation[84] implying that it may be an important regulator of cell fate.

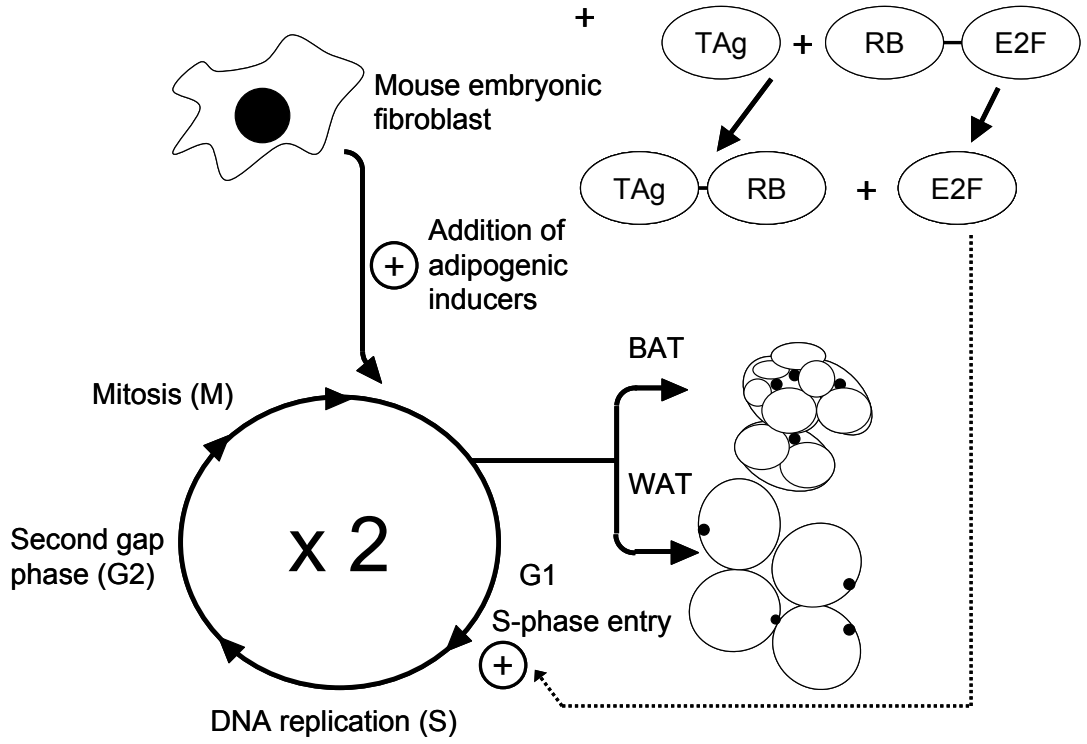

Figure 3. Rb and adipocyte differentiation.
When mouse embryonic fibroblasts are induced to differentiate into adipocytes, they re-enter the cell division cycle. They subsequently undergo two rounds of cell division, and exit the cycle [1]. Expression of Simian Virus 40 large T antigen (TAg) inactivates RB, and promotes a brown adipocyte phenotype[2].

3.2.3 RB

In addition to its role as a key regulator of the cell cycle, inhibition of RB function promotes brown versus white adipocyte differentiation in mouse embryonic fibroblasts implying that tight regulation of cell cycle can influence adipocyte cell fate [2]. In experimental adipogenesis, regulation of cell cycle is a critical step during maturation. In mature BAT and WAT adipocytes, as well as preadipocytes of epididymal WAT, pRB is clearly expressed. In contrast, preadipocytes of interscapular BAT lack pRB expression [2]. pRB may exert this "molecular switch" function on adipocyte fate through binding to the PGC1α promoter and repress transcription [85]. For example when mouse embryonic fibroblasts are induced to differentiate into adipocytes, they re-enter the cell division cycle. They subsequently undergo two rounds of cell division, and exit the cycle [1]. Expression of Simian Virus 40 large T antigen (TAg) inactivates RB, and promotes a brown adipocyte phenotype[2].

3.3 THE ORIGIN OF BROWN AND WHITE ADIPOSE TISSUE

Although several seemingly important receptors, signaling pathways, and transcription factors have been investigated in relation to brown and white preadipocyte differentiation, it is not entirely clear how the different phenotypical fates of these cell types are achieved. However, several lines of circumstantial evidence suggest their origin might not be the same: the anatomical location of BAT and WAT are relatively distinct in mice and other animals [55]. BAT can be found in for instance interscapular and axillary depots, whereas white fat can be found in the epidydimis [56]. Furthermore, in inguinal fat, that seems to be a mix between the two types, white fat cells arise independently of the brown lineage [86]. Preadipocytes, found in the stromal-vascular fraction of BAT and WAT, differentiate mainly into brown and white adipocytes, respectively [87]. During the completion of the studies in this thesis, more direct evidence of a distinctive origin for brown adipocytes has been published [88] which supported the findings I will present later on.

4 BREAST CANCER

4.1 SIGNIFICANCE

Globally, more than one million women are annually diagnosed with breast cancer, and it is the leading cause for cancer-related mortality [89]. In 2005 the number of diagnosed patients in Sweden was about 7000 per year (National Board of Health and Welfare, Sweden). Modern adjuvant hormonal and chemotherapy has a significant effect on survival after diagnosis; at 15-years post diagnosis, mortality has been estimated to be decreased by 50% for middle aged women with estrogen receptor (ER) positive disease through combined treatment with anthracyline-based poly-chemotherapy and the selective estrogen receptor modulator tamoxifen [90]. In Sweden, 5-year breast cancer survival has increased from 65% (1964-66) to 84% (1994-96;[91]). Recent data describing the current situation in Stockholm reports about 90% 5-year survival (Oncologic Centre, Stockholm). Considerable biological heterogeneity has long been recognized in breast cancer, and current prognostic and therapy predictive factors are not sufficient in describing this, which in turn means imprecise stratification of patients and under- as well as over-treatment. High-throughput methods for molecular characterization of breast cancers have attracted great interest, since they hold promise of greater insight into the molecular mechanisms leading to breast cancer development, improved prognosis and therapy response prediction as well as new molecular targets for treatment. Gene expression profiling with microarray technology has already identified subgroups of breast cancer with distinct patterns of gene expression and different prognosis [92-94].

4.2 PROGNOSTIC AND THERAPY PREDICTIVE FACTORS

By definition, a prognostic factor is informative with regards to outcome in untreated patients. By contrast, a predictive factor predicts the more likely response to some certain treatment. Prognostic factor are still quite influential in treatment decisions, due to limitations in currently used predictive factors.

4.2.1 Age

Several studies have demonstrated worse prognosis in young breast cancer patients [95-98]. Although this seems to at least partly be a reflection of increased risk of affected lymph nodes, negative hormone receptor status, and large tumors [99, 100], several studies have retained a negative effect after adjusting for confounding factors [101-103]. This may reflect previous under-treatment in this patient group, and age < 35 precludes assigning low risk in the St Gallen consensus of treatment of early breast cancer [104, 105]

4.2.2 Tumor size, lymph node status and stage

The size of the primary tumor and the number of affected axillary lymph nodes remain the most important prognostic factors, and are fundamental in clinical decision making. In a study of node-negative breast cancer, patients with tumors smaller than 2 cm who received no adjuvant treatment had a 20-year disease-free

survival (DFS) of 79% whereas patients with tumors larger than 2 cm had a 20-year DFS of 64% [106]. Nodal involvement is a strong prognostic factor. In the first National Surgical Adjuvant Breast and Bowel Project (NSABP), no lymph node metastasis was associated with a DFS of 85% at 5 years, whereas patients with ≥ 4 axillary lymph node metatsases had a 5-year DFS of 26% (tumors were ≤ 50 mm;[107]). Joint classification according to size, lymph node involvement and distant metastasis (the TNM staging system, table 1) has become the most important prognostic tool in breast cancer[108].

Table 1 Tumor stage and TNM classification. (Adapted from Regional Oncologic Center in Uppsala/Örebro region, 2006)

Stage	Tumor size (T)	Lymph node status (N)	Distant metastasis (M)
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1-2	M0
Stage IIIB	T4	N0-2	M0
Stage IIIC	Any	N3	M0
Stage IV	Any	Any	M1

Primary tumors size (T)
Tis=Carcinoma in situ, T1=Tumor ≤ 20mm, T2=Tumor 21-50mm in greatest dimension, T3=Tumor >50 mm, T4=Tumor of any size extending to chest wall or skin, and inflammatory carcinoma

Regional lymph nodes (N)
N0= No regional lymph node metastasis, N1=Moveable ipsilateral axillary metastasis, N2=Fixed ipsilateral metastasis, N3= Metastasis in ipsilateral supra- or infraclavicular lymph nodes, or internal mammary lymph nodes

Distant metastasis (M)
M0=No distant metastais, M1= Distant metastasis

4.2.3 Histological grade

The most commonly used system for histologic grading was originally presented by Bloom and Richardson, and later modified by Elston and Ellis [109, 110].

According to this system, grade is determined by adding individual scores for tubule formation, mitotic count, and nuclear pleomorphism, and was initially reported to be strongly correlated to prognosis[110]. Some have reported that they were unable to show that grade is of prognostic significance[111], and the reproducibility between laboratories has been questioned[112]. Interestingly, grade 1 and 3 tumors have been shown to harbor partially different recurrent chromosomal aberrations. Roylance and co-workers found that loss in the long arm of chromosome 16 (16q) was frequent in grade 1 carcinomas (65%), but not so common in grade 3 carcinomas (16%) in contrast to many other aberrations that were more common in high-grade tumors [113]. Recurrent chromosomal aberrations often confer some survival advantage to tumor cells (they harbor oncogenes or tumor suppressor genes), and different patterns reflect different paths of tumor progression. Thus, Roylance's *et al* finding suggests that grade can act as a proxy for phenotypical heterogeneity in breast cancer and be of biological significance. In contrast, loss of heterozygosity in 16q has been associated with distant metastasis in familial breast cancers, suggesting a different role for the underlying chromosomal aberration in this patient group [114]. Recently, grade has been acknowledged in the St Gallen consensus on treatment of early breast cancer, where it is one several factors used to discriminate between low and intermediate risk [104].

Table 2. Histopathological grading according to Elston and Ellis. (Adapted from Regional Oncologic Center in Uppsala/Örebro region, 2006)

Elston grading system		**Score**
Tubules (T) Percentage of tumor area composed of tubules	> 75% of the tumor	1
	< 10% **T** <75%	2
	<10%	3
Mitoses (M) Mitotic counts in 10 high power fields	<10	1
	10 < M < 20	2
	>20	3
Nuclear pleomorphism	Small nuclei, regular outlines, uniformity of nuclear chromatin	1
	Moderate variation in shape and size, visible nucleoli	2
	Pronounced variation in shape and size, large and abnormal nuclei	3

Summary:		
Elston score	**Differentiation**	**Grade**
3 – 5	Well differentiated	I
6 – 7	Moderately differentiated	II
8 – 9	Poorly differentiated	III

4.2.4 Estrogen receptors (ERs)

Two human estrogen receptors have been identified, ERα and ERβ. The terms Estrogen receptor or ER will be used in this text, reflecting the fact that distinction between ERα and ERβ has not been made previously[115]. In breast cancer ERα predominates [116], so ERα action is probably more relevant to previous findings.

Estrogen receptor activity was discovered in the late 1960s [117, 118], but despite the potential for assessing responsiveness to hormone treatment, the predictive capacity has not until recently become widely accepted, as is illustrated by inclusion of receptor negative patients in endocrine treatment studies in the mid nineties [119]. Development of drugs with anti-estrogenic properties and the discovery of tamoxifen in 1962 was to have a dramatic effect on research into the therapy for breast cancer [120, 121]. The classic route of action described for ER is dimerization as a consequence of binding to estrogen, followed by translocation to the nucleus. In the nucleus, ER binds to estrogen response elements – semi-specific DNA sequences – in association with other DNA-bound transcription factors and co-activators, and thus affects the transcription of estrogen responsive genes [122]. Non-classical (independent of DNA binding) actions of ER have also been described [123]. Importantly, estrogen can stimulate growth in breast cancer cell lines, and has been shown to control several key regulators of cell cycle progression [124, 125]. In an overview of randomized trials, treatment with tamoxifen for 1, 2 and about 5 years reduced proportional recurrence with 21%, 29% and 47% in patients with ER-positive or untested tumors. In contrast, no significant effect was seen in ER-negative tumors [126]. Early on, ER-expression was shown to be prognostic also [127]. However, later studies with longer follow-up suggest that the more favorable prognosis in ER-positive tumors may not be sustained [128], and that the relapse rate increases after a few years in ER positive relative to ER-negative tumors, so that the prognostic significance disappears [129]. Accordingly, ER expression is the most important treatment predictive factor in breast cancer.

4.2.5 The progesterone receptor

The progesterone receptor consists of two isoforms, PgR-A and PgR-B, is ER regulated, and mediates the effects of progesterone in both normal mammary gland and breast cancer [130]. The ratio of PR-A / PR-B has proven important for normal development of the mammary glands in rodents[131], and an increased PR-A / PR–B ratio has been described in breast cancer [132], and may be associated with resistance to tamoxifen [133]. Interestingly, polymorphism in the promoter, causing increased PR-B expression, has been associated with higher risk of developing breast cancer [134]. Although PgR similar to ER, is considered a weak prognostic factor that looses prognostic value with time [135], it may provide additional prognostic [136, 137] as well as tamoxifen-response predictive [135] information compared to ER.

4.2.6 HER2/neu (ERBB2)

The protein encoded by the geneERBB2 (located at 17q11.2-q12) is a transmembrane tyrosine kinase receptor and a member of the epidermal growth factor receptor family. It has no known ligand, but forms heterodimers with other family members, enhancing kinase-mediated activation of downstream signaling pathways, such as those involving mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K). Over-expression is frequently caused by amplification affecting ERBB2 and neighboring genes, and associated with worse prognosis in breast cancer[138-140]. Apart from being a prognostic factor, ERBB2

protein expression or amplification is assessed in order to choose patients suitable for trastuzumab therapy (a monoclonal antibody directed against ERBB2;[140]), and it has been suggested to have treatment predictive capacity for less obvious compounds such as anthracyclins[141], aromatase inhibitors[110] and – more controversially – tamoxifen[142, 143]. Of note, the predictive capacity in relation to anthracyclins may well be explained by genomic co-amplification of the neighboring gene topoisomeras II α [144]. Its role as a prognostic factor is reflected in the StGallen guidelines from 2005 [104].

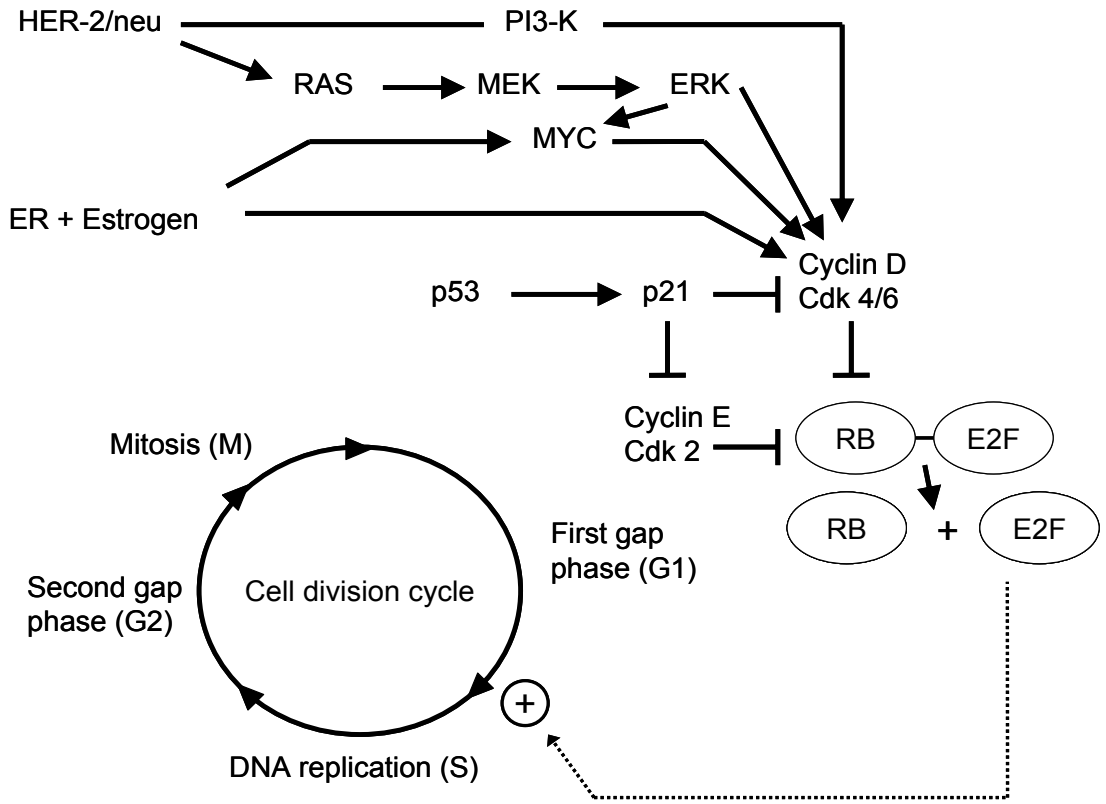

Figure 4. HER-2/neu and estrogen signaling.
HER-2/neu promotes proliferation via the PI3-K and RAS-MEK-ERK pathways, resulting in induction of Cyclin D, dissociation of phosphorylated RB and E2F. Estrogen and ER also induces Cyclin D, directly and indirectly.

4.2.7 The p53 tumor suppressor

p53 is a transcription factor that responds to several stimuli including DNA damage, oncogene activation, and hypoxia [145, 146]. It transcriptionally induces the cyclin-dependent kinase inhibitor p21 and pro-apoptotic proteins, resulting in cell cycle arrest or apoptosis [30]. p53 is a potent tumor suppressor, and function is lost in more than 50% of human cancers, mainly through mutations [147]. Mutations in the evolutionary conserved regions II and V have been associated with significantly worse prognosis in breast cancer [148]. In a meta-analysis of studies investigating p53 mutations in breast cancer, mutations were found in 20-30% of tumors, and the combined relative hazard was 2 (CI 1.7 – 2.5, overall survival) [149]. In a more recent study of 1,794 women with primary breast cancer, TP53 mutations within exons 5 to 8 conferred an elevated risk of breast cancer-specific death of 2.27 (relative risk)[150]. For prognostication, sequencing has been demonstrated to be superior compared to immunohistochemistry, that failed to detect 33% of mutations in one comparative study [151], and assessment of genomic DNA was more sensitive compared to RNA based methodology in another study[152]. Conflicting results with regards to the treatment predictive value of p53 mutations may be due to variable therapy regimens, different methods for assessing p53, and underpowered studies (overview in [153]). Recently, a p53 gene expression signature has been demonstrated to be of both predictive and prognostic value in breast cancer [154].

4.2.8 Angiogenesis

Angiogenesis is necessary for tumor growth beyond a certain size, oxygen and nutrient supply is not sufficient beyond 100 μm distance from capillary vessels [147]. The histological appearance of peritumoral vascular invasion has previously been considered of uncertain value [155-157], but is now, on the basis of new data included in assessment of risk for node-negative patients [104, 158]. The vascular endothelial growth factor (VEGF) has been extensively studied as an inducer of angiogenesis, and expression of VGEF has negative prognostic value in breast cancer, and correlates with mutant p53 [156, 159, 160].

4.2.9 Proliferation markers

Proliferation markers have been investigated in relation to prognosis with conflicting results [161-163], which may reflect the fact that several different methods to assess cell division have been used [164].

4.3 BREAST CANCER TREATMENT

4.3.1 Local therapy

Breast conserving surgery is a well established alternative to mastectomy for small (< 3 - 4 cm) non multi-centric tumors, and achieves comparable 20-year survival when combined with local radiotherapy [165, 166]. Radiotherapy is strongly recommended after breast conserving surgery to reduce loco-regional relapses [167-169]. Sentinel

lymph node dissection has emerged as an alternative to axillary lymph node dissection for small primary tumors [170].

4.3.2 Adjuvant therapy

The principal aim of adjuvant therapy is to target tumor cells that have escaped the primary tumor and are unavailable for local therapy (distant micro-metastases). Since prognostication on the basis of current factors lacks accuracy, present guidelines recommend adjuvant therapy in a vast majority of breast cancer patients.

4.3.2.1 Adjuvant endocrine therapy

Disruption of estrogen – estrogen receptor signaling is commonly achieved with tamoxifen, aromatase inhibitors and ovarian ablation or supression. Tamoxifen is a selective estrogen receptor modulator [171] with antagonist effects in breast cancer, but partial agonist effects in other tissues. An annual review of randomized trails reported 31% reduction of the annual death rate in estrogen receptor positive tumors, as a consequence of tamoxifen treatment for five years. This effect was seen both during the first five years and the following ten years [90]. In contrast, no significant benefit from tamoxifen is seen in patients with receptor negative tumors [172]. Aromatase inhibitors inhibit conversion of androgens to estrogens. In comparisons to tamoxifen, single treatment has not revealed more than marginal survival gain [173, 174], which also seems to be the case in sequential treatment (tamoxifen for 2-3 years, followed by aromatase inhibitor)[175, 176]. Ovarian ablation or suppression can be achieved with surgery, radiotherapy or gonadotropin-releasing-hormone (GNRH) agonists, and is associated with decreased breast cancer recurrence and mortality [90].

4.3.2.2 Adjuvant chemotherapy

Benefit of adjuvant systemic chemotherapy has been recognized for several decades [177]. In an overview of randomized clinical trials, anthracycline-based polychemotherapy reduced the annual breast cancer death rate by 38% in patients < 50 years old (when diagnosed), and 20% for patients 50-69 years old [90]. Further improvements seem achievable with docetaxel; in a randomized trial an improvement in disease-free survival (75%) was seen compared to anthracyclin-based polychemotherapy (68%) [178]. A comparable survival benefit for docetaxel was found in more recent trial also [179]. Results for adjuvant paclitaxel are less consistent. Disease-free survival has been reported to improve (70% in the paclitaxel group compared to 65% in the comparison group; [180]), as well as not improve [181]. Of note, tamoxifen and chemotherapy was administered simultaneously in the latter study.

4.3.2.3 Adjuvant trastuzumab

The monoclonal antibody trastuzumab targets the HER-2/neu receptor, and has been demonstrated to inhibit growth of HER-2/neu overexpressing tumor cells in vitro [182, 183]. Recent randomized trials have shown that trastuzumab improves outcome in patients with HER-2/neu positive tumors [184, 185].

4.3.3 Palliative treatment

Patients with hormone-receptor positive relapses are primarily offered hormonal therapy. Recent randomized trials favor aromatase inhibitors over tamoxifen [186-188].

Receptor negative or aggressive relapses are offered chemotherapy, and poly- compared to monotherapy is beneficial [189].

4.4 THE HETEROGENEITY OF BREAST CARCINOMAS

Variable expression of the estrogen receptor was an early indicator of heterogeneity in breast carcinomas [129]. Expression of ER, and lack thereof, seemed able to distinguish between groups of tumors with different clinical behavior. Apart from the prognostic potential of ER-expression already discussed, it has also been linked to differences in histologic grade [190], proliferation rate [191], and preferential metastasis site, where a tendency for metastasis to soft tissues and bone has been described for ER positive tumors, whereas ER-negative tumors frequently spread to visceral organs and the central nervous system [129]. That a significant degree of biological variability exists has subsequently found support in studies of histopathology and molecular biology in breast cancer. For some tumors, such as colorectal carcinomas, a well defined precursor lesion for invasive cancer has been described, indicating and ordered pathway of progression [192]. In contrast, a straightforward pathway of progression has been difficult to demonstrate in breast cancer. Some findings, such as clonal microsatellite alterations in atypical hyperplasia (AH) and concurrent ductal carcionoma in situ (DCIS) components, have been interpreted to support a serial progressive pathway [193]. The observation of small invasive cancers without accompanying atypical components on the other hand, seems to contradict this view[194]. Molecular heterogeneity seems to be the case throughout disease stages: in precursor lesions as well as invasive breast cancer, no pathognomonic cytogenetic abnormality has been observed, whereas a range of recurrent gains and losses have been described [195]. Furthermore, heterogeneity is not only evident between different patients, but frequently within a tumor from the same patient. In eight analyzed tumors, Teixeira and colleagues found two to six cytogenetically unrelated clones [196]. This polyclonality seems to extend to premalignant lesions [197], and lymph node metastases of breast cancer [198]. Similarly, the histopathology can be variable in both invasive lobular and ductal tumor samples, displaying areas of solid, tubulolobular, and alveolar variants, and cribriform, medullary and mucinous growth patterns, respectively [199]. The fact that the term breast cancer seems to comprise several biological entities has important clinical implications. A new treatment or prognostic marker needs to work in a significant fraction of covered (and not necessarily similar) tumor subtypes, for an overall effect or prognostic capacity to be evident. In tackling this, new methods such as expression microarrays and array comparative genomic hybridization (array-CGH) may turn out to be of considerable value.

5 ANALYSIS OF MICROARRAY DATA

5.1 INTRODUCTION

Microarray technology involves attaching thousands of probes (different DNAs with known sequence or identity) to a surface, and hybridizing RNA samples labeled with fluorescent dyes to this surface. The fluorescent dye is excited with laser, and the amount of emitted light provides an estimate of the degree of hybridization. Knowledge of the identity of a probe attached in a certain position allows estimation of hybridization to complementary transcripts, and thus the expression levels of thousands of genes can be assessed in parallel. Many different microarray platforms are manufactured, and there are some differences of importance for subsequent analysis.

In two color arrays (or two channel), two RNA samples are reverse transcribed and labeled with Cy3 and Cy5 fluorescent dyes respectively. Subsequent hybridization of the two samples is competitive, and Cy3 and Cy5 fluorescence is measured separately, yielding two estimates of hybridization for each probe. Estimation of gene expression is relative, and expressed as a ratio between the two samples (one is frequently a reference sample to allow comparisons to other hybridizations). In single channel arrays, only one sample is hybridized to each array and estimation of expression is absolute; comparisons to other hybridizations have to be made if change in gene expression is investigated. In the most widely used single channel arrays, manufactured by Affymetrix, the mRNA sample is converted to biotinylated cRNA and probes consist of 25 nucleotide oligonucleotides. Probes from pairs, with one perfect match (PM) and one mismatch (MM) probe, where the latter has a base in position 13 replaced by its complimentary base.

5.2 PREPROCESSING

5.2.1 Purpose

Preprocessing of microarray data aims at removing undesired sources of variation so that values given for individual genes will be as good a reflection of true changes - and non-changes - in mRNA abundance as possible. Many methods to achieve this have been proposed, but no clearly superior method has yet been identified. This is mainly due to the fact that there is no generally accepted test for preprocessing procedures. Frequently, tightly controlled calibration data sets have been used, but this way of assessing performance may not always be a good reflection of real data[200]. Also, what could be considered optimal preprocessing depends on subsequent analyses. There is often a bias-variance trade-off: a more complex procedure can remove more technical variation, but may at the same time introduce a new source of variation due to a more extensive assumption regarding the technical variation. If accuracy in actual changes is more important, this might be warranted, whereas if precision in non-changes is a priority, it might not. The choice between different methods and method settings for the optimal trade-off is not simple, and in practice it is currently mostly done manually and on an ad hoc basis.

Despite differences among the different platforms, there are tasks in preprocessing that are common to all microarray technology: Background adjustment, normalization, summarization, and quality assessment.

5.2.2 Background adjustment

Background adjustment is motivated by the fact that a fraction of the measured probe intensities are due to non-specific hybridization and noise in the optical detection system. This can be adjusted for to give more accurate values for specific hybridization. For both single and two channel platforms, local background can be estimated. In two-channel arrays, this is measured in areas of the glass slides not containing probe. In the Affymetrix Microarray Suite (MAS 5) software provided by the manufacturer of the most widely used single-channel arrays, local background is calculated as a weighted average of the lowest 2% of probe intensities in defined regions of the chip, and weights are reflecting the distance to these regions. Affymetrix microarrays also contain mismatch (MM) probes, where the 13^{th} of the 25 bases is replaced with its complement. Hybridization to these probes can be considered reflecting non-specific binding, and in an earlier version of the manufacturer's software (MAS 4), MM intensities were subtracted from perfect-match (PM) intensities. Since MM intensities are in fact higher than PM intensities in 30% of cases this frequently caused negative expression values, suggesting that the underlying assumption does not work [201].

In the MAS5 version, this was avoided by not using MM intensities that are higher than corresponding PM intensities, but rather an idealized mismatch (IM) based on the behavior of other MM intensities belonging to the same probe set. Also, to reduce the effect of outliers, an average is calculated with a one-step Tukey bi-weight algorithm, both for estimation of IM within a probe set, and the final summary of PM – IM intensities for the probe set [202]. This procedure involves a number of assumptions, and it has been demonstrated to introduce variance in lowly expressed genes. To avoid the PM - MM subtraction, the Robust Multichip Average (RMA) procedure was developed to yield expression estimates based on PM intensities only [201]. This method sacrifices some accuracy in reporting true changes – due to not utilizing MM intensities – for a significant gain in precision (reducing noise especially in lowly expressed genes). A further development has been described to achieve almost as much gain in precision in spite of utilizing the MM intensities to improve accuracy. This is achieved by incorporating a model of the relationship between the non-specific hybridization and the sequences of specific probes [203]. These commonly used procedures thus represent attempts to create optimal background adjustment with quite different aims regarding the accuracy-precision (bias-variance) trade-off. Many other methods have been proposed, but the decision to use or not use MM intensities for background correction seemed to be a major determinant in an assessment of 31 preprocessing algorithms, independent of subsequent approaches for normalization and summarization [204].

In background adjustment of two-channel microarray data, conclusions seem analogous. Background adjustment sometimes substantially reduces precision by increasing variability in low-intensity probes [205].

5.2.3 Normalization

Normalization is necessary to make measurements from different arrays comparable. Early approaches, using "house-keeping" genes (genes that can be assumed to not change between experiments) or spiked in RNA controls, have limitations. Small random changes in house-keeper gene transcription or spiked-in RNA amount can introduce significant variance in a microarray experiment; expression measures of thousands of non-changing genes are adjusted erroneously and may decrease precision. The global approach is less sensitive. Here, all genes are used, and a normalization coefficient is estimated on the basis of the mean or median of all expression measures on the array. This approach is used in Affymetrix MAS 4 and 5 versions. A more extensive assumption underlies the quantile normalization [206] used in RMA and GCRMA. The same empirical distribution is imposed on each array by rank ordering probe sets on individual arrays, calculating a mean for each probe set across arrays, and assigning it to the reordered probe sets. Thus, the global distributions are identical, but individual probe sets can have different assigned measures across arrays. For two-channel data, global normalization is often utilized to normalize "within chip": technical variation reflecting different labeling efficiencies and scanning properties of the Cy3 and Cy5 dyes need to be accounted for. Normalization coefficients reflecting both the center and the spread of the log-ratios ($\log_2$ [Cy5/Cy3]) are used, and the in the frequently used intensity dependent loess approach, the center is assumed to be a smooth function of the mean Cy5 and Cy3 measurements. The "within-chip" normalization centers log-ratios around zero, whereas further scale normalization can be performed between chips.

5.2.4 Summarization

Summarization is necessary in cases where a gene is represented by many probes on the array, as in the case of Affymetrix platforms. In MAS 5 software, the one-step Tukey bi-weight algorithm is used to summarize across probes, RMA and GCRMA uses a median polish procedure. A somewhat neglected aspect of summarization is the impact of accurately assigning probes to probe sets. The original Affymetrix probe set definition files (CDFs) were created when most genomes of respective species were not yet sequenced. Redefined probe set definition files have been proposed, better reflecting current knowledge [207], and increased precision as well as accuracy has been described [208]. This can also achieve another benefit compared to original Affymetrix CDFs, where two or more separate probe sets were frequently given for each gene. In analyses of gene sets, where genes relevant to some common biological process are considered together, individual genes are – from a statistical point of view – considered independent hits for the specific gene set. Assessing more than one gene set for each gene violates that assumption, so grouping all relevant probes into a singular probe set can be preferential, as in Dai's et. al. CDFs.

5.3 DIFFERENTIAL GENE EXPRESSION AND CONTROL FOR MULTIPLE TESTING

5.3.1 Differential gene expression

Fold-change is a popular way of describing change in gene expression between different experiments. If describes ratio of averages in the compared groups, and does not account for variability. It is now widely considered inappropriate for identifying genes for further validation [209, 210], but is useful for assessing the magnitude of gene expression change.

An alternative approach is to apply statistical tests to individual gene, and use the resulting test statistic as a basis for selection. A number of tests can be considered, including linear models, logistic models and Cox regression.

Since few data points are often available for individual genes, gene-specific variance estimates are imprecise. To increase power, especially for small experiments, several methods for modifying test statistics have been proposed [211-213]. The common principle is to add a term in the denominator (*t*-tests) based on all genes in the microarray. This procedure has been referred to as 'variance shrinkage', and has become popular [214].

5.3.2 Control for multiple testing

Testing thousands of genes in parallel is likely to yield hundreds of false positive findings in a typical micorarray experiment, if a threshold of $p = 0.05$ is used in individual significance tests. In order to account for this, control for multiple testing is necessary. A quite stringent approach is to use Bonferroni correction, controlling the family-wise error rate (FWER). The FWER describes the probability of making one error across all genes tested, and in many experiments this basis for correction would result in no genes being deemed differentially expressed.

An alternative measure was introduced by Benjamini and Hochberg: the false discovery rate (FDR)[215]. FDR describes the ratio of true versus false findings, and Benjamini and Hochberg also provided a procedure for controlling it. More powerful approaches for FDR assessment has subsequently been proposed and implemented [216-218]. In the popular Significance Analysis of Microarrays (SAM) algorithm [213], the FDR is estimated by analyzing permutations of the analyzed data, where case- and control labels are randomized to achieve an estimated distribution of test statistics for non-differentially expressed genes.

5.4 GENE SETS

Analyzing the result of a microarray experiment in terms of a list of differentially expressed genes has two important disadvantages: 1) small sample sizes, and efforts to maintain a low FDR often result in low power to detect differentially expressed genes. A consequence of this is unstable gene lists, which can be revealed by analyzing random subsets of the performed experiment [219]. In the breast cancer field, this is illustrated by several prognostic gene lists defined in different clinical cohorts, that in spite of being largely non-overlapping point towards the same biological processes (cell cycle control, DNA replication, apoptosis etc; [220]).

Interpretation of a long list of genes is challenging, and there is an obvious risk for investigator bias. In response to this several methods for considering the underlying

biology have been developed. An intuitive approach is to test the null hypothesis of no relationship between the set of differentially expressed genes, and sets of genes sharing some biological function, often Gene Ontology (GO) categories[221]. Over- or under-representation from specific gene sets can be assessed with Fisher's exact tests or $\chi 2$-tests (overview in [222]). This approach has been criticized for requiring a defined cut-off for differentially expressed genes, and alternative methods have been proposed (e.g. [223-226]). More fundamental criticism has been raised regarding the validity of these methods[214]. These methods treat the gene, rather than the case, as the sampled unit. The p-value in the gene sampling situation has a rather different interpretation compared to what we are used to; it addresses the reproducibility of findings on the same biological samples, with a new set of genes (for instance, a different array platform). What we normally take interest in is the reproducibility in a new set of biological samples, so the statistical test should treat the case as the sampled unit. This has the advantage of not disrupting the pattern of correlation between genes (i.e. not assuming that genes are independent), and one method adheres to this principle (Gene Set Enrichment Analysis, GSEA; [226])

5.5 CLASSIFICATION

5.5.1 Unsupervised classification

Unsupervised methods for identification of relevant phenotypes in microarray data have become very popular since the introduction in 1998 [227]. The most important advantage of such methods is the potential for unbiased classifications of samples and genes. If applied correctly, any pattern detected will be independent of the investigators preexisting view on the biology studied. In the case of the frequently used clustering algorithms, this is achieved by computing a distance between samples or genes, based on some distance measure such as Euclidean distance or one minus Pearson correlation [228]. Groups, or clusters, can then be defined on the basis of computed distances in several ways. This is often achieved with hierarchical clustering, where the most similar two samples (or genes) first form a cluster, then the second most similar and so forth, or with k-means clustering, where the number of clusters are predefined and determined by iteratively recalculating the cluster center. Other methods include partitioning around medioids (PAM; [229]) and self-organizing feature maps (SOFM; [230]).

The user needs to make several decisions when clustering data: 1) the distance measure to be used; 2) the linkage function (principally describing how the position for a formed cluster is to be determined); 3) the clustering algorithm. The range of options, and the fact that clustering algorithms always produce a result [231], have undermined the validity of these methods; users can frequently produce a clustering that seems to support their conclusions. This is further complicated by the fact that little has been established with regards to the relative merits of different clustering methods [232, 233], and a possible general lack of reproducibility in cluster analyses of microarray data sets of typical size[234, 235]. As a result, a consensus has emerged that resampling techniques can assess the reproducibility of unsupervised clustering, and that they should be used [214]. In these procedures, subsets are resampled from the original data,

unsupervised classification is applied, and consistency of results across the resamples is quantified [236-238]. In analogy to the situation in gene set analysis, resampling subsets of samples (microarrays) rather than genes seems to better address the important question; is the clustering reproducible in a new experiment, with new samples? Applying resampling on genes would seem to answer the question if the clustering would prove reproducible in the same biological samples with a different microarray platform, and is probably not the type of reproducibility we are primarily interested in[239].

Principal component analysis (PCA) and singular value decomposition (SVD) are useful methods for dimension reduction [240]. In these analyses, thousands of genes can be replaced by a number (equal to the number of samples) of uncorrelated meta-genes. Expression of meta-genes in different samples can reveal relationships between samples, and genes can be correlated to the meta-genes to reveal the underlying biology.

5.5.2 Supervised classification

Supervised classification involves defining a rule that predicts outcome on the basis of available predictor variables. This rule can be very simple, such as a threshold for a single variable (e.g. predicting ER status on the basis of a continuous variable for immunohistochemical staining intensity), or complex and based on many predictor variables. The outcome to be predicted can be dichotomous (e.g. ER status), continuous and possibly censored (e.g. survival), or have multiple levels (e.g. subtypes of cancer). The predictor variables can be any available data, typically expression levels for multiple genes in applications for microarray data.

5.5.2.1 Regression-based classification

Prediction rules can be defined in different ways, with different rationales. In regression modeling, a coefficient (acting as a "weight", reflecting importance) for each predictor variable is chosen so that the overall fit of the model defined by the coefficients is as good as possible. The quality of the fit is evaluated based on theoretical arguments (maximum likelihood, least squares) and not primarily concerned with predictive ability. An inherent underlying assumption for this approach is that of a specific distribution for the random variation in the data ("error").
In a classical setting with only a few predictor variables and a sufficient number of samples, the predictive power of the model can be assessed by considering the variation explained by the predictors in relation to random variation; if the random variation is small compared to the variation explained by the predictors, we can use the model for prediction.
In the same setting, regression also allows us to assess importance and replicability of individual predictors in the model. Based on the distributional assumptions of the model, we can calculate the probability of different values for an individual coefficient under specific scenarios. This allows us specifically the calculation of p-values for individual null hypotheses of type H_0 : coefficient=0. A small p-value for an individual coefficient leads us to expect that the observed weight is unlikely to be due to random

variation. This is also an indication that the variable corresponding to a small p-value in the current model will also work as predictor in an independent data set.
In the context of microarray data, with generally several thousand predictors and usually a few hundred samples at most, the regression based approach to prediction runs into problems. It is impossible to fit the full model with all predictor variables simultaneously. This can be addressed via feature selection (i.e. using only a suitably small subset of predictor variables), stepwise model fitting (i.e. variables are included or excluded in repeated model fitting steps), or shrinkage (i.e. the majority of coefficients is shrunk towards zero), or any other approach to reducing the dimensionality of the prediction problem. However, due to the inherent multiple testing character of the variable selection step, it is no longer possible to use the extent of model fit as an indicator of the predictive power of the model, or to use the p-value as an indicator of the importance of a predictor variable in the model. Spurious relationships between the random variation components in the data and the large number of available predictors will be included in the model; but as these relationships are just a necessary consequence of the high-dimensional predictor space, they will be impossible to replicate, and lead to clearly worse prediction results than would be expected from the model fit. In regard to assessing the importance of predictors based on p-values, all the reservations outlined in 5.3.2 apply. As a consequence, the predictive power of regression-based models, as well as the importance of individual predictors, is generally evaluated via cross-validation techniques.

5.5.2.2 Machine-learning based classification

Alternatively a number of techniques generally referred to as 'machine learning' methods, have been developed to accomplish prediction from large data sets such as typically found in microarray studies. Methods can be divided into two classes, those that make assumptions about the class-determined probability density functions (linear and quadratic discriminants, *k*-nearest neighbor), and those that do not (neural networks, support vector machines, and others) [228].
The *k*-nearest neighbor (*k*-NN) will serve as an example.
In *k*-NN classification, classification is achieved by calculating some measure for distance between observations, such as Euclidean distance. The distance measure is a reflection of the dissimilarity across the selected genes/features between two observations (two expression profiled tumors etc.). Given known class membership of some observations, prediction of class for each unknown observation is achieved by considering the *k* least distant observations with known class. The most frequent class among the *k* neighbors is accepted as the predicted class for the unknown sample. This simple method thus achieves classification of unknown samples based on the assumption that the correct class is the one most prevalent among other observations with similar gene expression profiles, i.e. in a local neighborhood of the high-dimensional predictor space.
In common with many machine learning approaches, the kNN method provides a straightforward algorithm for classifying new observations based on an existing data set with complete classification; furthermore, it has an appealing geometrical interpretation that can be visualized easily for low-dimensional prediction problems. In the same manner, kNN does not offer immediate answers for two crucial questions: 1) How well will it perform when applied to new data? 2) How should the model parameters (in this

case the size k of the neighborhood) be chosen? The characteristic solution for these problems in the machine learning setting is to sub-divide and re-use parts of the data for both choice of parameters (or model fitting) and model evaluation.

5.5.2.3 Cross-validation

It is often of fundamental interest to know if prediction would be accurate in new samples, and one way to achieve this is to simply split observations with known class into two, and use one half to train the classifier, the other to validate performance. In the *k*-NN example this means including half of the known samples as unknowns, applying the *k*-NN procedure, and then evaluating performance by comparing predictions in the anonymous cases to their true class memberships. This *hold-out* procedure is an example of cross-validation. The approach involves reducing the number of observations available for training, and may produce poor predictions.

Another option is to include one anonymous sample at a time, predicting its class, and repeat the procedure for all samples. This is called leave-one-out cross-validation, and often means more marginal loss in the estimation of predictive accuracy, but may be biased since almost the entire data at hand is used in training.

N-fold cross validation represents a trade off: in 10-fold cross validation, the data set is split into 10 equally sized parts, 9 are used for training and one for validation. This is repeated so that all parts have performed the function of validations sets (10 times).

It deserves mentioning that all aspects of the prediction algorithm design including the chosen genes, method for measurement, equations underlying the summary, and cutoff need to be defined solely in the training data to avoid 'information leakage' and a biased estimate of the true predictive accuracy. Furthermore, the inclusion criteria and the predicted end-point must be the same in training and validation sets [219]. Several early microarray papers failed to account for this and severely over-estimated predictive accuracy [241, 242].

6 AIMS OF THE THESIS

- To utilize microarrays to examine if gene expression changes are similar in aging and cellular senescence.

- To contrast gene expression changes, as assessed with microarray technology during early brown and white preadipocyte differentiation, to study regulation of cell fate.

- To use microarray data to study the connections between chromosomal positions and gene expression in primary breast cancers, and the relevance of such relationships for patient outcome.

- To test the possibility of predicting sites for distant recurrences with microarray profiles of primary breast cancers.

7 PATIENTS, MATERIALS AND METHODS

7.1 SENESCENCE AND AGING

7.1.1 Data collection and selection

PubMed was searched for studies of senescence or aging where microarrays had been used to monitor global changes in gene expression. Studies with less then 6,000 profiled genes were excluded, as subsequent cross-array and cross-species comparisons would become less reliable due to relatively few data points. From the 34 publications that were considered, we were able to use data from 10. Studies were excluded for the following reasons: raw or appropriately normalized data files could not be obtained (RMA or dChip normalized data for a number of reasons; authors did not reply, authors distributed files not belonging to any of the listed categories, data was lost; n = 17); not human, mouse or rat studies (n =5); technical problems, including anti-correlation between replicate experiments, no evident differential gene expression (n = 3)

Table 1 **Aging studies**

Authors	Chip type	Species	Tissue	Specifics
Welle S, et al [243]	HG U133 A+B	human	skeletal muscle	old (n=16) 66-77 years young (n =15) 21-24 years
Lu T, et al [244]	HG U95 Av2	human	frontal cortex of brain	old (n=11) 73-106 years young (n =10) 26-42 years
Rodwell GE, et al [245]	HG U133 A+B	human	kidney cortex and medulla	old (n=28) 72-92 years young (n =11) 27-45 years
Lee CK, et al [246]	MU 6500	mouse	cerebellum and neocortex of brain	old (n=2 / 3*) 30 months young (n=3) 5 months
Lee CK, et al [247]	MG U74 A	mouse	heart	old (n=5) 30 months young (n=5) 5 months
Blalock EM, et al [248]	RG U34 A	Rat	hippocampus, brain	old (n=10) 24 months young (n=9) 4 months

Table 2 **Senescence studies**

Authors	Chip type	Species	Tissue	Induction of senescence
Zhang H, et al [249]	Stanford, cDNA	human	fibroblasts	serial passaging
Schwarze SR, et al [250]	Stanford, cDNA	human	prostate epithelial cells	serial passaging
Zhang H, et al [251]	Stanford, cDNA	human	mammary epithelial cells	serial passaging
Larsson O, et al [252]	MG U74 Av2	mouse	thymus epithelial cells	heat inactivation of SV40 large T antigen

Data for the included studies was downloaded (http://www.ncbi.nih.gov/geo, or http://genome-www5.stanford.edu)or obtained directly from the authors, and are listed in tables 1 and 2. In some cases (Lee CK, et. al., both Zhang, H et. al.studies), we excluded samples from individual datasets if the correlations to replicate samples were dramatically different from other within-replicate correlations.

7.1.2 Normalization

We renormalized all datasets but one, for which we obtained dChip normalized data (Lu T et.al.). All Affymetrix data were normalized with RMA, all cDNA data were normalized using loess intensity dependent normalization with default settings in the Bioconductor package LIMMA.

7.1.3 Correlation in gene expression

The approach for assessing similarity in gene expression between different studies involved four steps; 1) calculation of change in gene expression; 2) mapping of probes across arrays and species; 3) calculating correlation across corresponding genes or orthologs; 4) assessing significance. In step 1, change in gene expression between cases (old tissue or senescent cells) and controls (young tissue or control cells), was calculated by subtracting average log2 expression in the control group from average log2 expression in the case group, for each probe. In step 2, the RESOURCERER tool (http://www.tigr.org/tigr-scripts/magic/r1.pl) and manufacturer annotation files (http://www.affymetrix.com) were used to map probes on different microarray platforms, to enable comparison between genes or orthologs. In step 3 all common genes or orthologs were used in calculation of Pearson, Spearman and Kendall correlations, to assess global similarity in gene expression change. In step 4), significance was assessed by disrupting true gene or ortholog relationships by randomization, and recalculating correlations. This was repeated 10 000 times to yield random distributions for assessing significance of the true correlation. The Zhang et al. [249] and Larsson et. al. [252] studies provided confluent cells as an alternative reference for senescent cells. To remove effects of cell proliferation differences, microarray data pertaining to these cells were used in step 1 above.

7.1.4 Gene ontology analysis

In the six comparisons of aging and senescence where confluent cells were available as controls for senescent cells, gene ontology (GO) analysis was performed. For all GO categories with more than two genes present in a specific comparison, the partial summation of the Pearson correlation was calculated:

$$rj = \sum (x_j - \mu_x)(y_j - \mu_y)/n\sigma_x\sigma_y,$$

where j denotes genes belonging to the specific GO category and μ_x, μ_y, and σ_x, σ_y, the means and standard deviations of expression ratios for all genes or orthologs in the

comparison. The partial summation thereby represents the contribution form the specific GO category to the overall correlation in the comparison.

7.2 BROWN AND WHITE ADIPOCYTE DIFFERENTIATION

7.2.1 Primary cell cultures and differentiation

Brown (interscapular, cervical, and axillary depots; BAT) and white (epidydimal; WAT) adipose tissue was isolated from male NMRI mice (3-4 weeks old; B&K, Stockholm, Sweden), as previously described (ref 1). Tissue was minced, pooled and incubated in a digestion solution (0.2% collagenase (type II; Sigma, St. Louis, MO) in a buffer consisting of 0.1 M Hepes, pH 7.4 / 123 mM NaCl / 5 mM KCl / 1 mM $CaCl_2$ / 4.5 mM glucose / 1.5% BSA) for 30 min at 37°C with continuous vortexing. Cells were filtered into 15-ml tubes utilizing a 250-μm pore nylon filter (Sintab, Oxie, Sweden), and kept on ice 20 min. The top layer of the suspension, containing mature adipocytes, was removed. After filtration through a 25-μm pore nylon filter (Sintab), cells were centrifuged for 10 min at 700 × *g*, suspended in 10 ml of DMEM, centrifuged for 10 min at 700 × *g* once more and suspended in 0.5 ml of culture medium per animal

Cell suspension was added (0.2 ml to each well) to 10-cm^2-well plates (Corning, Corning, NY) containing 1.8 ml of culture medium (DMEM with 10% (vol/vol) newborn calf serum (Invitrogen, Carlsbad, CA) / 2.4 nM insulin / 25 μg/ml sodium ascorbate / 10 mM Hepes, pH 7.4 / 4mM glutamine / 50 units/ml penicillin / 50 μg/ml streptomycin) per well. The cell phenotype was verified (using *ucp1* expression as a marker) by treatment with vehicle or 0.1 μM norepinephrine for 4 h. Medium was discarded and the cells were harvested from each well with 1 ml of Ultraspec (Biotecx Laboratories, Houston, TX, U.S.A.) as described in the manufacturer's protocol

7.2.2 Microarray hybridizations

Hybridization, washing, staining, and scanning of the arrays were performed according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA; www.affymetrix.com). RNA from six to eight primary culture preparations per time point were hybridized to Affymetrix U74A v2 array platforms. Implementations of the MAS 5 algorithm were used for normalization of internal data, as well as for comparison data. Quality was assessed by hierarchical clustering (for finding outliers), scaling factors, and 5':3' ratios.

7.2.3 Differentially expressed genes and gene set analysis

In comparisons of undifferentiated brown and white preadipocytes cultured for 4 days, the Significance Analysis of Microarrays (SAM) algorithm was used to identify genes expressed ≥ 2-fold in one cell type, at a FDR <1%. For identifying differentially expressed genes during brown and white preadipocyte differentiation, less stringent criteria were used; ≥ 2-fold change, 10% FDR, because some genes (e.g., oxidative

phosphorylation) genes relevant for brown adipocyte differentiation are not subject to large variations in transcript abundance; [253]). The Expression Analysis Systematic Explorer (EASE) analysis tool [254] was used to identify overrepresented GO categories.

7.2.4 Enrichment analysis of transcription factor binding site

Sequence information from the 1000bp upstream regions of genes on the U74A v2 microarray platform were aligned with corresponding upstream human ortholog sequences. Differences in frequency of predicted transcription factor binding sites were investigated in differentially versus non-differentially expressed genes.

7.2.5 Comparisons to external data

Gene expression changes in BAT and WAT differentiation was compared to external data sets [76, 84] in several ways. SAM analysis, and unmodified *t*-tests were used to define lists of genes differentially expressed between BAT and WAT, as well as a consequence of IRS1 knock-out and Sir2 (SIRT1) over-expression. Both differentially expressed genes and overrepresented Gene Ontology categories, based on identified genes (EASE), were compared. Overlapping gene lists were also assessed in EASE. Similarity was further investigated by correlation in gene expression changes (fold change) across all comparable genes. Principal component analysis (PCA) was applied to merged data sets (consisting of adipocyte as well as external data). To biologically annotate components in PCA analysis, the top 100 genes for individual components were analyzed with EASE.

7.2.6 RT-qPCR and Northern blot analysis

Total RNA was isolated using Ultraspec (Biotecx, Houston, TX) and the RNeasy mini kit column procedure (Qiagen, Valencia, CA) and reverse transcribed (Applied Biosystems, Foster City, CA). RT-qPCR was performed in triplicate from three independent cell cultures. For the muscle-specific transcription factors (MyoD, myogenin, Myf5 and Myf6) hindlimb skeletal muscle was obtained from 4-week-old animals to act as a positive controls. For Northern blotting, total RNA was separated on an agarose gel (1.25%).The RNA was transferred on to a Hybond-XL membrane by overnight blotting (Amersham Pharmacia Biosciences, Piscataway, NJ). Hybridization was performed with the addition of denaturated probe, [^{32}P]dCTP-labeled cDNA, corresponding to mouse uncoupling protein 1 mRNA, labelled by random priming (Amersham Pharmacia Biosciences).

7.3 BREAST CANCER

7.3.1 The Stockholm cohort

The Stockholm cohort consists of breast cancer patients that received therapy at the Karolinska Hospital during the years 1994 through 1996, identified through the

Regional Cancer Registry. From an initial set of 524 identified patients, gene expression profiles on available tumor material of sufficient quality were obtained for 159 patients. All profiled tumors had been frozen on dry ice or in liquid nitrogen and been stored in -70°C freezers. Patients were excluded for the following reasons: no frozen tumor (n = 231); degraded tumors (n = 42); insufficient amount of RNA (n = 36); profiled on U95A chips only (n = 14); had received neoadjuvant therapy (n =12); did not pass quality assessment for arrays (n = 12); living abroad (n = 7); refused to participate (n = 6); in situ cancer (n = 5); cancer in stage IV (n = 1). In the 231 patients without frozen tumors, the mean tumor diameter was lower, the mean number of affected axillary lymph nodes was lower, and the proportion of patients alive at the end of follow-up was lower, compared to the profiled group [220]. The ethical committee at the Karolinska Hospital approved this expression profiling project. The Regional Cancer Registry, complemented with patient records, was examined for information regarding tumor size, number of axillary lymph node metastases, hormonal receptor status, distant metastases, date and site of relapse, therapy, and date and cause of death. Sections from microarray profiled primary carcinomas were classified using Elston–Ellis grading [110] by a blinded pathologist.

7.3.2 The Uppsala cohort

The Uppsala cohort consists of breast cancer patients that received primary therapy from 1987 to 1989 in the county of Uppsala. From an initial set of 484 patients, gene expression profiles on available tumor material of sufficient quality were obtained from 243 frozen tumors. Tumors were excluded for the following reasons: no frozen tumor (n = 169); insufficient amount of tumor left (n = 16); insufficient amount or quality of RNA (n = 29); did not pass quality assessment for arrays (n = 10); could not match raw data files to patients (n = 10); no invasive cancer in histological reevaluation (n=7). Tumors were graded according to Elston–Ellis. This patient cohort has been described previously [148, 154, 220, 255].This RNA expression study was approved by the ethical committee at the Karolinska Institutet.

7.3.3 RNA preparation

Portions of the frozen tumors were cut into small pieces, contained in test tubes with RLT buffer (RNeasy lysis Buffer, Qiagen, Hilden, Germany), and homogenized for 30–40 s. During the project, treatment with Proteinase K for 10 min at 55°C was introduced since most initial RNA extractions not including this step produced either low RNA yield and or insufficient RNA quality. Qiagen microspin technology (Qiagen, Hilden, Germany) was subsequently used to isolate total RNA, and DNase was added to some samples to enhance RNA quality. Utilizing an Agilent 2100 bioanalyzer (Agilent Technologies, Rockville, MD, USA), the 28S:18S ribosomal RNA ratio was measured to assess the quality of RNA. Tumor RNA of high quality was then stored for microarray analysis at -70°C.

7.3.4 Microarray profiling

In vitro transcription, hybridization to microarrays, and scanning was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Two to 5 μg of total RNA was used for each preparation. The *in vitro* transcription reactions, generating biotinylated cRNA targets, were carried out in batches. Samples were then subjected to chemical fragmentation at 95°C for 35 min. cRNA (10 μg) was hybridized to Affymetrix U 133 A and B chips. The arrays were washed, and stained with streptavidin–phycoerythrin (final concentration, 10 μg/ml), and scanned according to the manufacturer's instructions. In cases of visual defects on inspection, samples were re-hybridized and rescanned on new chips.

7.3.5 Further analyses, paper III

7.3.5.1 Normalization

Redefined probe sets were used to achieve a one probe set – one gene relationship, and exclude unspecific probes[207]. Normalization was performed with the GCRMA algorithm[203].

7.3.5.2 Differential expression

Unmodified *t*-tests, as implemented in the EOC function in the OCplus R package [256]. Top ranking genes (200 and 500 genes long) for differential expression between groups defined on the basis of 5 year recurrence-free survival were extracted (false discovery rates: 0.17 – 3.1%).

7.3.5.3 Gene sets

Gene sets were defined on the basis of chromosomal arms and bands. Fisher's exact test was then applied to test the null hypothesis of independence between assignment to differentially expressed lists of genes, and all occurring bands and arms. Bonferroni and Benjamini–Hochberg[215] methods were used to control multiple testing for arms and bands, respectively.

7.3.5.4 A 16 q expression measure

To assess expression across the long arm of 16q, expression was averaged for each tumor, with and without per-gene mean and median normalization, yielding a single expression measure for each tumor. The effects of filtering genes on the basis of variance and average expression were also investigated (50% of genes discarded in both cases.

7.3.5.5 Molecular subtypes

Supervised clustering on the basis of correlation to centroids for molecular subtypes was preformed as previously described [257]. Informative "intrinsic genes" as described by Sørlie and co-workers were used to define the five centroids, one for each of the molecular subtypes in Sørlie's et. al. data. Subtype was then determined for each tumor in the present data with the nearest centroid method, on the basis of all overlapping intrinsic genes (by Entrez IDs).

7.3.6 Further analyses, paper IV

7.3.6.1 Metastasis patterns

Information regarding metastasis sites was obtained from patient records. Eighty-seven patients had distant metastases to at least one site. Fifty-eight patients had skeletal metastases, 26 had lung metastases, 17 had liver metastases, and ten had metastases to other sites (brain, pleura, distant lymph nodes, ovary, uterus, and lesser pelvis).

7.3.6.2 Normalization

Raw microarray data was normalized with the GCRMA [203] algorithm, utilizing redefined probe set definitions [207]. In comparisons to published skeleton and liver metastasis signatures, MAS 5 normalized data was used to increase comparability.

7.3.6.3 Metastasis signatures and validation

A simple approach was used for defining site-specific signatures. Genes were rank ordered on the basis of *t*-tests (comparing mean expression in the site-specific versus no site-specific metastasis groups), and the top 50 genes were chosen for a signature. Two centroids were defined in the training set as the average expression across signature genes in the site-specific versus no site-specific metastasis groups. Prediction was performed in the validation sets with the nearest centroid method; tumors were predicted according to the nearest of the two (site-specific and no site-specific metastasis) centroids. To assess robustness in prediction of metastasis sites, 500 random data sets (balanced for site-specific and no site-specific metastasis) were defined, each consisting of a split of available tumors into training and validation sets. For all possible proportions for the split (proportion of patients in the training set), 500 random data sets were produced. Signatures were defined in the training sets, and validated in the validation sets. For the 500 predictions in each validation set, accuracy in prediction was assessed as the number of expected minus empirical errors, or sensitivity and specificity.

7.3.6.4 Determination of genetic grade

Redefined histological grade was determined on the basis of a genetic grade signature provided by Ivshina and co-workers [258]. A simple score was calculated: for all signature genes over-expressed in high grade tumors the weight 1 was used, and for under-expressed genes -1 was used. For each tumor, gene expression measures across genetic grade signature genes were added after multiplying each gene with its weight. A cutoff for high genetic grade was set to 0 for the sum, on the basis of a bimodal distribution for the sum.G1 tumors were thus defined as being of low genetic grade in 92.5% of cases; G3 tumors were defined as being of high genetic grade in 89% of cases, the biologically heterogeneous G2 tumors were split into low genetic grade (71%) and high genetic grade designations (29%).

7.3.6.5 Determination of HER2/neu status

Good agreement between the HER-2/neu expression measure (as assessed with U133A and B arrays) and amplification has previously been demonstrated in 40 patients where both gene expression measures and FISH data was available (16 patients with HER-2/neu amplification; 16 without HER-2/neu amplification)[259]. A receiver operator

characteristic curve was used to determine an optimal cut-off (7.8) for determining amplification status (sensitivity 0.81, specificity 0.96).

7.3.6.6 Multivariate logistic regression

Multivariate logistic regression was performed with potential predictive variables, including genetic grade and HER-2/neu status.

7.3.6.7 Proposed skeletal and lung metastasis signatures

The utility in the present data of published [260, 261] skeletal and lung metastasis signatures was assessed in several ways. Hierarchical clustering with two different distance measures (Euclidean and 1 – Pearson correlation), and three different linkage functions (average, single and complete), on the basis of the respective signature genes was performed. SAM (two-class and censored survival applications), *t*-tests, and Cox proportional hazard ratios were calculated in relation to binary outcome (site-specific versus no site-specific metastasis) and censored site-specific survival, as appropriate. All approaches were tested both in the full set of 402 patients, and the subset of 87 patients with metastasis to at least one distant site.

8 RESULTS AND DISCUSSION

8.1 PAPER I

The contribution of cellular senescence to organism aging is controversial [262]. Due to limitations in morphological characteristics and specific molecular markers, it is difficult to establish whether senescent cells accumulate in aging tissues. Several microarray studies have been published describing the transcriptomes of aging tissues and senescent cells in vitro. We aimed to explore if gene expression signatures could be utilized to demonstrate similarity between cellular senescence and aging.

The overlap in regulated genes between similar microarray experiments is usually not great, although the agreement in biological processes implicated is generally good. To avoid a significant reduction of genes in a comparison across eleven microarray data sets, we made pair-wise comparisons of studies, by calculating the correlation between changes in gene expression. The correlations were assessed in two ways: first, they were compared to null-distributions of correlations achieved by randomizing gene-to-gene or gene-to-ortholog (in cross-species comparisons) relationships. In a second step, they were grouped together on the basis of the nature of the comparison: aging-aging comparisons (n= 21), aging – senescence comparisons (n=28), senescence-senescence comparisons (n= 6). In a final analysis, we investigated if any specific biological processes would be implicated by calculating the partial correlation contributed by specific gene ontology categories.

In aging-aging and senescence-senescence comparisons, individual pair-wise correlations were generally larger than the respective null-distributions (Bonferroni corrected p-value of 0.005), and t-tests supported a positive average correlation in these groups ($P< 0.001$, and $P= 0.032$, respectively). In contrast, the aging-senescence comparisons were with few exceptions within the null-distributions, with neither a positive or negative average, as assessed with a t-test ($P= 0.12$). Interestingly, when only two senescence studies were included, both of which had included an alternative reference, we had a different result. In these studies, gene expression profiles of senescent cells had been compared to proliferating as well as quiescent cells. When we made analogous comparisons (n= 14) with quiescent cells serving as a reference (rather than proliferating cells, as in our first analysis), the analysis seems to support an average similarity between cellular senescence and mouse aging, but not between human aging and cellular senescence. Here, several gene ontology categories involving mitochondria, protein folding, and RNA processing were implicated by similar change in expression.

How does this agree with previous findings? It seems clear that there are profound differences in how senescence is induced in human and mouse cells. Mouse telomeres (~150 kb) are many times longer than human telomeres (~10 kb). Mouse embryonic fibroblasts do however enter senescence after 10-15 population doublings in culture, seemingly independent of telomere length. This limitation can be removed by altering culturing conditions; low ambient oxygen or serum extends replicative lifespan

dramatically[32]. This has led some authors to entertain the possibility that replicative senescence does not exist in the mouse[263]. Other factors apparently present a limit, and that they may be of importance in organism aging is supported by increased expression of p16 and Arf in a variety of aging organs in mice[264]. This suggests that some source of in-vivo stress might be important in mouse aging, perhaps telomere independent senescence elicited by reactive oxygen species.

8.2 PAPER II

Induction of uncoupled oxidation of fuel substrates - a characteristic of brown fat cells - in white adipose tissue, is considered a plausible approach in combating obesity. The developmental relationships as well as specific molecular determinants of brown versus white adipocyte differentiation are however poorly characterized. With expression microarray technology, primary cultures of brown and white preadipocytes were contrasted at 4 and 7 days of culture, to capture transcriptional differences during differentiation. The major finding was expression of myogenin, MyoD, and Myf 5, previously identified as constituents of a myogenic transcriptional program, in brown but not white preadipocytes. Further potential mRNA markers for brown and white preadipocytes were also identified. The myogenic program appears suppressed during brown adipocyte differentiation. Another not unexpected difference between brown and white adipocyte differentiation, was the induction of a mitochondria-related transcriptional theme in brown adipocytes. Apart from this, significant similarity between transcriptional events during brown and white differentiation could be demonstrated: there was a 72% and 52% overlap in specific up- and downregulated genes respectively. Several approaches were used to detect similarity in transcriptional responses between the present data set and two data sets were potential molecular determinants of brown adipocyte differentiation had been altered. By combining principal component analysis and an approach to identify overrepresented gene ontology categories, a transcriptional theme changing inversely in maturing brown preadipocytes and SIRT1-overexpressing myoblasts was visualized. The implication of a myogenic transcriptional program in brown preadipocytes seems to link the origin of this specialized cell to myocytes, and may suggest a developmental relationship to skeletal muscle, where a capacity for oxidative metabolism is a crucial.

8.3 PAPER III

Several investigators have reported a pattern of correlation in expression between neighboring genes in cancers of different tissues that seems to reflect previously identified chromosomal aberrations [265-269]. We explored this with the dual aims of verifying that we can implicate certain chromosomal positions in relation to prognosis, and to investigate the relationship between implicated positions and recently defined molecular subtypes of breast cancer. In a collection of 402 primary breast cancers, profiled with expression microarray technology, we used a simple overrepresentation approach, based on Fisher's test, to identify disproportionate contributions from all occurring chromosomal arms and bands in lists of genes that could be associated with RFS. 16q, 20q, 1p, 13q were all implicated in consistency with previously described chromosomal aberrations of prognostic importance, loss of heterozygosity in 9p has

been associated with more rapid cell division and aneuploidy, but not survival. The most striking finding was a very significant overrepresentation of genes in 16q: this arm contributes 36 (18%) and 55 (11%) genes to lists negatively associated to RFS (set to sizes 200 and 500), which is a highly disproportionate contribution from the 313 (2%) genes in this arm represented on the used microarray platforms. Interestingly, loss in this arm has been shown to differentially affect tumor of different grade: 65% of grade I tumors had lost the long arm of chromosome 16 compared with only 16% of grade III tumors. This pattern of loss led the investigators to conclude that this difference probably reflect different pathways of tumor progression[113]. Further support for heterogeneous patterns of chromosomal aberrations with clinical implications has been provided by Rennstam and co-workers. Gains and losses were studied by comparative genomic hybridization in 305 primary invasive breast cancers. Three distinct patterns were identified: group A was defined by +1q, +16p, and -16q, group B by +11q, +20q, +17q, and -13q, and group C by -8p and +8q. Patients with aberrations in group A only had a significantly higher breast cancer survival rate than all other patients[270]. Having identified a likely gene expression correlate of 16q aberration, we were intrigued to find that no difference in expression in 16q could be demonstrated between basal, ERBB2-like, and luminal B subtypes. In contrast, they all displayed a significant difference in average expression compared to both luminal A and normal-like subtypes, which in turn were characterized by similarily low expression. Stratification across grades revealed the relationship demonstrated by Roylance et. al.: grade 3 tumors are characterized by high expression in 16q, compared to low expression in grade 1 tumors. This illustrates the potential in considering gene expression in cancer in a genomic positional context, and how such approaches are likely to identify connections between transcriptional networks and specific chromosomal aberrations of importance for tumor progression.

8.4 PAPER IV

In the population based cohorts of 402 patients, non-random capacity for prediction of skeletal, lung, and liver metastasis could be demonstrated utilizing a recently described multiple random validation strategy. This capacity was retained for lung and liver metastasis signatures (borderline in the liver case), but not for skeletal metastasis signatures, when testing predictive capacity in 87 patients who all had metastasis to at least one distant site. Skeletal signatures could not discriminate between tumors associated with skeletal metastasis and those with metastasis to other sites and are thus unspecific.

The genes most frequently included in lung and liver metastasis signatures were frequently located on the long arm of chromosome 16 (lung signatures) and 17q11 and 17q12 (liver signatures). When examining predictions made on the basis of defined lung and liver signatures, high grade (utilizing a redefined, gene-expression driven approach for tumor grading) and positive HER-2/neu status were more relevant characterizations of the tumors predicted. Furthermore, when cross-validated predictions where tested in multivariate regression with other predictors including redefined grade and HER-2/neu status, no independent ability was apparent.

We could not confirm that cell-line derived lung and skeletal metastasis signatures show predictive capacity in our clinical cohort of breast cancers, and with regards to the degree of correlation to the endpoints of lung and skeletal metastasis, individual genes in these signatures were either comparable to a random sample of genes or non-significant. We propose that the utility of metastasis-site gene expression signatures in primary breast cancers should be interpreted with considerable caution. We find that they can predict tumors at increased risk of metastasis, whereas prediction is confounded by grade and HER-2/neu related gene expression differences

GENERAL CONCLUSIONS

8.5 PAPER I

In collected microarray data addressing aging and senescence, we find that:

- Gene expression changes, occurring as a consequence of advancing age, display similarities in human, mouse, and rat.
- Cell cultures entering senescence display similarity in the alterations of gene expression.
- Similarity in gene expression changes between cell culture senescence and aging is only apparent when comparing senescence to aging in mouse, not to aging in human.

8.6 PAPER II

- Constituents of a previously identified myogenic transcriptional program - myogenin, MyoD, and Myf 5 – are active in brown but not white preadipocytes.

8.7 PAPER III

- By considering the relationship between chromosomal position and gene expression, we can implicate several genomic areas in relation to 5-year recurrence-free survival, known to undergo recurrent chromosomal aberrations of prognostic significance

- Among chromosomal arms, gene expression in the long arm of chromosome 16 stands out in its relationship to 5-year RFS, and expression in this arm suggests that five proposed molecular subtypes of breast cancer could be collapsed to form two groups in agreement with histopathological grade and survival (RFS) characteristics.

8.8 PAPER IV

- In two population derived cohorts, breast cancer patients recurring with lung and liver metastasis can be predicted with gene expression profiles of primary carcinomas, although prediction is characterized by poor sensitivity, numerous false positives, and dependence on biology underpinning grade and HER-2/neu status.

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