The effects of ageing on microenvironment-contextual epithelial cell signalling

Henriette Christie Ertsås

Avhandling for graden philosophiae doctor (ph.d.)
Universitetet i Bergen
2018

UNIVERSITETET I BERGEN

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No man is an island entire of itself; every man
is a piece of the continent, a part of the main.

John Donne

Scientific environment

This work was carried out at the Centre for Cancer Biomarkers (CCBIO), University of Bergen during the period 2011-2015, under the supervision of Professor James B. Lorens and Mark LaBarge. Professor Rein Aasland served as co-supervisor during the writing process. Collaborative work was performed in the lab of Mark LaBarge at the Lawrence Berkeley National Laboratory (LBNL), Berkeley, California. Financial support and a travel grant was provided by the Norwegian Cancer Society.

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NORWEGIAN **CANCER** SOCIETY

Acknowledgments

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I would like thank just anyone else in the lab and the general world who is interested and willing to contemplate big ideas such as the purpose of being. And at last, every single nerd: nerdy boys and nerdy girls.

Abbreviations

AGE	Advanced glycation end products
AKT	Protein kinase B
AUC	Area under the curve
BM	Basal membrane
BRAF	Rapidly Accelerated Fibrosarcoma; a serine–threonine protein kinase in the RAS-RAF-MEK-ERK signaling cascade
CCND1	Cyclin D1
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
COLI	Collagen I
COLIV	Collagen IV
CTL	Cytotoxic T-lymphocyte
DDR	DNA damage response
EC50	Half maximal effective concentration
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FN	Fibronectin
GAB1	GRB2-associated-binding protein 1 – adaptor protein
GRB2	Growth factor receptor-bound protein 2 – adaptor protein
GTP	Guanosine-5'-triphosphate
HEK293	Human Embryonic Kidney cells
HER2	Human Epidermal Growth Factor Receptor2 / ERBB2 / Neu
HIF-1	Hypoxia inducible factor 1
HMEC	Human mammary epithelial cell
LAM	Laminin
LEP	Luminal epithelial cells
LOX	Lysyl Oxidase
MAPK	Mitogen-Activated Protein Kinase
MCF10	Michigan Cancer Foundation-10 human breast cancer cell line
MEP	Myoepithelial cells
MMP	Matrix Metalloproteinases
mTORC1	Mechanistic Target of Rapamycin Complex 1
OIS	Oncogene-induced senescence
PI3K	Phosphoinositide-3 kinase
PIP	Phosphatidylinositol phosphate
PKC	Protein kinase C
RB	Retinoblastoma Protein
pre-stasis	Cells in a growing state before stasis
p16	Cyclin Dependent Kinase Inhibitor 2A, also abbr. CDKN2A
p21	Cyclin Dependent Kinase Inhibitor p21
PFA	Paraformaldehyde
PBS	Phosphate buffered saline

Rac	a GTPase of the Rho family
Ras	Short for Rat Sarcoma – a small G protein which binds to the cytoplasmic domain of RTK
Rho	a small G protein which regulate the formation of stress fibers
RTK	Receptor Tyrosine Kinase
RGD motif	Arg-Gly-Asp tripeptide
SASP	Senescence- Associated Secretory Phenotype
SHP2	non-receptor tyrosine phosphatase containing Src Homology 2 domains
Shc	Short for Src homology and collagen (Shc) family of adaptor proteins which function as a node for signaling proteins
Sos	Son of sevenless homolog – adaptor protein
Src	Short for Sarcoma – a non-receptor tyrosine kinase, a proto oncogene
TERT	Telomerase Reverse transcriptase
TDLU	Terminal Ductal Lobular Unit
TGF-β	Transforming Growth Factor-b
TP53	Tumour protein 53 = a tumour suppressor protein
VN	Vitronectin
y	years

Glossary

Agonescence	Senescence caused by telomere shortening
Agonist	A molecule which binds and stimulates the activity of a receptor
Anoikis	Cell death due to lack of cell anchorage to the extracellular matrix or neighbouring cells.
Antagonist	A molecule which binds and blocks the activity of a receptor
Apoptosis	Programmed cell death, as opposed to necrosis
Cell Strain	Finite life cells with have only gone through a limited number of population doublings.
Crisis	Telomer shortening leading to persistent growth arrest and apoptosis
Desmosome	Junctional protein complex that facilitates adhesion between epithelial cells
Finite life	The opposite of immortal life
Hemidesmosome	Junctional protein complex that facilitates adhesion between an epithelial cell and the surrounding extracellular matrix
Lineage	A group of cells descending from a common ancestor
Senescence	Living state of cell involving functional metabolism, but no cell division
Slug	Transcription factor known to induce EMT
Stasis	stress-associated senescence

Abstract

Age is the greatest risk factor for developing cancer. Two-thirds of cancer diagnoses occur in people over the age of 65. This increased vulnerability to tumourigenesis is attributed to intrinsic cellular changes, in particular the age-related telomere shortening and the accumulation of mutations over time and. We propose that extrinsic factors comprising age-related alterations in the tissue microenvironment are also important in cancer development. We sought to elucidate how the microenvironmental affects cells, and how this is related to cancer development. This knowledge can be utilized to improve prevention, diagnosis and treatment of cancer in our ageing population.

Cellular function is coordinated by microenvironmental factors such as growth factors, cytokines as well as extracellular matrix proteins. Soluble factor-mediated signal transduction is strongly influenced by microenvironmental context. To allow single cell level measurement of the microenvironmental contextual effect on cell signaling, we developed a novel flow cytometry method: microsphere cytometry. Single normal or neoplastic cells were adhered to uniform microspheres that display mimetic-microenvironments comprising surface combinations of extracellular matrix (ECM) in the presence of soluble agonists or antagonists. Temporal signaling responses were measured with fluorophore-conjugated antibodies that recognize response-dependent epitopes by multiparametric flow cytometry. Using this approach, we demonstrated that microenvironment-mimetic combinations of growth factors and extracellular matrix proteins generate distinct cellular signaling signatures from normal and patient biopsy-derived neoplastic cells.

We asked whether the ageing process affects how human mammary epithelial cells (HMEC) respond to microenvironmental signals, and if this altered response results in increased susceptibility to oncogenic transformation. Using microsphere cytometry we analyzed age-dependent changes in human

mammary myoepithelial and luminal epithelial cells exposed to different ECM and growth factors. We found that ECM–mediated MAP kinase and PI3K pathway activation levels in HMEC are attenuated with age, and that the diminished signaling magnitude in HMEC from ageing women correlated with reduced probability of activating oncogene-induced senescence.

Our results suggest that attenuated cell signaling response may reduce the likelihood of activating oncogene induced senescence, for cells in ageing women. We hypothesize this is the result of age-related changes to the microenvironment that support age-emergent cellular phenotypes with increased cancer susceptibility.

List of publications

Scientific

Paper I
Ertsås, H.C., Nolan, G.P., LaBarge, M.A., Lorens, J.B. (2017).
Microsphere cytometry to interrogate microenvironment-dependent cell signaling. Integr. Biol, 9(2), pp.123–134

Paper II

Ertsås, H.C., LaBarge, M.A., Lorens, J.B.
Microenvironment-contextual cell signaling is attenuated with age. [Manuscript]

Education and Public Outreach

Kronisk, men ikke dødelig (Chronic, but not deadly) 20.09.14, article in Bergens Tidende

Konroll, Kø og Kaos – Livet i cellen (Control, Queues and Chaos, inside a cell) 20.09.14 Popular science performance at Forskningsdagene (National Science Fair sponsored by the Norwegian Research Council)

Stopp kreftcellen Fråtse-Frida! (Stop the cancer cell Gloria Glutton!) 19.09.15 Popular science performance at Forskningsdagene (National Science Fair) , and as a part of CCBIO outreach program.

Kristine Kreftcelle (Cristine the Cancer Cell) Popular science lecture for young and adults frequently performed in schools and museums in 2016-2018, as a part of CCBIO outreach program

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1. Introduction

Humans are subject to increased mortality with age. The main theories of cellular ageing state that cell longevity is intrinsically programmed by certain genes (Werfel 2015) and is extrinsically determined by extracellular cues that determine when these genes are expressed (Kirkwood & Wellcome 2005). Hence in order to understand how ageing affects cells we must define the nature of these extrinsic factors and how they affect cellular functions. In particular, age is strongly correlated with increased cancer risk (White et al. 2014). Of all diagnosed cancers in Norway, 87% occur in people over the age of 50 (Kreftregisteret 2016). Congruently, post-menopausal women are more prone to breast cancer than younger women. The increased frequency of breast cancer with age is likely due to interactions between acquired gene mutations and age-dependent changes in the breast tissue microenvironment that affect mammary epithelial cells. In this thesis we address how ageing affects human mammary epithelial cell responses to microenvironmental signals, and how this corresponds to age-related cancer susceptibility.

1.1 Mammary gland

In this study we have used low passage primary human epithelial mammary cells (HMEC). The mammary gland and the ovaries are of particular interest as they go through monthly gland involution and apoptosis, followed by regeneration which involve a monthly mobilization of stem cells throughout fertile years followed by a forced retirement upon menopause (Ingthorsson et al. 2016; Rønnov-Jessen et al. 1996). Moreover, breast cancer is the most common female cancer worldwide, and healthy tissues are readily attainable from women who undergo reduction mammoplasty. We chose to study human mammary epithelial cells to address why cells in an older environment are more prone to develop into cancer cells.

The milk secreting compartment of the mammary gland – the parenchyme consists of myoepithelial and luminal epithelial cells (Fig.1). There is accumulating evidence for a bipotent mammary progenitor that gives rise to the two

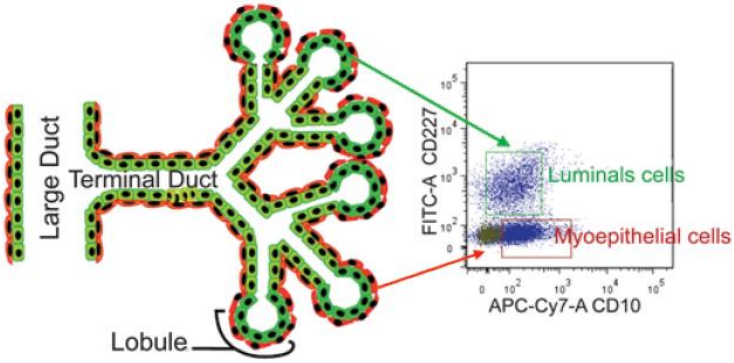


Fig.1: Tissue localisation and cell surface markers of the two mammary epithelial lineages: luminal and myoepithelial cells. Adapted from (LaBarge et al. 2007) with permission from Springer.

mammary epithelial lineages, but a quiescent stem cell subset is yet to be found. Only when there is a consensus on optimal subset markers can this be resolved (Visvader & Clevers 2016; Santagata & Thakkar 2014; Lim et al. 2009). The progenitor cells coexpress luminal and myoepithelial markers, EpCAM, CD49f, Cytokeratin 19 and 14, while the resulting myoepithelial cells are CD49f / CD14 positive, and luminal cells EpCam / CD19 positive (Spike et al. 2012; Stingl et al. 2001). The myoepithelial-luminal bilayer forms a hollow acinus inside which milk is produced. Luminal cells produce milk and secrete this into the lumen of the acinus. Myoepithelial cells wrap around the acinus, and contract to squeeze milk out through the ducts.

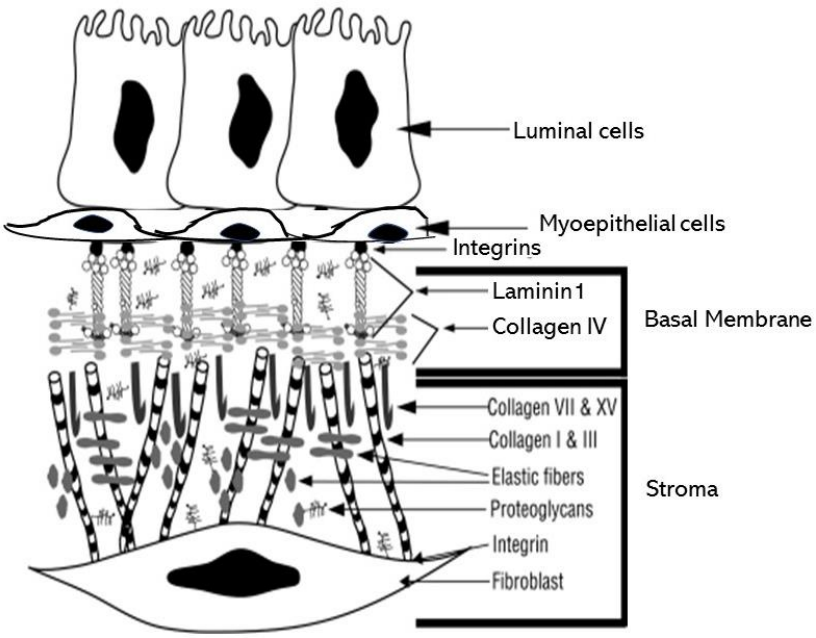


Fig.2 Basal membrane construction. Adapted from (Dunsmore 2008) with permission from Dovepress.

Myoepithelial cells are the main source of laminin α1 which is deposited along the apicobasal axis between the cell layers (Gudjonsson et al. 2005). Luminal cells attach to each other and to myoepithelial cells via desmosomes, and to the extracellular matrix (ECM) by hemidesmosomes. Maintenance of hemidesmosome distribution in the cell membrane is crucial for cell polarity, and consequently to function (Adriance et al. 2005). The laminin binding integrin $\alpha_6\beta_4$ regulates hemidesmosome assembly and therefore the polarity of both luminal and myoepithelial cells (Gudjonsson et al. 2002).

Epithelial cells in healthy mammary tissue are separated from the stroma by a basal membrane. It comprises the ECM proteins laminin and collagen IV (Fig.2). Laminin maintains cell differentiation and supresses growth (Spencer et al. 2007). Collagen IV keeps cell EGF signaling levels to a minimum (Ertsås et al. 2017) by inhibition through phosphatase SHP2 (Mattila et al. 2008; Deb et al. 1998). Myoepithelial cells orient one side towards the basal membrane, and the opposite side towards the luminal cells. Myoepithelial cells produce the ECM proteins and the cytokines necessary to instruct luminal differentiation and retain the distribution of integrin $\alpha_6\beta_4$ to the basolateral sides of luminal cells (Runswick et al. 2001; Deugnier et al. 2002). Groups of acini forms lobules surrounded by stroma (Fig.3). The breast stroma, which makes up 80% of the breast, contains matrix proteins, immune cells, fibroblasts, adipocytes, nerves and blood vessels (Rønnov-Jessen et al. 1996). The lobules proliferate, differentiate and then go through apoptosis with every menstrual cycle, and ultimately degenerate post-menopause (Milanese et al. 2006).

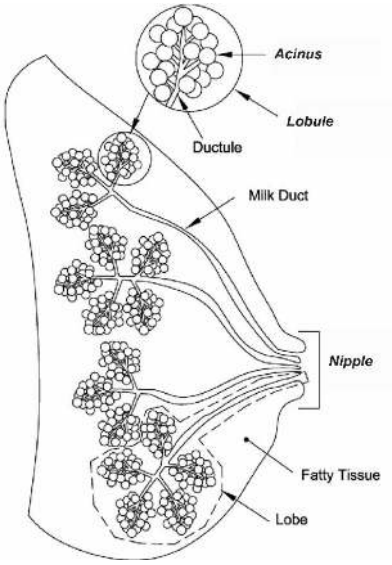


Fig.3: Illustration of the human breast with ducts and lobules consisting of epithelial bilayers. Adapted from (Mortazavi et al. 2015) with permission from American Society of mechanical engineers

1.2 Mammary Microenvironment

The fate of the progenitor mammary cell, whether it turns into a luminal or myoepithelial cell, is determined by a combination of soluble signals from other cells, as well as the surrounding extracellular matrix which together make up the microenvironment (LaBarge et al. 2009; Lui et al. 2012; Lim et al. 2009; Miyano et al. 2017). The sensing of the cellular microenvironment comprises cell-cell and cell-ECM interactions, as well as interactions with soluble and tensile factors (Glukhova & Streuli 2013). The microenvironment has 1) chemical properties conveyed through the ligand-receptor transition; 2) physical properties including the stiffness and elasticity of the tissue transferred through mechanoreceptors being pushed and pulled and 3) architectural properties which involve the distribution of cell surface proteins on particular sides of the cell, in polar patterns (Fig.4) (Marinkovic et al. 2016; Lee et al. 2015). The physical properties of the microenvironment that change with age are conveyed through mechanoreceptors such as integrins, they bind ECM proteins and translate the signal to the cell nucleus. Gene expression is consequently modified by the microenvironment, and the cell in turn modifies the microenvironment in a case of dynamic reciprocity (Bissell et al. 1982).

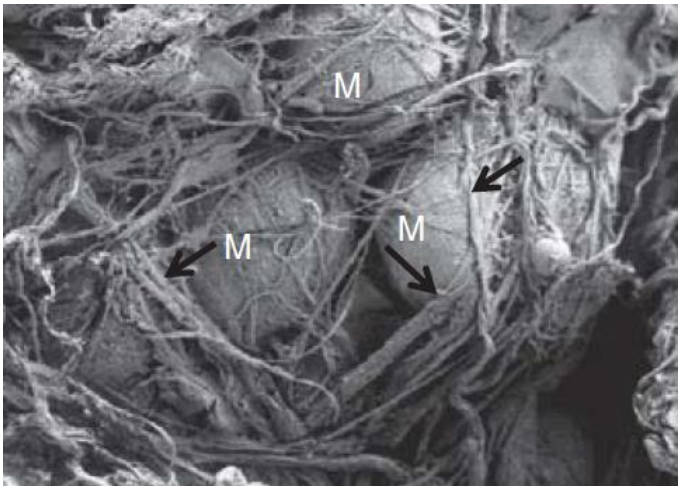


Fig.4: Fibrous extracellular matrix proteins (M) stretching around cells (Schedin & Keely 2011). With permission from Cold Spring Harbor Laboratory Press

1.2.1 Extracellular Matrix Proteins (ECM proteins)

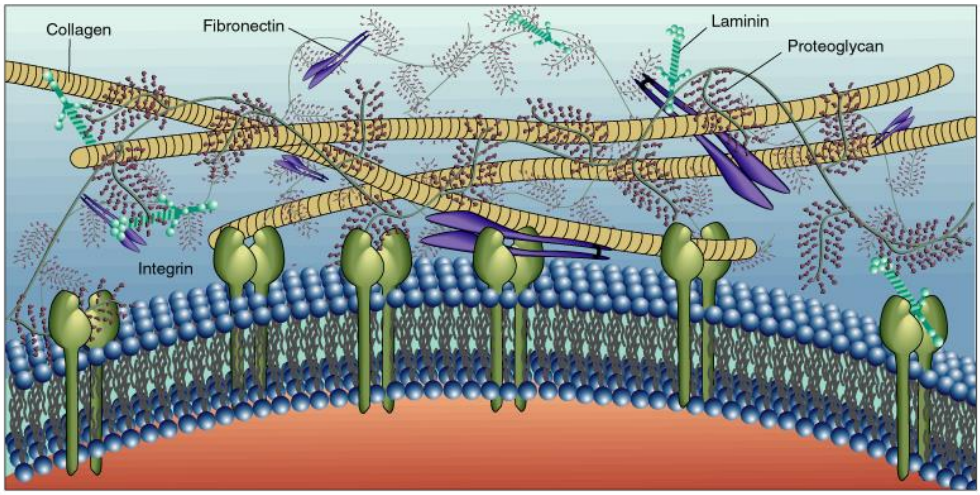


Fig.5: Extracellular matrix located on the outside of the cell membrane. Integrins, the ECM receptors are located in the membrane (Wiley 1999). With permission from John Wiley and Son.

The outer layer of the bilayer comprising myoepithelial and luminal cells, is in direct contact with the basal membrane, which consists of the matrix proteins laminin and collagen IV (Fig.5) (Fata et al. 2003). Other structural proteins include proteoglycans of the type heparan sulfate (Bonnans et al. 2014). Fibroblasts deposit collagen I outside of the basal membrane, in the interstitial area between the ducts and lobules. The interstitial tissue is also rich in collagen III, tenascin and proteoglycans (Bonnans et al. 2014).

Collagen

The matrix protein collagen is the most abundant protein in the body. There are at least 28 types, while the three types I, II and III make up the 80-90% of all collagens in the human body, and comprise the major part of bones and cartilage (Ricard-Blum 2011; Snedeker & Gautieri 2014). It contributes during wound healing, together with fibronectin, to build a rigid matrix that supports regrowth of cells (Midwood et al. 2004). When lysyl oxidase form cross links between neighbouring collagen I helices it creates a stiffer collagen fibril (Erler et al. 2006). Stiffer tissue tensile force in the interlobular stroma exert influence

on cell fate by activating integrin receptors in the underlying epithelium. Persistent high tensile force result in persistent integrin activation. The crosslinked collagen cluster together integrin $\alpha_2\beta_1$ among others, and form focal adhesions which trigger intracellular adaptors RhoGTPase and ROCK to increase intracellular stiffness and transcription of genes involved in proliferation, survival and cell motility (Mariotti et al. 2001; Morse, Brahme & D. A. Calderwood 2014; Sieg et al. 2000).

Fibronectin

The matrix protein fibronectin promotes proliferation and invasive behavior, while preventing differentiation from progenitor state into a functional cell (Roman et al. 2010). Normal interstitial fibronectin turnover in healthy mammary tissue is low, as detected by immunohistochemistry (Williams et al. 2008). The protein is degraded in the final steps of wound healing allowing collagen I deposition. The main role of fibronectin is during inflammation, embryonic development (Erler & Weaver 2009), and in the proliferative phase of the menstrual cycle (Williams et al. 2008),(Huveneers & Danen 2009), while fully developed mammary acini lose structure and polarity in the presence of persistent amounts of fibronectin (Williams et al. 2008). Proliferative signals, combined with the suppression of differentiation coming from persistent fibronectin in the tissue, prompt cells into becoming tumourigenic.

Laminin

The matrix protein laminin, induces cell differentiation into luminal and myoepithelial cells, and is responsible for maintaining the phenotypes of epithelial cells residing in intact tissue (Fig.6) (Spencer et al. 2010; Streuli et al. 1995). Laminin exogenically added to mammary epithelial cells in vitro restricts their production of fibronectin (Williams et al. 2008) , as laminin opposes the effect of fibronectin. In this manner laminin located in the basal membrane has a tumour-suppressive function. Cells remain differentiated and retain their function provided that the basal membrane is intact.

A major producer of ECM proteins are fibroblasts. Fibroblasts may also be the source of myofibroblasts, which appear in metastatic and inflammatory circumstances. The proposed theory is that fibroblasts morph into myofibroblast in these settings (Ueha et al. 2012). Finally, physical cues from the ECM, such as stiffness and topology, are as important as the biochemical component of soluble and fibrous ligands (Kass et al. 2007; Pouwels et al. 2012). We see potential for great variations in the breast microenvironment with increasing age and whenever homeostasis is challenged during the monthly hormonal cycles. We question how these variations are translated into gene expression in the residing cells of the tissue.

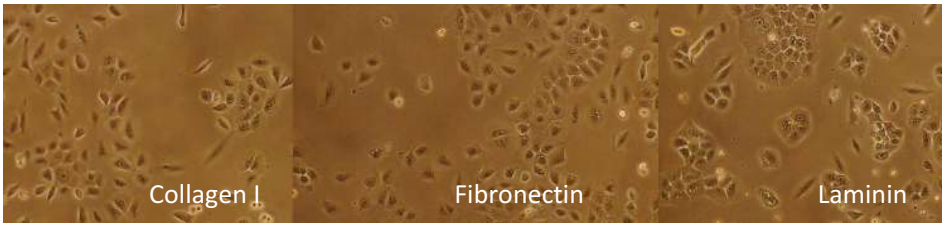


Fig.6: 2D growth of mammary fibrotic cell line MCF10 on collagen I, fibronectin and laminin, illustrates the proliferating effect of fibronectin and the differentiating (acinus-like structures form) effect of laminin.

1.3 Integrins

The cellular microenvironment imparts numerous physical forces such as stress applied parallel to the surface of the cell, compression of the cell, and pulling leading to expansion of the cell (Butcher et al. 2009). Cells demonstrate mechano-reciprocity to these inputs through mechanoreceptors such as

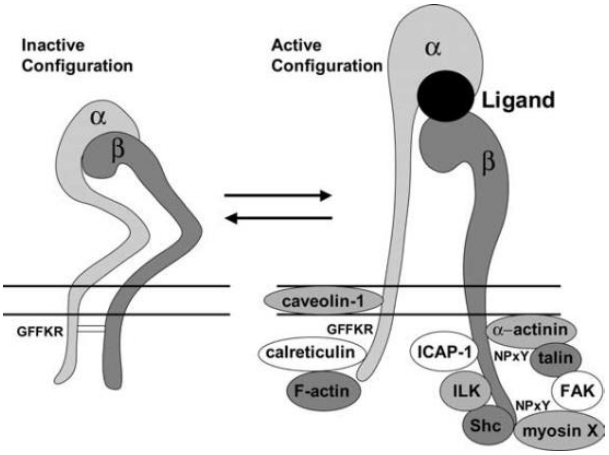


Fig.7: Integrin dimers are composed of an α and a β subunit which is inactive in the folded confirmation Several intracellular modulators are recruited to the intracellular moiety when the integrin is in the active configuration (Gilcrease 2007). With permission from Elsevier.

integrins (Hagios et al. 1998). Cell-cell or cell-ECM interactions are nanoscale forces that influence cell function through actomyosin contractility and actin dynamics (Butcher et al. 2009). Integrins are dimeric transmembrane proteins (α and β subunits) with an extracellular domain that binds ECM proteins, a transmembrane domain and an intracellular tail that is phosphorylated at either tyrosine (Tyr), serine (Ser) or threonine (Thr) by recruited kinases (Fig.7).

1.3.1 Ligand specificity of integrins

The level and type of integrins expressed, their distribution on the cell surface, as well as the type of ECM dominating the surrounding matrix will determine response. Fibronectin has affinity to $\alpha_5\beta_1$ and $\alpha_V\beta_3$, collagen I bind integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ (Hynes et al. 2011) while laminin binds to $\alpha_6\beta_1$, $\alpha_3\beta_1$ and has exclusive affinity to $\alpha_6\beta_4$ (Mercurio et al. 2001) (Fig. 8).

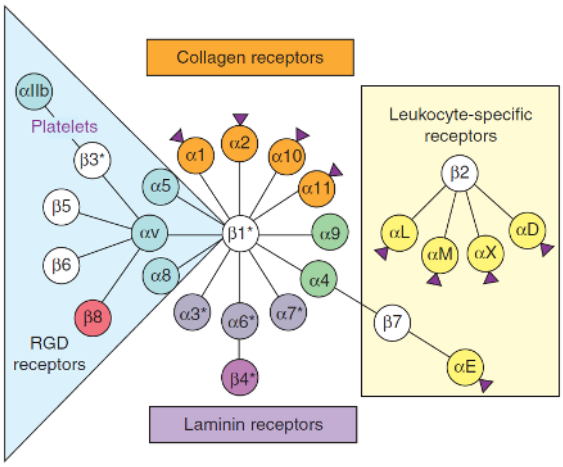


Fig.8: Integrin subunits (Hynes et al. 2011). With permission from Cold Spring Harbor Laboratory Press

1.3.2 Upstream and downstream of the integrin

Most receptors enter an active state upon ligand binding (Wegener & Campbell 2008; Paszek et al. 2009; Elosegui-Artola et al. 2014). However, integrin receptors are allosteric enzymes, and ligand affinity is influenced by the intracellular environment, to the extent that integrin activation can be initiated from both outside-in and inside-out, in a feed forward loop (Fig.9).

The inactivating clasp interaction between subunit α and can be released from the inside by Talin and Kindlin located on the inside leaflet of the plasma membrane. The release of subunit β from the clasp of subunit α make space around the extracellular domain for a ligand to bind (Pouwels et al. 2012; Legate et al. 2009).

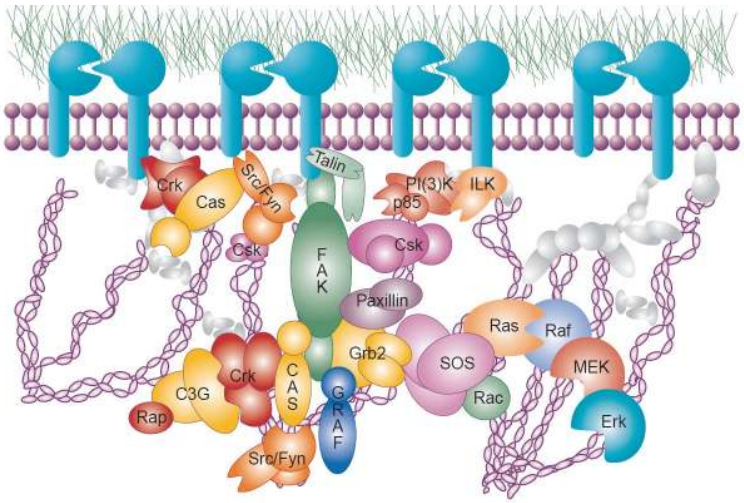


Fig.9: Integrin clusters (Miranti & Brugge 2002). With permission from Nature Publishing Group

Actin contraction on the inside increases affinity of the integrins on the outside and thereby increase stiffness around the cell (Trusolino et al. 2000). Independent of whether the integrin is activated by ligand binding or by the recruitment of intracellular adaptor talin or kindlin, the resulting adhesome complex comprise kinases, phosphatases, scaffold proteins, and signaling proteins such as Src, FAK, paxillin, Shc, Syk, Shr and Fyn among others (Fig. 9, Fig.12). All are involved in migration and motility (Roberts et al. 2002; Huveneers & Danen 2009; Legate et al. 2009) (Fig.12). The intracellular integrin binding proteins secondly activate nearby integrins and the intracellular moiety of nearby growth factor receptors. Vacant binding domains on the fibrous ECM adhere, and sequentially immobilize additional integrins into a cluster (Legate et al. 2009; Geiger & Yamada 2011; Morse, Brahme & D. A. Calderwood 2014). The cascade of assembled integrins and signaling proteins eventually mature into focal adhesions that integrate the cytoskeleton into the equation (Welf et al. 2012; Bonnans et al. 2014). FAK phosphorylated

at Tyr397 initiates cytoskeletal contraction and link focal adhesions to actin fibres of the cytoskeleton via paxillin and tensin (Morse, Brahme & D. A. Calderwood 2014) (Fig.12). Focal adhesions regulate cell shape and fate through interactions between the ECM on the outside and actin linker proteins on the inside, using RhoGTPases to remodel of the cytoskeleton (Riento & Ridley 2003; Kass et al. 2007). The Rho/Rock pathway triggers Cdc42 and Rac1 responsible for forming actin rich protrusions such as filopodia that induce random migration of cells in different directions, or lamellipodia which induce persistent cell migration of all cells in a sheet. The cell moves forward by the help of actin contractions, releasing integrin attachments at the rear end (Sieg et al. 2000). The result is cell motility and migration (Fig.12).

1.3.3 Integrin Interaction with growth factor (GF) receptors

Integrin induced signals provide an influential context for growth factor receptor- and G protein coupled receptor-induced signaling to occur within (Harburger & Calderwood 2009; Levental et al. 2009). Integrins fine-tune ongoing signaling (Morse, Brahme & D. A. Calderwood 2014), in part by increasing the plasma membrane proximity of signaling proteins. The intracellular moieties of integrins do not have their own kinase activity, but recruit kinases to sites that can be phosporylated. The recruited kinases phosphorylate residues on the integrins themselves, as well as nearby intracellular moieties of growth factor receptors. The kinase of the growth factor receptor autophosphorylate and activate downstream signaling, in particular the MAPK and PI3K pathways. Interestingly, integrin recruited kinases target other residues than the intrinsic kinase activity of the growth receptor, and therefore induce different downstream signaling compared to the growth factor alone (Kumar 1998; Balanis et al. 2011; Cabodi et al. 2004; Streuli & Akhtar 2009).

The growth factor receptor-integrin interaction is demonstrated by EGF stimulation of adherent versus suspension HEK293 (human embryonic kidney)

cells. Cells in suspension do not show changes in EGFR phosphorylation upon EGF stimulation. In contrast, cells adhered to a surface coated with fibronectin or laminin give a strong pEGFR response (Yarwood & Woodgett 2001) (Fig.10). This way ECM enhances growth factor signaling and modifies the requirement of cytokine binding to achieve receptor activation (Erler & Weaver 2009; Levental et al. 2009). The threshold to trigger receptor activation is lower when certain sites are already phosphorylated by help of adjacent integrins, allowing the recruitment of necessary kinases to phosphorylate remaining residues on the receptor.

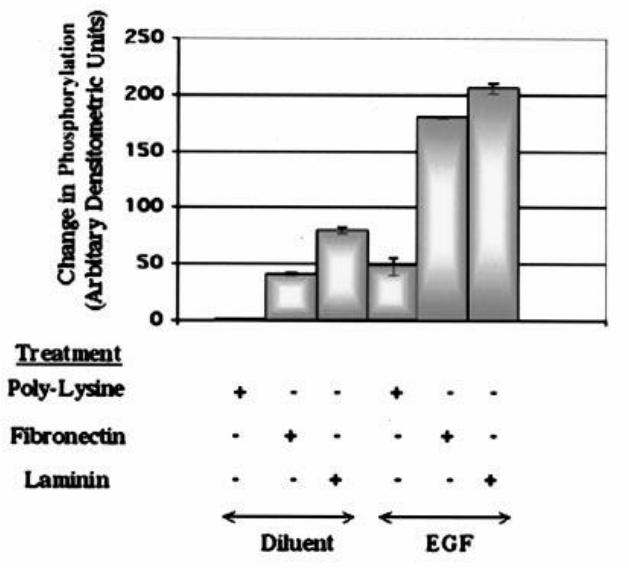
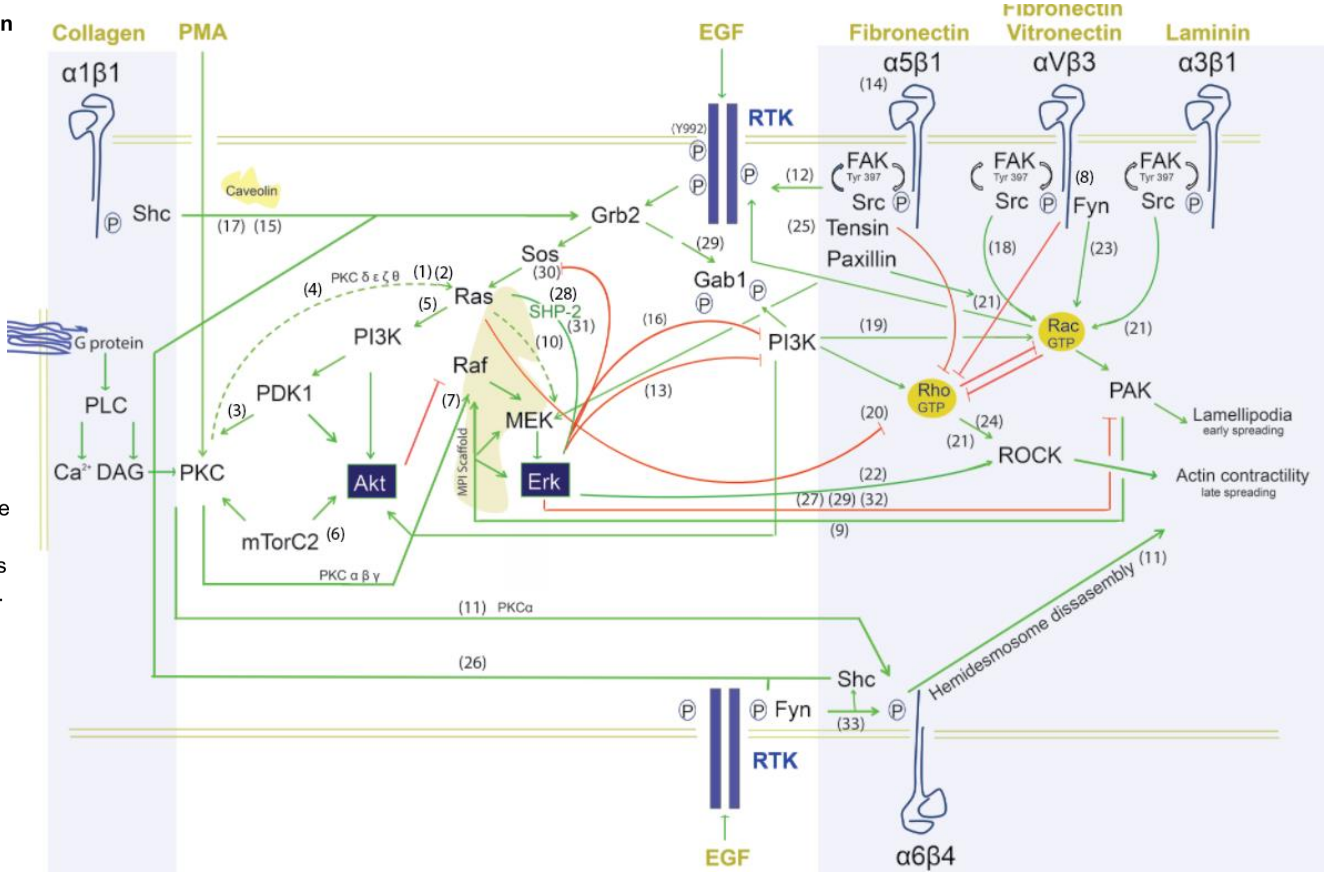


Fig.10 Phospho-levels of EGFR in HEK293 cells cultured on plastic (poly-Lysine), on fibronectin and on laminin, with or without EGF stimulation (Yarwood & Woodgett 2001). With permission from National Academy of Sciences

Fig.12: **Schematic presentation of downstream interaction between receptor tyrosine kinase (RTK) and integrins**. Clustering of integrins facilitate signaling cascades which eventually cause proliferation via MAPK, survival via PI3K or cytoskeletal contraction via Rho and Rock (Mariotti et al. 2001), as well as to surrounding RTK (Streuli & Akhtar 2009). p85 subunit of PI3K can be recruited directly to a phospho-site on the RTK (Liu et al. 2009), omitting the steps through Grb2 and Gab1. Negative feedback between pathways is labelled in red, positive feedback in green. Dotted lines represent multiple steps



Ref: (1) (Ginnan & Guikema 2006) (2) (Puente et al. 2006) (3) (Newton 2001) (4) (Kuriyama et al. 2004) (5) (Murillo et al. 2014) (6) (Riaz et al. 2012) (7) (Hong et al. 2008) (8) (Roberts et al. 2002) (9) (Sundberg-Smith et al. 2005) (10) (Chuderland & Seger 2005) (11) (Mercurio et al. 2001) (12) (Danilkovitch-Miagkova et al. 2000) (13) (Zhang et al. 2002) (14) (Hynes et al. 2011) (15) (Kikkawa et al. 2000) (16) (Tiganis et al. 1999) (17) (Wary et al. 1996) (18) (Moro et al. 2002) (19) (Tang et al. 1999) (20) (Balanis et al. 2011) (21) (Huveneers & Danen 2009) (22) (Paszek et al. 2005) (23) (Kostic & Sheetz 2006) (24) (Danen et al. 2005) (25) (Danen et al. 2002) (26) (Mariotti et al. 2001) (27) (Mendoza et al. 2011b) (28) (Wöhrle et al. 2009) (29) (Kolch et al. 2015) (30) (Birtwistle et al. 2007) (31) (Agazie & Hayman 2003) (32) (O'Reilly et al. 2006) (33) (Mariotti et al. 2001) (34) (Castellano & Downward 2011)
MPI=MEK scaffolding protein

1.3.4 Integrins can trigger GF Receptors independent of GF

Integrins can activate growth factor receptors in a ligand independent fashion (Fig.11). Balanis et al. demonstrated in murine mammary cells that EGFR was not activated through its ligand EGF, but rather through the activation of fibronectin-binding integrins $\alpha_V\beta_3$, or $\alpha_5\beta_1$ that lead to phosphorylation of Tyr992, Tyr1068, Tyr1173 and Tyr845 on the intracellular moiety of EGFR (Balanis et al. 2011). Adding EGF to the EGFR did not affect EGFR phospho-level, but affected the phospho-level of the intracellular moiety of β_4 (Mariotti et al. 2001) (Fig.12).

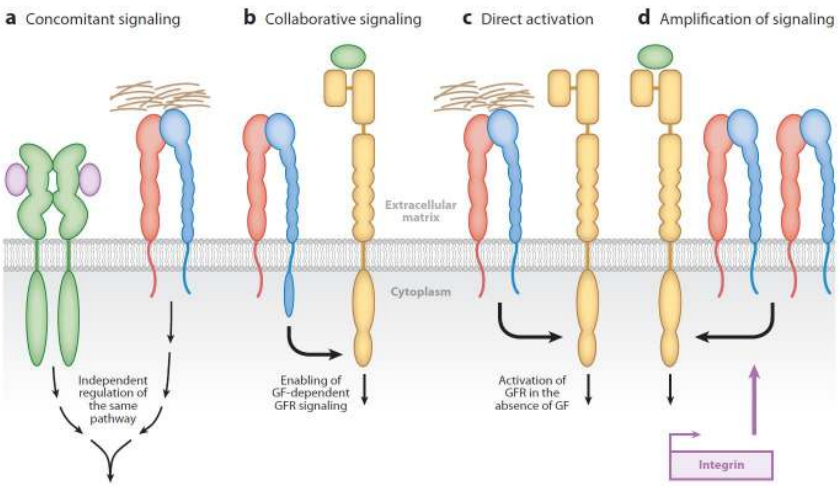


Fig.11: Integrin-Growth factor interaction (Ivaska & Heino 2011). With permission from Annual Reviews.

1.3.5 Crosstalk between MAPK and PI3K pathways

Crosstalk between the MAPK and PI3K pathways downstream of receptor tyrosin kinase (RTK) can alter the outcome of receptor activation. There are both positive and negative feedback interactions between signaling proteins in these pathways (Ref. 7 & 16 in Fig.12) that fine tune signal transduction in response to signal intensity or contextual factors (Ref. 12 & 17 in Fig.12). An example is recruitment of Shc–GRB2–SOS complexes that activate the RAS/MAPK pathway. This attenuate PI3K pathway activation when EGFR

stimulation increases. In fact, EGFR is found to be a potent activator of ERK, but a weak activator of the PI3K pathway (Kolch et al. 2015). EGFR has various ligands that generate different signals. When HGF binds to EGFR it triggers a different crosstalk pattern down-stream compared to when EGF binds. This is because HGF stimulation of EGFR does not initiate the tyrosine phosphatase SHP2 (Fig.12). SHP2 recruitment represents a negative feedback on the GAB1-PI3K pathway, and a positive feedback on the Ras-MAPK pathway (Yu et al. 2002). The MAPK substrate ERK5 is suggested to be responsible for SHP2 recruitment to GAB1 (Yu et al. 2002; Wöhrle et al. 2009). AKT activation is therefore retained low, while ERK remains high (Fig.12). Variations in signaling as a function of time may however not involve feedback at all, but rather diverging downstream pathways, acting on substrates in a series of different time points.

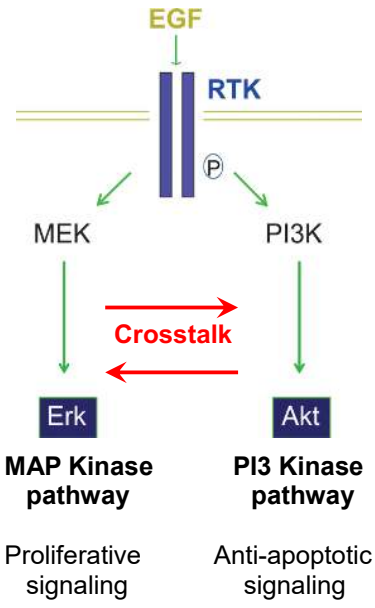


Fig.13: Crosstalk between pathways

An example is ERK which is phosphorylated after 5 minutes of growth factor stimulation, while AKT is not phosphorylated for another 45 to 90 minutes (Mendoza, Emrah Er, and Blenis 2011a). Crosstalk between MAPK and PI3K pathways is regulated by which and how many residues on RTK are phosphorylated upon stimulation (Fig.13). Signaling thresholds dictate whether feedback is positive, negative or diverging. pERK is involved in more negative feedback loops -back on itself and from other effectors - than pAKT (Birtwistle et al. 2007; Kumar et al. 2007). If distribution, rigidity and composition of the ECM changes due to age or pathology as described in section 1.2 will this

affect mechano- and growth factor signalling in cells residing in the tissue, and secondly will this effect be sufficiently long term to influence cell phenotype?

1.4 Ageing in the mammary gland

Ageing at the physiological level is a gradual reduction of tissue homeostatic function (Snedeker & Gautieri 2014). This is the result of changes in cellular function that affect the fidelity of cellular hierarchies and constitution of the ECM (Fig.14)

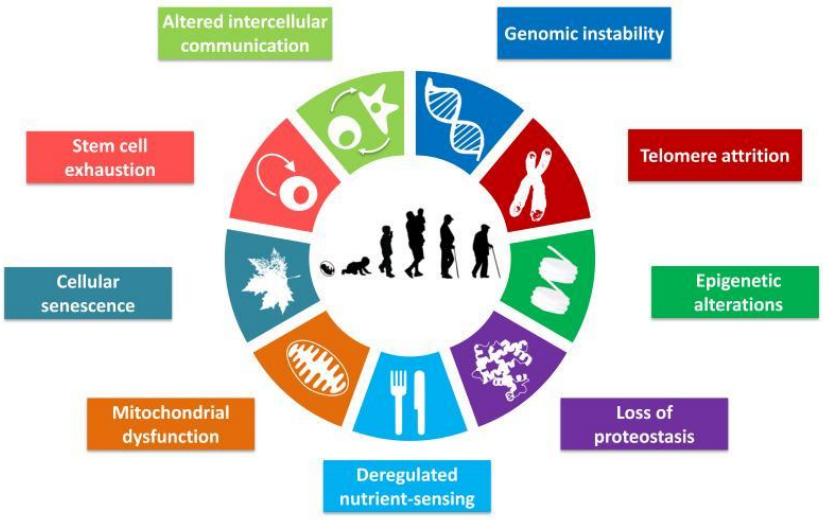


Fig.14: Hallmarks of ageing. A summary of reasons for ageing, physiological and cellular (López-Otín et al. 2013). With permission from Elsevier.

1.4.1 Extrinsic factors that promote ageing

The altered microenvironment potentiates the ageing process by supporting altered cellular phenotypes. The outcome is a reduction of cancer protective mechanisms including tumour-suppressive functions of the microenvironment (Mina J Bissell et al. 2002). How does these systemic changes of hormonal and structural nature (Table 1) trickle down to the microenvironment and into individual cells, to induce such long-term effects as cancer susceptibility?

Fibrosis
Fibrosis is an excess of fibrous connective tissue in an organ previously populated with cells (Zeisberg & Kalluri 2013). Fibrosis is considered an initiating stage of cancer development (Cox & Erler 2014). Mammography measures tissue density in the breast, and indicates the level of fibrosis. There is increased collagen I deposition and increased rigidity of the tissue. A stiffer microenvironment transforms interstitial fibroblasts into myofibroblasts (Bogatkevich 2015), and promote further secretion of collagen I in a feed forward loop (López-Nouoa & Nieto 2009).
Adipocytes replace epithelial cells
Invading adipocytes take over the space previously filled with epithelial cells (Machida & Nakadate 2015).
Reactive oxygen species (ROS)
Mitochondrial deterioration lead to reactive oxygen species ROS, which attack both DNA and proteins. Aberrant function of enzymes that deal with carcinogens in older people make them more sensitive to carcinogens (Hoffe 2012).
Collagen cross linking
Despite the resulting general decrease in breast density with age, individual loci have increased stiffness where collagen I is increasingly crosslinked. Lysyl oxidase (LOX) activity introduces crosslinks within collagen I fibrils, which increase rigidity and linearization (Egeblad et al. 2010). Accumulation of linearized collagen I with age allows more efficient cell migration, involving intensified integrin activation (Condeelis & Segall 2003)
Loss of immune function
Immune surveillance is less efficient in detecting and removing neoplastic cells. Simultaneously, inflammation is more frequent and more likely to become chronic giving rise to “the wound that never heals” (Dvorak 2016).

Disintegration of the Basal Membrane
Basal membrane experiences proteolytic degradation due to MMPs and inflammatory factors derived from fibroblasts and macrophages (Nguyen-Ngoc et al. 2012; Benz 2008; Milanese et al. 2006; Ueha et al. 2012). It becomes thinner and discontinuous. Epithelial cells are exposed to collagen I in the interstitium. This is interpreted by the epithelia as a wound, and wound healing is initialized (Xue & Jackson 2015; Midwood et al. 2004). Luminal cells cannot maintain differentiation when laminin is lacking in their microenvironment. Signaling from integrin receptors is initiated by the clustering of receptors, which explains why the epitopes need to occur in solid state, close to each other. This requirement is only fulfilled when ECM fibres are present in a matrix. Disintegration of the basal membrane result in fewer solid state ligands present to keep the integrin expression and localization in check (Sager 1993).

Table 1: tissue homeostatic function deteriorates with age

1.4.2 Intrinsic factors that promote ageing

Intrinsically determined ageing is genetically programmed, occurring independent of microenvironmental changes. It is exemplified by senescence which is the current prevailing theory of ageing (Childs et al. 2015). Senescent cells are in proliferative arrest, but remain metabolically active. They may infrequently revert to a proliferative state, apoptose or persist as a phenotype which creates an inflammatory microenvironment around themselves by secreting given cytokines (Kolch et al. 2015). Senescence links hyperplastic pathologies and ageing (Campisi 2013). The tumour suppressor TP53 protects the cells of young individuals against cancer, but will restrict life expectancy post-reproduction.

1.4.2.1 Senescence

Senescence occurs in response to excessive extracellular or intracellular stress (Coppé et al. 2010). Senescence-inducing stress includes telomere shortening following multiple cell divisions, exposure to oxidants, mitochondrial deterioration or disruption of chromatin organization.

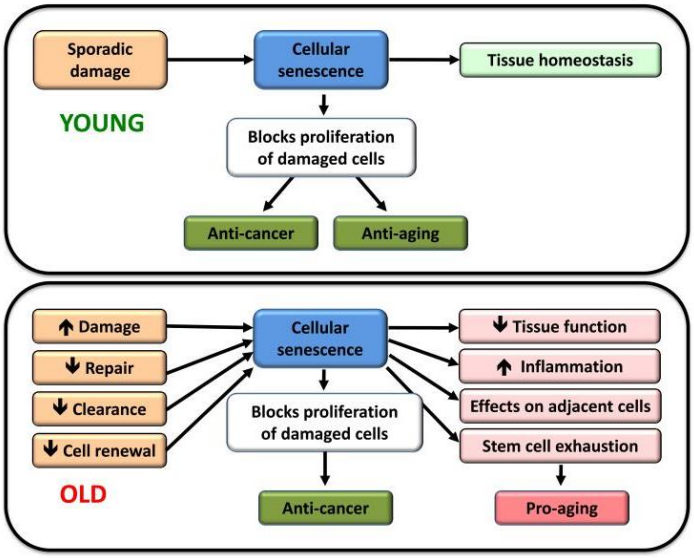


Fig.15: Impact of senescence in young and old tissue (López-Otín et al. 2013). With permission from Elsevier.

Alternatively senescence may be due to extrinsic exposures such as: DNA damaging chemotherapies, γ-irradiation and UVB light (Campisi & d'Adda di Fagagna 2007; van Deursen 2014; Hayflick & Moorhead 1961; Hornsby 2011).

Oncogenes causing inappropriate mitogen signaling can also lead to senescence. The fraction of senescent fibroblasts in humans have been found to increase with age (Faragher et al. 2017). A higher number of senescent cells can be due to chronic inflammation, a higher fraction of persistent non-acute senescent cells or less efficient elimination of senescent cells by an age ageing immune system (Shaw et al. 2010; Childs et al. 2014; Nikolich-Žugich 2014) (Fig.15). Senescence is a cancer-protective and tumour-suppressive mechanism but can in some cases have the opposite effect and promote cancer through senescence-associated secretory phenotype (SASP) which maintain inflammation (Campisi 2013; Hoare & Narita 2013) (Fig.16). The results of SASP are the reverse differentiation of epithelial into mesenchymal-like cells (epithelial to mesenchymal transition), microenvironmental changes and angiogenesis (Bavik et al. 2006; Parrinello 2005) (Fig.16).

1.4.2.2 Senescence in human epithelial cells

The senescence program is activated when a critical level of DNA damage is surpassed, and is implemented by TP53 or CDKN2A/p16 preventing entry into S-phase through the activation of transcriptional regulator Retinoblastoma (Rb) (Benz 2008). This inhibition is reversible upon DNA damage repair and is the dominant mechanism of senescence in human fibroblasts, astrocytes, keratinocytes and all murine cells. In general, mesenchymal-like cells demonstrate more DNA damage at stress-associated senescence (stasis) than do human epithelial cells. Stasis is the first of two steps that needs to be bypassed in order for cells to become immortal, which means they can divide an indefinite number of times (Olsen et al. 2002; Garbe et al. 2014). It is telomere independent, but is in similarity to agonescence characterized by vacuoled cells and β-galactosidase expression. Stasis is induced by DNA damage, oxidative stress etc. in contrast to agonescence, a type of senescence specifically induced by critically short telomeres. Human epithelial cells show evidence of a TP53 independent induction of stasis. In fact, neither TP53 nor CDKN2A/p16 ^{Ink4A} is required to induce stasis in human mammary epithelial cells (Olsen et al. 2002). Congruently, most mammary cancers still express wild type TP53 (Shachney & Silverman 2003).
Could the changes listed above can explain why ageing cells are more likely to develop into tumour cells.

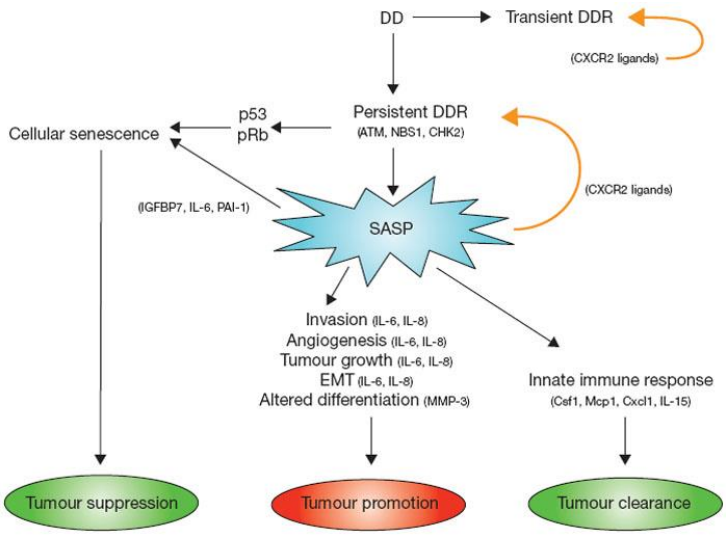


Fig.16 SASPs have both tumour-suppressive and tumour-promoting consequences (Fumagalli & d'Adda di Fagagna 2009). With permission from Nature Publishing Group.

1.5 Microenvironmental link between Ageing and Cancer

The common explanation for the increased incidence of cancer with increased age has been accumulation of sporadic mutations with time, the assumption being that when the number of oncogenes in the cell reaches a threshold, tumours will develop (Campbell et al. 2015; Vogelstein & Kinzler 1993). Vogelstein argues further that human organs demonstrating high numbers of stem cell divisions, are more likely to accumulate mutations, and therefore more prone to develop tumours compared to other organs (Tomasetti & Vogelstein 2015). However, several cancer types do not increase in direct correlation to age (Armitage, P;Doll 1954). Breast cancer incidence peaks at age 50 and then again around age 70, after which it decreases (Anderson et al. 2014). Most pre-malignant mutations occur before the age of twenty, and increase only slightly from then on (Degregori 2013) (Fig.17). Indeed, many of these mutations do not result in clinical cancer: 36% of people between the age of 50 and 70 years that died of non-cancer causes had detectable carcinomas in 2.5mm autopsy cross sections (Harach et al. 1985). In sum, the above imply that there is most likely a contextual parameter involved in age-related cancer in addition to the presence of mutations. Can this parameter, or the consequences of it, be detected through functional studies?

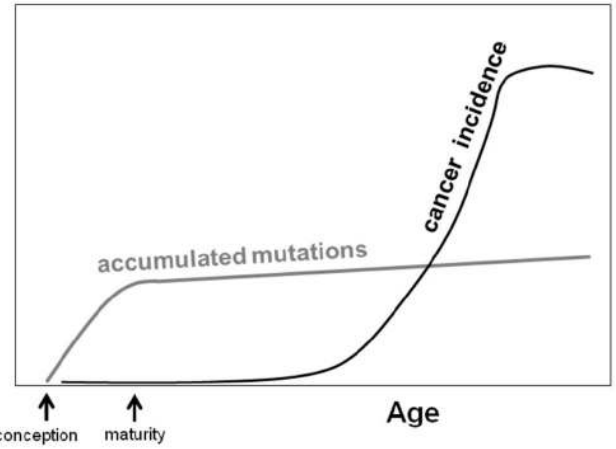


Fig.17: Cancer incidence and mutations as a function of age (Degregori 2013). With permission from Nature Publishing Group

1.5.1 Tumour microenvironment versus ageing microenvironment

The tumour microenvironment shows similarity to the ageing

microenvironment, indicating that the ageing microenvironment may have similar influence as a tumour microenvironment - on its residing cells. Here are some characteristics to recognize a tumour microenvironment. A metastatic niche and a tumour microenvironment induce malignancy in the residing cells.

1.5.1.1 Extracellular matrix composition and stiffness promote malignancy

A stiffer, cross-linked ECM is detected by cells through mechanosensing, increased numbers of focal adhesions and reorganization of the cortical actin cytoskeleton. This promotes cell migration and is implicated as a “highway” for metastatic cells to migrate along (Erler et al. 2006; Snedeker & Gautieri 2014). Cells respond to increased local tensile force by assembling focal adhesions, and to the relaxation of force by disassembling focal adhesions (Bershadsky et al. 2006; Paszek et al. 2009). The composition of the ECM changes during tumour development. Collagen I and fibronectin are produced in abundance by tumour cells and tumour associated cells (Kaplan et al. 2005; Ioachim et al. 2002). Tumours have been called: “the wound that never heals”, as they deposit fibronectin in the absence of actual injury (Polyak & Kalluri 2010). Hypoxia in the metastatic niche induces lysyl oxidase activity via hypoxia-inducible factor-1 (HIF-1), to form cross-links in collagen I (Erler et al. 2006). The deposition of collagen and fibronectin, and the crosslinks formed between collagen fibrils result in high stiffness in the tumour and surrounding stroma (Fig.18). Conversely, laminin tends to lack in tumourigenic tissue (Natali et al. 1992). These are all phenomena that we recognize in the ageing microenvironment, which prompts us to ask if the ageing ECM can have similar impact. Furthermore, could an ECM of lower stiffness rejuvenate old cells placed in it? The stiffness and ECM composition of the

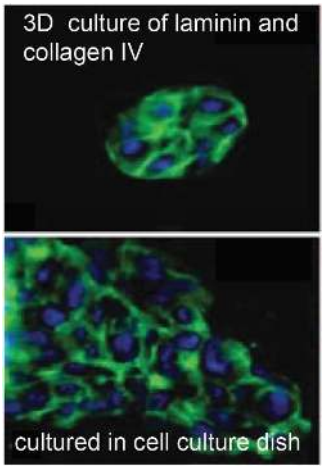


Fig.18 Cell morphology and phenotype is instructed by the surrounding ECM and its rigidity. Adapted from (Provenzano et al. 2008) with permission from BioMed Central Ltd..

mammary gland stroma is calibrated to maintain lobule conformation allowing lactation (Schedin & Keely 2011). Tumour development is prevented by physically forcing the cells into a native 3D orientation (Bissell et al. 2002; Nelson & Bissell 2005) (Fig.19). Normal mammary morphogenesis is associated with ECM turnover, processing and orientation. These changes occur at strictly regulated timepoints in tissue development and within the menstrual cycle. However, with ageing and during tumour development the ECM rearrangement becomes extensive and persistent (Schedin 2006).The ECM is no longer fulfilling its tumour-suppressive role.

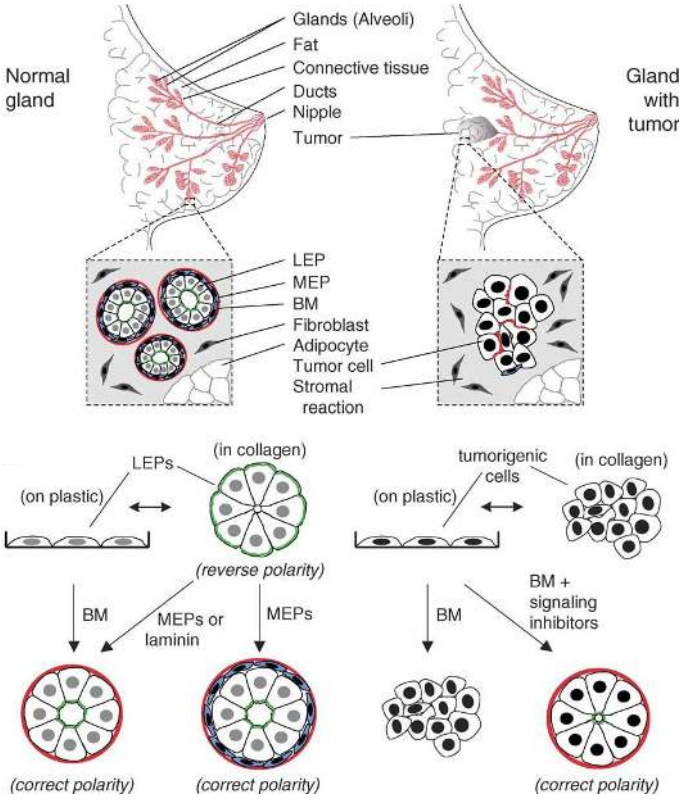


Fig.19: A malignant phenotype require a malignant microenvironment to manifest itself (Nelson & Bissell 2005). With permission from Elsevier.

1.5.1.2 Oncogene potential released by the microenvironment

Dolberg and Bissell demonstrated more than 30 years ago that tumours developed when Rous Sarcoma Virus (RSV) was injected into the wing web of a hatched chicken, due to the presence of inflammation in the puncture wound, while no tumours developed when the virus was put inside a developing embryo (Dolberg & Bissell 1984). Even if the genotype for

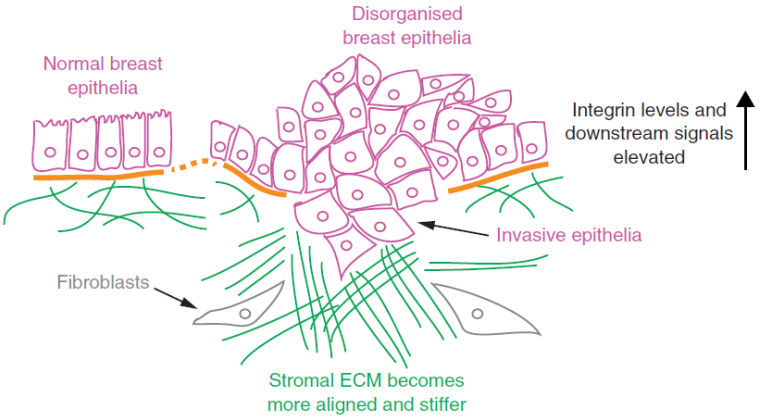


Fig.20: The tumour micro-environment informs the residing tumour cells into malignancy (Glukhova & Streuli 2013). With permission from Elsevier.

malignant behavior is present in the cell, we only see the phenotype manifested when promoted by changes in the microenvironment (Fig.20). Could mutations be suppressed in cells in young tissue, but be promoted by the inflammatory microenvironment in the ageing tissue? Cancer cell lines demonstrate malignant phenotype - protrusions, invasion and dissemination - in a stromal-like context (collagen I), but not in a context of basement membrane-like (laminin and collagen IV) ECM (Egeblad et al. 2010). Further, neoplastic cells can revert to a normal phenotype when placed back in stroma with stiffness equal to normal healthy breast tissue (Ingber 2008). Chronic inflammation in the tissue supplies a series of tumourigenic factors that promote cancer-related cellular phenotypes. In addition, inflammatory cytokines heavily remodel the non-cellular content of the stroma (López-Nouoa & Nieto 2009). Up to 90% of a pancreatic tumour may in fact be non-tumour stroma cells, recruited and mobilized in the context of proliferating tumour cells (Kong et al. 2012). Signals of tumour origin selectively recruit Th2 CD4 helper cells which facilitate tissue repair including angiogenesis, proliferation and anti-apoptosis (Denardo et al. 2009). Those are all pro-metastatic mechanisms. Th1 CD4 helper cells that promote neutralization and killing of tumour cells, on the other hand, are suppressed. Cancer is more than a collection of tumour cells: the surrounding context can both contribute and initiate the neoplasia.

Does the ageing microenvironment demonstrate enough of these tumour-initiating characteristics to produce tumour cells?

1.5.2 Oncogene induced senescence (OIS)

Finally, we propose a potential phenotype to be affected by the altered microenvironment in the ageing body. Oncogene induced senescence (OIS) is the first line of defense against cancer (Serrano M, Lin AW, McCurrach ME, Beach D 1997). This intrinsic mechanism serves to remove potentially oncogenic cells before they develop into tumours (Fig. 21). When an oncogene such as mutated Ras, Raf1 or EGFR is introduced into primary cells they do not become immortalized or tumourigenic. Most likely they go into senescence, followed by removal by NK cells (Childs et al. 2014). Could a loss of this mechanism make cells more prone to develop into tumour cells, and if so how does a cell lose this defence? An oncogene that introduces sustained mitogenic stimulation (MAPKinase signaling) triggers senescence, which is known as OIS. OIS involves overexcretion of GM-CSF, IL-6,-7.-8, -1β, G-CSF and INFγ; inflammatory factors that are less prominent in senescence induced by replicative exhaustion, γ-radiation, oxidative stress or extensive changes in chromatin structure (Nelson et al. 2014; Coppé et al. 2010). Moreover, CDKN2A/p16 induces OIS without SASP (Fig. 16), due to other signaling pathways being involved compared to replicative senescence (Adams 2009; Coppé et al. 2010). Where in the process of senescence may the microenvironment exert influence? Is the microenvironment necessary and sufficient for tumour cell initiation, or does it act in combination with other parameters?

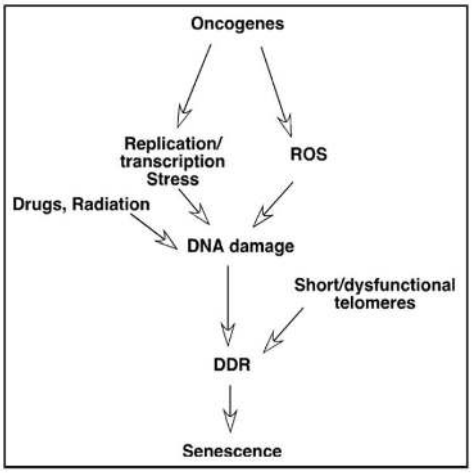


Fig.21: Oncogene induced senescence (Mallette & Ferbeyre 2007). With permission from Taylor & Francis.

2. Aims of study

Cancer incidence correlates with age. Post-menopausal women are more prone to breast cancer than younger women. The increased frequency of breast cancer with age is likely due to interactions between acquired mutations and age-dependent changes in the breast tissue microenvironment that affect mammary epithelial cells. The **central hypothesis** of this thesis is that ageing affects how human mammary epithelial cells respond to microenvironmental signals. To test this hypothesis, we developed a novel microsphere cytometry approach to measure microenvironment-contextual cell signaling at the single cell level. The **overall aim** of this project is to better understand the underlying mechanisms of age-related breast cancer.

The specific aims were to:

- Establish a flow cytometry method (microsphere cytometry) to measure ECM-contextual cell signaling in single adherent cells.
- Employ microsphere cytometry to investigate age-dependent changes in signaling responses to the microenvironment.
- Characterize age-related changes in mammary epithelial cells and how these contribute to cancer development.

3. Methodological considerations

3.1 Cell lines versus low passage primary cells

Our studies were performed on low passage primary human mammary epithelial cells (HMEC). Long term in vitro proliferation results in stress-associated senescence (stasis). Further, critically short telomeres result in telomere dysfunction-associated senescence (agonescence) (Stampfer et al. 2013). In contrast, cell lines are immortal: they have successfully bypassed both senescence and agonescence (Fig.22).

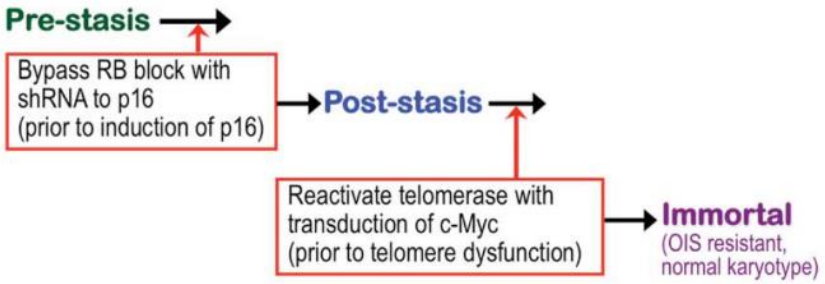


Fig.22: Immortalization is a two-step process (Garbe et al. 2014). With permission from Taylor & Francis.

3.1.1 MCF10A cell line does not represent primary human HMEC

The mammary cell line MCF10A, derived from fibrocystic mammary tissue, is frequently used by researchers to represent a normal epithelial cell (Qu 2015, Soule 1990). We found culturing of MCF10A cells to be EGF dependent. Acinus-like cell aggregates formed in the presence of laminin and the absence of EGF (Fig.23a). MCF10A are not known to be tumourigenic in mice. It is of luminal descent and forms milk-producing acini in 3D cultures with extracellular matrix proteins from the basal membrane (Matrigel) (Fig.23 a). However, they simultaneously express basal markers (Qu 2015), and demonstrate proliferation as predominant response to laminin, as opposed to differentiation (Petersen et al. 1992) (Fig.23a,b). When we employed microsphere cytometry to map the signaling signature of MCF10A cells compared to HMEC, MCF10A response to stimulation greatly varied from that

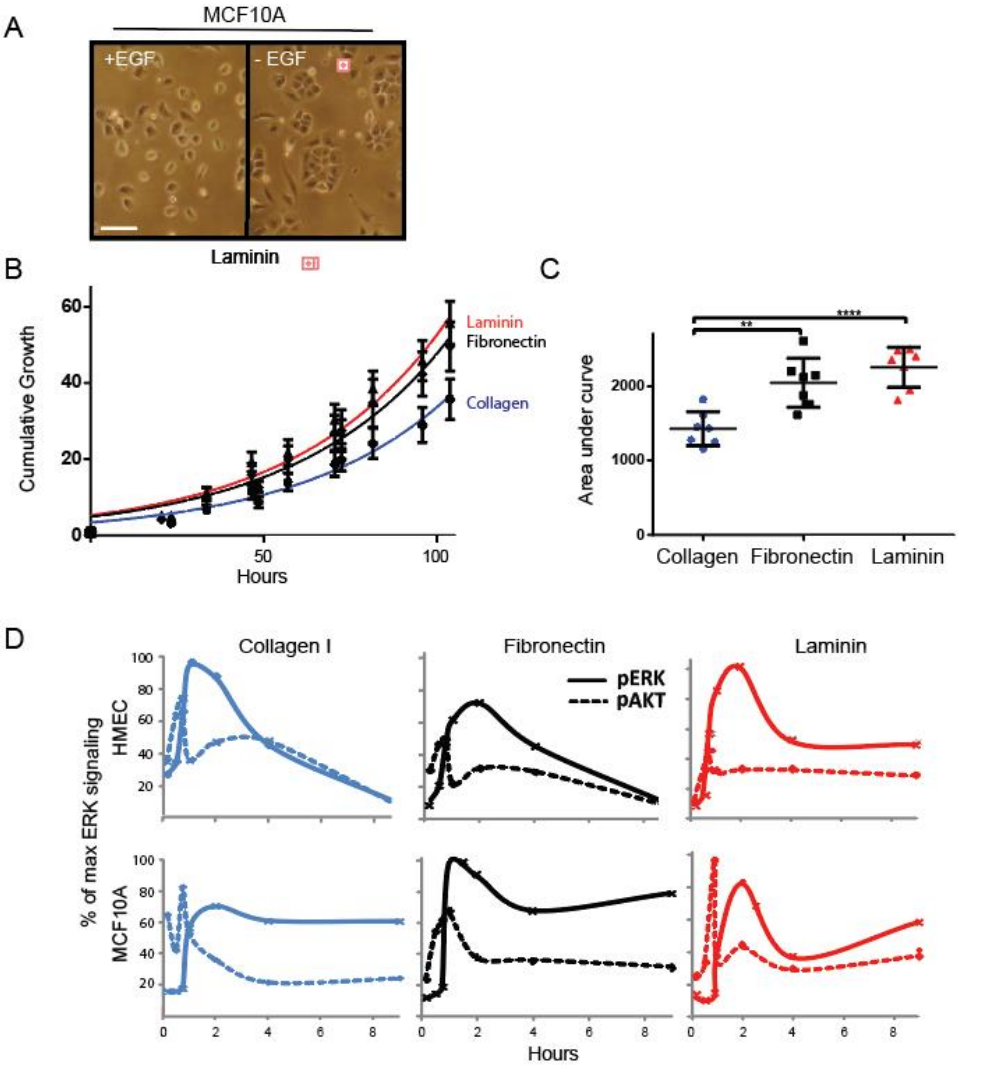


Fig.23: The MCF10A cell line does not represent primary HMEC (A) Phase contrast photos of cell growth in 2D cultures on different extracellular matrix (ECM) proteins revealed that cell morphology, but not cell proliferation, was dependent on EGF, on laminin in particular. In the absence of EGF, cells distributed themselves into structures similar to acini. Real acini may only form in a 3D substrate. Scale bar is 100 µm. (B) Cell proliferation was detected by the conversion of Resazurin into pink coloured Resarufin indicating the number of metabolizing cells present as a function of time. Among the ECM proteins tested, laminin mobilized the strongest growth, contrary to expectation. Laminin is known to direct differentiation at the expense of proliferation. In conclusion, MCF10A cells do not behave like primary HMEC. (C) AUC values of graph in (B). (D) Microenvironment-dependent cell signaling response patterns in MCF10A cells differs from those in HMEC. pERK and pAKT levels were measured longitudinally in HMEC and MCF10A, adhered to collagen, fibronectin or laminin-coated microspheres in growth factor supplemented culture medium. MFI (median fluorescence intensity) values for pERK (solid lines) and pAKT (dotted lines) plotted as a function of time (0-9 h). MCF10A signaling levels did not become normalized within the 9-hour period in comparison to HMEC, but remained high throughout. The graph is representative of three independent experiments.

of the HMEC (Fig.23d). Consequently, we chose to perform our experiments on HMEC rather than on MCF10A cells.

3.1.2 In vitro culture of Human Mammary Epithelial Cells (HMEC)

HMEC grow and disperse in cell culture dish from organoids which are microscopic tissue pieces - the products of enzymatic and mechanic processing of breast tissue following reduction mammoplasties. Samples can also be derived from non-tumour mastectomies or suspended cells from mother's milk. Cells are allowed to grow out of individual organoids placed in cell culture dishes. Contaminating fibroblasts are removed by partial trypsination as described in Paper I (Ertsås et al. 2017). Optimal HMEC growth is supported by culture medium M87A (insulin, EGF, estradiol, prolactin, somatotropin, thyroid stimulating hormone, adrenocorticotrophic hormone and vasopressin) combined with a low concentration of serum, and oxytocin with life extending effect (Stampfer 1985; Labarge et al. 2013; Garbe et al. 2009). Culture in M87A with oxytocin can support HMEC growth for 16 passages, which is equal to 50 population doublings. The biobank at Lawrence Berkeley National Laboratory contains HMEC derived from organoids isolated from 50 women ranging in age from 14 to 91 (hmec.lbl.gov). HMEC used in our studies were maximum 4^{th} passage.

Tissue culture of dissociated primary epithelial cells causes a number of adaptations, of which the most distorting is the tendency of cells to lose their luminal phenotype and become increasingly basal with prolonged in vitro culturing (Pechoux 1999). It is particularly important to confirm that the phenotypic changes detected in older HMEC are not technical artifacts, but indeed of biological nature. Therefore, we measured integrin expression on dissociated uncultured epithelial organoids from 9 women and compared these to 6^{th} passage HMEC. We found similar integrin expression with age in both uncultured and passage 6 HMEC (Paper 2, Suppl. Fig. 5c,d).

3.1.3 Selection of EGFR Del19 transformed cells

Human mammary epithelial cells (HMEC) were transformed with vector coupled EGFR with a constitutive active kinase activity due to a deletion in exon 19 of the EGFR gene. In order to select $EGFR^{Del19}$ transformed cells we performed selection by both antibiotics (Pyromycin) and by FACS flow. We found that either method gave cell populations with equally high EGFR expression levels. In conclusion, the method of selection is not critical to outcome, and HMEC selected by either method can be compared interchangeably (Fig.24)

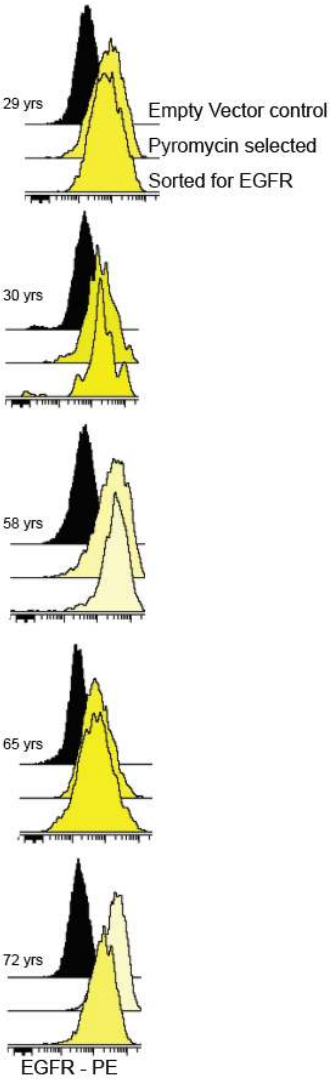


Fig.24 EGFR selection methods.

3.2 Isogenic series

In order to investigate changes in signalling response and integrin expression during malignant progression, we developed an isogenic transformation series. These are cell lines that range from normal to non-malignant immortal and finally tumourigenic. Tumourigenic cells are defined as cells capable of anchorage-independent growth in a gel like material. Malignant cells do not undergo anoikis even when lacking ECM anchorage. Pre-stasis normal cells

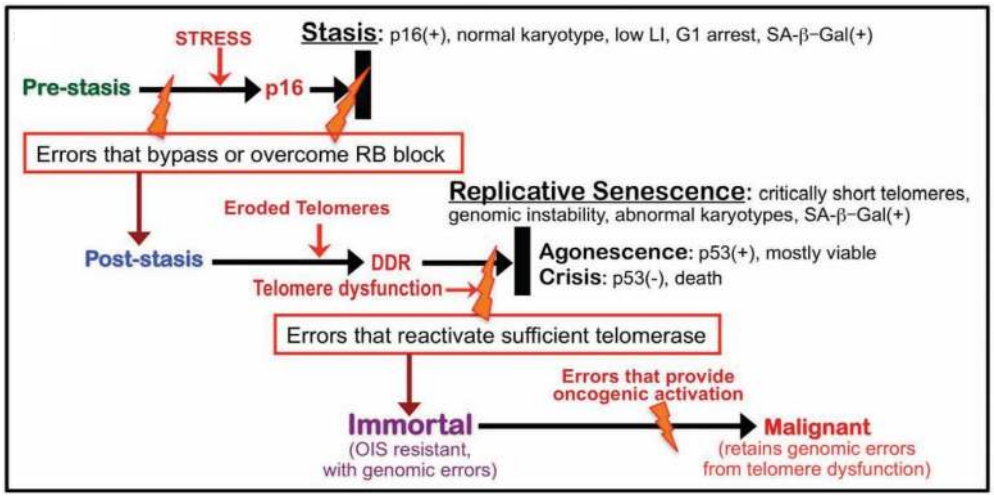


Fig.25: Two types of senescence that need to be overcome in order for cells to become immortalized (Garbe et al. 2014). With permission from Taylor & Francis.

(passage 3) were transduced with shRNA against CDKN2A/ p16, or overexpression of CCND1/CyclinD1 to allow them to bypass stasis by preventing Rb activation. CyclinD1 is necessary for self-renewal in mammary tissues, and more importantly luminal differentiation into functional lobules and ducts (Jeselsohn et al. 2010). CyclinD1 overexpression promotes transformation into a luminal phenotype, and increases tolerance to Neu/ERBB2 . Suppression of CDKN2A/p16 is less likely to give this result (Lee et al. 2015). Telomere shortening is overcome by ectopic MYC expression that induces transcription of the telomerase reverse transcriptase gene (TERT) (Kyo et al. 2008). Flow cytometric measurements revealed that myoepithelial or luminal cells were transduced with similar efficiency (Fig.26).

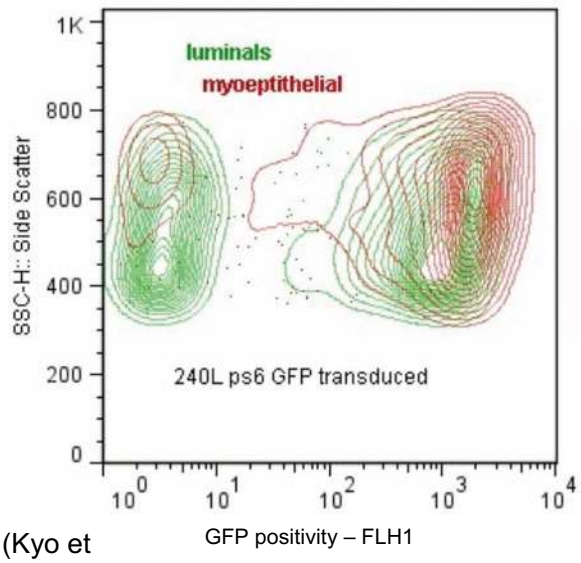


Fig.26: GFP transduction of luminal and myoepithelial cells. Subpopulations present immediately after transduction.

Hence, the low fraction of transformed luminal cells found in cell lines after prolonged passaging may be related to a myoepithelial-like phenotype, acquired post transformation. The isogenic series comprises pre-stasis, immortal and malignant human mammary epithelial cells. The genetic alterations from one step to the next are known as they were introduced by us

3.3 Flow cytometry

Flow Cytometry measures characteristics of cell populations at the single cell level, such as number, size, DNA content and immune phenotype. Cells are stained with fluorophore-conjugated antibodies, enter a laminar flow and are passed by a laser and fluorescence detector (Fig.27). Flow cytometry allows analysis of various cellular subpopulations within a sample. Fluorescence is proportional to the number of antibodies bound per cell. The median fluorescence of the cell population indicates the average number of epitopes in each cell. This can be used to calculate the percentage of cells demonstrating a change in fluorescence in response to a defined treatment. **Phospho Flow Cytometry** measures intracellular phospho-epitopes on signal transduction proteins. **Microsphere cytometry** described in Paper I (Ertsås et al. 2017) allows ECM contextual signaling measurement of subpopulations in the sample. Microspheres contribute a surface where a microenvironment can be mimicked (Fig.28). Each particle comprises both cell and context, and signaling is captured as the particles pass by the laser, (Fig.29). Flow cytometry is limited by available fluorescence wavelengths and their tendency

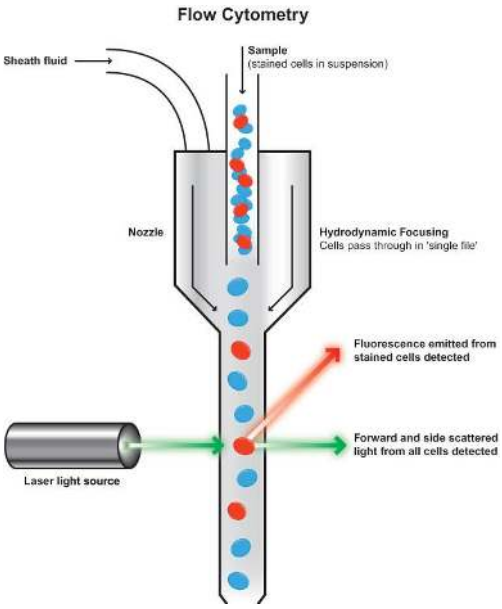


Fig.27: Abcam.com

to bleed into multiple detection channels. Compensation is conducted to account for spectral overlap.

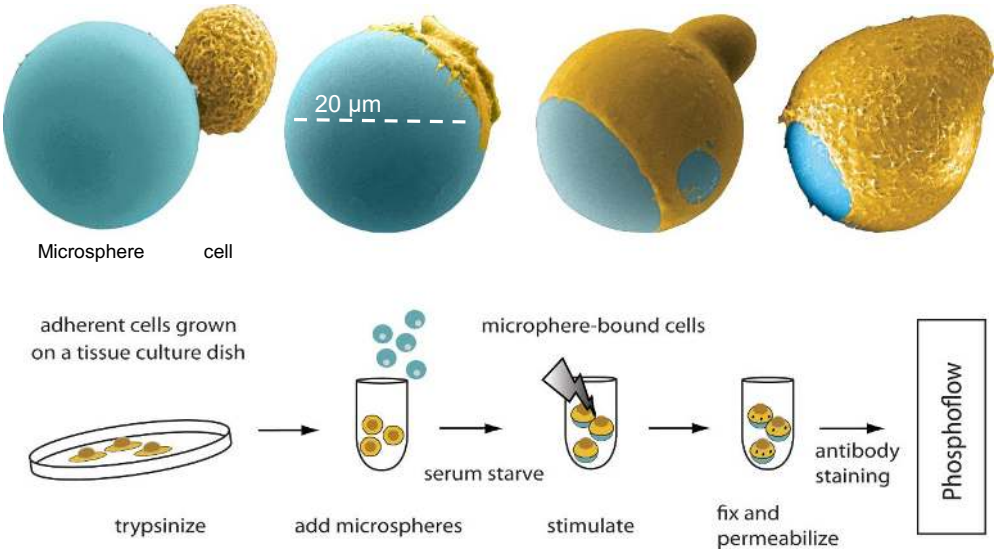


Fig.28: Electronmicroscopy images of a cell binding to a microsphere.
Flowchart of Microsphere Cytometry method (Ertsås et al. 2017). With permission from Royal Society of Chemistry.

3.3.1 Challenges of microsphere cytometry

A particular challenge in microsphere cytometry is avoiding aggregates in the sample. Aggregation is promoted by the ECM coating; collagen I-coated microspheres tended to clump more than laminin-coated particles . Aggregates do not enter the laminar flow and reduce the number of detectable events. It was therefore necessary to vortex the sample during analysis, every 30 seconds. Hence, the microsphere cytometry method requires more cells per sample compared to normal flow cytometry without microspheres, due to loss of particles during the running step

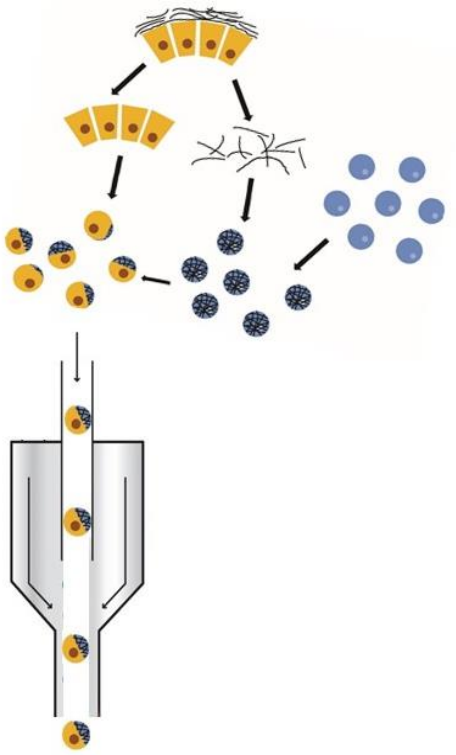


Fig.29: Microsphere cytometry: Microspheres (in blue) offer a surface where the microenvironment (black threads) can be mimicked. The cell (in yellow) wraps itself around the microenvironment.

3.4 Statistical considerations

Flow cytometry data analysis was performed in Cytobank or Flow Jo (Tree Star Inc.), and is presented either as colour coded histograms or plotted as sample fluorescence as a function of age or time. Fluorescence is presented as the log2 ratio of fluorescence post manipulation over pre-manipulation. Where “pre” is a control sample stained with a secondary antibody only (Paper 1 Fig. 2 and 5), or a sample were phosphorylation is inhibited (Paper 1 Fig. 3). A positive ratio in the histogram is illustrated in yellow and a negative ratio in blue (Fig.30).

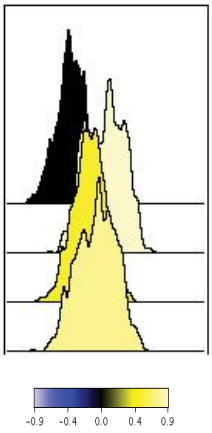


Fig.30: Histogram

.

Table 2. Definition of the Y axes unit

Paper	Figure	y axes or histogram width represents
1	Fig. 2,5	Log2 of fluorescence intensity (MFI) of samples relative to control stained with secondary antibody only
1	Fig. 4	Fluorescence intensity (MFI) of sample minus that of a control sample stained with secondary antibody onlym were ploted the percentage of signaling at given timepoints out of maximum signaling of total of timepoints measured.
1	Fig. 3	Log2 of fluorescence intensity (MFI) of sample relative to a control sample were phosphorylation was inhibited.
2	Fig. 1 C	GMFI of the responding population is defined as those cells that have a stronger fluorescence than a phospho-inhibited control.
2	Fig. 1 D	The GMFI of the responding population
2	Fig. 2	Log2 ratio of sample fluorescence over that of a control stained with secondary only. In the lower right corner of graph sample values were recalculated to find the ratio value when minimum value was set to 0 and maxium was set to 100, from this set up up EC50 values were determined.
2	Fig. 3 B	Fluorescence intensity (MFI) minus MFI of secondary only control, and its log2 value relative to the youngest individual among samples
2	Fig. 4	Log2 ratio of fluorescence intensity in cells with over cells without EGFR Del19-over expression
2	Suppl. Fig. 1	The GMFI of the responding population
2	Suppl . Fig. 2 B	The shift in fluorescence intensity (GMFI) induced upon EGF stimulation
2	Suppl. Fig. 2 C	Percentage of total cells that make up the responding population
2	Suppl. Fig. 3	Fluorescence intensity (MFI) of samples minus fluorescence of unstained cells, distributed on a log 2 scale on the y axes.
2	Suppl. Fig. 4	% bound cells out of all cells present in sample. Based on SSC-A intensity of the particle

Table 3. Choice of statistical tests

Paper	Figure	statistical test	Reason for choice of test
2	Fig. 1	linear regression	Differences in response changed gradually with increasing age. It did not separate into pre- and post- menopausal response. Regression was therefore prefered to student t-test
2	Fig. 2	non linear regression: dose - response curve (Stimulation)	The appearance of the graph representing the kinetics of signaling as a function of time is similar to a dose-response curve (log (agonist) vs normalized response)
2	Fig. 3	linear regression	Differences in integrin expression changed gradually with increasing age. It did not separate into pre- and post- menopausal response. Regression was therefore prefered to student t-test
2	Fig. 3 B ii)	linear regression of ratio LEP integrin expression over MEP integrin expression	A high expression of integrins is characteristic of MEP. Neighboring LEP expressing higher levels than MEP are defined as having a basal like nature.
2	Fig. 4	linear regression	Differences in oncogene induced signaling changed gradually with increasing age. It did not separate into pre- and postmenopausal response. Regression was therefore prefered to student t-test
2	Fig. 4 C	Kaplan-Meyer estimate	Each HMEC sample was considered an individual and death was defined as the point in time when the cell population was no longer proliferative.
2	Suppl. Fig. 3	non linear regression: Binding - Saturation	HillSlope fitting was selected because we assumed a binding - saturation relationship between cell signaling and time. (One site - specific binding with Hill slope)
2	Suppl. Fig. 4	non linear regression: Binding - Saturation	Hill Slope fitting was selected because we assumed a binding - saturation relationship between cell adsorption and time. (One site - specific binding with Hill slope) We chose to compare Area under the Curve rather than EC50 values because maximum and minimum adhesion percentage varies between the two women. Area Under the Curve captures both the speed with which cells bound and the total amount of cells bound. T-test is suited to test whether there is a significant difference in cell binding between two groups. In this case between parallels
2	Suppl. Fig. 6	non linear regression: dose - response curve (Inhibition)	We used the small molecule inhibitor erlotinib and measured survival. (log (inhibitor) vs - response) Variable slope

4.Summary of papers

Paper I

Microsphere cytometry to interrogate microenvironment-dependent cell signaling

Henriette Christie Ertsås, Garry P. Nolan, Mark A. LaBarge and James B. Lorens

Integr.Biol., 2017, 9 (2), pp. 123-134

Microenvironmental cues comprising surface-mediated and soluble factors control cellular signaling mechanisms underlying normal cellular responses that define homeostatic and diseased cell states. In order to measure cell signaling in single adherent cells, we developed a novel microsphere-based flow cytometry approach. Single normal or neoplastic cells were adhered to uniform microspheres that display mimetic-microenvironments comprising surface combinations of extracellular matrix (ECM) proteins in the presence of soluble agonists/antagonists. Temporal signaling responses were measured with fluorophore-conjugated antibodies that recognize response-dependent epitopes by multiparametric flow cytometry. Using this approach, we demonstrated that microenvironment-mimetic combinations of growth factors and extracellular matrix proteins generate distinct cellular signal networks that reveal unique cell signatures in normal and patient biopsy-derived neoplastic cells.

Paper II

Microenvironment-contextual cell signaling is attenuated with age.

Henriette C. Ertsås, Mark A. LaBarge and James B Lorens [Manuscript]

Post-menopausal women are more prone to breast cancer than younger women. The increased frequency of age-related breast cancers is likely due to interactions between mutations and age-dependent epigenetic changes that affect mammary epithelial lineage fidelity. We hypothesized that the ageing process fundamentally affects how human mammary epithelial cells (HMEC) respond to microenvironmental

signals, resulting in increased susceptibility to oncogenic transformation. In order to measure microenvironmental cell signaling in normal finite lifespan HMEC, we applied a novel microsphere-based flow cytometry technology. Microsphere cytometry allows multiparametric single cell quantification of signaling pathway activity and lineage-specific marker expression in cells adhered to surface-functionalized microspheres that mimic specific microenvironments. Using this approach, we analyzed age-dependent changes in human mammary myoepithelial and luminal epithelial cells exposed to various ECM and growth factors. We found that ECM–mediated MAP kinase and PI3 kinase activation levels in HMEC were attenuated with age. Older luminal cells displayed higher surface integrin levels consistent with acquired basal identity, albeit with decreased integrin phosphorylation and increased Src-phosphorylation relative to myoepithelial cells. We show that the diminished signaling magnitude in HMEC from older women correlated with reduced probability of activating oncogene-induced senescence. We propose that age-related alterations in ECM-mediated epithelial cell-regulation may impair protective tumor-suppression mechanisms and increase breast cancer susceptibility.

5. Discussion

Increased tumour incidence with age has been suggested to involve an attenuation of protective tumour-suppressive functions in the tissue (Fata et al. 2003; Bissel & Radisky 2001). Researchers propose that the post-reproductive microenvironment may select for cells with genetic or epigenetic changes. Transformed cells have higher fitness than healthy cells in the ageing body (Degregori 2013). These cells may be more efficiently removed during the reproductive period of life, but experience a selective advantage during ageing. Age-related alterations in immune surveillance facilitate emergent cellular phenotypes with altered senescence triggers that tolerate inflammation, tissue stiffness and a degraded basal membrane integrity (Rozhok et al. 2014)(Paper 2). Luminal mammary epithelial cells with acquired basal characteristics that facilitate interaction with different ECM proteins can derive a selective advantage in a non-native microenvironment that forms following basal membrane degradation. This new landscape favours transformed cells over healthy cells, and malignant phenotypes over benign (Rozhok et al. 2014).

Ageing phenotypes are thus the result of both intrinsic factors and extrinsic factors in the microenvironment (Hornsby 2002). Hence, we hypothesize that age-related microenvironmental changes in the human breast induce phenotypic modifications in epithelial cells (Paper 2).

5.1 Microsphere cytometry to interrogate microenvironment-dependent cell signaling.

In order to detect age-dependent phenotypic variations in mammary epithelial cells, manifested as cell signaling, we developed the microsphere cytometry approach. Microsphere cytometry permits quantification of cell signaling in single cells adhered to different ECM proteins. Commonly used methods, such as Western blot, can be used to measure protein expression changes in cells cultured on tissue culture plates coated with ECM proteins. However, these methods represent an average value for the cell population, masking

heterogeneity present in the sample (Jensen 2012) and variation in cell signaling in cellular subpopulations. Immunofluorescence-approaches can reveal subcellular locations of phosphorylated mediators, but throughput is low. The use of microspheres to conduct flow cytometry analysis of adherent cells was proposed by Bloch et al. more than 30 years ago (Bloch et al. 1983). We applied microspheres to quantify cell signaling in different ECM contexts that mimic the in vivo microenvironment (Fig.31).

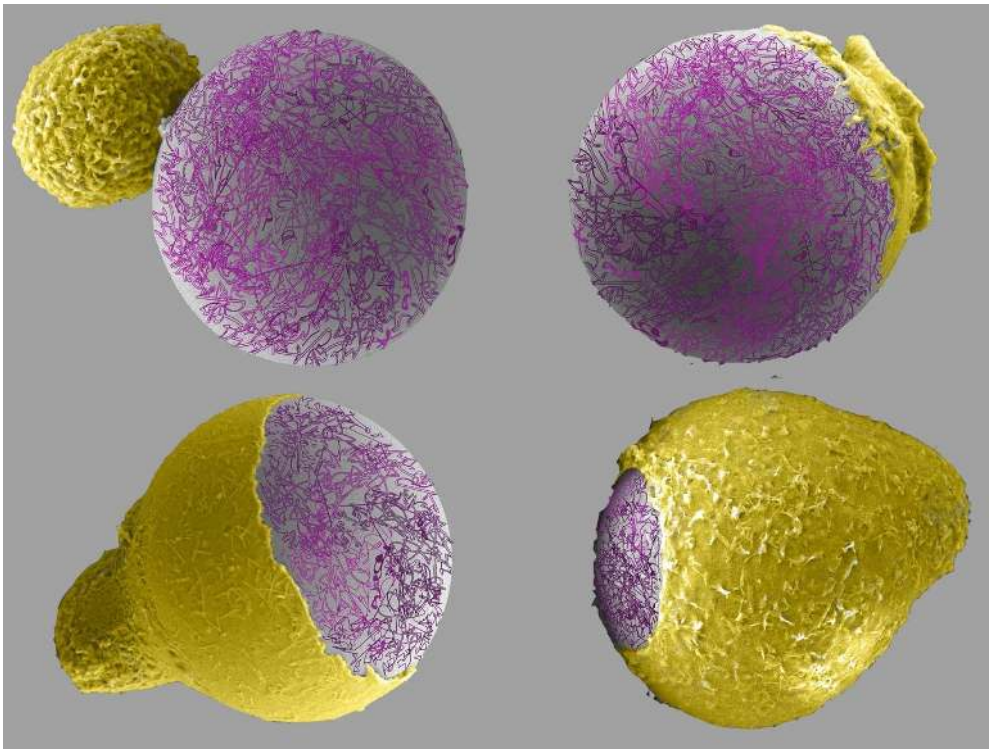
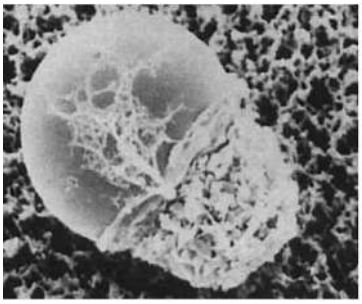


Fig.31: Illustration of ECM coating (in purple) on plastic microspheres. A mimicked microenvironment for cells to bind to, Paper I (Ertsås et al. 2017). With permission from Royal Society of Chemistry

Phospho-flow cytometry as a quantitative single-cell methodology offers high throughput, but requires that cells are in a suspension (Irish et al. 2006)(Fig.27). Single cells bound to 20 µm diameter microspheres coated with ECM, are amenable to flow cytometry analysis and treatment with soluble factors (Fig.29). Currently we are limited by the physio-chemical attributes of

the microspheres. Plastic microspheres are far stiffer than tissues. above physiological levels. It is feasible to derive microspheres of uniform diameter with varying stiffness using alternative chemistries such as hydrogel based microdroplets. Physiologically relevant stiffness in microdroplets can be achieved by adjusting the concentration and increasing the crosslinking of PEG (poly ethylene glycol) or agarose (Allazetta et al. 2013). Further it is feasible to encapsulate cells within agarose to provide a 3D environment for individual cells (Hammill et al. 2000).



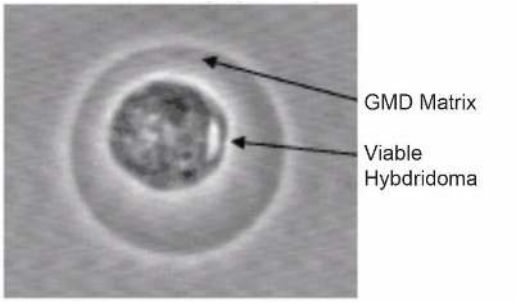


Fig.32 Scanning Electron Microscopy
Cell Microsphere (uncoated)
(Bloch et al. 1983)
With permission from Wiley and Sons

Phase Contrast Microscopy
Gel Microdrop (GMD)
One Cell Systems

Microdroplets can be made with a diameter between 10 to 100 µm (Fig.32). The cell is encapsulated inside the agarose gel droplet of a given stiffness and supplemented with different ECM. One Cell Systems™ have the equipment to make gel-encapsulated gel droplets in the lab to run on the flow cytometer. A microdroplet with ECM embedded in the gel may be less sticky than an ECM coated microsphere. Microdroplets could therefore be convenient for use in a mass cytometer, with lower risk of clogging the internal capillary of the nebulizer.

5.2. Contextual response of cells in an ageing micro-environment is attenuated with age

Our data indicate that human mammary epithelial cells (HMEC) from older women have reduced MAPK signaling responses to different ECM (Paper 2 Fig.1, Suppl. Fig.1). The increase in p(Thr202/Tyr204) ERK level following growth factor stimulation was delayed in older HMEC bound to collagen I or

laminin compared to HMEC derived from younger women (Paper 2 Fig.2). We found a significant reduction in p(Tyr1510) levels of the integrin β_4 subunit in luminal cells (Paper 2 Fig.3c). Integrin β_4 influences laminin signal transduction, supporting the notion that the laminin-mediated signaling associated with HMEC differentiation is diminished with age (Glukhova & Streuli 2013; Streuli & Akhtar 2009; Li et al. 2003). Proliferative and migratory behaviours supported by fibronectin and collagen I (Carey et al. 2017) are similarly downregulated in older HMEC. Notably, the breast basal membrane becomes discontinuous with age in part due to hormonal changes during menopause (LaBarge et al. 2015; Sympson 1994). Concurrently, collagen I and fibronectin deposition increases with age (Schedin & Keely 2011). We did not detect significant differences in PI3K-AKT pathway activation in HMEC from older versus younger women (Paper 2 Fig.1d). This could relate to the observation that adhesion-activated PI3K is largely ECM independent (S. J. Yarwood & Woodgett 2001). Other researchers have found greater variation in the ECM-induced transcriptome of HMEC within a group of ageing women compared to the variation found among a group of young women. This suggests a deregulation of gene expression with age (Miyano et al. 2017; Yau et al. 2007). Our data confirm a loss of regulation manifested by the lack in response to ECM in HMEC derived from ageing women.

5.3 Attenuated cell signalling responses with age

5.3.1 Integrin expression and cell adhesion

Extrinsic factors mediate cell signalling that determine cellular behaviours. Contextual ECM information is largely mediated through integrin receptors. We therefore asked whether the age-dependent, attenuated, cell signaling was correlated with a change in surface expression of integrin, as measured by flow cytometry. Surface integrin expression levels were higher on myoepithelial than on luminal cells, a ratio that did not differ with age. Luminal cells from older women showed increased expression of integrin α_6, β_4, α_2 and β_1, while α_5 and α_V remained unchanged (Paper 2 Suppl. Fig.5). These

findings correspond to previous immunohistochemistry analysis of cartilage cells (Shakibaei et al. 1993). The results were consistent for both uncultured primary, and HMEC up to passage 5, and showed that integrin expression levels were not affected by short term in vitro culturing under our conditions.

We confirmed that the older luminal cells adhered to fibronectin, both measured as the absolute number of cells adhered within a 2.5-hour period, and as the percentage of adhering cells per minute (Paper 2 Suppl. Fig.4a,b, Suppl. Data Fig.1c). This corresponded with higher surface integrin β_1 levels on older cells (Paper 2 Suppl. Figure 5). Tissue stiffness is likely an age-dependent factor that influences integrin expression and/or distribution. Epithelial cells grown in vitro in a rigid 2D cell culture dish have previously demonstrated higher integrin expression compared to cells grown in a compliant 3D matrix (Delcommenne & Streulis 1995). Cell invasiveness is known to be promoted by higher levels of integrin β_1 receptors (Ganguly et al. 2013; Berry et al. 2003). Increased $\alpha_6\beta_4$ expression found in certain carcinomas also correlates with invasive properties (Gordon et al. 2003). 60% of primary breast carcinomas show persistent β_4 expression (Davis et al. 2001), and α_6 levels are high in several tumours (Ding et al. 2013; Natali et al. 1992). This contrasts with our findings using isogenic progression series, where in fact surface expression of all measured integrins (β_1, β_4, α_6, α_2, α_5 and α_V) decreased with increasing malignancy (Suppl. Data Fig.2).

It is paradoxical that HMEC from older women adhere more efficiently to fibronectin (through their β_1, α_5 and α_V subunits), but show lower levels of adhesion-induced phosphorylated ERK compared to HMEC from younger women (Paper 2, Fig.1d right side). This was consistent for all ECM proteins tested (Paper 2 Suppl. Fig.1). One explanation may be that integrin dimers can be redistributed without altering expression. During ageing and fibrosis $\alpha_6\beta_4$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ on luminal cells tend to relocate from the basolateral surface to spread into the luminal, lateral and basal surface of the cell (Fig.33) (Ding et

al. 2013). For example, when hemidesmosomes disassemble following chemotactic stimuli, integrins redistribute to more diffuse F-actin interaction in the plasma membrane (Mercurio et al. 2001). Increased tissue stiffness is associated with reduced integrin β_4 polarity (Paszek et al. 2005; Ding et al. 2013). The depolarisation and spread of $\alpha_6\beta_4$ throughout the membrane facilitates detachment from the substrate and subsequent migration (Natali et al. 1992; Stewart & O'Connor 2015). Interestingly, integrin α_6 is reported to be tumour-suppressive when polarized, but to promote a malignant phenotype when delocalized (Natali et al. 1992). Flow cytometry analysis of integrin expression does not distinguish integrin localization. In conclusion, HMEC integrin surface expression levels and cellular adhesion did not correlate with MAPK signalling responses in our analysis.

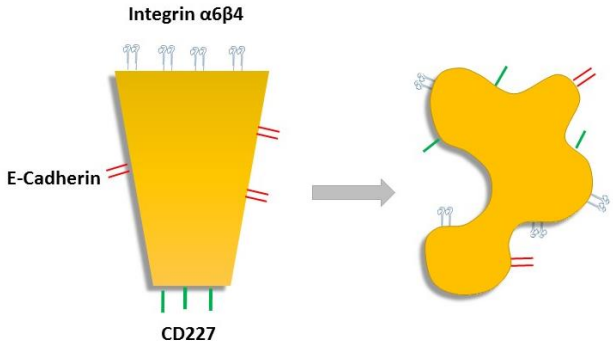


Fig.33: Integrin depolarization on epithelial cells induces loss of architecture and change in morphology

5.3.2 Reduced integrin activation with age

We asked whether the altered ECM-mediated MAPK signaling response in the ageing phenotype could be related to changes in integrin activation. Our data demonstrate that activated Src pTyr418 and integrin β_4 pTyr1510, as well as the activated conformation of integrin β_1 in myoepithelial cells decreases with age (Paper 2 Fig.3bi)). The fraction of integrin β_4 pY1510 versus total integrin β_4 is reduced in luminal cells from older women (Paper 2 Fig.3c), indicating reduced integrin β_4 activation, in spite of overall higher surface integrin levels. Tyrosine residues on β_4 are thought to be substrates for the Scr family kinase, Fyn (Mariotti et al. 2001). Thus, the observed attenuated ECM-induced MAPK

signaling in older human mammary epithelial cells is likely not due to lower integrin levels or reduced adhesion, but rather to integrin redistribution and reduced phosphorylation by Scr family kinases.

5.4 Extracellular matrix and Integrins play a role in oncogene induced senescence

5.4.1 Mechanotransduction is affected by age

Integrin-mediated cell signalling is suggested to fine-tune growth factor receptor signal transduction (Morse, Brahme & D. a. Calderwood 2014). This ensures that ECM context in the microenvironment can influence growth factor cell signaling characteristics (Giancotti & Tarone 2003; Legate et al. 2009), such as survival through the PI3K pathway, proliferation through the MAP kinase pathways or cytoskeletal tension via ERK – Rho pathway (Kass et al. 2007; Levental et al. 2009). The MAP kinase pathway can drive cell senescence when mitogenic overload induces a DNA damage response and mobilization of tumour suppressors P53 and CDKN2A/p16 (Childs et al. 2015; Lin et al. 1998; Di Micco et al. 2006) (OIS see section 1.5.2). Previous studies have found an age-dependent effect on the ability of cells to respond to stiffness by influencing lineage determination into either myoepithelial or luminal phenotype (Pelissier et al. 2014), while we quantified age-dependent MAPK pathway activity as the number of phospho-epitopes on ERK. Our results indicate that the amplitude and duration of pERK activation in response to ectopic expression of a constitutively-active $EGFR^{Del19}$ is reduced (fig.34 and fig.35 [orange dotted line]) in older HMEC. This appears to influence cell division. HMEC derived from older women grew more slowly in vitro than HMEC from younger women. Studies show that it is the cumulative time, rather than the frequency of the pERK activation that determines entry into S-phase (Kolch et al. 2015).

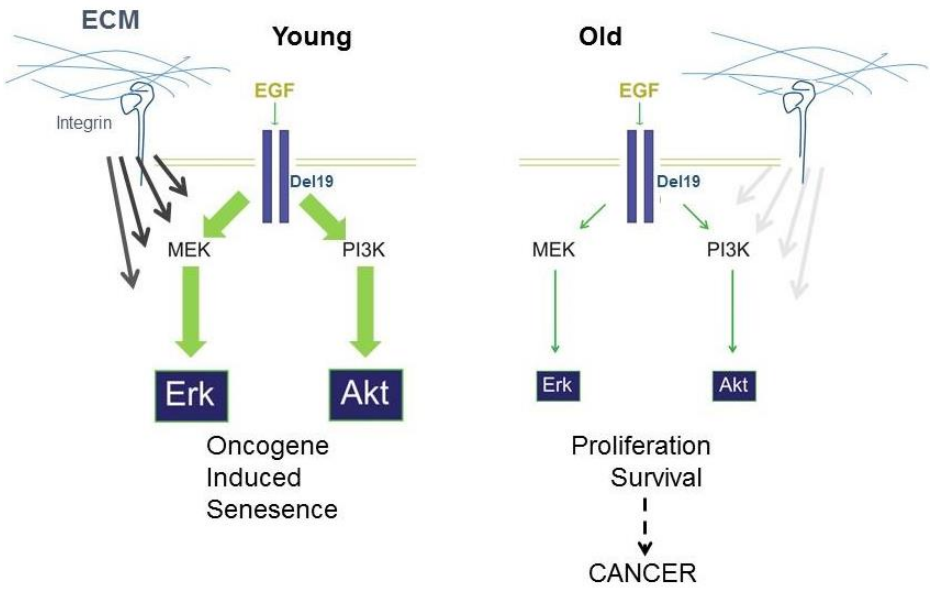


Fig.34. Schematic model of age-dependent attenuated signaling.

Birtwistle et al 2007 suggested the pre-peak response curve of pERK results primarily from EGFR-Ras Raf MEK activity, while the post-peak period is maintained via PI3K-PIP3-Gab1-Grb2-SOS-Ras activity (Birtwistle et al. 2007). However, pERK is involved in many negative feedback loops; this is consistent with our data showing normalized pERK levels within 2 hours after stimulation in younger HMEC (Paper 2, Suppl. Fig. 3b). Sustained pERK stimulation is required to trigger OIS (Lin et al. 1998). This might be due to the integration of several signaling inputs or a single constitutively active input such as mutant EGFR

Combined integrin and growth factor signaling form a sigmoidal response through an “AND” gate. An example of a response through an “AND” gate is a feed forward reaction comprising two or more pathways that converge on a node. They may have different kinetics, but both must be activated for the converging node to be triggered. Output varies depending on whether it is one, the other, or both branches that are activated (Alon 2007; Dueber et al. 2007).

For example, different phosphorylated tyrosines on receptor tyrosine kinase domains that recruit cognate SH2-signaling proteins signal can converge on a downstream signalling node; thus a phosphorylation threshold must be surmounted to trigger a downstream signalling event (Giancotti & Tarone 2003). Integrins also contribute to downstream signalling that could trigger or inhibit alternative signaling pathways (Moro et al. 1998; Balanis et al. 2011; Danen et al. 2005; Huveneers & Danen 2009). The converging nodes include Src, Shc, RhoA, FAK and Rac (section 1.3.4 Fig.12). The attenuated ECM response found in older HMEC can consequently be due to attenuated integrin signaling as we did not observe a significant effect on growth factor signaling (Paper 2 Suppl. Fig.2b)

5.4.2 The tumour-suppressive function of senescence is lost with age

Senescence is a hallmark of ageing. The fraction of senescent cells is higher in the ageing body (Dimri 1995, Faragher 2017). Paradoxically, our data suggest that older HMEC are resistant to oncogene induced senescence introduced by $EGFR^{Del19}$ with constitutive active kinase activity. (Paper 2 Fig.4). The mitogenic overload following the introduction of the Ras oncogene will in most cases lead to cell cycle arrest (A. Kilbey*, A. Terry, E.R. Cameron 2008). In our study, HMEC derived from postmenopausal women demonstrated tolerance to constitutive active EGFR, proliferating for several passages in vitro (Paper 2 Fig.4c, Suppl. Data Fig.3a). In contrast, younger HMEC expressing $EGFR^{Del19}$ became enlarged, displayed vacuoles, expressed β-galactosidase and stopped proliferating (Paper 2 Fig. 4, Suppl. Data Fig. 3b). In vivo they would eventually be removed by the innate immune system (Shaw et al. 2010). This suggests that HMEC from postmenopausal may be less likely to activate senescence in response to oncogenic EGFR due to attenuated signal transduction comprising slower kinetics and a lower overall amplitude. $EGFR^{Del19}$ induced pERK and pAKT levels were different in older and younger HMEC, in spite of similar EGFR expression levels (Paper 2

Suppl. Fig.6). HMEC selected by antibiotics expressed similar high EGFR levels as flow cytometry sorted HMEC (Fig.24).

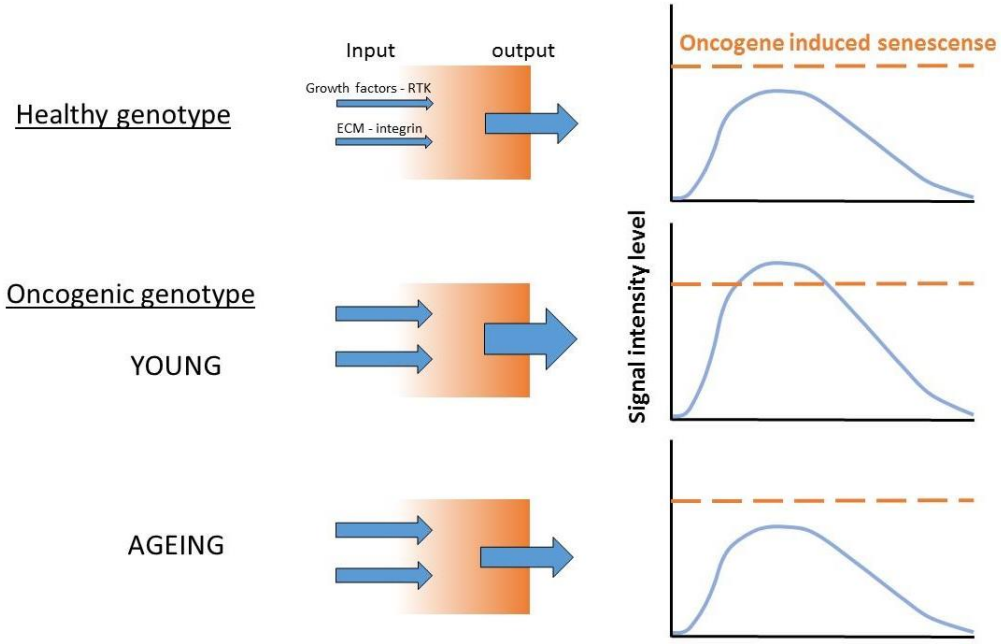


Fig.35: Schematic model of the signaling intensity level necessary to induce OIS

It has been shown that MAPK pathway activation must transcend a threshold for a certain amount of time in order to induce senescence via TP53 (Olsen et al. 2002). We suggest that mitogenic overactivation in older HMEC does not transcend this threshold and therefore do not mobilize TP53 (Fig.35 [orange line]). The cell continues to live (Suppl. Data Fig.3a). The ageing microenvironment may limit MAPK and PI3K activity, and hence reduce OIS triggering (Paper 2 Fig.1, Fig.2, Suppl. Fig.3). A similar correlation between loss of mTOR activity and increased lifespan of the nematode *C. Elegans* was previously reported (Vellai et al. 2003; Lamming et al. 2013). Resistance to OIS could increase the likelihood of accumulating oncogenic and epigenetic changes, which explains how an ageing microenvironment increases cancer susceptibility.

5.5 Ageing luminal cells exhibit basal-like traits

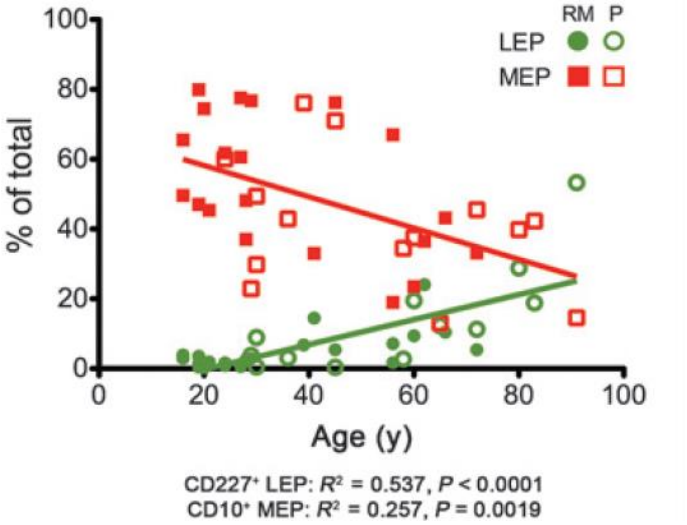


Fig.36: Myoepithelial and luminal epithelial cell fraction as a function of age (Garbe et al. 2012)
P= peripheral non-tumour mastectomy tissue
RM= reduction mammoplasty tissue. With permission from American Association for Cancer Research.

It is previously shown that the proportion of luminal cells increases in ageing mammary tissue, while myoepithelial cells decrease (Garbe et al. 2012) (Fig.36). Normally, luminal cells undergo frequent cell division during the menstrual cycle and pregnancy, while myoepithelial cells have a lower turnover (Clarke et al. 2005). However, luminal cells from older breast tissue differ. In vivo samples from ageing women demonstrate that luminal MUC-1 ($CD227^+$) cells express lower levels of cytokeratin 19, another common luminal marker. Certain MUC-1 cells are entirely negative for K19 (Garbe et al. 2012).

We found increased basal characteristics in luminal cells from older women. Normally, luminal cells express less than half the level of integrins compared to myoepithelial cells (Paper 2, Suppl. Fig.5b). Longitudinal monitoring of cell adhesion to laminin showed that myoepithelial cells generally adhere faster and at higher percentages than luminal cells (Suppl. Data Fig.1b). Secondly, luminal cells generally proved to be more responsive to fibronectin - measured as the level of phospho-epitopes of AKT- than myoepithelial cells (Suppl. Data Fig.4). In contrast, myoepithelial cells were more responsive to EGF stimulation, as measured by pAKT levels (Suppl. Data Fig.4), consistent with a higher level of EGF receptors (Colemann et al. 1988). This finding is congruent

with myoepithelial cells interfacing the basement membrane, while luminal cells reside in a microenvironment created entirely by homo- and heterotypic cell-cell interactions with myoepithelial cells (Adriance et al. 2005; Deugnier et al. 2002). Myoepithelial cells are hence responsible for conveying information from the matrix to the luminal cells (Gudjonsson et al. 2005).

Our data shows that only luminal cells from older women demonstrated a significant increase in integrin expression of subunit α_2, α_6, β_1 and β_4, which could reflect exposure to new extracellular matrix contexts (Paper 2 Suppl. Fig.5b,d). As a woman ages, basement membrane integrity is degraded and the number of myoepithelial cells is reduced (Fig.36), increasing the likelihood for luminal cells to be exposed to new matrix proteins (Deugnier et al. 2002). The loss in total myoepithelial cells and structural alterations in the basement membrane favour luminal cells with basal traits that can benefit from ECM interactions (Warburton et al. 1981; Gudjonsson et al. 2005).

Congruent with Garbe et al. we further discovered an age-dependent increase in luminal progenitor cells among the human mammary epithelial cells (Suppl. Data Fig.5). LaBarge et al. reported that fate decisions of epithelial progenitors are regulated by specific microenvironmental features, in particular laminin that induces quiescence and impairs differentiation (LaBarge et al. 2009). As the laminin and collagen IV rich mammary basement membrane disintegrates with age (Amano 2009; Sympson 1994), progenitor differentiation into luminal or myoepithelial lineage is affected. Age-dependent gene silencing is found to particularly target those promoters which maintain stem cell differentiation. Thus, the residing cells in ageing tissue might therefore not be able to become differentiated (Teschendorff et al. 2010). cKit+ mammary progenitors from postmenopausal women fail to differentiate into either luminal or myoepithelial lineage, instead retaining expression of both K14 and K19 (Garbe et al. 2012). Mammary epithelial progenitors have demonstrated an age-dependent loss of response to stiffness variations, where the progenitor cells of the ageing

phenotype differentiate into luminal cells regardless of substratum stiffness (Pelissier et al. 2014). This is in line with our findings of older luminal cells with increased basal characteristics including increased adhesive properties (Suppl. Data Fig.1b-e), increased integrin expression (Paper 2, Suppl. Fig.5b,d) and lower response to ECM (Paper 2 Fig.1d, Suppl. Data Fig.4) as mentioned above. Our data and that of Garbe et al. 2012 show an increasing fraction of cells in the ageing mammary gland retaining both luminal and basal characteristics. The altered nature of ageing mammary cells promote involution (Milanese et al. 2006); the ageing mammary gland is no longer able to produce milk nor facilitate contraction of the lobule. The older HMEC does not become fully differentiated or undergo epigenetic changes related to epithelial to mesenchymal transition (EMT) (Teschendorff et al. 2010). Indeed, the ageing microenvironment is associated with inflammation, fibrosis, increased stiffness, oxidative stress and loss of immune function (see section 1.4.1) - all factors known to promote EMT (Cox & Erler 2014; Nieto 2013; Thiery et al. 2009).

Thus, luminal cells may acquire basal-like traits via different mechanisms such as skewed differentiation or EMT. This is supported by the fact that HMEC from older women demonstrate methylation patterns associated with retained stem cell characteristics (Widschwendter et al. 2007). Different lines of evidence suggest that luminal progenitor cells are the cell-of-origin for breast cancer. BRCA1 mutants have been found to give rise to immature epithelial cells with basal differentiation characteristics and basal tumours assumed to be of basal origin. However, emerging data assert that they originate from luminal progenitors, and that these luminal progenitors are prone to survive oncogenic transformation due to their basal-like traits (Choudhury et al., 2013; Lim et al., 2009; Molyneux et al., 2010; Proia et al., 2011). It might be a similar mechanism acting in progenitors residing in ageing tissue, that cause them to develop into luminal cells with basal traits. We ask whether the undifferentiated state unveiled in mammary luminal cells in post-menopausal women is the feature that makes them more prone to develop into cancer cells.

5.6 Ageing and immortalization: Shared traits?

In considering how ageing HMEC avoid OIS, it is useful to consider cells immortalized in vitro which appear to share the trait. Previous studies suggest that low-level oncogene expression is the reason that immortalized HMEC do not undergo OIS following oncogenic transformation, while finite lifespan HMEC become senescent (Olsen et al. 2002). Olsen et al. found no β-galactosidase activity, and TP53 or CDKN2A/p16 did not need to be inactivated in order for benzo(a)pyrene immortalized cells to survive in the presence of the Raf-1 oncogene. The immortalized HMEC evaded growth arrest by maintaining Raf-1 expression, and the downstream pERK levels, low (Olsen et al. 2002). We found an attenuated cell response in immortalized HMEC (overexpressing constitutive CCND1/Cyclin D1 and MYC) compared to isogenic cells with a finite lifespan (Suppl. Data Fig.7). The mitogenic signal resulting from transformation of the Cyclin D1/ MYC immortalized cells with the oncogene Neu/ERBB2 was insufficient to trigger OIS. The cells survived (Section 3.2 Fig.25) in spite of CDKN2A/p16, TP53 or other tumour-suppressive mechanisms (Stampfer et al. 2013; Lin et al. 1998). It has been suggested that immortalization suppresses mitogenic activation (Stampfer et al. 2013), our data suggest that the process of ageing has similar effect on HMEC.

Interestingly, immortalization by overexpression of CCND1/Cyclin D1 rather than CDKN2A/p16 inhibition gave rise to a subpopulation of luminal cells, which was not found among CDKN2A/p16 inhibited cells (Suppl. Data Fig.6). Moreover, Lee et. al. 2015 demonstrated a larger population of luminal cells in CCND1/Cyclin D1 immortalized cells from postmenopausal cells, compared to premenopausal cells. Postmenopausal CCND1/Cyclin D1 immortalized cells comprised cells expressing a mix of both luminal and basal markers, while premenopausal CCND1/Cyclin D1 immortalized cells formed cells which were mainly negative for the luminal marker CD227 and positive for the basal marker CD10 (Lee et al. 2015). This could indicate that alterations in signaling

in older HMEC may give different results upon oncogene expression. We can only speculate whether suppression of the cell cycle regulators CCND1/Cyclin D1 is linked to the higher prevalence of luminal breast cancers in ageing women.

5.7 Do OIS resistant cells eventually succumb to agonescence?

Overcoming stress-associated-arrest (stasis) and agonescence by CDKN2A/p16 silencing, CCND1/Cyclin D1 overexpression and telomerase reactivation leads to immortalization (Section 3.2 Fig.25). Stasis is the type of senescence related to stress, including mitogenic overload, while agonescence is the type of senescence related to telomere shortening (Stampfer et al. 2013). Agonescence comprises certain viable cells, and is reversible in contrast to crisis that entails full blown cell death (Garbe et al. 2007). p53 suppression is not required for HMEC to bypass stasis, agonescence, or to evade OIS. This is in contrast to human keratinocytes and fibroblasts, as well as murine cells in general, which are comparably more vulnerable to DNA damage and stress mediated by TP53 - they transform more easily than human epithelial cells (Stampfer et al. 2013; Adams 2009).

Fibroblasts with repressed tumour suppressor gene TP53 allow senescent cells to re-enter the cell cycle, but this does not occur in HMEC once CDKN2A/p16 is activated (Beauséjour et al. 2003). The CDKN2A/p16 promoter is methylated in spontaneously immortalized cell lines leading to lower transcription levels. CDKN2A/p16 promoter methylation is also detectable in human breast tissues (Novak et al. 2009). If CDKN2A/p16 expression is impaired in older HMEC, senescence, but not agonescence, will be affected. Senescence appeared reversible in older HMEC compared to young HMEC (Suppl. Data Fig.3b). This could account for the lesser degree of immediate OIS in older HMEC. Neither cell type displayed anchorage-independent growth (data not shown). However, when telomeres eventually become critically short, even OIS-tolerant older HMEC will undergo

agonescence. Our data demonstrate that HMEC from young women senesced upon EGFR Del^{19} overexpression, while HMEC from ageing women continue proliferating (Suppl. Data Fig.3a). In other words, $EGFR^{Del19}$ transformed older cells were not immortalized, as they did eventually go into agonescence, with the exception of two cases. $EGFR^{Del19}$ expression in HMEC derived from an 80-year-old woman were cultured more than 19 passages; cryopreservered cells further re-established cell growth. Supplemental functional studies are needed to confirm whether the HMEC have become immortalized.

In conclusion, a majority of the HMEC from postmenopausal women analysed demonstrated prolonged growth upon ectopic expression of the EGFR Del^{19} oncogene, but they remain sensitive to telomere shortening. Hence full transformation can be, at least theoretically, accomplished with additional genetic changes.

6. Concluding remarks

60% of all cancer cases in US are diagnosed in people over the age of 65 (Berger et al. 2006), but there is no consensus to how ageing increases cancer susceptibility beyond a simple accumulation of genetic changes. We found that HMEC derived from older women show increased basal traits, altered signal transduction in response to extracellular matrix proteins and evidence of reduced senescence following oncogene expression. We suggest that this is the result of epigenetic changes imposed on HMEC by an age-related attenuation of the tissue microenvironment (Hashizume et al. 2015). This may lead to increased susceptibility to breast cancer with age.

7. Future perspectives

7.1 Epigenetic modulations as targets for cancer treatment

Age-dependent deregulation of gene expression and signaling can be a result of the increased DNA methylation in regions containing CpG islands combined

with a decrease in overall DNA methylation (Weidner et al. 2014; Christensen et al. 2009). Methylation of CpG islands in promoters silence gene expression (Jones et al. 2015; Lam et al. 2012). The notion that cancer susceptibility increases with age due to these epigenetic changes suggests that drugs targeting epigenetic mechanisms can be used as cancer chemoprotective agents.

The cancer-preventive effect of demethylating drugs has been demonstrated in mouse models and individuals with higher cancer risk due to lifestyle or age (Issa 2008). Considering that age-dependent gene silencing is skewed towards genes that govern development and maintain differentiation (Boland et al. 2014; Teschendorff et al. 2013), could epigenetic targeted therapy block tumourigenesis by reviving silenced tumour-suppressor genes such as CDKN2A/p16, $p21^{Cip1/Waf1/Sdi1}$ and TP53? As stated by Azad et al: “Reverting the software package of epigenetic abnormalities should be easier than repairing the hard drive that harbours the upstream mutations” (Azad 2013). In addition to being a target for treatment, age-related epigenetic signatures can be useful biomarkers of increased cancer susceptibility, exemplified by hypermethylation of CpG islands in promoters predicting future tumour risk (Teshcendorff 2013).

7.2 Adjustments to experimental set-up

Anderson et al. demonstrated in 2014 that breast cancer risk does not increase with age in a linear manner (Fig.37)(Anderson et al. 2014). There is a peak at 50 and 70 years, after which cancer risk declines. A similar peak is found around the age of 80 for all cancers in general, among both genders (Pavlidis et al. 2012). These peaks in cancer risk are reflected in our data and might have contributed to the observed lack of significant correlation between age and response to growth factor stimulation, in particular. Two out of 10 individuals in our study were older than 70, and may have confounded our results. We did indeed see that cell signaling was higher in these 2 individuals compared to the other older women (Paper 2 Fig.1d). Including these

individuals in our measurements of growth factor stimulation response as a function of age made the calculated correlations non-significant (Paper 2 Suppl. Fig.2b). In follow-up studies we would like to include more women in their sixties rather than in their seventies.

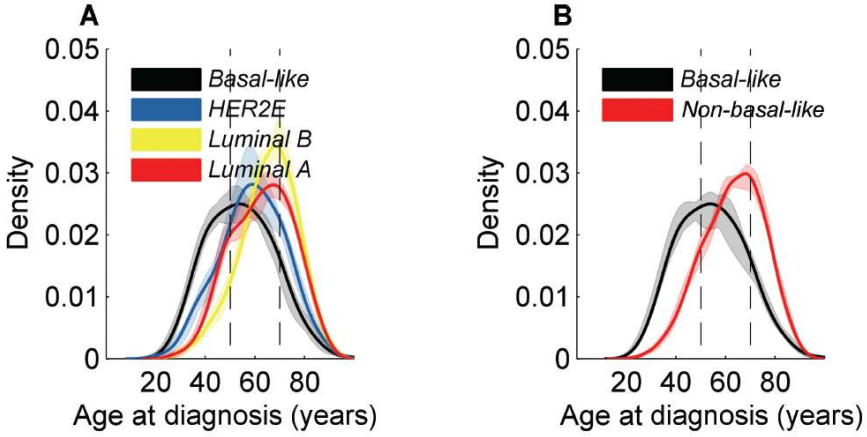


Fig.37: Incidence of breast cancer subtypes by age (Anderson et al. 2014). With permission from Oxford University Press.

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III

10. Supplementary data

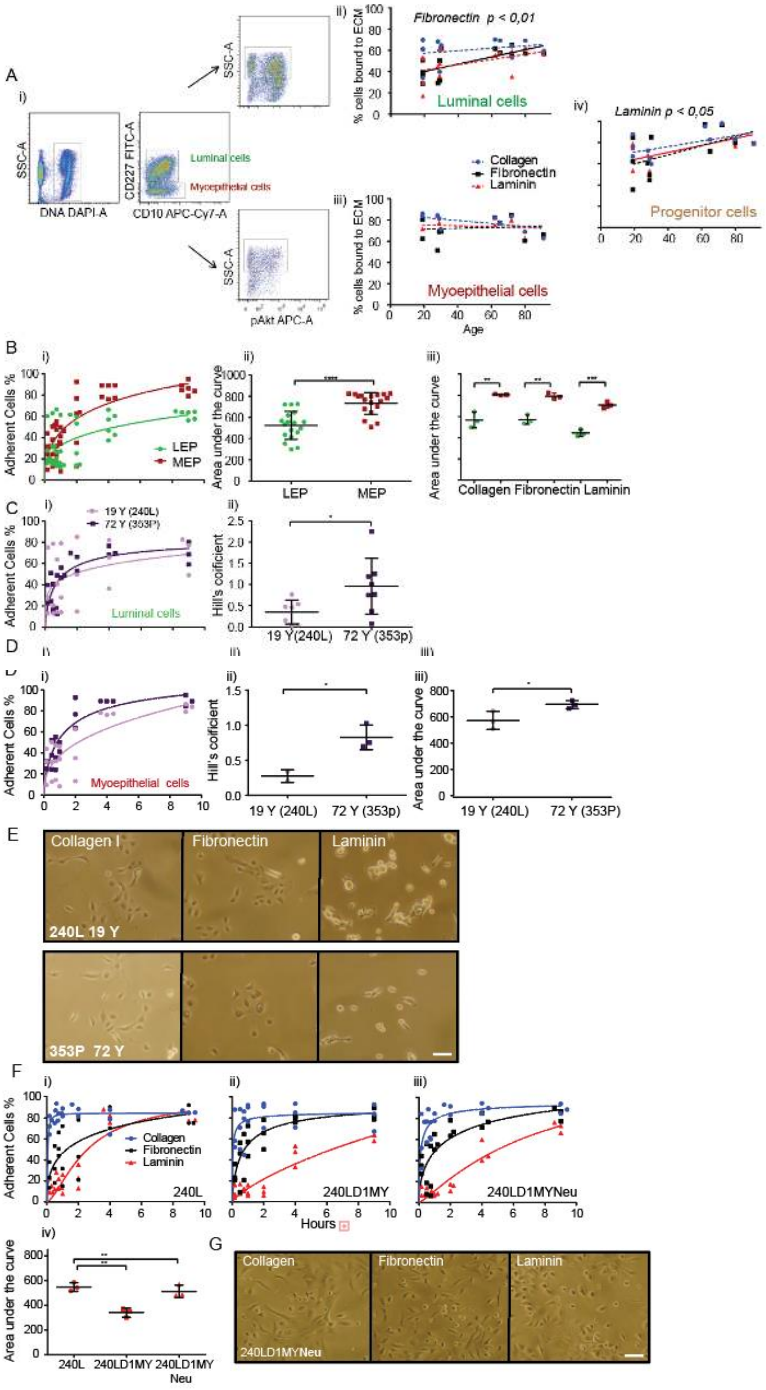

Fig.1 Cell adhesion efficiency distinguish human mammary cells according to lineage, age and immortalization. *(A) The p*ercentage of cells adhered to different ECM-coated microspheres by 2,5 hours of incubation was plotted as a function of age. HMEC from 10 women 19-91 y were included. i) Illustration of gating scheme to obtain luminal cells (LEP) ($CD227^+/CD10^-$) and myoepithelial cells (MEP) ($CD227^-/CD10^+$). Side scatter values combined with DNA staining distinguish cells from naked microspheres. ii) LEP, iii) MEP and iv) progenitor ($c\text{-}Kit^+$ /$CD227^+$) cells are presented separately. Significant linear regression correlations are marked as solid lines. A significant linear regression correlation between age and cell adhesion abilities was found for LEP bound to FN. (B) Cell adhesion kinetics of LEP and MEP to FN-coated microspheres was monitored for a period of 9 hours i) Data were fitted into a Hill's curve. Comparison of AUC values of the Hill's curves demonstrates significant difference in cell adhesion between LEP and MEP of ii) all ECM in total iii) and on individual ECM. (C) Cell adhesion kinetics of young and old LEP (19 y, 240L / 72y, 353P) on FN coated microspheres. i) Hill's curve of cell adhesion ii) Comparison of Hill's coefficients demonstrates significant difference in cell adhesion between young and old LEP. (D) Cell adhesion kinetics of young and old MEP (19 y, 240L / 72y, 353P) on LAM coated microspheres. i) Hill's curve of cell adhesion. ii) Comparison of Hill's coefficients of cell adhesion between young and old MEP iii) Comparison of AUC values of the Hill's curves. We demonstrated significant difference in cell adhesion between young and old MEP. (E) Phase-contrast images of young and old HMEC illustrating adhesion intensity to different ECM proteins. (F) Cell adhesion kinetics of an isogenic HMEC progression series (240L). The series comprised normal i) HMEC (240L), ii) immortalized (240LD1MY) and iii) oncogene-transformed (240LD1MYNeu) cells. iv) Comparison of AUC values of the Hill's curves on LAM. The data demonstrate a significant decrease in cell adhesion to LAM-coated microspheres with increasing tumourigenicity Tukey's multiple comparisons test confirms significant difference in adhesion between the three graphs, p=0,008. (G) Phase-contrast images of the isogenic HMEC progression series illustrating adhesion intensity to different ECM, after 24 hours of growth. (n=3 for each cell type). Error bars are ±SD. Scale bars are 100 µm. Remaining p -values were calculated by Student's two tailed t-test comparing AUC-values * P<0,05 ** = P<0,01. **** p< 0,0001 .

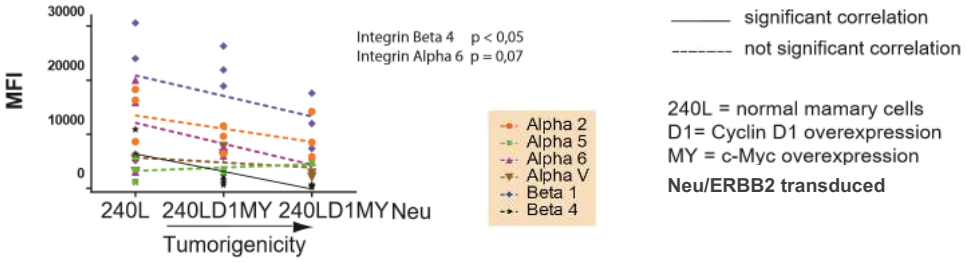

Fig.2 Integrin expression during cell transformation

Linear regression analysis of integrin expression on an isogenic human mammary epithelial cell progression series (n=3 for each cell line). Significant correlations ($P<0.05$) are demarcated by solid lines; non-significant correlations are represented by dotted lines.

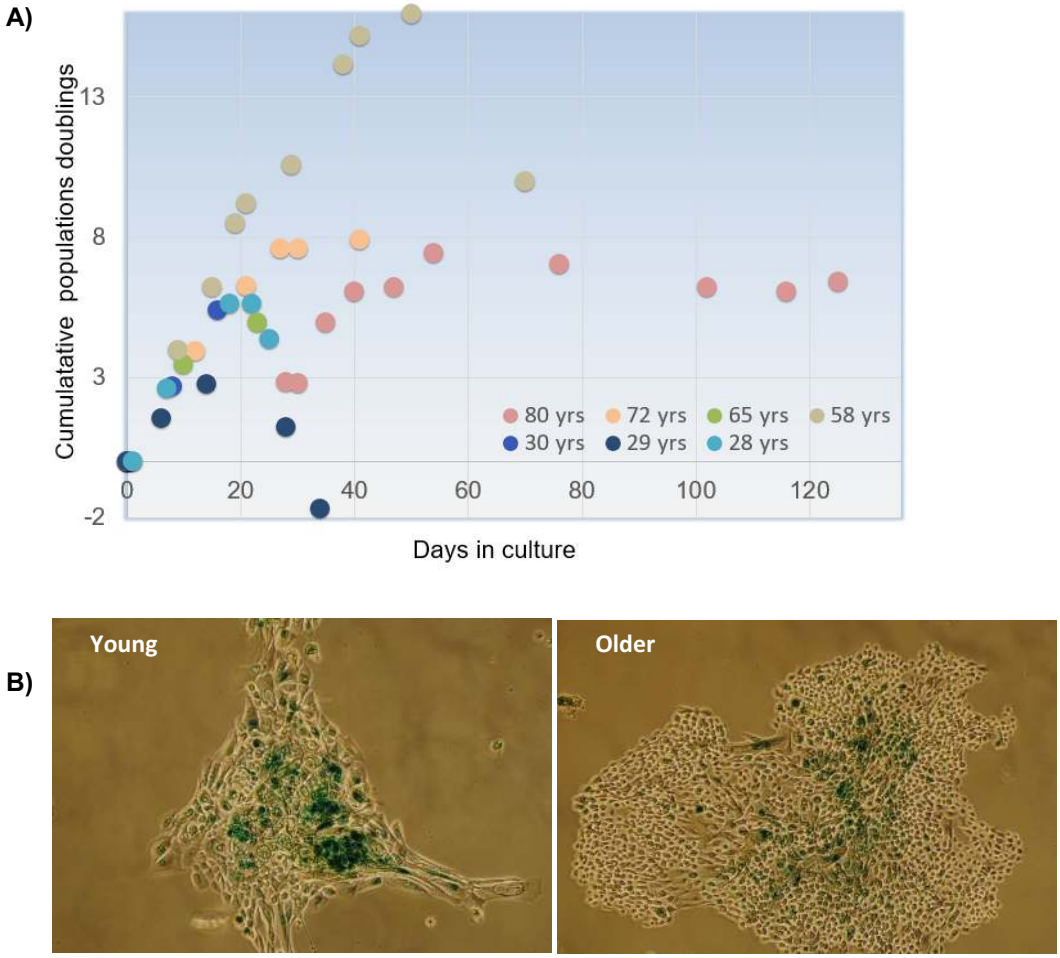

Fig.3 Cell population growth of oncogene transformed cells (EGFR ^{Del19}).
Overexpression of EGFR with a deletion in exon 19, making it constitutively active, leads to oncogene induced senescence in cells derived from a younger microenvironment (28, 29 and 30 y). A) The cells barely survive a couple of population doublings, while cells derived from older microenvironments (80, 72, 65 and 58 y) tend to survive a longer period of time. Growth is detected from the timepoint that selection of EGFR positive cells is initiated, either by flow cytometry sorting or antibiotic selection. B) β-Galactosidase activity in young and old cells upon EGFR transduction as they go into senescence. The young cells go into senescence within 5 population doubling after EGFR is introduced, ageing cells survive at least 18 population doublings before showing signs of growth arrest. Scale bar is 100 μm.

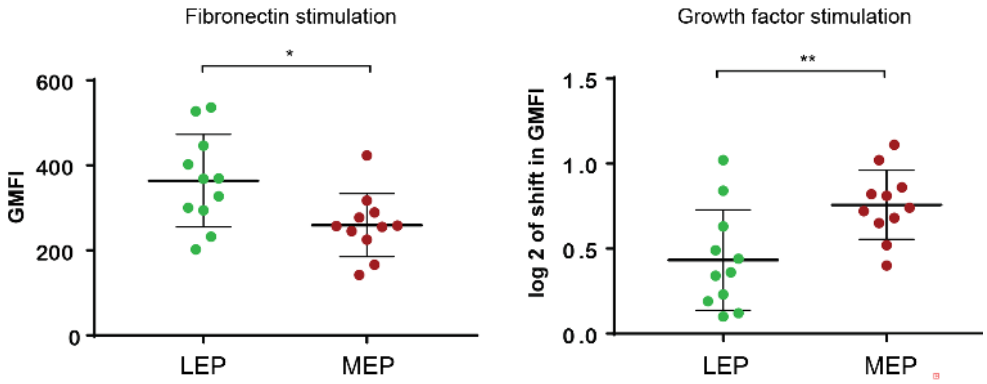

Fig.4 Cell activity levels differ between luminal (LEP) and myoepithelial (MEP) cells. Comparison of pAKT levels between LEP and MEP. A student t test of grouped data shows lower ECM induced signaling in MEP compared to LEP, but higher response to growth factors in MEP compared to LEP. This is likely because the low FN- induced signaling level in MEP allow for a stronger shift in phospho-level upon growth factor stimulation. LEP are more responsive to FN, while MEP save their response until GF stimulated. GMFI = geometric mean fluorescent intensity. * $p < 0,05$ ** $p < 0,01$

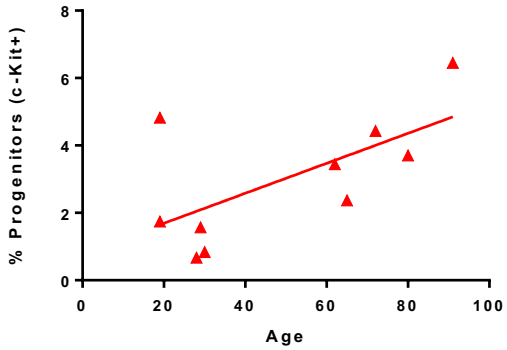

Fig.5 Luminal progenitor (c-Kit+) population increases with age

Linear regression analysis of c-Kit positive population among HMEC from 10 women 19-91 years, $p<0.05$.

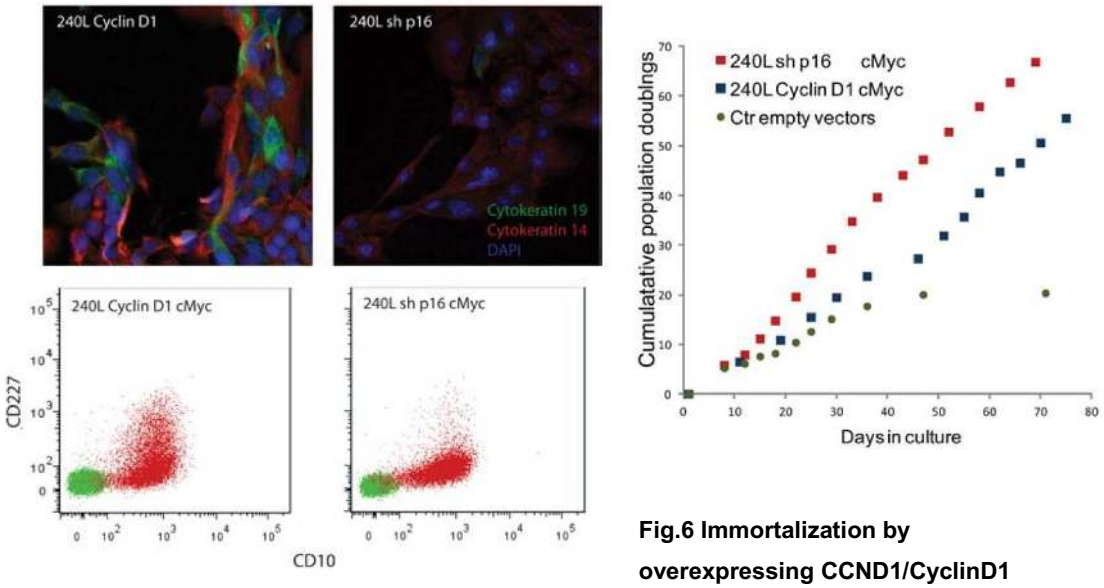

Fig.6 Immortalization by overexpressing CCND1/CyclinD1 results in a luminal subpopulation not obtained when immortalizing through the suppression of CDKN2A/p16. Telomer dysfunction was circumvented by overexpressing MYC which reactivates telomerase and recuperate telomere length (Kyo 2008). A control population was transduced with a retroviral vector that contained neither shRNA against CDKN2A/p16, nor overexpressed CCND1/CyclinD1 or MYC (Kolch 2015). Transduced cells that grew beyond the control population were defined as having obtained immortality.

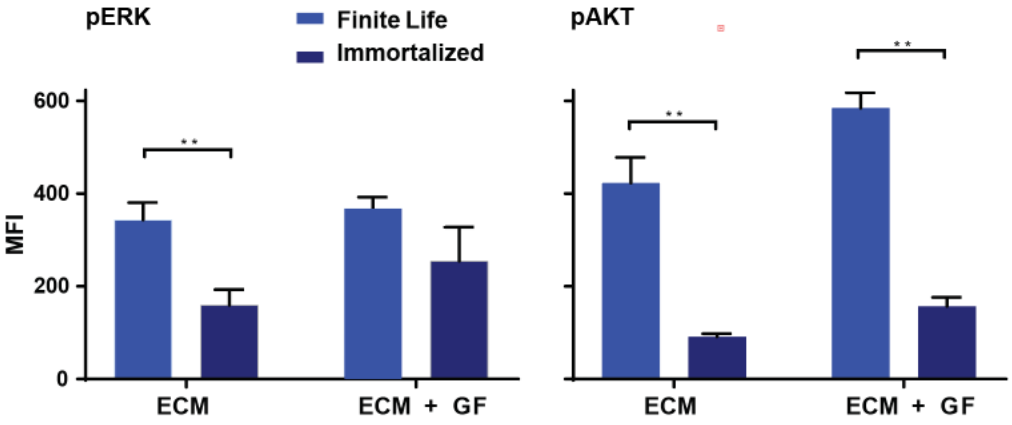

Fig.7 Immortalized cells demonstrate reduced response to ECM and growth factor stimulation. Response to solid ECM and soluble growth factors demonstrated as shift in phospho-protein levels (pERK and pAKT) upon stimulation. We compared finite life HMEC (240L) to immortalized (240LD1MY) cells. Student t-test two tailed unpaired ** p<0,01 MFI = mean fluorescent intensity.

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